PURIFICATION AND PROPERTIES OF RAT BRAIN PYRUVATE CARBOXYLASE

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percent sign
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
Caps	cyclohexylaminopropanesulfonic acid
PEG	polyethylene glycol

CHAPTER I

INTRODUCTION

CO_2 Fixation in the Brain

Only in the past decade has CO_2 fixation in brain tissue been seriously investigated, since relatively little CO_2 fixation occurs in brain tissue as compared to liver or kidney which have substantial anabolic activity, for example gluconeogenesis. Brain tissue has little gluconeogenic capacity (1-3). Nevertheless, investigation of CO_2 fixation in brain tissue has provided much information concerning the metabolism of this organ, even though, the exact reaction or reactions responsible for the fixation of CO_2 has not been established nor has the role or roles which this reaction plays in the metabolism of the brain been fully explored.

CO_2 fixation in brain tissue was implicated as early as 1953 in experiments by Moldave, Winzler, and Pearson (4). They found that HCO_3^- labeled aspartate and glutamate of brain proteins synthesized during long term incubations of brain slices. Furthermore, glutamate was labeled primarily in C-1 and aspartate in C-4. This is as predicted if CO_2 fixation occurred with pyruvate or phosphoenolypyruvate to form a four carbon citric acid cycle intermediate.

Berl *et al.* (5, 6) and Waelsch *et al.* (7) studied the incorporation of label into brain free amino acids under normal conditions and during ammonia toxicity by intercarotid infusion of cats with HCO_3^-. Under

normal conditions the order of the specific activities of the brain free amino acids from highest to lowest was aspartate, glutamine, and glutamate. Approximately 95% of the label in glutamate was in C-1 while 63% of the label in aspartate was in C-4. During ammonia intoxication the glutamine content of the brain was increased while the content of aspartate, glutamate, and the citric acid cycle intermediates remained the same. Also, the specific activity of glutamine increased such that the order of the specific activities of the brain free amino acids from highest to lowest was glutamine, aspartate, and glutamate. These results were interpreted to indicate that CO_2 fixation produces a four carbon citric acid cycle intermediate and that this CO_2 fixation is increased during ammonia toxicity to replace intermediates consumed by the de novo synthesis of glutamine. The latter interpretation has been challenged (8).

McMillan and Mortensen (9), and Koeppe et al. (8, 10) investigated the labeling pattern of brain glutamate and aspartate using 2-^{14}C-pyruvate and 2-^{14}C-glucose, respectively, as precursors. There was a significant amount of CO_2 fixation at the four carbon level of the citric acid cycle as indicated by the percent of label in carbons 2 and 3 of glutamate. Also, the amount of label in C-5 of glutamate was the highest of any tissue measured, probably indicating that a greater proportion of pyruvate was utilized via pyruvate dehydrogenase than in any other tissue. During stress by ammonia intoxication (8, 9) or thiamine deficiency (10) the percent of label in C-5 of glutamate did not decrease. This indicated that under the conditions studied there was no decrease in the proportion of pyruvate utilized via pyruvate dehydrogenase or increase in CO_2 fixation, implying that CO_2 fixation

may possibly be working at full capacity under normal conditions.

The de novo synthesis from citric acid cycle intermediates or the leakage of these intermediates from the mitochondria necessitates anaplerotic replenishment. The extent of de novo synthesis from citric acid cycle intermediates and the anaplerotic synthesis of these intermediates within the brain is unknown but they must balance each other. A small amount of anaplerotic synthesis via CO_2 fixation occurs normally in the brain, approximately 13 to 17% (9). Conditions which stimulate this synthesis have not been demonstrated conclusively. If CO_2 fixation is taking place at maximal rates in the brain under normal conditions then the role of this CO_2 fixation system may be quite general and not related to any specific metabolic event.

CO_2 Fixation in the Brain at the Enzymatic Level

The enzymes capable of CO_2 fixation have been reviewed by Wood and Utter (1). However, because of the nature of CO_2 fixation occurring in brain tissue only those enzymes which can fix CO_2 into malate or oxalacetate from pyruvate or phosphoenolpyruvate need be discussed in relation to brain metabolism. These are the malic enzyme, phosphoenolypyruvate carboxykinase, and pyruvate carboxylase. Recently, the malic enzyme and phosphoenolpyruvate carboxykinase from brain tissue have been studied in some detail.

The intracellular distribution of the malic enzyme is tissue specific (12-17). Approximately 75% of the rat brain enzyme is mitochondrial whereas in the liver the enzyme is soluble (12). The malic enzymes from bovine brain have been separated and purified (18). In this

tissue the mitochondrial enzyme and the soluble enzyme are isozymes differing in electrophoretic and kinetic properties. The thermodynamic equilibrium for the reaction catalyzed by the malic enzyme favors decarboxylation and also, the rate of the carboxylation reaction is very slow, especially with the mitochondrial enzyme. These observations suggest that this enzyme does not participate significantly in the carboxylation of pyruvate.

Rat brain phosphoenolpyruvate carboxykinase has been partially purified by Cheng and Cheng (19). In rat brain tissue the enzyme has a catalytic capacity of about 0.3 μ moles/minute/gram of tissue and is primarily mitochondrial in contrast to that of rat liver (20). The brain and liver enzymes also differ in their kinetic properties, reactivity toward sulfhydryl inhibitors, and molecular weight. These differences indicate that the brain and liver enzymes may be isozymes. The high apparent K_m, 5 mM, of the brain enzyme for phosphoenolpyruvate, much higher than that of the liver enzyme, severely limits the capacity of this enzyme to carboxylate phosphoenolpyruvate under physiological conditions.

Pyruvate carboxylase (EC 6.4.1.1) is widely distributed throughout nature, being found in many microorganisms (21) and in most mammalian tissues (22). The discovery of this enzyme in 1960 by Utter and Keech (23) was stimulated when it was found that avian liver mitochondria could synthesize significant amounts of phosphoenolpyruvate, even though, the mitochondria were devoid of the malic enzyme. The synthesis of phosphoenolypruvate from pyruvate in gluconeogenic tissues was considered to occur through a pathway involving malate and oxalacetate, the dicarboxylic acid shuttle, the reversal of the pyruvate kinase

reaction being inadequate to account for gluconeogenesis (24). In the absence of malic enzyme, some other route of formation of malate or oxalacetate from pyruvate was necessary. The search for pyruvate carboylase was prolonged because of its absolute requirement for the allosteric activator acetyl - CoA[1].

In vertebrate tissues the intracellular location of the enzyme is exclusively mitochondrial (25-27). Among the vertebrate tissues, pyruvate carboxylase is present in the highest concentration in the liver and kidney, tissues with large capacities for gluconeogenesis. Indeed, pyruvate carboxylase plays an important role in gluconeogenesis (28). However, the enzyme has been implicated in several other roles including lipogenesis (26), glycerogenesis (26), and anaplerosis (29). Scrutton (28) suggests that "the anaplerotic role probably represents the original function of pyruvate carboxylase and may account for the presence of the enzyme in many microorganisms, and, at low levels in such tissues as brain, heart muscle, and intestinal mucosa".

Pyruvate carboxylase has been purified from several sources, including chicken (30, 31), turkey (21), calf (21), and rat liver (32, 33), sheep kidney cortex (34), bakers yeast (35, 36), and Pesudomonas citronellolis (37). The basic properties of the enzyme pertaining to the general catalytic mechanism appear to be similar regardless of the species from which the enzyme is obtained (21, 22, 24, 28). Pyruvate carboxylase (EC 6.4.1.1) catalyzes the reaction:

$$\text{Pyruvate} + [\text{MgATP}]^{-2} + HCO_3^- + \underset{\text{oxalacetate} + \text{MgADP}^- + PO_4^{=}.}{\overset{\text{acetyl-CoA},Mg^{+2},K^+}{\rightleftharpoons}} \qquad (1.1)$$

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[1]The author will use without definition those abbreviations accepted by (1973) J. Biol. Chem. 248, 1-8, throughout the thesis.

The enzyme from mammalian or avian sources is a tetramer with a molecular weight of 500,000 composed of identical subunits. Each subunit contains one molecule of bound biotin and a divalent cation, generally Mn^{++}. The enzyme from vertebrate sources requires acetyl-CoA for maximal activity. The requirement of the avian liver enzyme for an acyl-CoA allosteric activator is absolute, acetyl-CoA being the most effective (38). On the basis of exchange reactions the following minimal mechanism has been established.

$$\text{E-biotin} + \text{MgATP}^{-2} + \text{HCO}_3^- \xrightleftharpoons{\text{acetyl-CoA},Mg^{++},K^+} \text{E-biotin-CO}_2 + \text{MgADP}^- + \text{PO}_4^{=} \quad (1.2)$$

$$\text{E-biotin-CO}_2 + \text{pyruvate} \rightleftharpoons \text{E-biotin} + \text{oxalacetate} \quad (1.3)$$

Acetyl-CoA activates the carboxylation reaction (Equation 1.2) of every vetebrate enzyme studied; it also slightly activates the transcarboxylation reaction (Equation 1.3) of the rat liver enzyme. Initial rate studies of the overall reaction for chicken liver (39), rat liver (40), and Aspergillus niger (41) pyruvate carboxylases indicates that this enzyme utilizes a non-classical two site Bi Bi Uni Uni Ping Pong mechanism.

Although the basic catalytic properties of the various pyruvate carboxylases are similar, the regulatory properties of the enzyme from different species are often very diverse (42). This is due partly to differences in the arrangement and interactions between the subunits of the enzyme among the various species as is illustrated by differences in activation by acyl derivatives of coenzyme A, and inhibition by acetoacetyl-CoA and L-aspartate (22).

Salganicoff and Koeppe (12) established that pyruvate carboxylase is present in rat brain tissue in very small quantities, 0.2 units per

gram of tissue, and is mitochondrial in location. Felicioli et al. (43) have studied the subcellular distribution of ox brain pyruvate carboxylase and have measured the content of this enzyme in the brain tissue of several mammalian species. Patel et al. (44, 45) working with intact brain mitochondria have shown that CO_2 fixation by pyruvate carboxylase is inhibited by phenylpyruvate but not avidin.

A small amount of preliminary work has been done directly on very crude brain pyruvate carboxylase preparations. Felicioli et al. (46) found that ox brain pyruvate carboxylases exhibited substrate inhibition when $MgATP^{-2}$ was above 2 mM and that free Mg^{++} was required for maximal activity. Patel et al. (44, 47) reported that the apparent K_m for pyruvate with both human and rat brain pyruvate carboxylase is 0.2 mM. They have also shown inhibition of pyruvate carboxylase by phenylpyruvate and malate.

Pyruvate carboxylase is present in rat brain tissues in very small quantities, about 1/18 that of rat liver on a unit per gram of tissue basis (25). Since liver weight is about ten times that of brain, the total pyruvate carboxylase content of rat liver is 150 to 200 times that of rat brain. In fact, when Keech and Utter (31) originally assayed brain tissue for pyruvate carboxylase they would not commit themselves as to whether or not brain tissue contained pyruvate carboxylase activity.

The purpose of this thesis was to purify pyruvate carboxylase from rat brain tissue and study its properties to gain insight concerning the function of this enzyme in nervous tissue. We chose rat brain as a source despite the difficulties mentioned, for two reasons. First, we wished to compare the properties of pyruvate carboxylase from liver and

brain tissues of the same species. The recent work by McClure et al. (32, 40, 48, 49) and Seufert et al. (33) on rat liver pyruvate carboxylase have given us a good basis of comparison. Secondly, a great body of work has been accomplished with the rat as an experimental animal and we wanted to add to this growing body of information. Therefore, the work was done with rats rather than animals with larger and cheaper brains.

CHAPTER II

MATERIALS AND METHODS

Materials

Tris·ATP, Trizma Carbonate, Trizma HCl, pyruvic acid, 5-5'-dithiobis-(2-nitrobenzoic acid), NADH, NAD, lactic and malic dehydrogenases were purchased from Sigma Chemical Co. Sodium pyruvate, Na_2·ATP, coenzyme A, dithiothreitol, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, sodium phenylpyruvate and oxalacetate were purchased from Calbiochem. Sucrose, $(NH_4)_2SO_4$, mannitol, Tris base, Na_2EDTA, and $MgSO_4$ were obtained from Schwartz/Mann. Ficoll was obtained either from Sigma or Pharmacia Fine Chemicals Inc. Polyethylene glycol, average molecular weight 6,000, was obtained from Matheson Coleman and Bell. Cyclohexylaminopropanesulfonic acid, avidin, and biotin were purchased from Nutritional Biochemical Corporation. Alcohol dehydrogenase and catalase were purchased from Worthington Biochemical Corporation. Citrate synthetase was obtained from Boehringer Mannheim Biochemicals. Acetoacetyl-CoA was purchased from P-L Biochemicals, Inc. Celite 545 was obtained from Curtin Scientific Co. Albino male rats, 150-250g, were obtained from Holtzman and fed a 4% Teklad mouse and rat diet until use. $NaH^{14}CO_3$ was purchased from Amersham/Searle Corporation.

Methods

Preparation of Reagents

Acetyl-CoA used in early experiments was prepared from CoA and acetic anhydride by the method of Simon and Shemin (50) in 100 mM Tris base. Preparation of acetyl-CoA in this manner produces a significant amount of diacyl-CoA which has been reported to be a potent inhibitor of pyruvate carboxylase with respect to acetyl-CoA (51). Large amounts of the diacyl-CoA are prevented by using only a 15% excess of acetic anhydride and by maintaining the pH between 7.0 and 7.5 during the course of the reaction (51).

Ficoll solutions were prepared from the commercially supplied reagent without dialysis (52). Celite 545 was washed extensively prior to equilibration with the appropriate buffers. Tris·HCO_3 buffers were prepared either from the commercial Trizma carbonate reagent or by titration of a solution of Tris base to pH 8.0 by the addition of solid CO_2. Both the Tris·HCO_3 and the cyclohexylaminopropanesulfonic acid buffers required Millipore filtration before use.

Substrate Assays

Concentrations of adenine nucleotides were determined spectrophotometrically as described by McClure et al. (32). Pyruvate concentrations were determined in 100 mM Tris·HCl, pH 8.0 as described by Bucker et al. (53). Acetyl-CoA concentrations were determined as described by Srere (54). The bicarbonate concentrations of the stock solutions used when bicarbonate was the variable substrate in the subsequent kinetic studies were determined manometrically (55) and the

endogenous concentrations of bicarbonate in kinetic assay reagents were corrected as described by McClure et al. (32). Protein was determined by the spectrophotometric method of Kalckar (56):

Protein concentration (mg/ml) = $1.45(A_{280})- 0.74 (A_{260})$.

Enzyme Assays

Alcohol dehydrogenase activity was ascertained spectrophotometrically (340 nm) at pH 10.3 using the method described by Bonnichsen (57). Catalase activity was assayed at a wavelength of 230 nm under the conditions described by Luck (58). Citrate synthetase was assayed exactly as described by Srere (51). Lactic dehydrogenase was assayed as described by Clark and Nicklas (52) and malic dehydrogenase was assayed by measuring the decrease in absorbance at 340 nm in a reaction mixture of 100 uM oxalacetate, 250 uM NADH, and 100 mM Tris·HCl, pH 8.0 (59). Acetyl-CoA deacylase activity was determined by following the increase in absorbance at 412 nm in a reaction mixture of 250 uM acetyl-CoA, 100 uM 5,5'-dithiobis-(2-nitrobenzoic acid), and 100 mM Tris·HCl, pH 8.1 (54).

ATPase was assayed in a reaction mixture of 2 mM Na_2ATP, 3 mM $MgCl_2$, 75 mM KCl, and 50 mM Tris-acetate buffer, pH 7.5 (60, 61). The enzyme sample assayed was incubated in the 0.5 ml reaction mixture for 15 minutes at room temperature. After addition of 10 ml of 10% (w/v) of trichloroacetic acid containing 10 mg of thiourea and 30 mg of ferrous ammonium sulfate hexahydrate, the mixture was centifuged to remove precipitated protein and the phosphorous liberated was determined by the method of Goldenberg and Fernandez (62).

The following two assays were used to determine pyruvate carboxy-

lase activity, unless otherwise indicated. The first is a radioisotope assay used for monitoring pyruvate carboxylase activity in crude preparations of brain tissue. The reaction mixture of 0.25 ml, pH 8.0, had the following millimolarities: Tris·Cl, 100; Na_2ATP, 1.0; $KH^{14}CO_3$ (~4 uC), 21; acetyl-CoA, 0.16; sodium pyruvate, 8.0; $MgSO_4$, 9.0. Also present in the reaction mixture was NADH, 0.16 mM, and 5 units of malic dehydrogenase to convert labile oxalacetate to the more stable malate. The reaction was run at room temperature for 1 minute and under these conditions approximately 1 mg of protein could be accommodated per assay. The reaction was terminated by the addition of an equal volume of 6N HCl and the acidified samples were evaporated to dryness by a stream of air. The residues were suspended in H_2O and centrifuged, and the supernatant fraction was assayed for ^{14}C in a Packard liquid scintillation counter using Bray's (63) scintillation cocktail. The second assay, which followed the decrease in absorbance at 340 nm in a recording spectrophotometer thermostated at 25°, was employed to determine the activity of the more purified fractions when the interfering activity of lactic dehydrogenase was low. The reaction mixture of 1.0 ml, pH 8.0, had the following millimolarities: Na_2ATP, 1.0; sodium pyruvate, 5.0; $KHCO_3$, 30.0; $MgSO_4$, 8.0; acetyl-CoA, 0.16; Tris-Cl, 100. Also present in the reaction mixture was NADH, 0.16 mM, and 5 units of malic dehydrogenase. The optimum amount of malic dehydrogenase was ascertained as described by McClure (32, 64). A unit of pyruvate carboxylase activity catalyzes the production of 1 umole of oxalacetate per minute in the above reaction mixtures.

Analysis of Kinetic Data

The design of the kinetic experiments, analysis of results, and nomenclature of kinetic terms are as outlined by Cleland (65). When ATP^{-4} was the variable substrate in a kinetic experiment it was added as a 1:1 molar solution of ATP^{-4} and $MgSO_4$. All spectrophotometric measurements were made with a Model 124, Hitachi Perkin-Elmer double beam spectrophotometer thermostated at 25°C. Initial rates were expressed as ΔA_{340}/minute.

Initially plots of $\frac{1}{v}$ versus the reciprocal of concentration of the varied substrate or activator were made and when these plots were linear the data were fitted to Equation 2.1.

$$v = \frac{VA}{Ka + A} \tag{2.1}$$

Statistical fits to Equation 2.1 were obtained by using Fortran computer programs obtained from either Cleland (66) or Schulz (67) employing an IBM 360/65 model computer. A final fitting of the data was made to Equations 2.2 to 2.4 depending upon the overall appearance of the reciprocal plots and the secondary plots of the slopes and intercepts. When biphasic kinetics were apparent in the Lineweaver-Burk plots and the substrate concentration over each linear portion of the plot defined, each region was analyzed separately and independently of the other according to Equation 2.2.

$$v = \frac{VAB}{KiaKb + KbA + KaB + AB} \tag{2.2}$$

$$v = \frac{VAB}{KiaKb + KbA + AB} \tag{2.3}$$

$$v = \frac{VA}{Ka(1 + I/Kis) + (1 + I/Kii)A} \tag{2.4}$$

Equation 2.2 corresponds to the sequential order of addition and release of substrates and products. Equation 2.3 corresponds to the equilibrium ordered addition of the first substrate followed by the addition of the second and Equation 2.4 refers to noncompetitive inhibition. Computer programs for direct fits to equations 2.2 to 2.4 may also be obtained from Cleland (66). When an acyl-CoA derivative was a varied component of a kinetic experiment all kinetic constants were determined as described by Scrutton and Fung (68).

CHAPTER III

EXPERIMENTAL AND RESULTS

Catalytic Capacity of Rat Brain Tissue

Pyruvate carboxylase activity of rat brain tissue was determined by homogenization and treatment of the tissue as described by Ballard et al. (25) by use of the radioisotope assay described. The cerebral hemispheres of a rat brain were homogenized in 5 volumes (w/v) of freshly prepared 0.25 M sucrose and lyophilized. The lyophilized residue was extracted with approximately 5 ml of 50 mM Tris-acetate, pH 7.0, 5 mM in ATP, 5 mM in $MgSO_4$, and 0.5 in mM EDTA. The pH of this extract was brought to 7.4 with 0.5 M Tris base and assayed for pyruvate carboxylase activity. The activity of rat brain averaged 0.21 units per gram of wet tissue which agrees with the value reported by Salganicoff and Koeppe (12) and corresponds to 0.002 units per mg of protein. Similarly the activity of rat testes, pig and beef brain have been determined to be 0.13, 0.10, and 0.04 units per gram of tissue, respectively. Higher values have been reported for rat brain of 0.5 and 0.88 units per gram of tissue by Scrutton and Utter (69) and Ballard et al. (25), respectively. However, these assays were incubated at temperatures much higher than the room temperatures used here, 30° was used by Scrutton and Utter (69) and 37° by Ballard et al. (25).

Purification of Rat Brain Pyruvate Carboxylase

Phase I - Preparation of Mitochondria

Mitochondria were isolated essentially according to the procedure of Clark and Nicklas (52). All centrifugations for the preparation of mitochondria were carried out in a Servall RC 2-B centrifuge refrigerated to approximately 0° in a SS-34 head using 50 ml polyethylene centrifuge tubes.

Rats were killed by decapitation and the cerebral hemispheres were rapidly removed and placed in cold isolation medium, a solution 0.25 M in sucrose, 10 mM in Tris, pH 7.4, 0.5 mM in EDTA and dithiothreitol. The cerebral hemispheres of 16 rats were quartered and then washed at least twice with a small volume of isolation medium. Homogenization was carried out in 40 ml of isolation medium using a Thomas size C homogenizer. Two passes were made with a standard teflon pestle turning at 640 rpm. Additional isolation medium was then added to the homogenate to bring the volume to 120 ml. Routinely, the above procedure was repeated until a combined homogenate of 240 ml containing the cerebral hemispheres of 32 rats was obtained.

The homogenate was centrifuged at 2,000g for 3 minutes in six equal portions. The supernate fractions were decanted and pooled. During the decantation process care was taken to remove as much of the supernate as possible and in so doing a small amount of the loosely packed white fluffy material of the upper portion of the pellet was usually decanted with the supernate. Each pellet was suspended with a stirring rod in about 15 ml of isolation medium, pooled, and centrifuged again at 2,000g for 3 minutes. The supernate fractions were pooled along with those of

the previous centrifugation and centrifuged at 2,000g for 3 minutes to remove the white fluffy material previously mentioned. The supernate from this centrifugation was then centrifuged in six equal portions for 8 minutes at 12,500g to obtain a crude mitochondrial pellet.

Each mitochondrial pellet was suspended in 8 ml of a 3% Ficoll solution 120 mM in mannitol, 30 mM in sucrose, 0.25 mM in EDTA and dithiothreitol using a pipette with a large orifice and layered onto 20 ml of a 6% Ficoll solution 240 mM in mannitol, 60 mM in sucrose, 0.5 mM in EDTA and dithriothreitol. Then the gradient was centrifuged 11,500g for 30 minutes. The supernatant portion of each tube was partially decanted and the light fluffy layer above each pellet was removed by swirling the contents of the tube gently against the pellet. The contents of each tube was drained and the six pellets were combined by resuspending in 20 ml of 10^{-4}M EDTA and lyophilized.

The lyophilized residue was stored evacuated over Drierite at -29° until use. Stored in this manner there is no detectable loss in pyruvate carboxylase activity over a several week period.

Phase II - $(NH_4)_2SO_4$ Fractionation

The lyophilized residue was extracted with 30 ml of 50 mM Tris-Cl buffer, pH 7.4, 0.5 mM in EDTA and dithiothreitol, Buffer A, and homogenized as described previously. When necessary the pH of the extract was adjusted between 7.2 and 7.4 by the addition of solid Tris base. The extract was centrifuged at 100,000g for 30 minutes in a refrigerated Beckman Spinco model L preparative ultracentrifuge. The supernate fractions were pooled and allowed to come to room temperature.

The 100,000g supernate fraction was then brought to 33% saturation

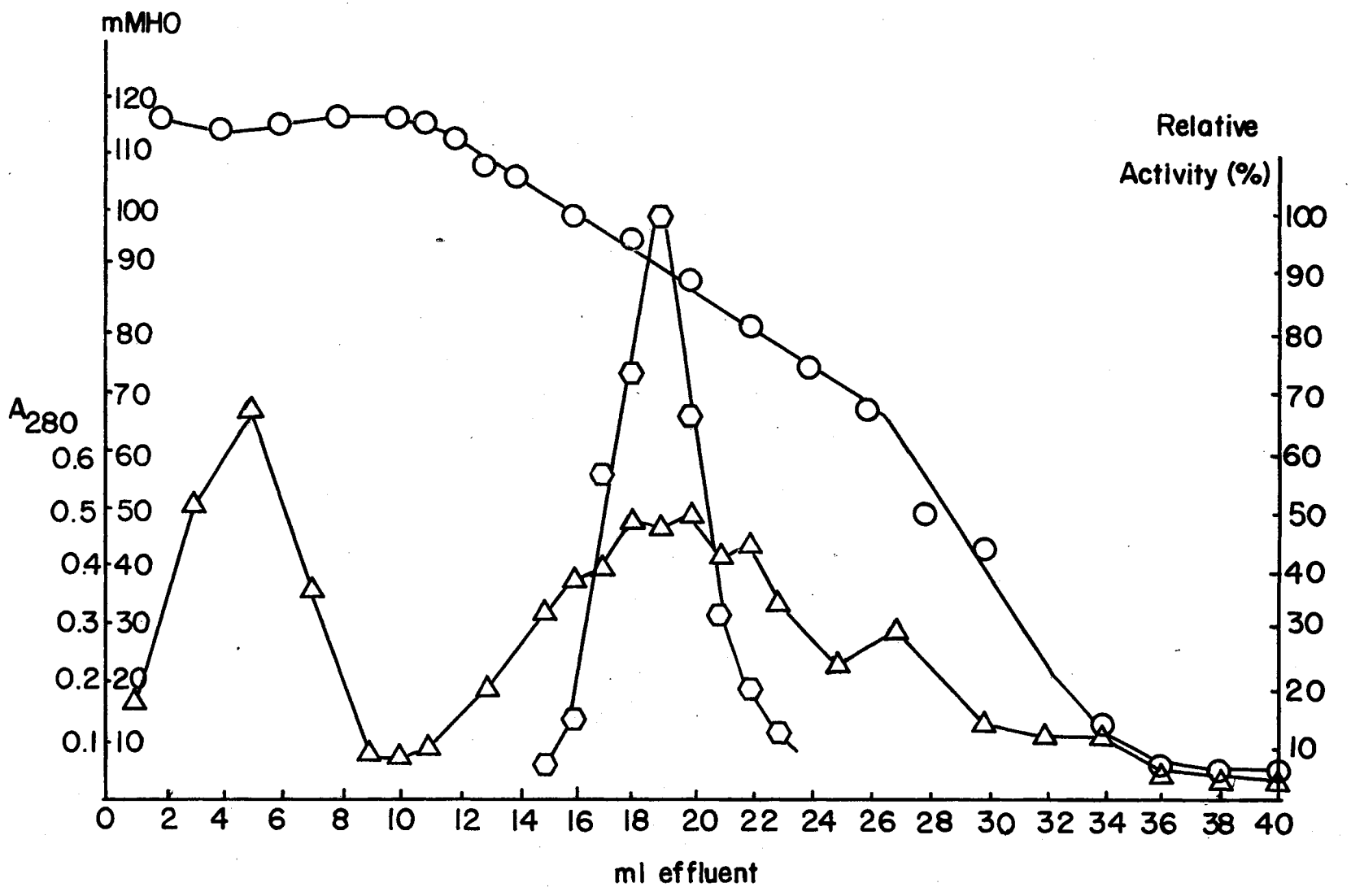
(196 grams/liter) by the slow addition of solid $(NH_4)_2SO_4$ over a 30 minute period. The pH was continually maintained between 7.2 and 7.4 by the addition of solid Tris base. The pyruvate carboxylase activity was then pelleted by a 10,000g centrifugation for 20 minutes at 20°.

At this stage of purification rat brain pyruvate carboxylase was not cold labile. The enzyme was as stable at 4° as at room temperature. For convenience, further purification steps were carried out at room temperature.

The next step of purification was patterned after the Celite $(NH_4)_2SO_4$ gradient solubilization procedure of King (70). The 33% $(NH_4)_2SO_4$ pellet was suspended with a pipette in 1-2 ml of 50 mM Tris-Cl buffer, pH 7.4, 0.5 mM in EDTA and in dithiothreitol, and 33% saturated with $(NH_4)_2SO_4$, Buffer B. Approximately 0.5 ml of wet Celite, equilibrated with Buffer B, was added to the suspension and the slurry was layered on a 1 x 9 cm Celite column equilibrated with Buffer B. During this time it was sometimes necessary to slurry the top centimeter or so of the column to hasten its flow rate, thus preventing protein precipitation from clogging the column. The column was eluted with a 40 ml linear gradient of decreasing ionic strength from that of Buffer B to that of Buffer A. The flow rate of the column was maintained at 0.33 ml per minute with the aid of a peristalic pump and 2 ml fractions were collected. An elution profile of the column is shown in Figure 1.

The tubes containing pyruvate carboxylase activity were pooled and brought up to an $(NH_4)_2SO_4$ concentration in excess of 33% saturation as determined by the conductivity of the solution. Following a 30 minute 100,000g centrifugation the pellet was dissolved in 5 ml of Buffer A. Enzyme purified through this phase is referred to as Fraction A material.

Figure 1. $(NH_4)_2SO_4$ Gradient Solubilization of Pyruvate Carboxylase on a Column of Celite 454. Conductivity (o—o) in mMHO, protein (Δ—Δ) as measured at 280 nm, and enzyme activity (⬡—⬡) expressed as percent of fraction #19 were determined.


mMHO
120
110
100
90
80
70
60
50
40
30
20
10
Relative
Activity (%)
100
90
80
70
60
50
40
30
20
10
A280
0.6
0.5
0.4
0.3
0.2
0.1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
ml effluent

Phase III - Polyethylene Glycol Fractionation

Fraction A was brought to 10% (w/v) concentration of polyethylene glycol by dropwise addition of 1.25 ml of a 50% (w/v) polyethylene glycol solution in Buffer A (71, 72). The suspension was stirred gently for 20 to 120 minutes and then centrifuged at 100,000g for 30 minutes. The supernate was then brought to a final concentration of 18% (w/v) polyethylene glycol by dropwise addition of 1.55 ml of the 50% solution and allowed to stand with gentle stirring for 20 minutes. Following a 30 minute 100,000g centrifugation the pellet was dissolved carefully in 1.0 ml of Buffer A and called Fraction B.

The pellet formed during the centrifugation following the second addition of polyethylene glycol was often so minute as to go unnoticed. Great care was necessary in orienting the centrifuge tubes such that the location of the pellet could be ascertained.

Fraction B was centrifuged an additional 100,000g for 20 minutes to remove dust and other debris which caused lightscattering during the spectrophotometric determination of protein concentration. The supernate from this centrifugation was used for the following studies and was routinely stored at 4°.

The time required to complete phase II and III purifications was between 10 and 12 hours. An outline of the purification scheme is shown in Figure 2 and the results of a typical purification through phases II and III appears in Table 1. The specific activity of pyruvate carboxylase purified through phase III ranged between 2.7 and 7.8 units per mg of protein with an average of 4.6 units per mg of protein for 15 purifications. The average number of units of pyruvate carboxylase obtained in Fraction B from a purification starting with 32 rat brains was 1.14

Figure 2. Purification Scheme for Rat Brain Pyruvate Carboxylase. P = pellet; S = supernate.

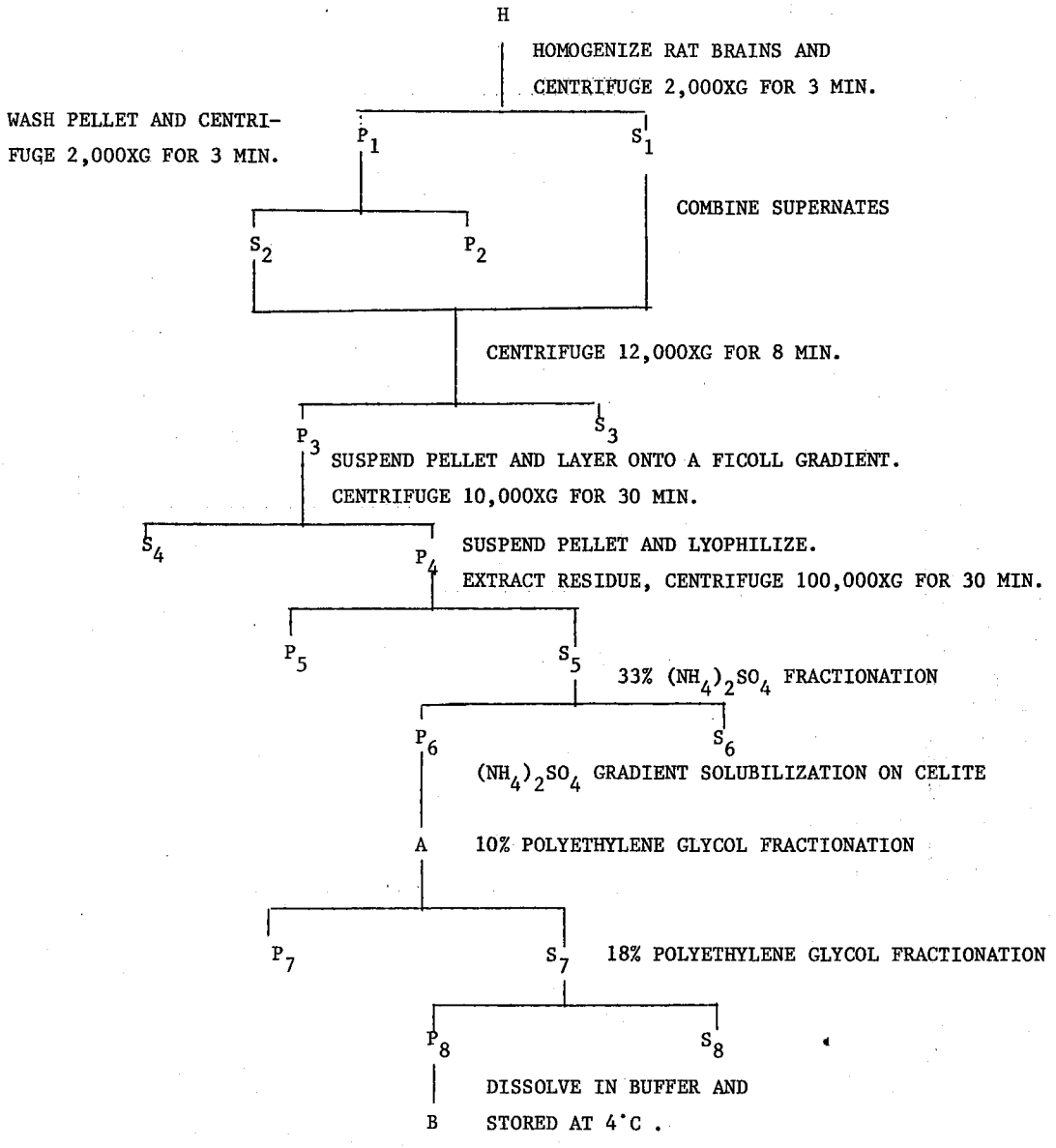

H
HOMOGENIZE RAT BRAINS AND
CENTRIFUGE 2,000XG FOR 3 MIN.
WASH PELLET AND CENTRI-
FUGE 2,000XG FOR 3 MIN.
P1
S1
COMBINE SUPERNATES
S2
P2
CENTRIFUGE 12,000XG FOR 8 MIN.
P3
S3
SUSPEND PELLET AND LAYER ONTO A FICOLL GRADIENT.
CENTRIFUGE 10,000XG FOR 30 MIN.
S4
P4
SUSPEND PELLET AND LYOPHILIZE.
EXTRACT RESIDUE, CENTRIFUGE 100,000XG FOR 30 MIN.
P5
S5
33% (NH4)2SO4 FRACTIONATION
P6
S6
(NH4)2SO4 GRADIENT SOLUBILIZATION ON CELITE
A
10% POLYETHYLENE GLYCOL FRACTIONATION
P7
S7
18% POLYETHYLENE GLYCOL FRACTIONATION
P8
S8
DISSOLVE IN BUFFER AND
B
STORED AT 4°C .

TABLE I

PURIFICATION OF RAT BRAIN PYRUVATE CARBOXYLASE

Treatment	Vol. ml	Protein mg/ml	Pyruvate Carboxylase			Acetyl-CoA Deacylase		
			Units/ml	Total Units	Units/mg	Units/ml	Total Units	Units/mg
Homogenate*	240	19.2	0.04	9.6	0.002	-	-	-
Celite column	15.5	0.24	0.26	3.9	1.1	0.46	7.1	1.9
Fraction A	5.0	0.52	0.53	2.7	1.0	0.90	4.5	1.7
10% PEG Supernate	6.3	-	0.36	2.3	-	0.03	0.16	-
10% PEG Pellet	1.0	0.51	0.63	0.63	1.24	1.1	1.1	2.3
18% PEG Pellet (Fraction B)	1.0	0.32	1.6	1.6	5.1	0.05	0.05	0.14

* Values 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.

and varied between 1.9 and 0.6 units. This represents an average yield of 42% and a 4 fold purification from the material pooled following the $(NH_4)_2SO_4$ solubilization on Celite.

When a crude mitochondrial fraction was obtained from fresh pork brains using the same methods as described for the rat brain tissue and lyophilized, it was found after subsequent extraction of the residue that the majority of the pyruvate carboxylase activity remained particulate. Sonification of the particulate fraction apparently solubilized the enzyme but in poor yield. If the pyruvate carboxylase of pig liver mitochondria is also very tightly bound then this may account for the comment by Scrutton (28) during the purification of the pig liver enzyme that the final yield of enzyme was low even though the initial yield of mitochondrial material was high.

Purity and Stability

Contaminating Enzymes

The various fractions in the purification procedure were assayed, as described in the materials and methods section, for the following enzyme activities in addition to pyruvate carboxylase; ATPase, citrate synthetase, lactic dehydrogenase, malic dehydrogenase, and acetyl-CoA deacylase. In Fraction A ATPase and citrate synthetase activities were not detectable. However, the activities of lactic and malic dehydrogenases were 0.3% and 2.5%, respectively, that of pyruvate carboxylase. Unfortunately, in Fraction A the activity of acetyl-CoA deacylase was often 5 times that of pyruvate carboxylase. Remarkably, polyetheylene glycol fractionation removed the majority of this serious contaminant. As shown in Table 1, in Fraction B the acetyl-CoA deacylase activity

averaged only 3.5% that of pyruvate carboxylase. Furthermore, the acetyl-CoA deacylase activity was much less stable than the pyruvate carboxylase activity under the conditions used for storage.

Electrophoresis

Disc gel electrophoresis of Fraction B material with a specific activity of 5.0 on a 4% polyacrylamide gel at pH 9.5 (73) revealed at least four distinct protein bands.

Stability on Storage

When Fraction B material with protein concentration of 0.32 mg/ml was stored at 4° the loss of enzyme activity over a period of time followed first order kinetics with a half-life of approximately 7 days and first order rate constant of 0.004 $hr.^{-1}$ (Figure 3). Protein concentration of Fraction B ranged from 0.11 to 0.39 mg/ml and averaged 0.24 mg/ml. If the protein concentration is much below 0.1 mg/ml the stability of pyruvate carboxylase is greatly reduced (25, 32).

pH Profile

The activity of rat liver pyruvate carboxylase at various pH's and in several buffers is shown in Figure 4. The pH optimum for this enzyme lies between 8.0 and 8.5.

Density Gradient Centrifugation

A zonal centrifugation in a 5 to 20% linear sucrose density gradient was carried out on Fraction A as outlined by Martin and Ames (74). The 4.5 ml gradient was 50 mM in Tris-Cl, pH 7.4, and 0.5 mM in EDTA

Figure 3. Stability of Pyruvate Carboxylase in Fraction B. Rat brain pyruvate carboxylase was purified through Phase III (Fraction B), with a protein concentration of 0.32 mg/ml, and assayed after storage at 4° for the times indicated.

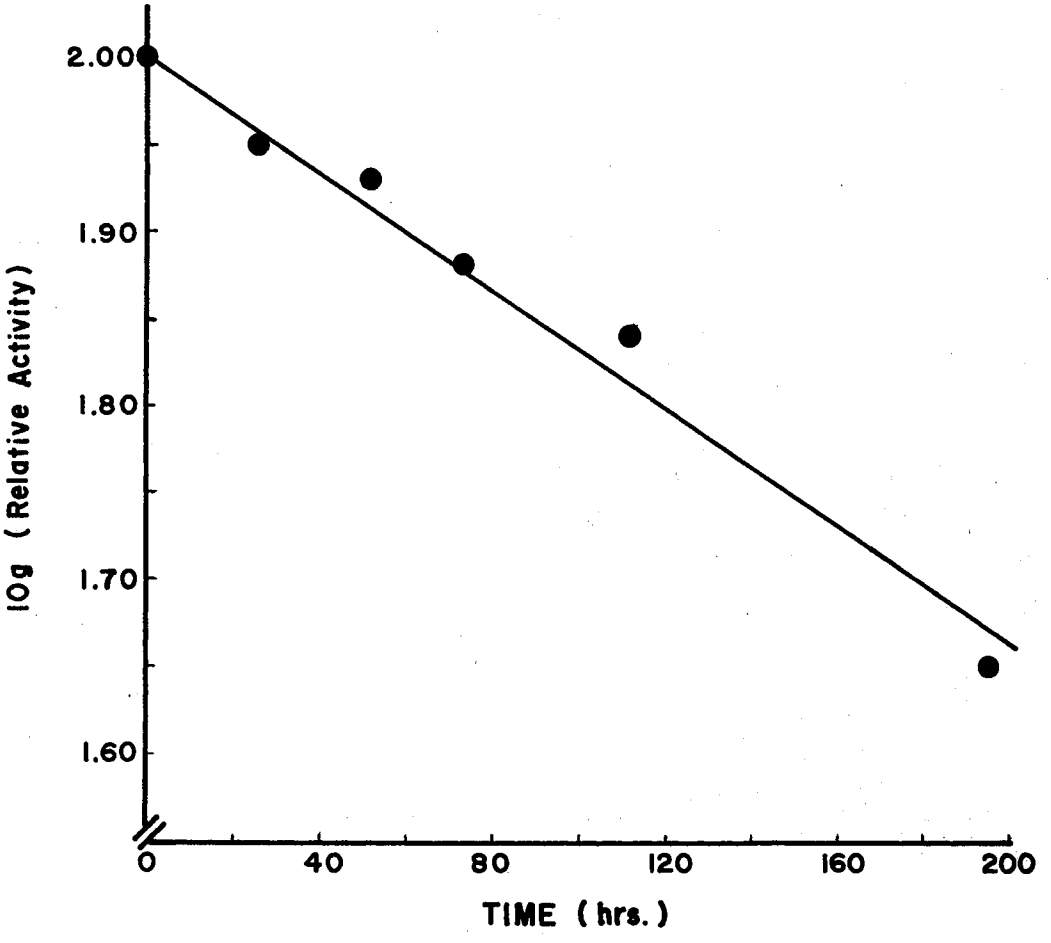
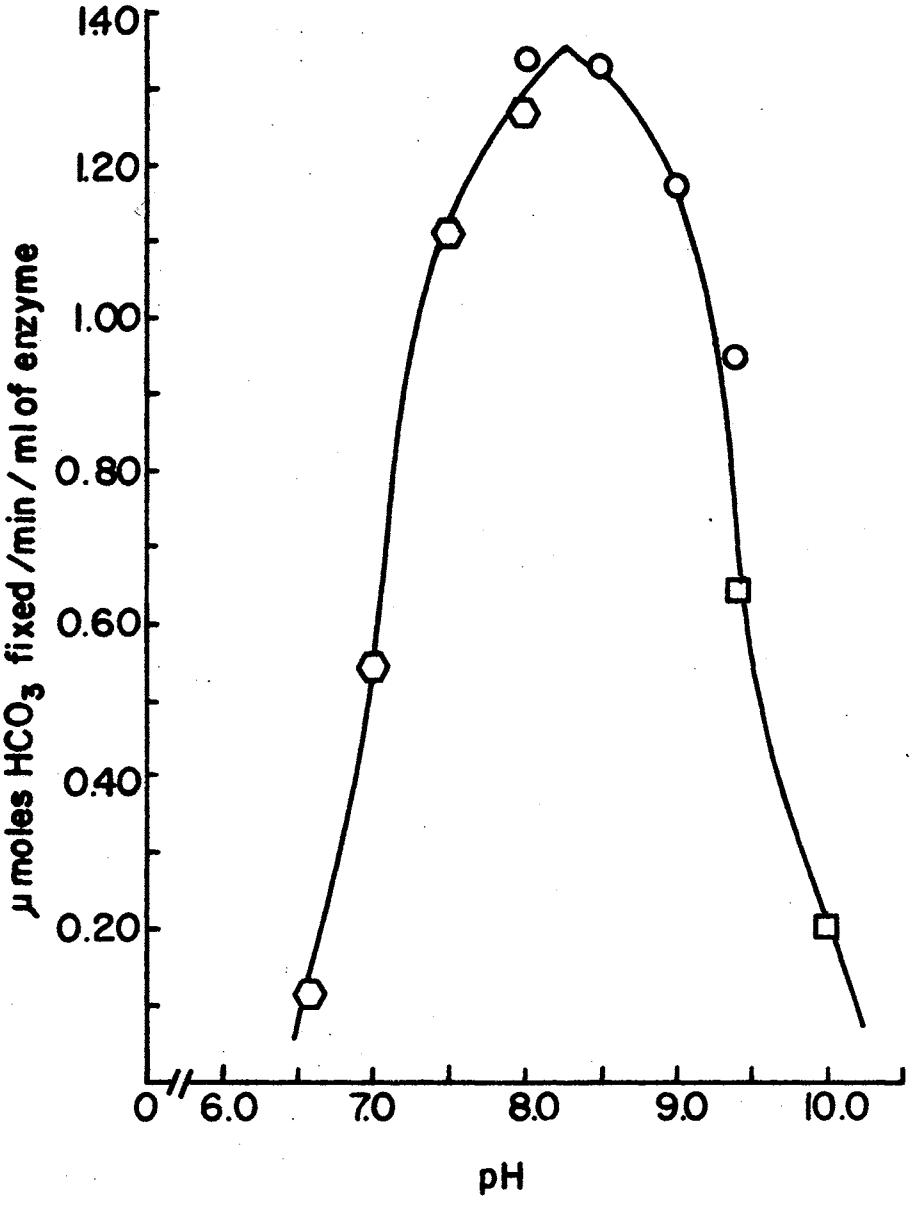

2.00
1.90
1.80
1.70
1.60
log (Relative Activity)
0
40
80
120
160
200
TIME (hrs.)

Figure 4. Effect of pH on Pyruvate Carboxylase Activity. Pyruvate carboxylase activity was measured, at the pH indicated, in 100 mM Hepes (⬡—⬡), Tris (o—o), or Caps (□—□) buffer.


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0
μ moles HCO_3 fixed /min / ml of enzyme
6.0
7.0
8.0
9.0
10.0
pH

and dithriothreitol. The gradient loaded with Fraction A material and the marker enzymes alcohol dehydrogenase and catalase was centrifuged 7 hours at 45,000 rpm in a Beckman Spinco SW 65L rotor maintained at 15°. The gradient was separated into approximately thirty fractions following centrifugation. An activity profile of the enzyme activities measured is shown in Figure 5. Using alcohol dehydrogenase and catalase as standards the average $S°_{20,w}$ values calculated for acetyl-CoA deacylase and pyruvate carboxylase were 8.0 and 14.8, respectively (74).

Inhibition Studies

Avidin

One unit of avidin, that amount which will bind 1 ug of biotin, completely inhibited 0.02 units of pyruvate carboxylase in the standard 1.0 ml assay. However, prior incubation of avidin with 25 ug of biotin in the standard assay completely prevented the avidin inhibition.

Phenylpyruvate

Phenylpyruvate is a noncompetitive inhibitor of pyruvate carboxylase as shown in Figure 6. In this experiment the pyruvate concentration was varied at several fixed concentrations of phenylpyruvate, all other substrates and activators being maintained at saturating conditions. The K_{is} for phenylpyruvate was determined to be 2.3 mM while the K_{ii} was 13.4 mM. The K_a for pyruvate in this experiment was 0.14 mM.

Unfortunately, the commercial malic dehydrogenase preparation rapidly reduced phenylpyruvate with the concurrent production of NAD^+. To obtain the data shown in Figure 6 it was necessary to subtract a high background from the initial rate data.

Figure 5. Enzyme Activity Profile of Fraction A Material Following Density Gradient Centrifugation. Fraction A was centrifuged, as described in text, after addition of alcohol dehydrogenase and catalase as standards. Enzyme activities are expressed in ΔA_{230}/0.66 min/0.01 ml for catalase (◇—◇), ΔA_{340}/27 min/0.05 ml for pyruvate carboxylase (○—○), ΔA_{340}/3 min/0.02 ml for alcohol dehydrogenase (o—o), and ΔA_{412}/3 min/0.02 ml for acetyl-CoA deacylase (△—△).

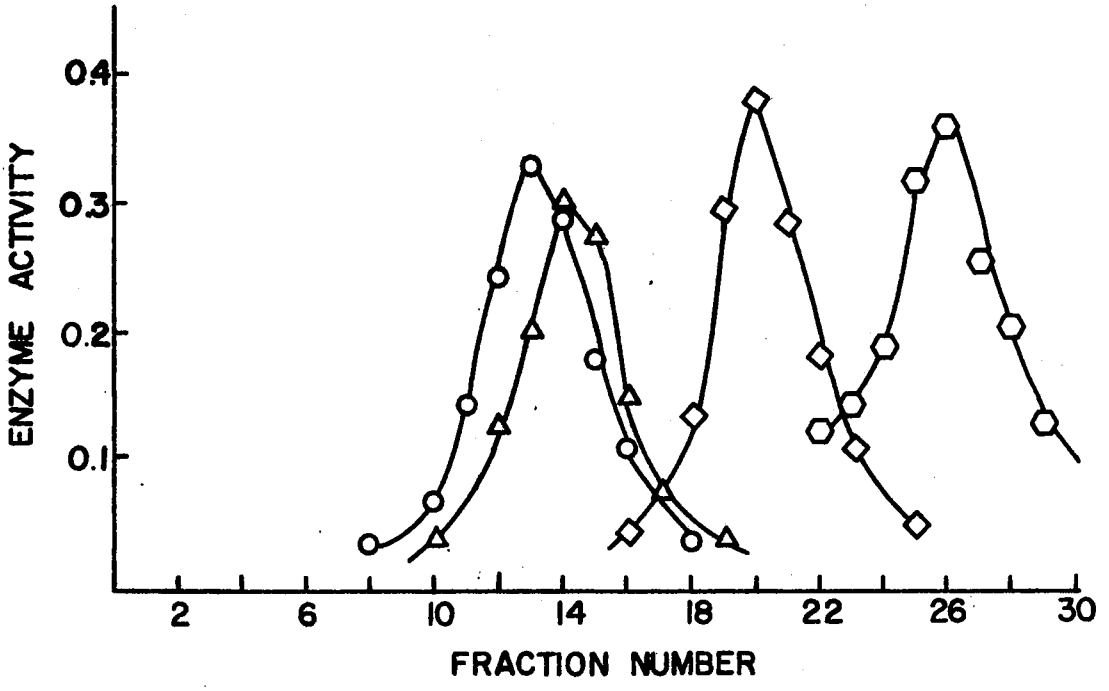
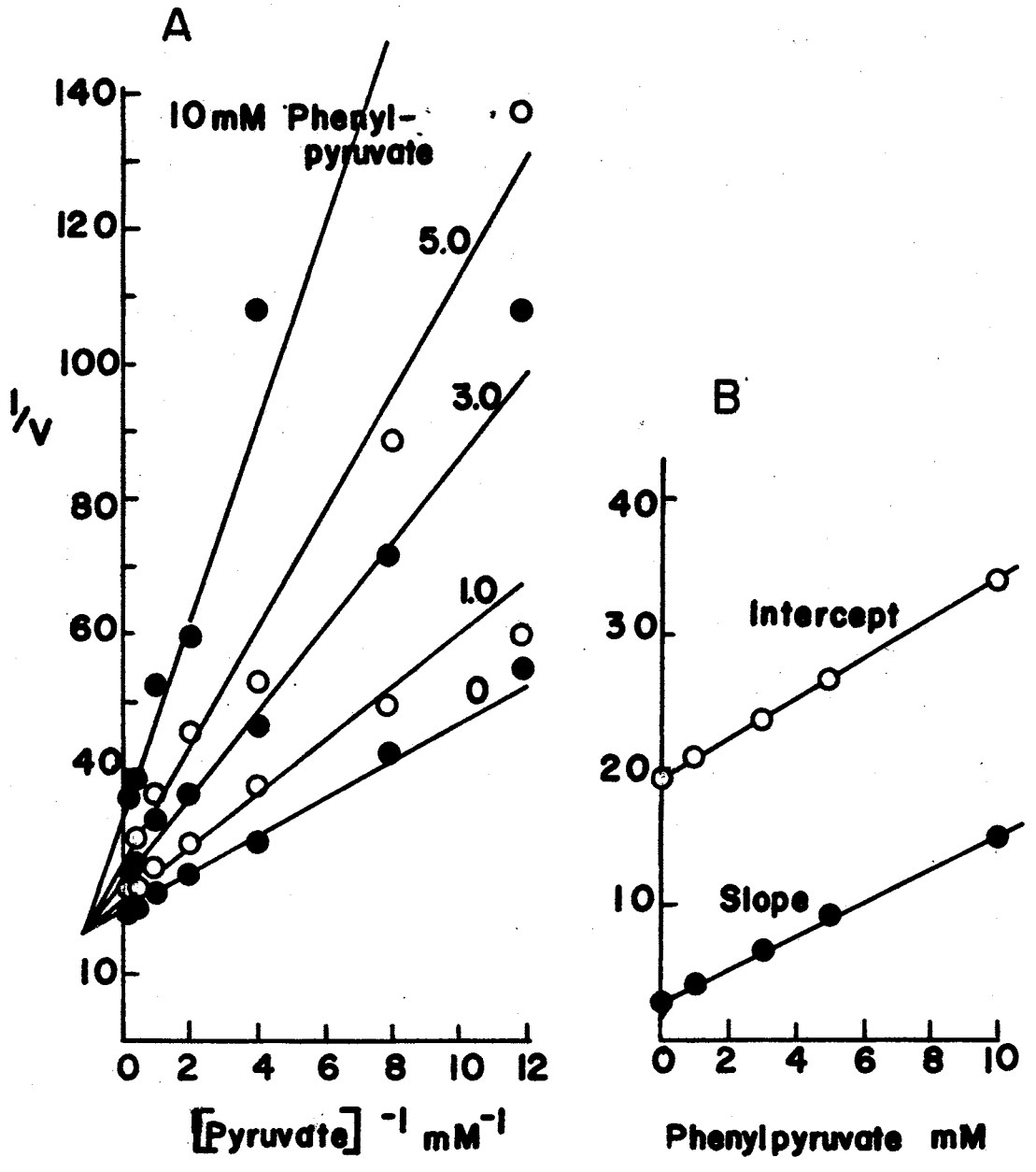

ENZYME ACTIVITY
0.4
0.3
0.2
0.1
2
6
10
14
18
22
26
30
FRACTION NUMBER

Figure 6. Phenylpyruvate Inhibition of Rat Brain Pyruvate Carboxylase

A, reciprocal velocities are plotted as a function of pyruvate concentrations at fixed concentrations of phenylpyruvate indicated.
B, secondary plot of slopes (●—●) and intercepts (o—o).

The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles: phenylpyruvate, as indicated; pyruvate, as indicated; ATP, 1.0; $KHCO_3$, 30; $MgSO_4$, 6.0; acetyl-CoA, 0.15; NADH, 0.16; Triethanolamine-Cl, 70; and 5 units of malic dehydrogenase.


A
140
120
100
80
60
40
10
1/v
10 mM Phenyl-
pyruvate
5.0
3.0
1.0
0
0 2 4 6 8 10 12
[Pyruvate] -1 mM-1
B
40
30
20
10
Intercept
Slope
0 2 4 6 8 10
Phenylpyruvate mM

$SO_4^=$ Inhibition

The sulfate ion is a competitive inhibitor of chicken liver pyruvate carboxylase with respect to acetyl-CoA (68). However, sulfate concentrations up to 25 mM did not inhibit rat brain pyruvate carboxylase at any concentration of acetyl-CoA between 6 and 190 uM.

Acetoacetyl-CoA Studies

Acetoacetyl-CoA activated rat brain pyruvate carboxylase slightly in the presence of subsaturating levels of acetyl-CoA, Figure 7A. Acetoacetyl-CoA at a concentration of 290 uM activated rat brain pyruvate carboxylase to approximately 50% of the level attained by the same saturating concentration of acetyl-CoA. Also, as is shown in Figure 7B, the acetoacetyl-CoA kinetics were very sigmodial with a Hill coefficient of 2.9. An apparent K_a of activation for acetoacetyl-CoA has been estimated from these data of 150 uM. This corresponds to a Hill coefficient of 2.0 and an apparent K_a of activation of 17 uM for acetyl-CoA.

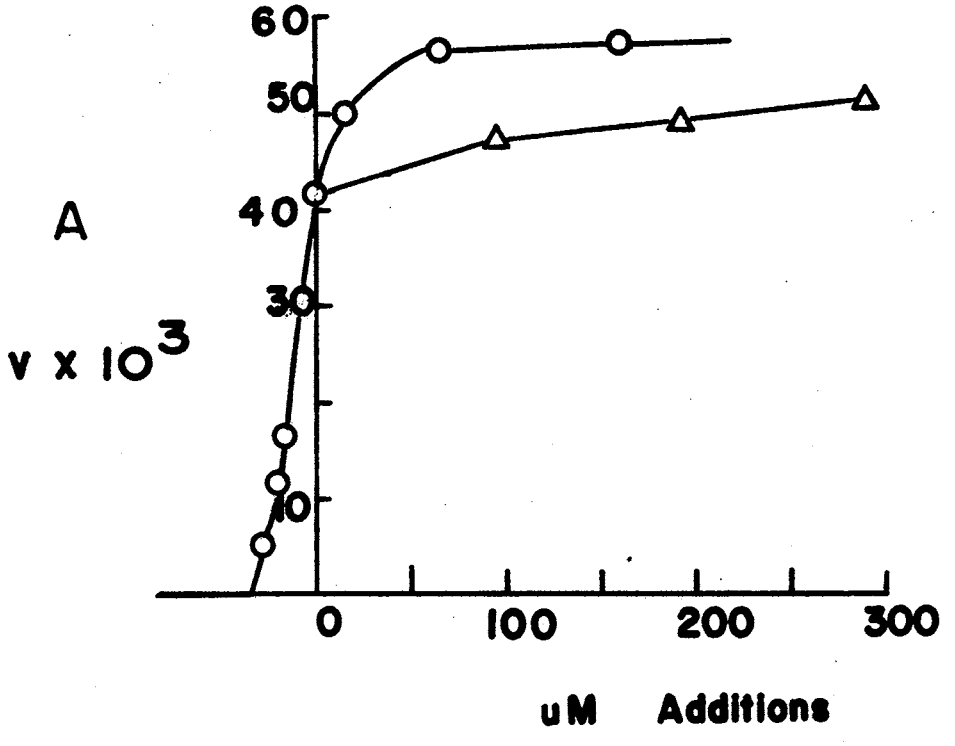
Kinetic Studies

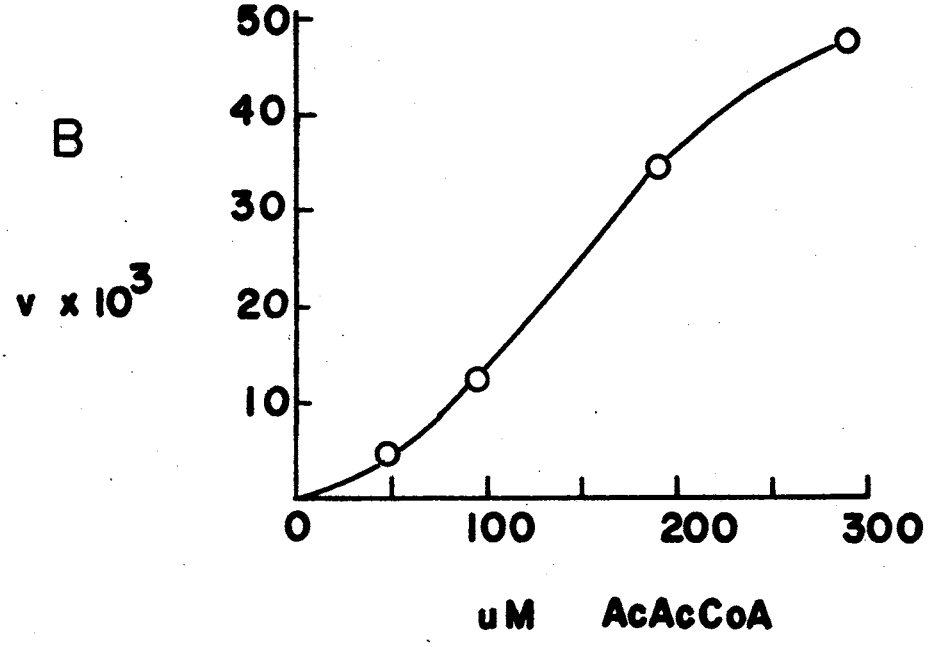
The kinetic parameters of the reaction catalyzed by rat brain pyruvate carboxylase have not been previously examined in detail. However, a great volume of material is available in the literature concerning chicken liver pyruvate carboxylase kinetics (22, 28, 39, 75). Recently McClure *et al*. (32, 40, 48, 49) have published detailed kinetics of the rat liver enzyme. The kinetics presented here are modeled after these studies in anticipation of comparing the results.

Figure 7. Acyl-CoA Activation of Rat Brain Pyruvate Carboxylase

A, influence of acetoacetyl-CoA on the activation by acetyl-CoA, circles indicate control curve with acetyl-CoA, and triangles additions of acetoacetyl-CoA to the basic system containing 28 uM acetyl-CoA. The abscissa scale for acetyl-CoA concentration is identical with that for the additions but originates 28 uM to the left. B, activation of rat brain pyruvate carboxylase by acetoacetyl-CoA alone.

The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles: pyruvate, 5.0; ATP, 1.0; $KHCO_3$, 30.0; $MgSO_4$, 8.0; acyl-Coa derivatives, as indicated; Tris-Cl, 100; NADH, 0.16 mM; and 8 units of malic dehydrogenase.


A
v x 10^3
60
50
40
30
10
0
100
200
300
uM Additions


B
v x 10^3
50
40
30
20
10
0
100
200
300
uM AcAcCoA

K^+ Activation

Monovalent cations activate pyruvate carboxylase from several sources, and generally, K^+ is the most effective activator (28, 32). The relationship of K^+ activation to the overall reaction was studied by varying each substrate at fixed concentrations of KCl while the remaining substrates and activators were at saturating concentrations. The concentrations of the non-varied substrates and activators are indicated in the legend of each figure in the illustrations to follow. When the kinetics of cation activation were investigated the malic dehydrogenase preparation used was dialyzed against the appropriate reaction buffer and the salts of the basic medium were altered appropriately. The values of the various kinetic parameters for K^+ activation obtained are listed in Table II where K_a and K_{ia} are, respectively, the Michaelis and inhibition constants for the indicated substrate, and $K_k{}^+$ and $K_{ik}{}^+$ are the activator and inhibition constants for K^+. These are apparent constants because they have not been extrapolated to infinite concentrations of the other substrates and activators.

When pyruvate is the varied substrate in a kinetic experiment involving pyruvate carboxylase the double reciprocal plots are nonlinear (32, 76, 77). Such as biphasic plot is shown in Figure 8A. The lines drawn through the experimental points approximate the true asymptotes described by a higher than first order equation. Following previous convention pyruvate I refers to the low (< 0.2 mM) concentration range of this substrate and pyruvate II refers to the high (≥ 0.2 mM) concentration range (32). The slopes and intercepts of Figure 8A yield linear replots as a function of KCl concentration in both regions.

TABLE II

APPARENT MILLIMOLAR KINETIC CONSTANTS RELATED TO K^+ ACTIVATION

A. Constants related to the substrate or activator indicated

Varied Substrate	K_a	K_{ia}	K_k^+	K_{ik}^+
HCO_3^-	3.38	8.09	0.93	2.22
Pyruvate I (< 0.2 mM)	0.10	0.05	2.69	1.29
Pyruvate II (> 0.2 mM)	0.12	0.28	2.01	4.78
Acetyl-CoA	0.02	--	1.5	5.0

B. Constants with $MgATP^{-2}$ as the variable substrate

KCl Range	K_{mgATP}	K_{imgATP}	K_k^+	K_{ik}^+
KCl I (< 3.0 mM)	0.07	0.24	0.60	3.43
KCl II (> 3.0 mM)	0.07	0.18	1.83	4.66

In Figure 8B the data are plotted as a function of KCl concentration at fixed concentrations of pyruvate. In the secondary plot of Figure 8B, Figure 8C, it is evident that the slope function undergoes a very distinct change while the intercept function remains almost linear.

When KCl was the varied substrate at fixed levels of $MgATP^{-2}$ the double reciprocal plot was unexpectedly biphasic as shown in Figure 9A. The convention used in Table IIB is that KCl I and KCl II refer respectively, to the low (< 3.0 mM) and high (> 3.0 mM) concentration ranges of KCl. It is evident from examination of Table IIB and Figure 9B that the kinetic parameters for $MgATP^{-2}$ have not changed in the low and high ranges. Here the lines of the double reciprocal plot with $[MgATP^{-2}]^{-1}$ as the variable substrate intersect even though the data pertaining to each range of KCl were analyzed separately. The data in the first two examples examined thus far fit a sequential equation, Equation 2.2 and were analyzed accordingly.

The kinetics of K^+ activation when HCO_3^- or acetyl-CoA was the variable substrate were analyzed according to Equation 1.1. The kinetics of K^+ activation with HCO_3^- as the variable substrate are shown in Figure 10A and the secondary plot of the slope and intercept functions in Figure 10B. The kinetics of K^+ activation with acetyl-CoA as the fixed substrate are irregular in that the double reciprocal plot, Figure 11A, is linear, but the secondary plot of the slope and intercepts, Figure 11B, is parabolic. The kinetic parameters K_{ik}^+ and K_k^+ were determined in the manner as described by McClure, Lardy, and Kniefel (32).

Mg^{++} Activation

Pyruvate carboxylase also requires a divalent cation for total

Figure 8. Effect of Pyruvate on the Activation of Pyruvate Carboxylase by K^+.

A, reciprocal velocities are plotted as a function of the pyruvate concentration at the fixed concentrations of KCl indicated.
B, reciprocal velocities are plotted as a function of KCl concentrations at the fixed concentrations of pyruvate indicated.
C, secondary plots of the slopes (●—●) and intercepts (o—o) of B.

The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles: pyruvate, as indicated; ATP, 0.8; HCO_3^-, 28; $MgSO_4$, 6.0; KCl, as indicated; acetyl-CoA, 0.13; Tris-Cl, 100; NADH, 0.16; and 6 units of malic dehydrogenase.

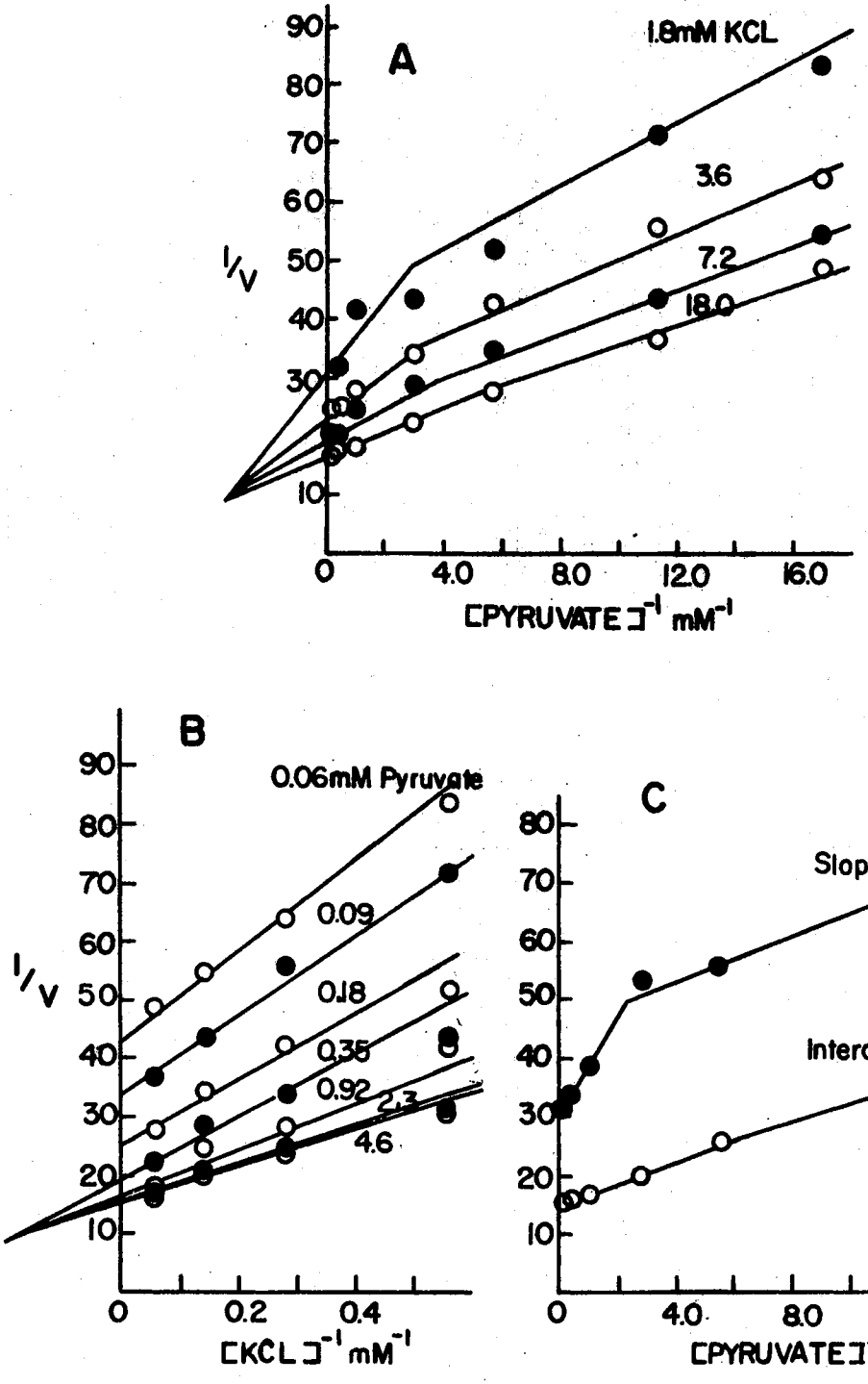

A
1.8mM KCL
3.6
7.2
18.0
1/V
[PYRUVATE]⁻¹ mM⁻¹
B
0.06mM Pyruvate
0.09
0.18
0.35
0.92
2.3
4.6
1/V
[KCL]⁻¹ mM⁻¹
C
Slope
Intercept
[PYRUVATE]⁻¹mM⁻¹

Figure 9. 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 of MgATP indicated.
B, reciprocal velocities are plotted as a function of the MgATP concentration at the fixed concentrations of KCl indicated.
Inset, secondary plot of slopes (●—●) and intercepts (o—o) of B.

The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles: pyruvate, 4.1; MgATP, as indicated; HCO_3^-, 28; $MgSO_4$, 6.0 above MgATP indicated; KCl, as indicated; acetyl-CoA, 0.13; Tris-Cl, 100; NADH, 0.16; and 6 units of malic dehydrogenase.

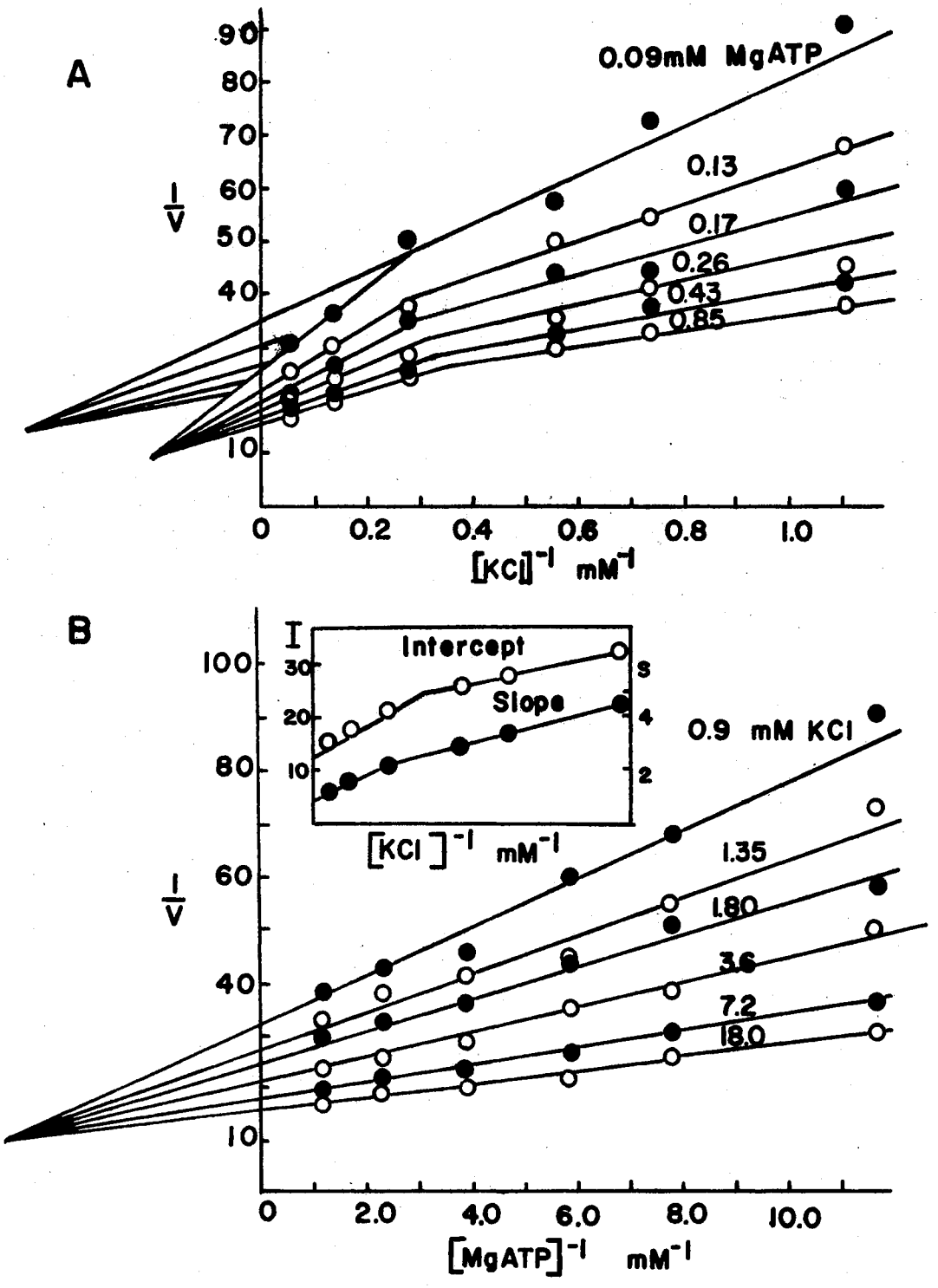

A
0.09mM MgATP
0.13
0.17
0.26
0.43
0.85
1/V
90
80
70
60
50
40
10
0
0.2
0.4
0.6
0.8
1.0
[KCl]⁻¹ mM⁻¹
B
Intercept
Slope
I
30
20
10
S
4
2
[KCl]⁻¹ mM⁻¹
0.9 mM KCl
1.35
1.80
3.6
7.2
18.0
1/V
100
80
60
40
10
0
2.0
4.0
6.0
8.0
10.0
[MgATP]⁻¹ mM⁻¹

Figure 10. Effect of HCO_3^- on the Activation of Pyruvate Carboxylase by K^+.

A, reciprocal velocities are plotted as a function of the HCO_3^- concentrations at the fixed concentrations of KCl indicated.
B, secondary plot of slopes (●—●) and intercepts (o—o) of A.

The 1.0 ml reaction mixtures contained in micromoles: pyruvate, 7.1; ATP, 2.0; HCO_3^-, as indicated; $MgSO_4$, 8.0; acetyl-CoA, 0.14; KCl, as indicated; Tris-Cl, 100; NADH, 0.16; and 6 units of malic dehydrogenase.

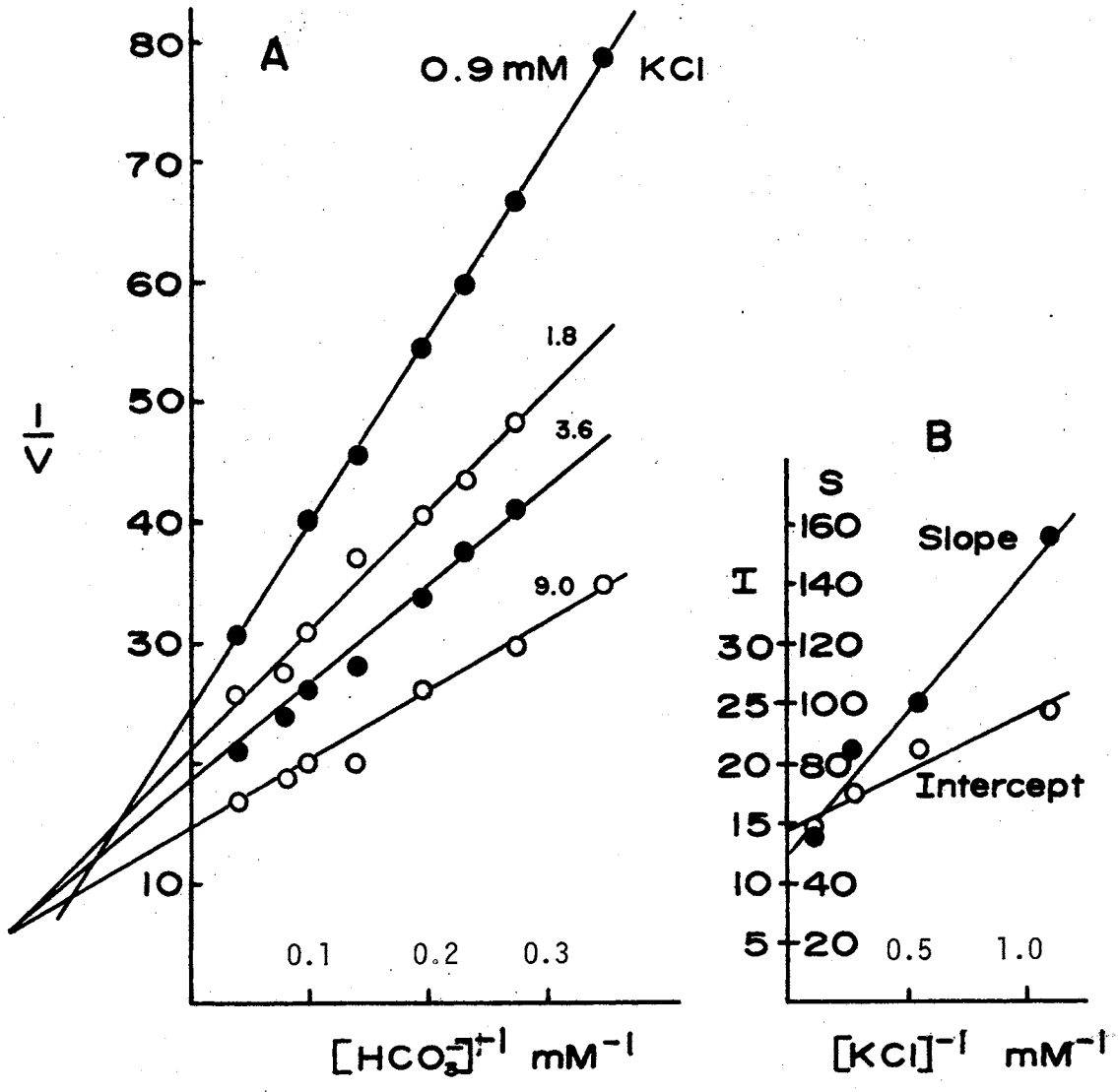
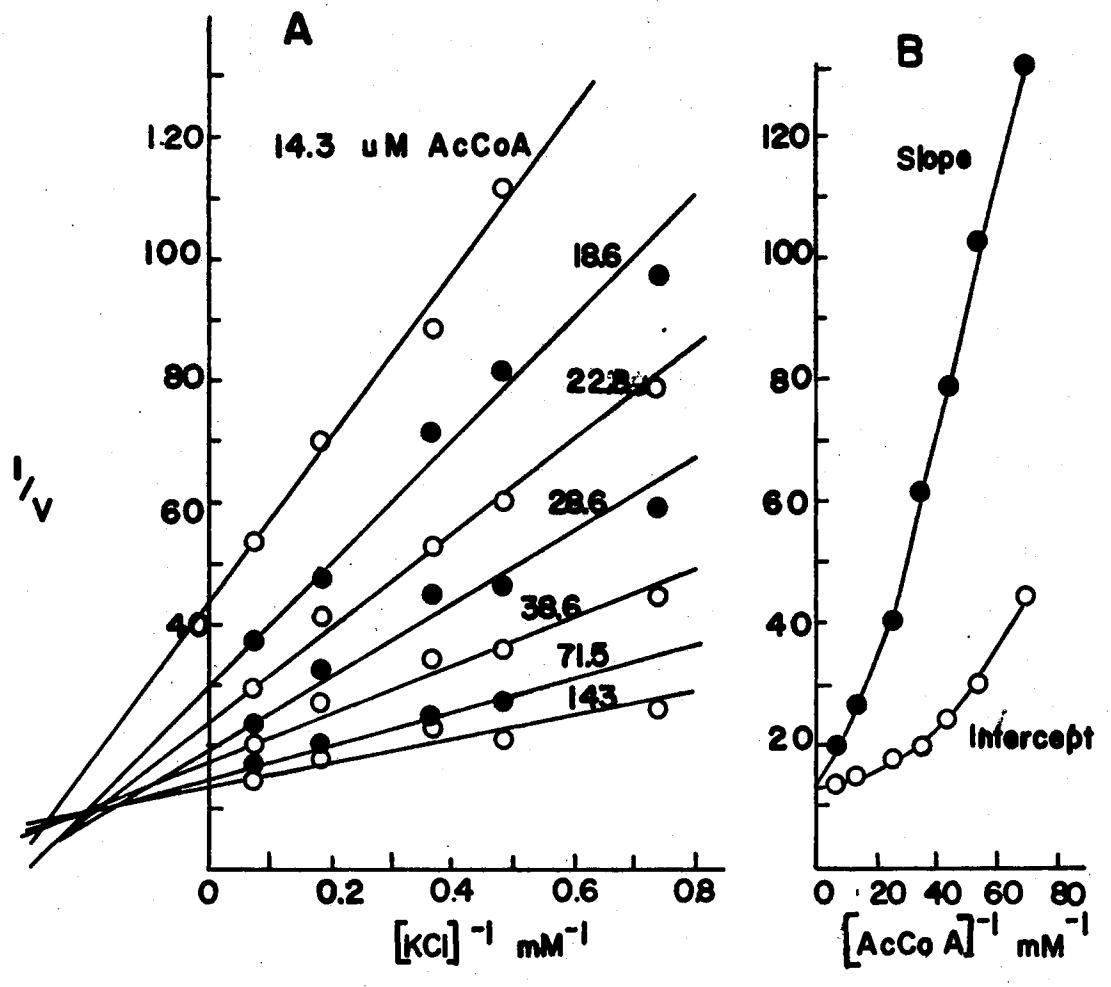

A
0.9 mM KCl
1.8
3.6
9.0
1/V
80
70
60
50
40
30
10
0.1
0.2
0.3
[HCO3-]-1 mM-1
B
S
I
Slope
Intercept
160
140
120
100
80
40
20
30
25
20
15
10
5
0.5
1.0
[KCl]-1 mM-1

Figure 11. Effect of Acetyl-CoA and K^+ on the Activation of Pyruvate Carboxylase.

A, reciprocal velocities are plotted as a function of the KCl concentration at fixed concentrations of acetyl-CoA indicated.
B, secondary plots of slopes (●—●) and intercepts (o—o) of A.

The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles: pyruvate, 7.4; ATP, 2.0; HCO_3^-, 25; $MgSO_4$, 9.0; KCl, as indicated; Tris-Cl, 100; acetyl-CoA, as indicated; NADH, 0.16; and 6.5 units of malic dehydrogenase.


A
14.3 uM AcCoA
18.6
22.8
28.6
38.6
71.5
143
1/V
120
100
80
60
40
0
0.2
0.4
0.6
0.8
[KCl]⁻¹ mM⁻¹
B
Slope
Intercept
120
100
80
60
40
20
0
20
40
60
80
[AcCo A]⁻¹ mM⁻¹

activation, Mg^{++} being the most effective (22). The $MgATP^{-2}$ chelate is the actual substrate for the enzyme. However, there is an additional requirement for Mg^{++}, above that necessary to form the chelate, for total activation of the enzyme (22, 32). The apparent kinetic parameters for Mg^{++} activation are shown in Table III, the notation used being the same as those in the previous tables.

The relationship between $MgATP^{-2}$ as a substrate and Mg^{++} as an activator is shown in Figures 12A and 12B. In this experiment Mg^{++} was varied at fixed concentrations of ATP^{-4}. The concentration of free Mg^{++} was calculated as outlined by Blair (78) taking into account complex formation between ATP^{-4}, Mg^{++}, and the monovalent cations Na^{+} and K^{+}. The formation constants used were 70,000 M^{-1} for $MgATP^{-2}$ and 15 M^{-1} for both Na^{+} and K^{+} complexes of ATP^{-4} (79). Because the $MgATP^{-2}$ concentration is a function of the concentration of Mg^{++}, the concentration of $MgATP^{-2}$ shown as the fixed substrate in Figure 12A actually varied slightly over the concentration range of Mg^{++} used. However, because this variation was insignificant when viewed as a fraction of the total ATP^{-4} it was ignored. These data were analyzed and fitted to the equilibrium ordered equation, Equation 2.3.

The double reciprocal plot obtained when the Mg^{++} concentration was varied at fixed concentrations of HCO_3^- was biphasic, as shown in Figure 13A. Biphasic kinetics with these substrates have not been reported previously. In Table IIIB, $MgSO_4$ I and $MgSO_4$ II refer, respectively, to the low (< 0.4 mM) and high (> 0.4 mM) range of Mg^{++} concentrations. The biphasic kinetics observed when pyruvate was varied at fixed concentrations of Mg^{++} are shown in Figure 14A. The data for both biphasic situations were analyzed and fitted to Equation 2.2.

TABLE III

APPARENT MILLIMOLAR KINETIC CONSTANTS
RELATED TO Mg^{++} ACTIVATION

A. Constants related to the substrate indicated

Varied Substrate	K_a	K_{ia}	$K_{mg^{++}}$	$K_{img^{++}}$
MgATP	0.05	∞	0	3.32
Pyruvate I (< 0.2 mM)	0.09	0.02	0.29	0.07
Pyruvate II (> 0.2 mM)	0.09	0.16	0.20	0.37

B. Constants with HCO_3^- as the variable substrate

$MgSO_4$ Range	$K_{HCO_3^-}$	$K_{iHCO_3^-}$	$K_{mg^{++}}$	$K_{img^{++}}$
$MgSO_4$ I (< 0.4 mM)	4.78	3.86	0.14	0.11
$MgSO_4$ II (> 0.4 mM)	3.46	5.03	0.29	0.40

Figure 12. Effect of MgATP on the Activation of Pyruvate Carboxylase by Mg^{++}.

A, reciprocal velocities as a function of the free Mg^{++} concentration at the fixed concentrations of MgATP indicated.
B, secondary plot of slopes (●—●) and intercepts (o—o) of A.

The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles: pyruvate, 9.2; $KHCO_3$, 20; $MgSO_4$, as indicated; Triethanolamine-Cl, 100; and 5.6 units of malic dehydrogenase.

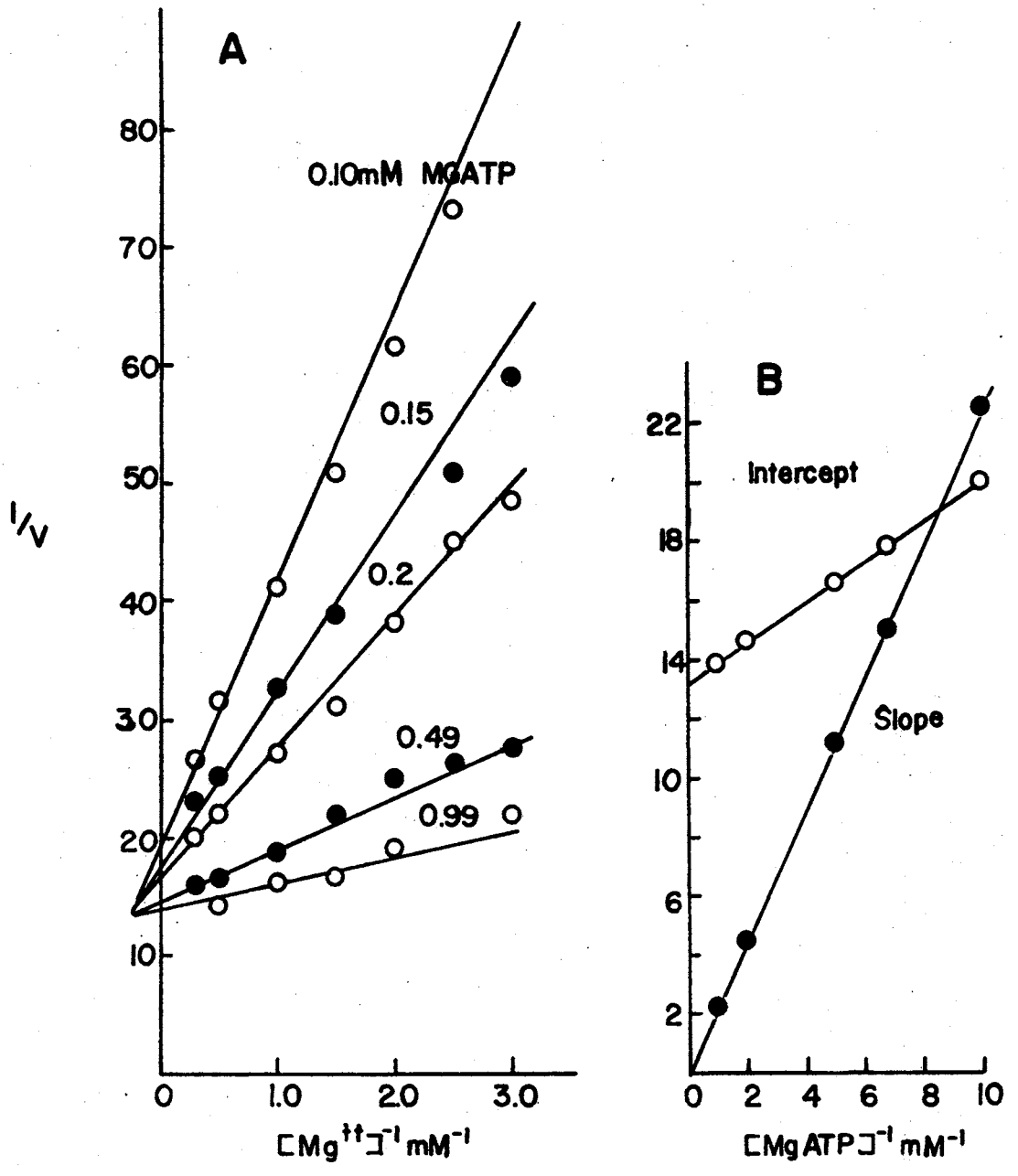

A
0.10mM MGATP
0.15
0.2
0.49
0.99
1/V
80
70
60
50
40
30
20
10
0
1.0
2.0
3.0
[Mg++]-1 mM-1
B
Intercept
Slope
22
18
14
10
6
2
0
2
4
6
8
10
[MgATP]-1 mM-1

Figure 13. Effect of HCO_3^- as the Activation of Pyruvate Carboxylase by Mg^{++}.

A, reciprocal velocities as a function of the free Mg^{++} concentration at fixed concentrations of $KHCO_3$.
B, reciprocal velocities as a function of the $KHCO_3$ concentration at fixed concentrations of free Mg^{++}.
C, secondary plot of slopes (●—●) and intercepts (o—o) of B.

Total K^+ was maintained at 28.3 mM by varying KCl. The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles pyruvate, 4.6; MgATP, 0.8; $KHCO_3$, as indicated; acetyl-CoA, 0.12; $MgSO_4$, as indicated; Triethanolamine-Cl, 100; NADH, 0.16; and 5.4 units of malic dehydrogenase.

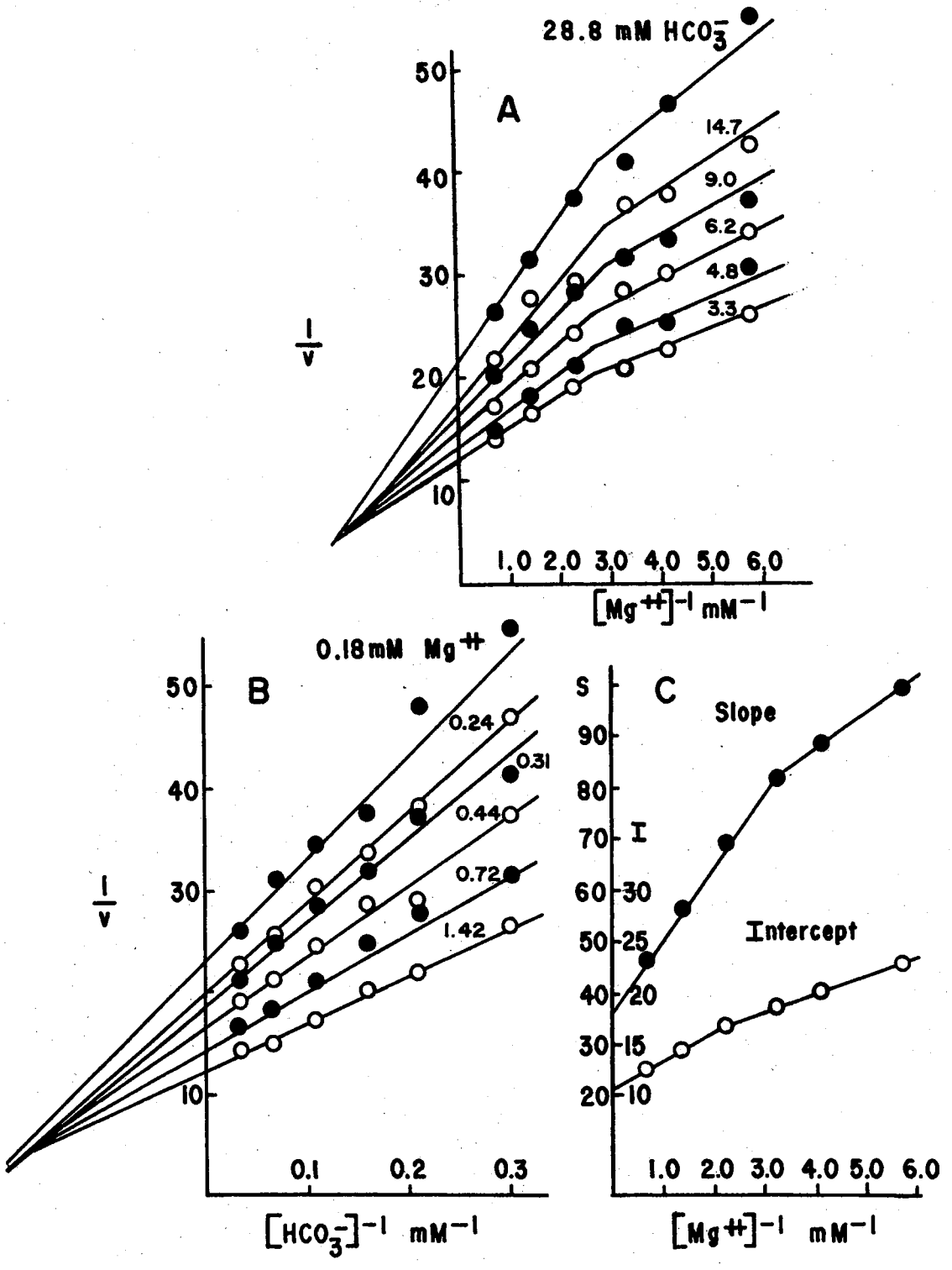
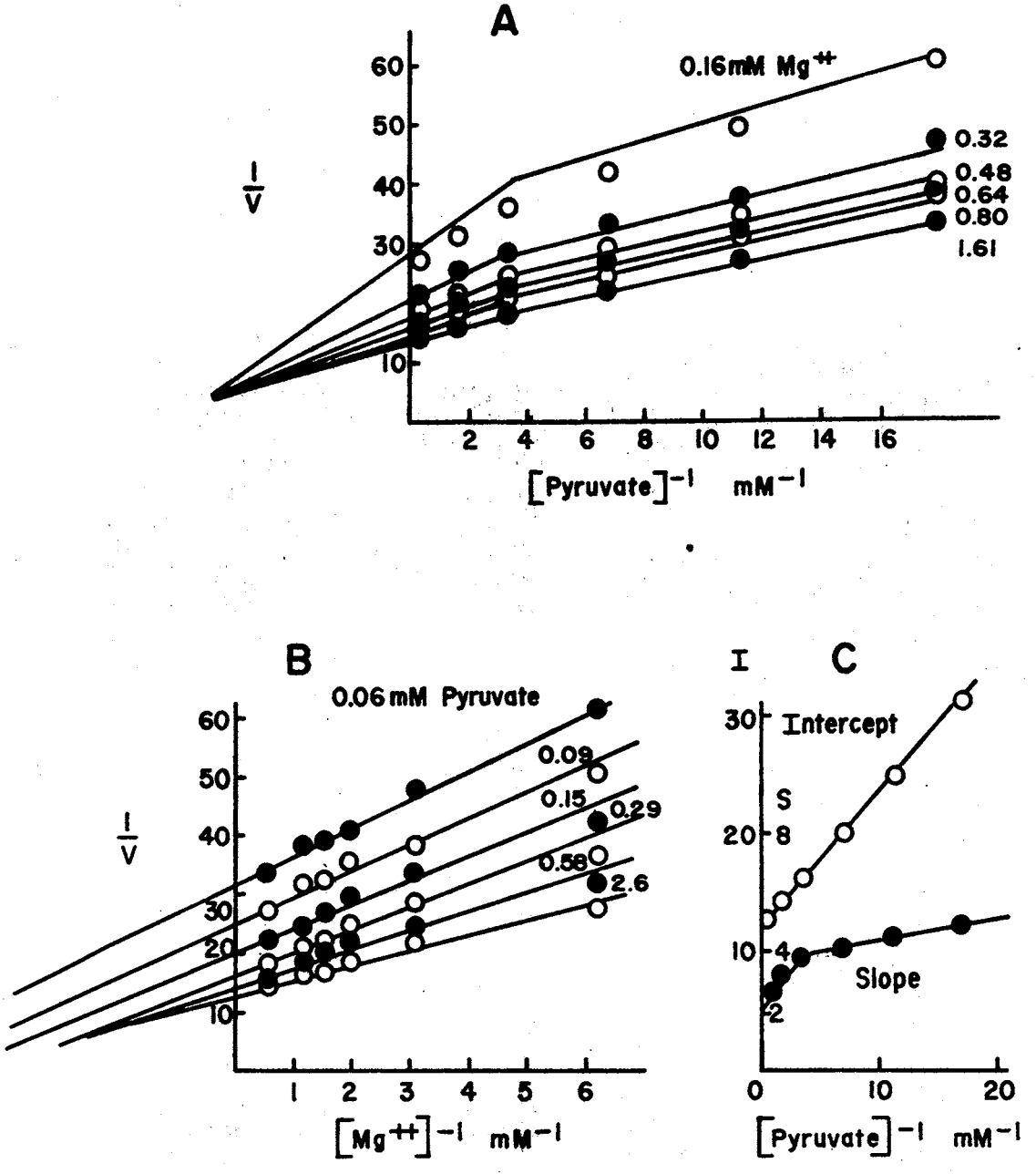

28.8 mM HCO3-
A
14.7
9.0
6.2
4.8
3.3
1/v
50
40
30
20
10
1.0 2.0 3.0 4.0 5.0 6.0
[Mg++]-1 mM-1
0.18mM Mg++
B
0.24
0.31
0.44
0.72
1.42
1/v
50
40
30
10
0.1 0.2 0.3
[HCO3-]-1 mM-1
C
Slope
Intercept
S
I
90
80
70
60
50
40
30
20
30
25
20
15
10
1.0 2.0 3.0 4.0 5.0 6.0
[Mg++]-1 mM-1

Figure 14. Effect of Pyruvate on the Activation of Pyruvate Carboxylase by Mg^{++}.

A, reciprocal velocities are plotted as a function of the pyruvate concentration at fixed levels of free Mg^{++}. B, reciprocal velocities are plotted as a function of the free Mg^{++} at fixed concentrations of pyruvate. C, secondary plots of slopes (●—●) and intercepts (o—o) of B.

The 1.0 ml reaction mixture contained in micromoles: pyruvate, as indicated; MgATP, 0.8; $KHCO_3$, 30; $MgSO_4$ as indicated; acetyl-CoA, 0.15; Triethanolamine-Cl, 100; NADH, 0.16; and 6.4 units of malic dehydrogenase.


A
0.16mM Mg++
0.32
0.48
0.64
0.80
1.61
1/V
60
50
40
30
10
2 4 6 8 10 12 14 16
[Pyruvate]-1 mM-1
B
0.06 mM Pyruvate
0.09
0.15
0.29
0.58
2.6
1/V
60
50
40
30
20
10
1 2 3 4 5 6
[Mg++]-1 mM-1
I C
30
Intercept
S
20
8
10
4
Slope
2
0 10 20
[Pyruvate]-1 mM-1

Substrate Kinetics

When 1/v was plotted against $[MgATP]^{-1}$ at different fixed concentrations of HCO_3^- an intersecting pattern was observed which corresponds to the sequential addition of two substrates. Accordingly, the data were fitted to Equation 2.2. The apparent kinetic constants so determined are presented in the legend of Figure 15. At high $MgATP^{-2}$ concentrations a slight amount of substrate inhibition is apparent.

The double reciprocal plot obtained when pyruvate was varied at fixed concentrations of $MgATP^{-2}$ was, as expected, biphasic and conformed to previous patterns. As seen in Figure 16A, in the low range of pyruvate, the lines are almost parallel, but at high levels of pyruvate they intersect. This pattern is similar to the one observed with the rat liver enzyme (40). The kinetic data were fitted to Equation 2.2 and the kinetic constants are presented in Table IV.

When 1/v versus $[MgATP^{-2}]^{-1}$ was plotted at fixed concentrations of acetyl-CoA, figure 17A, the lines were linear and fit Equation 1.1. However, the secondary plots of the slopes and intercepts were parabolic (Figure 16B). This type of pattern is similar to that obtained in experiments using the rat (33) and chicken liver (38, 68) enzyme. Actually the apparent K_a for acetyl-CoA, which is approximately 0.01 mM, was found to vary only slightly over the range of $MgATP^{-2}$ concentrations used.

Figure 15. Initial Velocity Patterns for Pyruvate Carboxylase with MgATP and $KCHO_3$ as Variable Substrates.

A, reciprocal velocities are plotted as a function of the MgATP concentration at fixed concentrations of $KHCO_3$ indicated.
B, reciprocal velocities are plotted as a function of the $KHCO_3$ concentrations at fixed concentrations of MgATP indicated.
Insets, secondary plots of slopes (●—●) and intercepts (o—o) of the corresponding primary plot.

The total K^+ concentration was maintained at 30 mM by varying KCl. The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles: pyruvate, 5.5; MgATP, as indicated; $KHCO_3$, as indicated; $MgSO_4$, 6.0 above MgATP; acetyl-CoA, 0.12; Triethanolamine-Cl, 70, NADH, 0.16; and 6 units of malic dehydrogenase. Apparent kinetic constants from a fit to Equation 2.2 are; $K_{HCO_3^-}$, 2.0 mM; $K_{iHCO_3^-}$, 2.98 mM; K_{MgATP}, 0.075 mM; K_{iMgATP}, 0.11 mM.

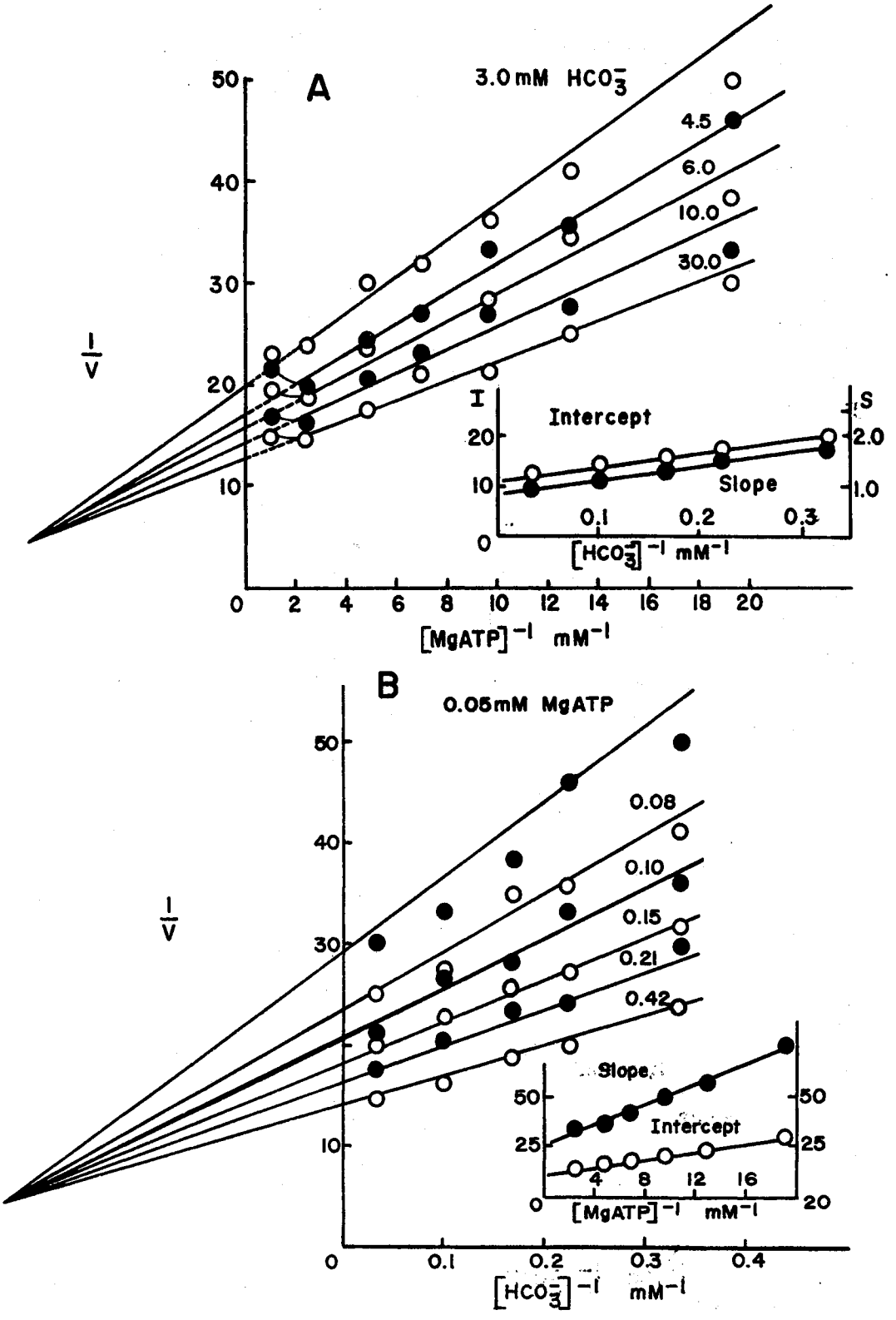

A
3.0 mM HCO_3^-
4.5
6.0
10.0
30.0
$\frac{1}{V}$
$[MgATP]^{-1}$ mM^{-1}
Intercept
Slope
I
S
$[HCO_3^-]^{-1}$ mM^{-1}
B
0.05 mM MgATP
0.08
0.10
0.15
0.21
0.42
Slope
Intercept
$[MgATP]^{-1}$ mM^{-1}
$[HCO_3^-]^{-1}$ mM^{-1}

Figure 16. 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 at 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 8.0) contained in micromoles: pyruvate, as indicated; MgATP, as indicated; $MgSO_4$, 6.0 above MgATP; acetyl-CoA, 0.12; Triethanolamine Cl, 70; NADH, 0.16; and 6.8 units of malic dehydrogenase

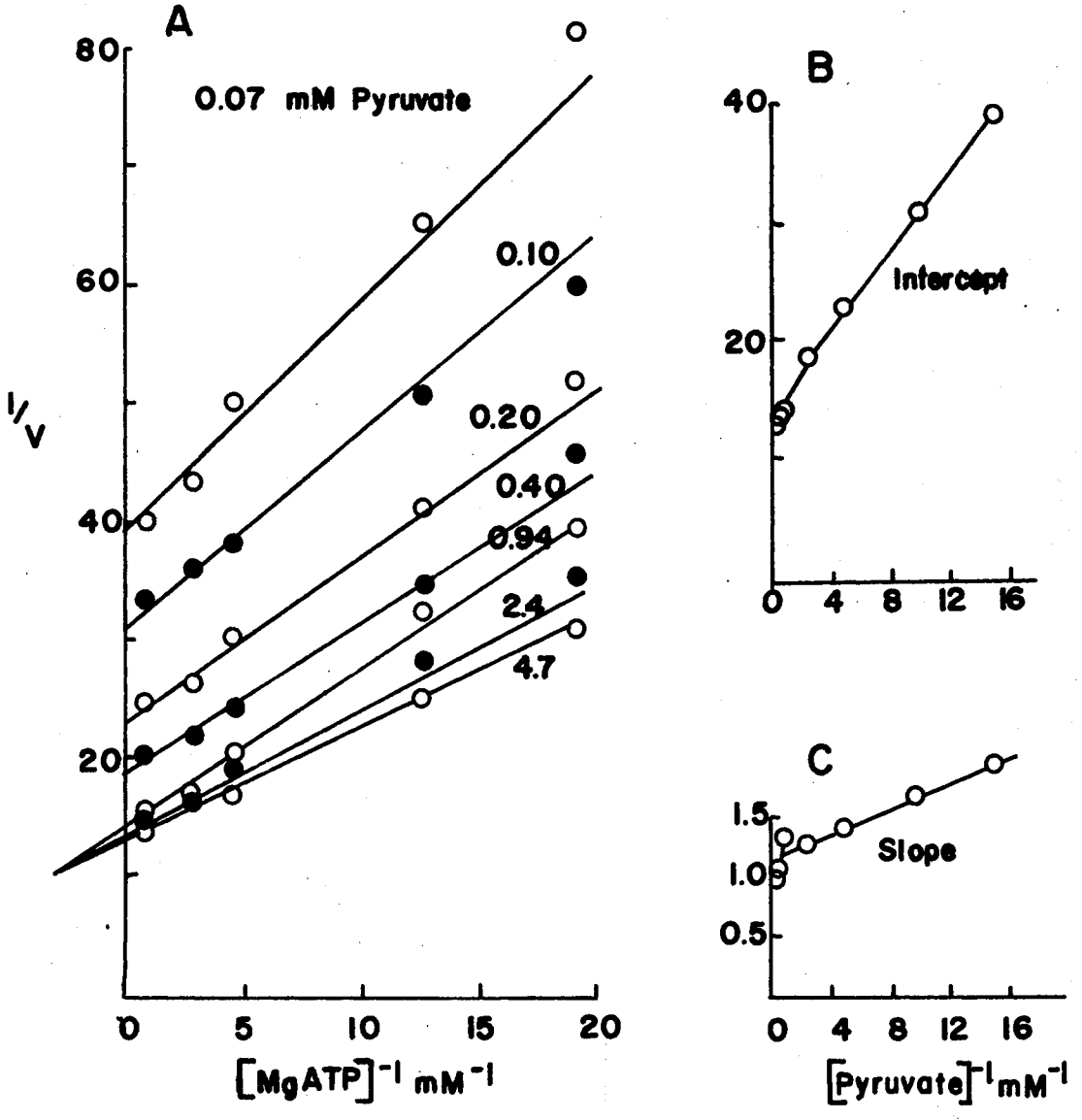

A
0.07 mM Pyruvate
0.10
0.20
0.40
0.94
2.4
4.7
1/V
80
60
40
20
0
5
10
15
20
[MgATP]⁻¹ mM⁻¹
B
Intercept
40
20
0
4
8
12
16
C
Slope
1.5
1.0
0.5
0
4
8
12
16
[Pyruvate]⁻¹mM⁻¹

TABLE IV

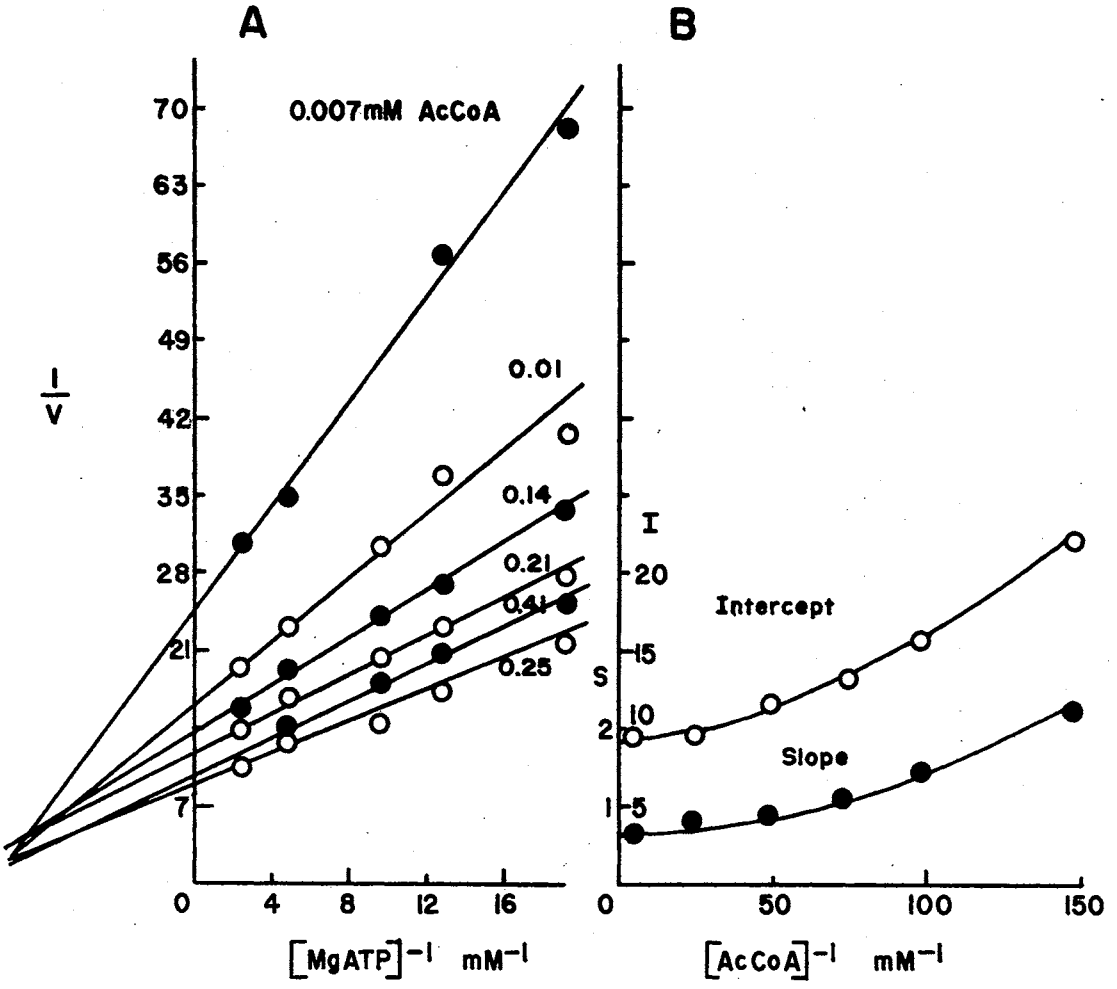
APPARENT MILLIMOLAR KINETIC CONSTANTS FOR
RAT BRAIN PYRUVATE CARBOXYLASE

Constant	Pyruvate I	Pyruvate II
K_{MgATP}	0.08	0.07
K_{iMgATP}	0.03	0.34
$K_{pyruvate}$	0.11	0.10
$K_{ipyruvate}$	0.05	0.52

Figure 17. Effect of MgATP on Activation of Pyruvate Carboxylase by Acetyl-CoA.

A, reciprocal velocities plotted as a function of MgATP concentration at the fixed concentrations of acetyl-CoA indicated.
B, secondary plot of slope (●—●) and intercepts (o—o) of A.

The 1.0 ml reaction mixtures (pH 8.0) contained in micromoles: pyruvate, 5.0; $KHCO_3^-$, 30; MgATP, as indicated; $MgSO_4$, 6.0; acetyl-CoA, as indicated; Triethanolamine-Cl, 70; NADH, 0.16; and 7.7 units of malic dehydrogenase.


A
B
0.007mM AcCoA
0.01
0.14
0.21
0.41
0.25
1/V
70
63
56
49
42
35
28
21
7
0
4
8
12
16
[MgATP]⁻¹ mM⁻¹
I
20
15
10
5
S
2
1
Intercept
Slope
0
50
100
150
[AcCoA]⁻¹ mM⁻¹

CHAPTER IV

DISCUSSION

Purification

Previous purification schemes for chicken (30, 68) and rat liver (32) pyruvate carboxylase were unsuccessful when used to isolate the small amount of enzyme present in rat brain tissue. Considering the catalytic capacity of rat brain tissue (Chapter III) and assuming a maximal specific activity of pyruvate carboxylase of 25 units per mg of protein, the brains of 32 rats (30g) would have an estimated 250 to 400 ug of pyruvate carboxylase protein (32). Most procedures for the purification of this enzyme use kilogram quantities of starting material containing thousands of units of enzyme. Purification steps such as DEAE ion exchange chromatography greatly diluted the rat brain enzyme, to the point of instability, even though the experiments were carried out on a micro scale. Therefore, a new procedure for the purification of rat brain pyruvate carboxylase has been developed. Though simple, it has resulted in significant purification, permitting the preliminary characterization of this enzyme.

The Clark and Nicklas (52) isolation procedure provides mitochondria in good yield when slightly modified for this study (Chapter III). The mitochondrial fraction obtained by this procedure is reported to come from the glial cell bodies and comparable to fraction E of the De Robertis procedure (44). The Celite solubilization step used in the

purification of rat brain pyruvate carboxylase was found to be very reliable and consistent. The usefulness of this technique was suggested by the purification scheme of Seufert et al. (33) in which rat liver pyruvate carboxylase was brought to near homogeneity by extensive and repetitive $(NH_4)_2SO_4$ fractionation alone. The employment of polyethylene glycol to separate pyruvate carboxylase and acetyl-CoA deacylase activities indicates the usefulness and versatility of this simple technique, one that is often forgotten or overlooked during protein purification procedures.

Assuming a maximal specific activity similar to that of the rat liver enzyme, 25 units per mg of protein, the rat brain pyruvate carboxylase preparation reported here is approximately 20% pure (32). In view of the heterogeneous electrophoretic pattern of our enzyme preparation (Fraction B), there is no reason to believe that the theoretical maximum specific activity of rat brain pyruvate carboxylase is not in the range of 25 units per mg of protein. However, in making such comparisons it is necessary to consider carefully the often large discrepancies in the determination of protein concentration due to choice of method and arbitrary standards. The high specific activity, reported for the rat liver enzyme by Seufert et al. (33), of 40 units per mg of protein is probably because of the differences in their assay temperature and method of protein determination from those of McClure et al. (32).

General Properties

Following the first $(NH_4)_2SO_4$ fractionation of chicken liver (30) and sheep kidney (34) pyruvate carboxylase, the enzymes are cold labile.

Neither the rat brain (Chapter III) nor liver enzyme is cold labile (32). Winhurst and Manchester (80) have reported rat liver pyruvate carboxylase to be cold labile. However, their enzyme rapidly lost activity under all conditions, probably due to the lack of a thiol reducing agent, such as dithiothreitol, in their buffers.

Rat liver and sheep kidney pyruvate carboxylase catalyze a measurable acyl-CoA independent carboxylation that has been well characterized respectively by Scrutton and White (81), and Ashman et al. (82). Rat brain pyruvate carboxylase catalyzes an acyl-CoA independent carboxylation of pyruvate but the extent of this carboxylation varied considerably between preparations and was never well characterized, being always less than 5% of the acetyl-CoA dependent carboxylation.

The broad pH optima of rat brain pyruvate carboxylase, between 8.0 and 8.5, is similar to the optima of the enzyme from other sources; 8.3 for the yeast enzyme (83), 7.8-8.0 for the chicken liver enzyme (74), 8.0-8.4 for the sheep kidney enzyme (34), and 8.0-8.5 for the rat liver enzyme (32, 33).

As determined by density gradient centrifugation rat brain pyruvate carboxylase has a $S^{\circ}_{20,w}$ of 14.8, close to the value of 14.5 reported for the rat liver enzyme using the same technique (32). The values obtained by analytical ultracentrifugation of the rat (32) and chicken liver (30) enzyme are 15.1 and 14.8, respectively. The subunits of rat liver pyruvate carboxylase have a molecular weight of 130,000 as determined by electrophorsis on 5% acrylamide gels at pH 7.1 in 1% sodium dodecyl sulfate (32). All pyruvate carboxylases examined thus far are large enzymes with molecular weights slightly greater than 500,000, the exception being the enzyme from Pseudomonas citronellolis which has a

molecular weight of approximately 270,000 (22). Data reported here indicate that rat brain pyruvate carboxylase has a molecular weight of approximately 500,000.

All pyruvate carboxylases contain biotin, one molecule covalently bound per subunit, and are inhibited by avidin, a protein which binds biotin almost irreversibly. Rat brain pyruvate carboxylase is no exception; it is rapidly and totally inhibited by avidin (Chapter III). Interestingly, avidin does not inhibit CO_2 fixation in intact rat brain mitochondria (45), indicating that the rat brain enzyme is probably located on the inner membrane of the mitochondria as is reported for the rat liver enzyme (32).

Noncompetitive inhibition of rat brain pyruvate carboxylase by phenylpyruvate was first demonstrated by Patel et al. (44, 45) and is confirmed here. A K_i of 0.48 mM for phenylpyruvate (84) has been reported for the chicken liver enzyme compared to 2.3 mM reported here. The difference between these values may not be significant because of the experimental difficulties we had in the kinetic studies with the rat brain enzyme (Chapter III).

Sulfate ion is a competitive inhibitor of chicken liver pyruvate carboxylase with respect to acetyl-CoA having a K_i between 1.0 and 1.3 mM (68); it is not an inhibitor of the rat liver (49) or rat brain (Chapter III) enzyme.

Acetoacetyl-CoA is a noncompetitive inhibitor of chicken liver pyruvate carboxylase with respect to all reaction components, including acetyl-CoA, with a K_i between 0.1 and 0.5 mM (28). B-hydroxybutyryl-CoA, the other component of this redox pair, is a weak activator of the chicken liver enzyme. A combination of inhibition and activation by

these acyl-CoA derivatives has been proposed as a possible control for this enzyme by the NADPH/$NADP^+$ ratio (28). However, acetoacetyl-CoA has a negligible effect on rat liver pyruvate carboxylase (49). The physiological significance of the activation of rat brain pyruvate carboxylase by acetoacetyl-CoA reported here is dubious considering the high K_a.

Activation of pyruvate carboxylases by acyl-CoA derivatives is characterized not only by the apparent K_a for activation but also by the Hill coefficient which indicates the degree of cooperativity in the activation. The Hill coefficient of 2.0 for rat brain pyruvate carboxylase for acetyl-CoA is similar to the values of 2.0 (32) and 2.1 (28) reported for the rat liver enzyme. The cooperativity of the avian liver enzyme is slightly greater than that of the mammalian liver enzyme, the Hill coefficient for chicken liver pyruvate carboxylase being 2.9 (21). The K_a for activation of pyruvate carboxylase is much lower for the avian liver enzyme, 2-3 uM, than for the mammalian liver enzymes, 20-30 uM (28). The value of 17 uM for the rat brain enzyme (Chapter III) approximates those obtained with the rat liver enzyme, 20 to 25 uM (32). As noted in many publications and confirmed here, acetyl-CoA activation is independent of the concentration of substrates and other activators, but is very sensitive to conditions such as pH and ionic strength (28, 32, 33, 49).

Kinetics

McClure et al. (32) were the first to extensively study the kinetics of monovalent cation activation of pyruvate carboxylase. The monovalent cation activation of chicken liver pyruvate carboxylase, the

most extensively studied enzyme, went unnoticed because this enzyme is the only known pyruvate carboxylase to be activated by the Tris cation, the acid component of the assay buffer most commonly used. Monovalent cations activate many enzymes which catalyze the phosphorylation of a carboxyl group or enolate anion or other reactions in which an enol-keto tautomer is an intermediate (85), K^+ activates the first partial reaction in the pyruvate carboxylase reaction, i.e., the carboxylation step (Equation 1.2).

If the kinetic terms K_a and K_{ia} are respectively, the concentration of substrate resulting in half maximal activity at saturating K^+, and the concentration of substrate required to half saturate the enzyme as the concentration of K^+ approaches zero, the relative magnitude of these two parameters as well as the complimentary constants, $K_k{}^+$ and $K_{ik}{}^+$ indicate how the binding of a substrate affects the binding of K^+ and vice versa (32). The ratio K_{ia}/K_a is illustrated in reciprocal plots of initial velocity against substrate concentration at changing fixed concentrations of substrates as activator (Figures 8 thru 17). If the family of lines thus produced intersect above the abscissa then the K_{ia} is greater than the K_a, the ratio of the two being greater than one. If the K_a is greater than the K_{ia}, the lines intersect below the abscissa whereas intersection on the abscissa indicates a K_{ia}/K_a ratio of one. The farther above the abscissa that the family of lines intersects, indicating a larger K_{ia}/K_a ratio, the more effect the concentration of the changing fixed substrate or activator has on the binding of the varied substrate or activator. As shown in Table II, the K_{ia}/K_a ratio for every substrate except pyruvate I, is in excess of 2.3 as are the $K_{ik}{}^+/K_k{}^+$ ratios. Thus, K^+ greatly enhances the binding of each

substrate and vice versa, except in the low concentration range of pyruvate. This is in slight contrast to the K^+ activation of the rat liver enzyme where the monovalent cation has a greater effect on the binding of HCO_3^- than the binding of other substrates. The values reported here for K_{ia} and K_{k^+} are slightly lower than those reported by McClure et al. (32) for the rat liver enzyme. However, the magnitude of most of the kinetic constants of the rat liver and brain enzymes are similar.

Free Mg^{++} is a requirement for many $MgATP^{-2}$ dependent carboxylases being required for acetyl-CoA, proprionyl-CoA, and B-methylcrotonyl-CoA carboxylases in addition to pyruvate carboxylase (32). The zero intercept plot on the slope replot in Figure 12 indicates an equilibrium ordered addition of Mg^{++} followed by $MgATP^{-2}$. The value of zero reported for $K_{Mg^{++}}$ in this experiment means that $MgATP^{-2}$ effectively traps the metal on the enzyme. With the rat liver enzyme the results are similar to those reported here using pH 8.0 and 25° (32). However, at pH 7.3 and 37° the addition of Mg^{++} and $MgATP^{-2}$ to the rat liver enzyme is reversed, with $MgATP^{-2}$ adding in an equilibrium ordered manner followed by Mg^{++} (49).

Biphasic double reciprocal plots have been observed in kinetic experiments with pyruvate carboxylase using pyruvate as a variable substrate (28, 32). Scrutton (28) reported that the biphasic nature of pyruvate kinetics decreases with the chicken liver enzyme with increasing temperature, linearity being achieved at 44°. In contrast, McClure et al. (49) found biphasic plots with pyruvate in studies with the rat liver enzyme at pH 7.3 and 37°. Attempts have been made to explain the abrupt transitions in the Lineweaver-Burk plots in terms of negative

and positive cooperativity (79, 80). Also, Delvin et al. (86) have proposed that the biphasic plots of human liver pyruvate carboxylase are caused by the presence of two forms of the same enzyme with different K_a's for pyruvate.

The additional biphasic kinetics reported here (Tables IIB and IIIB), those with K^+ as the variable substrate at changing fixed cocentrations of $MgATP^{-2}$ and with Mg^{++} as the variable substrate at changing fixed concentrations of HCO_3^- further complicate matters. The importance of these new observations is not clear. However, some analogies can be made concerning all of the biphasic kinetics reported here. As indicated in Tables II thru IV and in the replots of the slopes and intercepts of the corresponding data, the apparent K_a for a particular substrate remains constant while the other kinetic constants undergo transition, the change being confined primarily to the corresponding inhibitor constants, i.e. K_{ia} and $K_{ik}+$. An exception is in the biphasic plot where K^+ is the variable substrate (Figure 8), the greatest change being in the K_{ia} and K_k+ values. In contrast, in the biphasic behavior of the kinetics of rat liver pyruvate carboxylase, the change in kinetic constants is confined primarily to the K_a for pyruvate. Also, in the low concentration range of the variable substrate exhibiting biphasic kinetics regardless of the source of the enzyme, the binding of the variable substrate is not affected by the concentration of the changing fixed substrate or activator and vice versa, as is indicated by the corresponding ratio of K_{ia}/K_a. This situation is reversed in the high concentration range of the variable substrate.

As shown in Figure 15, in contrast to the rat liver enzyme, with rat brain pyruvate carboxylase there is slight substrate inhibition by

$MgATP^{-2}$ even at 30 mM HCO_3^-. Rat liver pyruvate carboxylase exhibits substrate inhibition by $MgATP^{-2}$ at low levels of pyruvate (40). The unusual pattern in Figure 16A where the concentration of $MgATP^{-2}$ is varied at fixed concentrations of pyruvate, which theoretically should be a series of parallel lines if the addition of substrates and release of products conformed to a simple ping pong mechanism, indicates that pyruvate somehow affects the affinity of the enzyme for $MgATP^{-2}$ in addition to its action as a substrate.

CHAPTER V

SUMMARY AND CONCLUSION

Pyruvate carboxylase is present in rat brain in very small quantities, 0.5 units per gram of wet tissue. We have purified this rat brain enzyme 2,500 fold to approximately 20% of its probable maximal purity. The procedure involves isolation of purified mitochondria, lyophilization, and buffer extraction of the residue to solubilize the particulate enzyme. After centrifugation the supernate is fractionated with $(NH_4)_2SO_4$ employing a gradient solubilization on a Celite column. Finally the contaminant enzyme, acetyl-CoA deacylase, is removed by polyethylene glycol precipitation.

The rat brain enzyme is similar to pyruvate carboxylase from other tissues and species; its properties closely resemble those of rat liver pyruvate carboxylase. Rat brain pyruvate carboxylase is not cold labile, is irreversibly inactivated by avidin, has a pH optima of 8.0 to 8.5 and an apparent molecular weight of slightly greater than 500,000. Phenylpyruvate is a noncompetitive inhibitor of the enzyme, but $SO_4^{=}$ does not inhibit over a wide range of concentrations. Acetoacetyl-CoA is a weak activator with a K_a of approximately 150 uM.

Only subtle differences in certain kinetic parameters distinguish rat brain from rat liver pyruvate carboxylase. It is difficult to assess the importance of these differences, but the possibility exists that the enzymes from these two tissues may be isozymes. The following apparent

millimolar kinetic constants support the proposition that this enzyme catalyzes most of the fixation of CO_2 into oxalacetate in rat brain: $K_{pyruvate}$, 0.1; K_{MgATP}, 0.07; $K_{HCO_3^-}$, 2.0; K_{k^+}, 1-2; $K_{Mg^{++}}$, 0.2-0.3; $K_{acetyl\text{-}CoA}$, 0.017-0.025. Considering these kinetic constants and the *in vivo* concentrations of the substrates and activators of this enzyme in the rat brain it is probable that pyruvate and acetyl-CoA, 91.0 and 3.9 n moles per gram of tissue, respectively, limit the activity of this enzyme *in vivo* (87, 88).

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