Pyruvate Carboxylase from Chicken Liver

STEADY STATE KINETIC STUDIES INDICATE A "TWO-SITE" PING-PONG MECHANISM*

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SUMMARY

On the basis of initial velocity and product inhibition studies a nonclassical Ping Pong Bi Bi Uni Uni mechanism has been proposed for pyruvate carboxylase from chicken liver. The nonclassical feature of this mechanism is the proposal that each active site on the enzyme is composed of two separate and functionally distinct catalytic sites, i.e., a separate catalytic site exists for the reactants of each partial reaction. The two catalytic sites are presumably linked by the biotinyl residue which functions as the carboxyl carrier. The Bi Bi partial reaction, in which MgATP₂⁻, MgADP⁻, HCO₃⁻, and phosphate are the substrates, is proposed to utilize a rapid equilibrium random Bi Bi mechanism which includes the formation of two abortive complexes, E-HCO₃⁻-Pi and E-HCO₃⁻-MgADP⁻. A rate equation has been developed for the proposed mechanism by employing a combination of rapid equilibrium and steady state methodology. The proposed mechanism is directly analogous to the modified Ping-Pong Bi Bi mechanism previously described for the biotin-enzyme methylmalonyl-CoA transcarboxylase (D. B. Northrop (1969) J. Biol. Chem., 244, 5808).

Pyruvate carboxylase (pyruvate:CO₂ ligase (ADP), EC 6.4.1.1) purified from chicken liver catalyzes the following reaction:

Pyruvate + MgATP₂⁻ + HCO₃⁻ → E-biotin + MgADP⁻ + Pi + oxalacetate + acetyl-CoA, Mg²⁺, K⁺ (1)

This enzyme is visualized as a tetrameric molecule when examined in the electron microscope after negative staining (1) and contains biotin and tightly bound manganese in equimolar ratio at a level approximating 4 moles per mole of enzyme (2, 3). Besides the substrates listed in Reaction 1, optimal enzymatic activity requires the presence of Mg²⁺ in excess of that required for formation of the metal-nucleotide complex (4) and the presence of a univalent cation such as K⁺ or Tris⁺. In addition, the enzyme exhibits an absolute requirement for activation by an acyl-CoA (e.g. acetyl-CoA) (5).

A minimal reaction mechanism for pyruvate carboxylase from chicken liver has been proposed (Reactions 2 and 3) on the basis of isotope exchange studies and of the isolation of the carboxylated biotin-enzyme intermediate (6).

E-biotin + MgATP₂⁻ + HCO₃⁻ → E-biotin + oxalacetate (2)

According to the terminology of Cleland (7) the minimal mechanism represented in Reactions 2 and 3 is described as Ping Pong Bi Bi Uni Uni.

Recently, Northrop (8) and Northrop and Wood (9) have performed detailed kinetic studies on the related biotin-enzyme methylmalonyl-CoA transcarboxylase (methylmalonyl-CoA:oxalacetate transcarboxylase (EC 2.1.3.1)) which catalyzes carboxyl transfer between an acyl derivative of coenzyme A and an α-keto acid (Reactions 4 and 5).

E-biotin + methylmalonyl-CoA → E-biotin + propionyl-CoA (4)

It will be noted that pyruvate carboxylase and methylmalonyl-CoA transcarboxylase share a common partial reaction (Reactions 3 and 5). Although the initial velocity studies of the transcarboxylase reaction indicated conformity to the expected Ping Pong Bi Bi mechanism (8), anomalous results were obtained during studies of (a) the properties of the inhibition of the over-all reaction by products and dead end inhibitors (8) and (b) the specificity of inhibition of Reactions 4 and 5 (as measured by isotope exchange techniques) by various substrate analogs (9). These data were interpreted to suggest the operation of a modified Ping Pong Bi Bi mechanism in which separate sites exist on the enzyme for catalysis of the two partial reactions (Reactions 4 and 5). Northrop and Wood (9) suggested that the two separate sites are linked by a mobile biotinyl residue, which in this and other biotin-enzymes is mounted on a long flexible side chain (10).

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Comprehensive initial velocity and product inhibition studies are reported here for the acyl-CoA-dependent pyruvate carboxylase purified from chicken liver which are consistent with the proposal that catalysis by this enzyme is described by a nonclassical Ping Pong Bi Bi mechanism. Similar conclusions have been reached from recent studies of pyruvate carboxylase from rat liver by McClure et al. (11,12) although the evidence is somewhat different in nature. Steady state kinetic studies of the acyl-CoA-independent pyruvate carboxylase from Aspergillus niger (13) have also been reported.

A preliminary report of some of the data has appeared (14).

**EXPERIMENTAL PROCEDURE**

### Materials

Tris, ATP, ADP, NADH, and oxalacetate were obtained from Sigma; malate dehydrogenase, lactate dehydrogenase, glucose-6-P dehydrogenase, and hexokinase were purchased from Boehringer. HEPES was obtained from Calbiochem, enzyme A from P-L Biochemicals, acetic anhydride and pyruvic acid from Eastman, and 5,5'-dithiobis-(2-nitrobenzoic acid) from Aldrich.

Other chemicals used in these studies were reagent grade.

### Methods

The commercial preparations of malate dehydrogenase and lactate dehydrogenase were equilibrated with 0.01 M Tris-Cl, pH 7.8, on a Sephadex G-25 column, 20 x 1 cm. This procedure is necessary in order to remove SO$_4^{2-}$, which is a potent inhibitor of pyruvate carboxylase from chicken liver.

Acetyl-CoA was prepared from coenzyme A and acetic anhydride by the method of Simon and Schemin (15). The thiolester content of acetyl-CoA preparations was determined with citrate synthase. The release of CoA was estimated with 5,5'-dithiobis-(2-nitrobenzoic acid) (16).

Pyruvic acid was vacuum distilled and stored frozen as 0.1 M solutions adjusted to pH 6.5 with KOH. Pyruvate concentrations were determined with lactate dehydrogenase (17) and oxalacetate concentrations with malate dehydrogenase (18).

Protein was determined by the spectrophotometric method of Warburg and Christian (19). Concentrations of adenine nucleotide solutions were determined spectrophotometrically, assuming a molar absorptivity of 15,400 (20). The concentrations of free and complexed nucleotides were calculated by assuming that the apparent stability constants of MgATP$^-$ and MgADP$^-$ were 70,000 and 4,000 M$^{-1}$, respectively, at pH 7.8 (21). In assays of oxalacetate decarboxylation the free Mg$^{2+}$ was maintained at 5 mM and MgADP:ADP at 20. At these levels of Mg$^{2+}$, phosphate concentrations were limited to 50 mM or below, to avoid the formation of precipitates.

Pyruvate carboxylase was purified through Stage 5 and stored as described by Scrutton et al. (22), except that most preparations were further purified by chromatography on a DEAE-Sephadex A-50 column. Specific activities (determined as described in Reference 22) are reported as micromoles of product formed at 25°C per min per mg of protein. Pyruvate carboxylase preparations used in these studies varied in specific activity from 15 to 30 i.u. per mg.

The initial rate of CO$_2$ fixation by pyruvate carboxylase was measured by estimating the rate of oxalacetate production in the presence of malate dehydrogenase and NADH (5). When oxalacetate was added as a product inhibitor the initial rate of the reaction was estimated from the increase in absorbance at 290 nm due to oxalacetate production. Where appropriate the observed rate was corrected for breakdown of added oxalacetate by measuring the change in absorbance in the absence of acetyl-CoA. When HCO$_3^-$ was the variable substrate all solutions were freshly prepared from water which had been boiled for 30 min and then cooled and stored under N$_2$. The actual concentration of HCO$_3^-$ in the cuvette was taken as the sum of the concentration of added HCO$_3^-$ plus the measured endogenous HCO$_3^-$ concentration. The endogenous HCO$_3^-$ concentration (always <0.4 mM) in the assay mix was calculated with the equation $A = (o) K_a/V$, where $K_a$ and $V$ are apparent constants estimated graphically with velocities at high concentrations of added HCO$_3^-$ and $o$ is the velocity measured in the absence of added HCO$_3^-$. The initial rate of oxalacetate decarboxylation by pyruvate carboxylase was measured by estimating the rate of pyruvate production in the presence of lactate dehydrogenase and NADH (2). The rates were corrected for the decarboxylation of oxalacetate which was not due to catalysis of the over-all reaction by measuring the rate of pyruvate production in a system lacking acetyl-CoA. When pyruvate was added as product inhibitor the initial rate of oxalacetate decarboxylation was determined by measuring ATP production in the presence of hexokinase, glucose, glucose 6-phosphate dehydrogenase, and NADP (2).

In all cases specific reaction conditions for each experiment are given in the appropriate figure legend. After equilibration of the assay systems to 25°C, the reactions were initiated by addition either of pyruvate carboxylase (CO$_2$ fixation) or of pyruvate carboxylase + Mg$^{2+}$ (oxalacetate decarboxylation).

The change in absorbance at the appropriate wave length (290 or 340 nm) was recorded continuously with Gilford spectrophotometers, model 2000 or 240, each of which was equipped with a Radiometer constant temperature apparatus maintained at 25°C. Full scale deflections in the range 0.1 to 1.0 absorbance unit were used. The chart speed was adjusted to give slopes of approximately 40".

**Data Analysis**—The statistical analysis of the kinetic data was based on the procedures developed by Cleland (23). Reciprocal velocities were plotted graphically against the reciprocals of substrate concentrations. When these plots were linear, the data were fitted to Equation 6.

$$v = \frac{V S}{K_a + S}$$

Least squares fits were performed on an IBM 1620 digital computer using FORTRAN computer programs obtained from W. W. Cleland of the University of Wisconsin. These programs provide values for the constants in the fitted equation, standard errors of their estimates, and weighting factors for further analysis. The form of the over-all rate equation was determined from secondary plots in which slopes ($K_a/V$) and intercepts ($1/V$) obtained from Equation 6 were plotted against either the inhibitor concentration or the reciprocal of the changing fixed

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1 The abbreviation used is: HEPES, N-2-hydroxyethylpipera-

2 The abbreviation used is: HEPES, N-2-hydroxyethylpipera-

3 C. H. Fung and M. F. Utter, unpublished observations.
substrate concentration. Equations 7 to 11 were employed to fit data for which the double reciprocal plots conformed, respectively, to an intersecting initial velocity pattern, a parallel initial velocity pattern, linear competitive inhibition, linear uncompetitive inhibition, and linear noncompetitive inhibition (24).

\[ v = \frac{V_{AB}}{K_A + (1 + K_i/A)B} \]  
\[ v = \frac{V_{AB}}{K_B + (1 + K_i/B)A} \]  
\[ v = \frac{V_A}{K_A + (1 + K_i/A)A} \]  
\[ v = \frac{V_A}{K_B + (1 + K_i/B)B} \]  
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Assays were performed in duplicate and the points drawn in figures showing double reciprocal plots are the average of the two assays. Precision of the duplicate assays was within 3% with the coupled assay of CO₂ fixation and within 5% for the measurements at 290 nm and for the coupled assays of oxalacetate decarboxylation. Observed initial velocities which deviated markedly from the indicated pattern were discarded. The lines drawn through the points in double reciprocal plots are calculated from a least square fit of the data (Equation 6) unless otherwise stated in the figure legend. The slopes and intercepts of these lines then provided the data points in the secondary plots. However, the lines shown in the secondary plots were calculated from a fit of the original experimental data to the appropriate over-all rate equation.

RESULTS AND DISCUSSION

**Definition of Enzyme Species under Study**—An acetyl-CoA, e.g. acetyl-CoA, is an essential cofactor for pyruvate carboxylase from chicken liver. To simplify the interpretation of the present studies, all experiments were conducted at an essentially saturating concentration of acetyl-CoA (50 μM). As shown in Fig. 1, this is more than twice the concentration of activator required to obtain the optimum maximum velocity with each of the substrates tested. Furthermore, the apparent \( K_m \) values of the substrates are independent of the acetyl-CoA concentration when the acetyl-CoA concentration exceeds 20 μM. Since a saturating acetyl-CoA concentration was employed, in the present studies the catalytic properties observed are those of the enzyme-modifier complex in which all kinetically significant modifier sites are occupied.

In addition to the requirement for activation by an acetyl-CoA, expression of maximal catalytic activity by pyruvate carboxylase from chicken liver also requires the presence of a monovalent cation, e.g. K⁺, Tris⁺. The studies reported here were performed in the presence of optimal concentrations of the cation activator. Thus in assay systems buffered with Tris⁺ the concentration of Tris cation was approximately 85 mM and small variations in the K⁺ concentration in this system did not affect the initial reaction rate. When K⁺ HEPES was employed as a buffer for the CO₂ fixation assay system the K⁺ concentration was maintained at 100 to 125 mM by addition of KCl. Oxalacetate decarboxylation appeared insensitive to variations in the K⁺ concentration over the experimental range employed (100 to 180 mM).

**Initial Velocity Studies of CO₂ Fixation and Oxalacetate Decarboxylation**—Previously reported isotope exchange experiments (6) indicated that MgATP₂⁻, HCO₃⁻, MgADP₋, and phosphate are involved in a partial reaction (Reaction 2) with a sequential Bi Bi kinetic mechanism. This conclusion is based on experiments showing that isotope exchange between ATP and P₃ is dependent on the presence of MgADP (6). Pyruvate and oxalacetate are involved in a separate partial reaction (Reaction 3) since the exchange of [³¹P]pyruvate with oxalacetate occurs in the absence of the other reaction components (6). Thus, the over-all reaction catalyzed by pyruvate carboxylase appears to be the sum of two partial reactions, one with a sequential Bi Bi kinetic mechanism and the other with a Uni Uni mechanism.

Cleland has defined procedures for predicting the initial velocity patterns which will be observed if the experimental data are consistent with the mechanism under consideration (25). For the kinetic mechanism proposed for pyruvate carboxylase on the basis of isotope exchange studies, i.e. Ping Pong Bi Bi Uni Uni, double reciprocal plots of the data should consist of a family of parallel lines when the varied pair of substrates is composed of one substrate from each partial reaction. If both of the varied substrates are involved in the sequential Bi Bi partial reaction, the initial velocity pattern will be a family of lines intersecting in both the direction of CO₂ fixation and the direction of oxalacetate decarboxylation. Observed initial velocities which deviated markedly from the indicated pattern were discarded. The lines drawn through the points in double reciprocal plots are calculated from a fit data for which the double reciprocal plots conformed, respectively, to an intersecting initial velocity pattern, a parallel initial velocity pattern, linear competitive inhibition, linear uncompetitive inhibition, and linear noncompetitive inhibition (24).

**FIG. 1.** Variation of the apparent maximum velocity of CO₂ fixation as a function of the acetyl-CoA concentration. The substrate—MgATP₂⁻ (□) or pyruvate (○)—was varied at different fixed levels of acetyl-CoA and the apparent \( V_{\text{max}} \) for each experiment was computed by fitting the data to Equation 6. The percentage of \( V_{\text{max}} \) values were obtained by normalizing the computed maximum velocities after setting the optimum maximum velocity equal to 100%. These studies were performed in duplicate and the points drawn in figures showing double reciprocal plots are the average of the two assays. Precision of the duplicate assays was within 3% with the coupled assay of CO₂ fixation and within 5% for the measurements at 290 nm and for the coupled assays of oxalacetate decarboxylation. Observed initial velocities which deviated markedly from the indicated pattern were discarded. The lines drawn through the points in double reciprocal plots are calculated from a least square fit of the data (Equation 6) unless otherwise stated in the figure legend. The slopes and intercepts of these lines then provided the data points in the secondary plots. However, the lines shown in the secondary plots were calculated from a fit of the original experimental data to the appropriate over-all rate equation.

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In addition to the requirement for activation by an acetyl-CoA, expression of maximal catalytic activity by pyruvate carboxylase from chicken liver also requires the presence of a monovalent cation, e.g. K⁺, Tris⁺. The studies reported here were performed in the presence of optimal concentrations of the cation activator. Thus in assay systems buffered with Tris⁺ the concentration of Tris cation was approximately 85 mM and small variations in the K⁺ concentration in this system did not affect the initial reaction rate. When K⁺ HEPES was employed as a buffer for the CO₂ fixation assay system the K⁺ concentration was maintained at 100 to 125 mM by addition of KCl. Oxalacetate decarboxylation appeared insensitive to variations in the K⁺ concentration over the experimental range employed (100 to 180 mM).

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The initial velocity pattern for each possible pair of substrates in both the direction of CO₂ fixation and the direction of oxalacetate decarboxylation has been determined and the results are summarized in the first three columns of Table I. For each case in which both of the varied substrates are members of the pro-
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TABLE 1

| Varied substrate | Changing fixed substrate | Observed pattern | Apparent $K_a$ | Apparent $K_i$ | Fixed substrate |
|------------------|--------------------------|------------------|----------------|----------------|----------------|
| MgATP$^-$        | HCO$_3^-$                | Intersecting     | $0.13 \pm 0.01$ | $0.15 \pm 0.02$ | Pyruvate, 3.0 |
|                  | Pyruvate                 | Parallel         | $0.20 \pm 0.01$ | $1.6 \pm 0.2$   | Pyruvate, 3.0 |
| HCO$_3^-$        | MgATP$^-$                | Intersecting     | $1.3 \pm 0.1$   | $1.6 \pm 0.1$   | MgATP$^-$, 0.5 |
|                  | Pyruvate                 | Parallel         | $1.6 \pm 0.1$   | $1.6 \pm 0.1$   | MgATP$^-$, 0.5 |
| Pyruvate*        | HCO$_3^-$                | Parallel         | $0.49 \pm 0.02$ | $0.19 \pm 0.05$ | HCO$_3^-$, 16 |
|                  | MgATP$^-$                | Parallel         | $0.35 \pm 0.01$ | $0.35 \pm 0.01$ | Oxalacetate, 0.65 |
| Phosphate        | Phosphate                | Intersecting     | $0.19 \pm 0.02$ | $7.9 \pm 2.2$   | Phosphate, 40  |
| Oxalacetate      | Oxalacetate              | Parallel         | $0.23 \pm 0.01$ | $0.23 \pm 0.01$ | Oxalacetate, 0.65 |
| MgADP$^-$        | MgADP$^-$                | Parallel         | $7.7 \pm 0.8$   | $7.7 \pm 0.8$   | MgADP$^-$, 1.2 |
|                  | Phosphate                | Parallel         | $11 \pm 1$      | $11 \pm 1$      | MgADP$^-$, 1.2 |
|                  | Oxalacetate              | Parallel         | $0.20 \pm 0.01$ | $0.20 \pm 0.01$ | Oxalacetate, 0.65 |

* With pyruvate as the varied substrate, a double reciprocal plot over a wide range of pyruvate concentrations shows nonlinearity at high concentrations, i.e., a curvature concave downward. This has been noted with pyruvate carboxylase from chicken liver (26), rat liver (11), and sheep kidney (27). With the enzyme from chicken liver deviation from linearity is noted only at concentrations for pyruvate above 3 mM and at temperatures below 30°C (28). All the initial velocity experiments reported here have been carried out at pyruvate concentrations of 3 mM, or less, to avoid this complicating feature.

The data for each experiment were fitted to the appropriate rate equation (cf. "Methods") to obtain the apparent $K_a$ and $K_i$ for the various substrates. These constants are summarized in Table I. According to Cleland, if the standard errors of the fitted constants are 10% or less of the fitted constants themselves, one can be quite confident that the proper rate equation was employed (23). For the data in Table I the standard errors of the apparent $K_a$ values are all <10% or less of the calculated values. These constants therefore reflect a good internal consistency between the rate equation employed and the experimental data tested.

The results of the isotope exchange experiments reported previously (6) and the initial velocity studies presented here are entirely consistent with the proposed Ping Pong Bi Uni Uni mechanism depicted in Equations 2 and 3. Thus, the kinetically significant events in the reaction catalyzed by pyruvate carboxylase are

\[ \text{MgATP}^2- + \text{HCO}_3^- + \text{P} \rightarrow \text{Phosphate} + \text{HCO}_3^- + \text{MgADP}^2- + \text{CO}_2 + \text{P} \]

\[ \text{MgADP}^2- + \text{P} + \text{CO}_2 \rightarrow \text{MgATP}^2- + \text{Phosphate} \]

The primary data from which the constants in Tables I and III were calculated have been deposited with the National Auxiliary Publications Service. To obtain a copy, request NAPS Document No. 01745 from the ASIS National Auxiliary Publications Service, C.C.M. Information Corp., 886 Third Avenue, New York, N.Y. 10022, remitting in advance (payable to CCMI-NAPS) $2.00 for each microfiche or $5.00 for each photocopy.

$^*$ Six different substrate pair combinations and 16 different product-substrate combinations were examined during the course of these studies. Only representative examples of the primary data are presented in this paper because presenting all of the primary data would produce an unnecessarily bulky paper. Also, in the opinion of the authors most of the primary data is of minimal interest to many readers. Therefore, except for data shown in the paper, the primary data from which the constants in Tables I and III were calculated have been deposited with the National Auxiliary Publications Service. To obtain a copy, request NAPS Document No. 01745 from the ASIS National Auxiliary Publications Service, C.C.M. Information Corp., 886 Third Avenue, New York, N.Y. 10022, remitting in advance (payable to CCMI-NAPS) $2.00 for each microfiche or $5.00 for each photocopy.
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![Graph](image)

**FIG. 3.** Initial velocity study of oxalacetate decarboxylation with phosphate and oxalacetate as the varied substrates. Each cuvette contained the following components in a total volume of 1.0 ml (in micromoles): K\(^+\) HEPES, pH 7.8, 200; MgADP\(^{2-}\), 1.2; free Mg\(^{2+}\), 5; oxalacetate and K\(^+\) phosphate, as indicated; lactate dehydrogenase (20 μg) and pyruvate carboxylase (specific activity = 32, 24 μg). Other conditions were as listed for Fig. 2. The lines in this graph are drawn as determined by a fit of the data to Equation 8.

The initial velocity patterns shown in Table I are equally consistent with two fundamentally different types of Ping Pong Bi Bi Uni Uni mechanisms. The distinction between the two types of mechanisms depends on the number of separate and independent catalytic sites which are assumed to be operative within each active site. Classical mechanisms assume that the active site functions as a single catalytic site, e.g. the glutamate-oxalacetate transaminase mechanism (29). An active site which functions as a single catalytic site in the pyruvate carboxylase reaction is represented schematically in Fig. 4A. On the other hand, nonclassical mechanisms assume that two separate and functionally independent catalytic sites are present within each active site; or more specifically, a separate catalytic site exists for the reactants of each partial reaction. A schematic representation of an active site of this type is shown for pyruvate carboxylase in Fig. 4B. For the type of active site illustrated in Fig. 4B it is assumed that the biotinyl residue, which functions as a carboxyl carrier, can link the two separate catalytic sites and thus provide the element which unifies the active site.

The classical concept of the active site has been incorporated into the reaction mechanism of methylmalonyl-CoA transcarboxylase (S), and this enzyme exhibits several catalytic and structural parameters which are analogous to those described for pyruvate carboxylase, e.g. the presence of bound metal ions and biotin residues in equimolar ratio (30). It was therefore of great interest to determine whether pyruvate carboxylase also possesses a reaction mechanism which is consistent with the existence of an active site composed of two catalytic sites.

**Product Inhibition Studies: Reaction Mechanism of Pyruvate from Chicken Liver**—The two different types of Ping Pong Bi Bi Uni Uni mechanisms described above can be distinguished by product inhibition studies. If an ordered sequence is assumed with MgATP as the first reactant and P\(_i\) as the first product for the Bi Bi portion of a classical ping-pong sequence, the predicted product inhibition patterns are those shown in Column 3, Table

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**ACTIVE SITES: ILLUSTRATIVE SCHEMES**

**A. CLASSICAL**

![Diagram](image)

**B. NON-CLASSICAL**

![Diagram](image)
Comparison of product inhibition patterns predicted by four different Ping Pong Bi Bi Uni Uni mechanisms to the experimentally observed patterns (see text)

| Varied substrate | Product inhibitor | Classical mechanism | Classical rapid equilibrium | Nonclassical rapid equilibrium | Nonclassical with abortive complexes | Observed |
|------------------|-------------------|---------------------|-----------------------------|--------------------------------|-------------------------------------|----------|
| MgATP<sup>−</sup> | MgADP<sup>−</sup> | UC                  | UC                          | C                              | C                                   | C        |
|                  | Phosphate         | NC                  | UC                          | C                              | C                                   | C        |
|                  | Oxalacetate       | C                   | NC                          | NC                             | NC                                  | NC       |
| HCO<sub>3</sub><sup>−</sup> | MgADP<sup>−</sup> | UC                  | UC                          | C                              | NC                                  | NC       |
|                  | Phosphate         | NC                  | UC                          | NC                             | NC                                  | NC       |
|                  | Oxalacetate       | C                   | NC                          | NC                             | NC                                  | NC       |
| Pyruvate         | MgADP<sup>−</sup> | C                   | C                           | UC                             | UC                                  | UC       |
|                  | Phosphate         | C                   | C                           | UC                             | UC                                  | UC       |
|                  | Oxalacetate       | NC                  | NC                          | C                              | C                                   | C        |
| MgADP<sup>−</sup> | MgATP<sup>−</sup> | UC                  | UC                          | C                              | C                                   | C        |
|                  | HCO<sub>3</sub><sup>−</sup> | NC                  | UC                          | C                              | NC                                  | NC       |
| Phosphate        | MgATP<sup>−</sup> | UC                  | UC                          | C                              | C                                   | C        |
|                  | HCO<sub>3</sub><sup>−</sup> | NC                  | UC                          | C                              | NC                                  | NC       |
| Oxalacetate      | MgATP<sup>−</sup> | C                   | C                           | UC                             | UC                                  | UC       |
|                  | HCO<sub>3</sub><sup>−</sup> | UC                  | C                           | UC                             | NC                                  | NC       |
|                  | Pyruvate          | NC                  | C                           | NC                             | NC                                  | NC       |

<sup>a</sup> Ordered Bi Bi sequence assumed as MgATP<sup>−</sup>, HCO<sub>3</sub><sup>−</sup>, E, MgADP<sup>−</sup>.
<sup>b</sup> The abortive complexes which form at the site of the Bi Bi partial reaction are E-HCO<sub>3</sub><sup>−</sup>-P; and E-HCO<sub>3</sub><sup>−</sup>-MgADP<sup>−</sup>.
<sup>c</sup> NC designates noncompetitive; UC, uncompetitive; and C, competitive.

II. If the Bi Bi partial reaction is assumed to have a rapid equilibrium random mechanism in the classical ping-pong reaction, the predicted patterns are those shown in Column 4, Table II. With a nonclassical mechanism (Fig. 5B), the predicted patterns are those shown in Column 5. It is apparent that the inhibition patterns predicted for the nonclassical mechanism differ for each possible combination of varied substrate and product inhibition from those predicted for the two versions of the classical mechanism. The latter two show a fair degree of correspondence.

The classical and nonclassical mechanisms are most readily distinguished by examination of certain key pairs of product inhibitor and varied substrate which should lead to competitive inhibition patterns. For the classical mechanisms (Fig. 5A) a competitive interaction is predicted between pyruvate and MgADP<sup>−</sup> and also between oxalacetate and MgATP<sup>−</sup> because in each case the varied substrate and the product inhibitor combine at the same site with the same form of the enzyme. Thus the classical mechanism predicts competitive interactions only when the varied substrate and the product inhibitor are involved in different partial reactions. On the other hand the nonclassical mechanism (Fig. 5B) predicts competitive interactions only between substrates and products which are in the same partial reaction, e.g. between pyruvate and oxalacetate. The kinetic behavior predicted for the nonclassical mechanism is a direct consequence of the assumption that a separate catalytic site exists for the reactants of each partial reaction.

The patterns observed with the various product inhibitors are shown in Table II, Column 7. The apparent kinetic constants, which were computed as described under "Methods," are shown in Table III and 5 of the 16 sets of data are presented in Figs. 6 to 10.
Table III

| Variable substrate | Apparent $K_m$ | Product inhibitor | $K_i$ | $K_{ia}$ | Pattern | Fixed substrates |
|--------------------|---------------|------------------|------|---------|---------|-----------------|
| MgATP$^-$          | 0.11 ± 0.01   | MgADP$^-$        | 0.21 ± 0.01 | C | Pyruvate, 3.0; HCO$_3^-$, 20.00 |
|                    | 0.070 ± 0.004 | Phosphate        | 13.00 ± 1.00 | C | Pyruvate, 3.0; HCO$_3^-$, 16.00 |
|                    | 0.071 ± 0.01  | Oxalacetate      | 0.23 ± 0.05 | NC | Pyruvate, 3.0; HCO$_3^-$, 20.00 |
| HCO$_3^-$          | 1.02 ± 0.05   | MgADP$^-$        | 1.56 ± 0.36 | NC | MgATP$^-$, 0.5; pyruvate, 3.00 |
|                    | 0.88 ± 0.09   | Phosphate        | 12.00 ± 2.00 | NC | MgATP$^-$, 0.2; pyruvate, 3.00 |
|                    | 1.63 ± 0.09   | Oxalacetate      | 0.13 ± 0.01 | NC | MgATP$^-$, 2.0; pyruvate, 10.00 |
| Pyruvate           | 0.53 ± 0.03   | MgADP$^-$        | 0.26 ± 0.05 | UC | MgATP$^-$, 0.5; HCO$_3^-$, 20.00 |
|                    | 0.28 ± 0.01   | Phosphate        | 25.00 ± 1.00 | UC | MgATP$^-$, 4.0; HCO$_3^-$, 10.00 |
|                    | 0.37 ± 0.02   | Oxalacetate      | 0.13 ± 0.01 | C  | MgATP$^-$, 2.0; HCO$_3^-$, 10.00 |
| MgADP$^-$          | 0.24 ± 0.04   | MgATP$^-$        | 0.20 ± 0.02 | C  | Phosphate, 45.0; oxalacetate, 0.65 |
| Phosphate          | 16.00 ± 1.00  | HCO$_3^-$        | 10.10 ± 1.70 | NC | Phosphate, 45.0; oxalacetate, 0.65 |
| Oxalacetate        | 13.50 ± 1.20  | HCO$_3^-$        | 0.39 ± 0.03 | C  | MgADP$^-$, 1.2; oxalacetate, 0.65 |
|                    | 0.333 ± 0.002 | MgATP$^-$        | 8.70 ± 1.60 | NC | MgADP$^-$, 1.2; oxalacetate, 0.65 |
|                    | 0.044 ± 0.003 | HCO$_3^-$        | 2.10 ± 0.03 | UC | MgADP$^-$, 2.0; phosphate, 40.00 |
|                    | 0.051 ± 0.002 | Pyruvate         | 20.00 ± 3.00 | UC | MgADP$^-$, 1.0; phosphate, 45.00 |
|                    |               |                  | 0.24 ± 0.01 | C  | MgADP$^-$, 5.0; phosphate, 50.00 |

Fig. 6. Product inhibition of oxalacetate decarboxylation by MgATP$^-$ with oxalacetate as the varied substrate. Each cuvette contained the following components in a total volume of 1.0 ml (in micromoles): Tris-Cl, pH 7.8, 60; K$^+$ phosphate, pH 7.8, 40; MgADP$^-$, 2.0; free Mg$^{2+}$, 5; MgATP$^-$ and oxalacetate, as indicated; lactate dehydrogenase (12.5 µg) and pyruvate carboxylase (specific activity = 17,600 pg). Other conditions were as listed for Fig. 2. Inset, secondary plot of intercepts versus the concentration of MgATP$^-$.

Identification of the reaction mechanism as nonclassical is further suggested by the observation of competitive product inhibition when the product inhibitor and variable substrate are involved in separate partial reactions. The assignment of a specific nonclassical mechanism to pyruvate carboxylase from chicken liver requires identification of the sequential mechanism which characterizes the Bi Bi partial reaction. The competitive patterns observed when MgATP$^-$ interacts with MgADP$^-$ or phosphate are consistent with the sequential model and indicate that the binding of reactants at the Bi Bi site can be described by a rapid equilibrium random type of mechanism. However, a simple random mechanism does not distinguish between a rapid equilibrium random Bi Bi mechanism and a random Bi mechanism but the former has been assumed here because the procedures which have been employed to derive the rate equation
Fig. 8. Product inhibition of CO₂ fixation by phosphate with MgATP₂⁻ as the varied substrate. Each cuvette contained the following components in a total volume of 1.0 ml (in micromoles): K⁺ HEPES, 100; K⁺ pyruvate, 3; KHCO₃, 16; free Mg²⁺, 4; phosphate and MgATP₂⁻, as indicated; malate dehydrogenase (20 μg) and pyruvate carboxylase (specific activity = 4.3 μg). Other conditions as listed for Fig. 2. Inset, secondary plot of slopes versus phosphate concentration.

Fig. 9. Product inhibition of CO₂ fixation by oxalacetate with pyruvate as the varied substrate. Each cuvette contained the following components in a total volume of 1.0 ml (in micromoles): K⁺ HEPES, pH 7.8, 200; MgATP₂⁻, 2; free Mg²⁺, 3; NaHCO₃, 10; acetyl-CoA, 0.05; and pyruvate carboxylase (specific activity = 18.5, 60 μg). Velocity is change in absorbance per min at 290 nm. Inset, secondary plot of slopes versus the concentrations of oxalacetate.

Fig. 10. Product inhibition of oxalacetate decarboxylation by bicarbonate with phosphate as the varied substrate. Each cuvette contained the following components in a total volume of 1.0 ml (in micromoles): K⁺ HEPES, pH 7.8, 200; oxalacetate, 0.05; MgADP⁻, 1.2; free Mg²⁺, 5; bicarbonate and phosphate, as indicated; and lactate dehydrogenase (17 μg) and pyruvate carboxylase (specific activity = 22, 43 μg). Other conditions were as listed for Fig. 2. Inset, secondary plot of slopes and intercepts versus HCO₃⁻ concentration.

The major features of the reaction mechanism proposed for pyruvate carboxylase from chicken liver are therefore the following: (a) the over-all reaction is the sum of two partial reactions and can be described as Ping Pong Bi Bi Uni Uni; (b) separate catalytic sites exist for the reactants of each partial reaction; (c) the two catalytic sites are connected by a biotinyl residue which isomerizes back and forth, carrying the carboxyl group from one site to the other; (d) the mechanism of the Bi Bi partial reaction is rapid equilibrium random Bi Bi with the additional existence of two kinetically significant abortive complexes (E-HCO₃⁻-P₁ and E-HCO₃⁻-MgADP⁻).
FIG. 11. A, the reaction mechanism of the Bi Bi partial reaction. This scheme is suitable for analysis by the rapid equilibrium method of Cha (32). The solid lines connect "productive" enzyme reactant complexes and the broken lines show pathways by which "abortive" enzyme reactant complexes may form. The reaction mechanism of the Uni Uni partial reaction is shown in Equation 15. A = MgATP-, B = HCO₃⁻, P = Pᵢ, and Q = MgADP--; B, a scheme for the pyruvate carboxylase reaction with a steady state analysis by the King-Altman procedure (33) (see text).

Derivation of Rate Equation for Proposed Two-Site Mechanism—The procedure employed by Northrop (8) was used to derive a rate equation for the mechanism proposed for pyruvate carboxylase. The derivation of rate equations for such complex reaction mechanisms has been greatly facilitated by the development of a procedure which combines features of the steady state and rapid equilibrium methods (32). Application of this method for derivation of the rate equation for pyruvate carboxylase had to take into account the general features of the mechanism as described above. The additional assumption is required that the substrates and products bind equally well to their respective sites whether or not the biotinyl residue is carboxylated.

In the derivation four species are assumed to contribute to the steady state distribution of the enzyme (Fig. 11B). Of these species [ER] and [EC] represent enzyme with free and carboxylated biotin, respectively, at the site for the Bi Bi partial reaction, while [EP] and [EB] represent the enzyme with the free and carboxylated biotin at the site for the Uni Uni partial reaction. Each of these species represents the sum of free enzyme plus all enzyme-reactant complexes which have the appropriate form of biotin at the indicated binding site.

The procedure for determining the fraction of the total enzyme which is present as a given enzyme-reactant complex is based on the rapid equilibrium method. These fractions are called "fractional concentration factors" (32), and for the purpose of nomenclature are represented by F with a subscript denoting the reactants involved in the complex, e.g. \( F_{AB} \). A separate set of fractional concentration factors is required for each catalytic site, since the two sites behave independently. The scheme in Fig. 11A must be analyzed in order to obtain the necessary fractional concentration factors for the Bi Bi half-reaction, \( F_{AB} \) and \( F_{PQ} \). The expressions obtained for these factors are shown in Equations 13 and 14.

\[
F_{AB} = \frac{(k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) A B}{(k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) A B + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) B + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) A + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) P + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) Q + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) A + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) B + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) P + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) Q}
\]

\[
F_{PQ} = \frac{(k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) PQ}{(k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) PQ + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) A R + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) R + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) A + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) P + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) Q + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) B Q + (k_1 k_2 k_3 k_4 k_5 k_6 k_7 k_8 k_9) B Q}
\]

For the α-keto acid catalytic site the relevant fractional concentration factors are obtained by analysis of the following scheme:

\[
ER \xrightarrow{k_{ER}} E \xrightarrow{k_{EC}} EC
\]

The fractional concentration factors for this partial reaction are shown in Equations 16 and 17.

\[
F_C = \frac{k_{ER} C}{k_{ER} C + k_{EC} C + k_{ER} R}
\]

\[
F_R = \frac{k_{ER} R}{k_{ER} R + k_{EC} C + k_{ER} C}
\]

The derivation of analogous equations for methylmalonyl-CoA transcarboxylase has been described in the Appendix of Reference 8.

The complete rate equation may then be obtained by analysis of Fig. 11B using the steady state procedure described by King.
...substrates. The expressions for the fractional concentration factors (Equations 13, 14, 16, and 17) may then be substituted into the relationship obtained by the Kin-Alman analysis to give the complete rate equation. After defining the kinetic constants (7) the following rate equation is obtained.7

\[ \tau = \frac{V_1V_2ABC - V_1V_2/K_{eq}PQR}{K_{eq}K_1V_1 + K_1V_2AB + K_1V_2BC + K_1V_3AC + V_1ABC} \]

The complete rate equation can be modified for initial velocity studies of CO\(_2\) fixation by setting those terms containing the concentrations of the products \(P, Q,\) and \(R\) to zero. The reciprocal form of the resulting equation is identical with Equation 12. Hence Equation 18 predicts the observed initial velocity patterns. In order to test whether Equation 18 predicts the product inhibition patterns which are observed (cf. Table III), those terms containing the two products which are not present in the experiment are set equal to zero. Such an analysis has been conducted for each of the reaction as product inhibitor and the predicted patterns coincide with the experimental data, except for the case in which HC\(_2\)O\(_3\) is the product inhibitor and oxalacetate is the varied substrate (see Table II, Column 5 and 6). A rationale for the observed inconsistency is presented below.

On the basis of the results of these studies and of the studies with methylmalonyl-CoA transcarboxylase by Northrop (8), prediction of the product inhibition patterns expected for a two-site mechanism appears to conform to the following principles. (a) Competitive patterns are observed only when the product inhibitor and the varied substrate are members of the same partial reaction. (b) Noncompetitive and uncompetitive patterns are observed when the product inhibitor and the varied substrate are members of different partial reactions; the pattern is uncompetitive if the product inhibitor is a member of a bireactant (or higher reactancy) partial reaction, the pattern is noncompetitive if the product inhibitor is a member of a unireactant partial reaction. (c) Substrate analogs which are true dead end inhibitors show competitive or uncompetitive inhibition; competitive inhibition versus the reactants to which they are structurally related and uncompetitive inhibition versus the reactants of the other partial reaction (cf. References 8 and 11).

**Concluding Discussion**—The proposal of a two-site mechanism for pyruvate carboxylase from chicken liver is supported by the present kinetic studies and also by previous studies on the properties of the interaction of substrates and inhibitors with this enzyme.

The two sites involved in catalysis of the partial reactions of pyruvate carboxylase appear to act independently of each other. For instance rate constants for interconversion steps at one site do not appear to be influenced by the presence or absence of a reactant at the other site. Such interaction would give rise to an apparent activation or inhibition which would be expressed as nonlinearity in the double reciprocal plots. No such deviations from linearity were observed in the studies reported here. The proposed independence in function of the two catalytic sites is further supported by previous observations. First, the properties of the interaction of the bound manganese with the substrates of the UniUni partial reaction (Equation 3) are unaffected by the presence of the substrates and cofactors of the Bi Bi partial reaction (Equation 2), which fail to show any significant interaction with the bound metal (35, 36). Second, oxalate acts as a specific inhibitor of the exchange of \[^{14}C\]pyruvate with oxalacetate (Reaction 3) and has no significant effect on the rate of exchange of \[^{14}P\]phosphate with ATP (Reaction 2) (35).

The independence in function of the two catalytic sites implies a spatial separation between these sites. In this case the required intersite linkage may be provided by the biotinyl residue which has an essential role in catalysis at both sites (6). If this residue is attached in peptide linkage to the e-NH\(_2\) group of a lysine residue in apopyruvate carboxylase, as has been shown for other biotin carboxylases (10) movement between catalytic sites as much as 28 A apart would be possible if the flexible arm is fully extended. Such movement may be facilitated by the change in net charge on the biotinyl residue which accompanies carboxylation.

The simplifying assumption that the carboxylation of the biotinyl residues has no significant effect on the interaction of substrates and products with pyruvate carboxylase is less well documented. Oxalate acts as an uncompetitive inhibitor of CO\(_2\) fixation when pyruvate is the varied substrate, but as a competitive inhibitor of oxalacetate decarboxylation when oxalacetate is the varied substrate (35). These inhibition patterns, which indicate preferential binding of oxalate to the noncarboxylated form of the enzyme, show that this inhibitor does discriminate between the two forms of the enzyme. Similar conclusions have previously been deduced from the properties of the oxalate inhibition of exchange of \[^{14}C\]pyruvate with oxalacetate catalyzed by methylmalonyl-CoA transcarboxylase (9). However, in the over-all reaction catalyzed by this latter enzyme a complex pattern of inhibition is observed when pyruvate is the varied substrate (9). The properties of this inhibition have been interpreted as indicating that oxalate has the capacity to trap the biotinyl residue at the \(\alpha\)-keto acid site (9). Somewhat similar data have been obtained in studies of the inhibition of pyruvate...
carboxylase from *Saccharomyces cerevisiae* by oxalate when pyruvate is the varied substrate. Equation 18 predicts that HCO₃⁻ will be an uncompetitive inhibitor of oxalacetate decarboxylation when oxalacetate is the varied substrate, but instead a noncompetitive pattern is observed (Fig. 7, Table II). This apparent inconsistency can be explained if the rate of the reaction in the direction of oxalacetate decarboxylation is not limited solely by the interconversion of the central complex but also in part by release of MgATP from the enzyme. Under such circumstances HCO₃⁻ could react with E-MgATP and biotin (cf. Fig. 5B) to regenerate the carboxyl biotin form. This will result in an inhibition of the reaction which cannot be overcome by increasing the concentration of oxalacetate, hence yielding a noncompetitive pattern. A similar F-MgATP complex has been reported for the rat liver enzyme by McClure et al. (12).

Direct evidence that an active site may be separated into two different catalytic sites has been obtained in structural studies of the related biotin enzyme acetyl-CoA carboxylase from *Escherichia coli*. This enzyme may be separated into three classes of subunits all of which are required for catalysis of the over-all reaction (37). The biotinyl residues of this protein are carried on a small subunit, the biotin carrier protein (37). Another subunit (E₈) catalyzes carboxylation of free biotin in the presence of ATP + HCO₃⁻ and must therefore carry a binding site for this cofactor (37). A binding site for biotin also exists on the third subunit (E₉) (38) which is required for transcarboxylation for Y-N-carboxybiotinyl enzyme to acetyl-CoA (39). These observations provide definitive proof that each active site of acetyl-CoA carboxylase from *E. coli* is composed of two separate and distinct catalytic sites. A similar separation of an enzyme into subunits responsible for catalysis of different portions of the over-all reaction has been shown for pyruvate dehydrogenase complexes obtained from both mammalian and microbial sources by Reed (cf. Reference 40). However a thorough steady state kinetic analysis has not been reported for either acetyl-CoA carboxylase or pyruvate dehydrogenase complexes responsible for catalysis of different portions of the over-all reaction mechanism for any given enzyme.

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Pyruvate Carboxylase from Chicken Liver: STEADY STATE KINETIC STUDIES INDICATE A "TWO-SITE" PING-PONG MECHANISM
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