Kinetic Studies of Citrate Synthase from Rat Kidney and Rat Brain*

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SUMMARY

Crystalline citrate synthase (citrate oxalacetate lyase (coenzyme A-acetylating) EC 4.1.3.7) has been prepared from rat kidney and rat brain. These enzymes are homogeneous as judged by acrylamide gel electrophoresis, and immunologically identical with the rat heart citrate synthase. Steady state kinetic studies including product inhibition experiments indicate that the substrates are added in a random order, the products come off in random order, and that two dead end complexes are formed. When acetyl-CoA concentrations are varied over wide range, a nonlinear Lineweaver-Burk plot is obtained suggesting apparent substrate activation. One interpretation of this observation is that some cooperativity exists between two sites on the enzyme.

Two interpretations for the nonlinear behavior are considered. One is that cooperativity exists between two sites on the enzyme and the other is that the rate of the binary complex formation between acetyl-CoA and enzyme is slower than the rate of ternary complex interconversion.

EXPERIMENTAL PROCEDURE

Materials—Bio-Gel A-0.5m was obtained from Bio-Rad Laboratories, Richmond, Calif.; DEAE-cellulose (DE-52) from Reevo Angel Co., New York, N. Y.; hydroxylapatite from Clarkson Chemical Co., Williamsport, Md.; Sephadex G-100 from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden; NAD, NADH, oxalacetate, and malate from Calbiochem, Los Angeles, Calif.; DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) from Sigma, St. Louis, Mo.; malate dehydrogenase from Boehringer, Germany; and coenzyme A from P-L Laboratories. Acetyl-CoA was prepared by the method of Simon and Shemin (8). Assay—Citrate synthase activity was determined at 412 nm by measuring the initial rate of reaction of liberated CoA-SH with DTNB as described by Sere et al. (8). For routine assay, the reaction mixture contained 0.1 μmole of DTNB, 0.3 μmole of acetyl-CoA, 0.5 μmole of oxalacetate, 100 μmole of Tris-CI buffer, pH 8.1, and enzyme solution in a total volume of 1.0 ml. The reaction was carried out at 25° and initiated by the addition of oxalacetate. The measurements were made in a Hitachi No. 124 double beam spectrophotometer with the attachment of a Hitachi 165 recorder. One unit of enzyme is the amount of enzyme that catalyzes the liberation of 1 μmole of CoA-SH per min under these conditions.

Specific activity is expressed as units per mg of protein. Protein is determined in crude fractions by the phenol reagent method (9), in pure fractions by the biuret method (10), and in column chromatography fractions according to the procedure of Warburg and Christian (11). These methods were standardized with crystalline bovine serum albumin.

Acrylamide Gel Electrophoresis—Acrylamide gel electrophoresis of the enzyme was carried out according to the procedure of Davies (12). Electrophoresis was performed in 7.5% acrylamide gel, with two different electrode buffers; 0.05 M Tris-glycine containing 10 mM sodium citrate, pH 8.3, and 2.5-M-β-alanine-acetate, pH 4.3. The enzyme sample to be analyzed was layered on top of the gel with an equal volume of 0.2 M sucrose containing bromophenol blue (13).

The abbreviation used is: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).
thymol blue in the electrode buffer. A current of 3 mA per gel was applied for 2 hours at room temperature. The gels were stained in 1% Amido black in 7% acetic acid.

**Kinetic Studies**—Most kinetic studies were carried out in 1.0-cm cuvettes maintained at 28°C, and initial velocities were measured using the highest sensitivity on the recorder (full scale was 0.1 A). The rate of the forward reaction was measured by the DTNB method as described above except for the variation in the acetyl-CoA and oxalacetate concentrations. Since the $K_m$ values for both substrates are low, the change of absorbance with time approximates linearity for only a short time. Under the conditions of the assay using 1-cm cuvettes and a 0.1-A scale when either substrate is 2.5 μM the total reaction is only 0.034 A and linearity can only be approximated for about one-fourth of this span or about 10% of full scale. In spite of this limitation triplicate analyses of initial velocities were within 15% of each other. It was suggested that we could increase the sensitivity and measure initial velocities at concentrations below the $K_m$ value by using cells with a 10-cm light path rather than 1-cm cells. We therefore repeated the kinetics of acetyl-CoA and oxalacetate using 10-cm cells in a Cary 15 spectrophotometer using the 0.1-A slide wire. Under these conditions the reactions were linear for about full scale when the limiting substrate concentration was 2.5 μM. Experiments were performed in the Cary from 0.5 to 5 μM and in the Hitachi at concentrations greater than 2.5 μM. The same kinetic behavior was observed in Lineweaver-Burk plots with data obtained in both instruments, i.e. lines intersecting on the $x$ axis.

The rate of reaction of citrate and CoA (the reverse reaction) was followed in a system coupled to malate dehydrogenase and NADH (13). The assay medium for measuring the reverse reaction contained 100 μmoles of Tris-HCl buffer, pH 8.1, 0.2 to 5 μmoles of potassium citrate, 50 to 500 μmoles of CoA (assayed according to the method of Stern and Kiesicki (14)), 200 units of malate dehydrogenase, and 200 μmoles of NADH in a total volume of 1.0 ml. The kinetic constants for citrate and CoA were also determined at pH 6.1 in 0.1 M imidazole acetate using this method. The reactions were started by the addition of citrate synthase. Control assays were performed with K$_2$SO$_4$ to differentiate between changes in rates due to ionic strength effects and changes in rate due to specific substrate effects.

The DTNB assay could not be used to test the inhibitory effect of CoA on the forward reaction since CoA would react with the DTNB. To measure the effect of CoA on the forward reaction we used the coupled malate dehydrogenase assay for citrate synthase (14). Since this assay follows the conversion of malate to oxalacetate, the oxalacetate concentration was varied by varying the amount of added malate to displace the initial malate dehydrogenase equilibrium (4). Duplicate or triplicate initial velocities were determined at each of four or more concentrations of the variable substrate (~10-fold range of concentration) at each of at least three fixed concentrations of the second substrate. When product inhibition was studied, at least two fixed concentrations of inhibitor were used at seven concentrations of the variable substrate and at two fixed concentrations ($K_m$ and 10 × $K_m$) of the second substrate.

**Analysis of Kinetic Data**—After hand-plotting the data according to Lineweaver-Burk (reciprocal velocities against reciprocal concentrations), best fit straight lines were drawn. For each of the substrates an intersecting pattern was observed indicating a sequential mechanism. Such kinetic results can be described by the equation

$$v = \frac{V_{A B}}{K_v + K_{s A} + K_{s B} + K_{in}K_{s}}$$

so that the results were then fitted to this equation using a computer program of Cleland (15) (see below for definition of terms). The intersection occurred on the $x$ axis indicating $K_v = K_{in}$. One of the patterns obtained in the double reciprocal plots of product inhibition studies was that of lines intersecting on the $y$ axis and these were analyzed using the equation for linear competitive inhibition (Equation 2).

$$v = \frac{VA}{K(1 + I/K_{A}) + A}$$

The other pattern obtained with double reciprocal plots of some product inhibition studies was that of lines intersecting on the $x$ axis and these were analyzed using the equation for linear noncompetitive inhibition (Equation 3).

$$v = \frac{VA}{K(1 + I/K_{A}) + A(1 + I/K_{B})}$$

Most kinetic plots in this paper show experimental points and best fit lines as determined from the computer fits of the data to the appropriate equation (15). Only when deviation from linearity was observed with low concentrations of acetyl-CoA were the lines treated as if they were composed of two linear portions and plotted by hand. Citrate inhibition of acetyl-CoA was corrected for an ionic strength effect by using rates of citrate synthase in the presence of an equivalent ionic strength of K$_2$SO$_4$ as control rates.

**Dissociation Constant for Oxalacetate**—When citrate synthase is placed in urea, it unfolds exposing its sulfhydryl groups to react with DTNB. This denaturation can be prevented by oxalacetate. A plot of log $k_0 - k_1/k_1$ where $k_0$ is the first order rate constant of DTNB reaction in urea alone and $k_1$ is the first order rate constant in the presence of oxalacetate, against log (oxalacetate) yields a straight line. The concentration of oxalacetate where log $k_0 - k_1/k_1$ is equal to 0 is the $K_{diss}$ for oxalacetate (16). The slope of that line is the number of oxalacetate molecules binding per site on the enzyme.

**Immunological Studies**—Antiserum to rat heart citrate synthase was obtained as described previously (1). Double diffusion studies were carried out at 4°C for 48 hours according to the method of Marcus and Grollman (17).

**RESULTS**

**Procedure for Purification of Citrate Synthase from Rat Tissues**

**Step 1: Homogenization**—Frozen rat kidneys (300 g) were put into 1.5 liters of an extraction solution which was 40% saturated with ammonium sulfate, 1 mM EDTA, 5 mM citrate, 20 mM potassium phosphate buffer, pH 7.4, and contained 5 ml of Antifoam-60. This mixture was homogenized in a large Waring Blender for four 1-min periods at full speed, cooling the solution between homogenization periods in an ice bath. The homogenate was centrifuged at 27,300 × g for 30 min at 4°C and the precipitate was discarded.

**Step 2: First Ammonium Sulfate Precipitation**—The supernatant solution (assumed to be 40% saturated ammonium sulfate) was brought to 50% saturation with solid ammonium sulfate. The precipitate was removed by centrifugation as described.
above and discarded. The supernatant solution was brought to 75% saturation of ammonium sulfate with solid ammonium sulfate, stirred for an hour, and centrifuged at 27,300 × g for an hour. The precipitate was dissolved in 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA.

Step 3: Bio-Gel A-0.5m Column Chromatography—The solution of the 75% ammonium sulfate precipitate was centrifuged at 27,300 × g for 15 min to remove any insoluble material. The supernatant solution was applied to a Bio-Gel A-0.5m column (5.0 × 80 cm) previously equilibrated with 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, and the protein was eluted with the same buffer. Citrate synthase was eluted at a buffer volume from 1000 to 1300 ml.

Step 4: Second Ammonium Sulfate Precipitation—The eluate from Bio-Gel A-0.5m column was brought to 50% saturation with solid ammonium sulfate. After stirring for 30 min, the solution was centrifuged at 27,300 × g for 30 min. The precipitate was discarded, and the supernatant solution was brought to 75% saturation with solid ammonium sulfate. The precipitate was dissolved in 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA. Insoluble protein was removed by centrifugation at 12,100 × g for 15 min, and the supernatant solution was dialyzed for 24 hours against 10 liters of 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA. The external solution was changed four times.

Step 5: DEAE-cellulose Column Chromatography—Insoluble protein formed during dialysis was removed by centrifugation at 12,100 × g for 15 min, and the supernatant solution was applied to a DE-52 cellulose column (5.0 × 35 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The column was washed with the same buffer followed by a linear gradient of 0 to 0.2 M KCl in the same buffer. Citrate synthase was eluted from the column at approximately 0.1 m KCl. The fractions containing the enzyme were combined and the protein precipitated with solid ammonium sulfate (80% saturation) and collected by centrifugation at 30,900 × g for an hour. The precipitate was dissolved in 20 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA.

Step 6: Hydroxylapatite Column Chromatography—The enzyme solution was dialyzed as described previously, centrifuged to remove insoluble protein, and the supernatant solution applied to a hydroxylapatite column (2.5 × 35 cm) previously equilibrated with 20 mM potassium phosphate buffer, pH 7.4. The column was washed with the same buffer, and eluted with a linear gradient between 5 mM and 0.2 M potassium phosphate buffer, pH 7.4. Citrate synthase was eluted from the column at approximately 0.1 M buffer concentration. Citrate synthase fractions were collected and precipitated with solid ammonium sulfate as before. The precipitate was collected by centrifugation at 30,900 × g for an hour and dissolved in 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA.

Step 7: Sephadex G-100 Column Chromatography—The citrate synthase preparation was then applied to a Sephadex G-100 column (2.0 × 100 cm) previously equilibrated with 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, and eluted with the same buffer. Citrate synthase was eluted from the column between 400 to 460 ml of buffer eluent. Citrate synthase in these fractions were precipitated by addition of solid ammonium sulfate. The precipitate was collected by centrifugation and dissolved in a small amount of 20 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA.

Step 8: First Crystallization—A small amount of solid ammonium sulfate was added slowly to the enzyme solution with continuous stirring until a slight turbidity appeared. The enzyme solution was stirred by a magnetic stirrer for 3 days at 4°, during which time crystals appeared as judged by the silkiness of the solution.

Table I: Purification procedure for citrate synthase from rat kidney

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total activity</th>
<th>Protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate in 40%</td>
<td>1,400</td>
<td>7,000</td>
<td>22,900</td>
<td>0.30</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 to 75% (NH₄)₂SO₄</td>
<td>120</td>
<td>5,300</td>
<td>18,400</td>
<td>0.20</td>
</tr>
<tr>
<td>Bio-Gel A-0.5m</td>
<td>20</td>
<td>4,700</td>
<td>770</td>
<td>6.1</td>
</tr>
<tr>
<td>DE-52 cellulose</td>
<td>24</td>
<td>3,820</td>
<td>118</td>
<td>33</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>3</td>
<td>3,000</td>
<td>46</td>
<td>65</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>1.2</td>
<td>1,900</td>
<td>10</td>
<td>132</td>
</tr>
<tr>
<td>Second crystallization</td>
<td>1.6</td>
<td>890</td>
<td>6.55</td>
<td>138</td>
</tr>
</tbody>
</table>
the diluted solution of rat liver, rat heart, rat kidney, and rat brain crystalline citrate synthase were added to each outer well. Diffusion was allowed to proceed at 4°C for 24 hours. On the stained plate, a single precipitin band was visible against all citrate synthases, and the precipitin bands were fused to each other at their ends, with no spur formation observed between the bands.

The ability of the antiserum prepared from the treated rabbit in the 5th week after injections to yield a precipitate with each enzyme was estimated as previously described (1). Approximately 10 μg of citrate synthase of rat kidney or rat brain were incubated with the indicated amount of the antiserum in 10 mM potassium phosphate buffer, pH 7.4, containing 0.85% sodium chloride at 4°C for 24 hours. The reaction mixture was centrifuged at 17,300 × g for 30 min and enzyme activity determined on an aliquot of the supernatant solution. The combining capacities of both enzymes with the antiserum were apparently identical, and their activities could be 90% precipitated with 40 μl of the antiserum (Fig. 1).

**Kinetic Studies**—The kinetic data of the forward and reverse citrate synthase reaction were examined with crystalline citrate synthase from rat kidney and with the enzyme from rat brain. Since the results with the two enzymes were the same within experimental error whenever compared, only the rat kidney enzyme was used for all kinetic studies. Variation of one substrate at a series of fixed levels of cosubstrate gave apparent Michaelis constants for acetyl-CoA and oxalacetate independent of the concentration of cosubstrate. In rat kidney synthase, the apparent $K_m$ for acetyl-CoA was 5.0 μM as determined with oxalacetate concentrations of 2.5, 5.0, and 10 μM (Fig. 2) and the apparent $K_m$ for oxalacetate was 4.5 μM as determined with acetyl-CoA concentration of 2.5, 5.0, and 10 μM (Fig. 3). The kinetic constants of acetyl-CoA and oxalacetate for rat brain citrate synthase were 4.8 and 5.0 μM, respectively. The kinetics for citrate and CoA in the reverse reaction was measured in the malate dehydrogenase-coupled system as previously described (11) at pH 6.1 and 8.1. The apparent $K_m$ for each substrate was essentially independent of the concentration of its cosubstrate at both pH values, the $K_m$ values for CoA were 32 μM at pH 6.1 and 39 μM at pH 8.1 (Fig. 4). The apparent $K_m$ values for citrate were 199 μM at pH 6.1 and 30 mM at pH 8.1 (Fig. 5).

The use of the 10-cm cell enabled us to examine a lower range of concentrations of acetyl-CoA and oxalacetate. In this range the $K_m$ for oxalacetate decreased only from 4.5 μM (Fig. 3) to 3.0 μM (Fig. 6) while the $K_m$ of acetyl-CoA dropped from 5.0 μM (Fig. 2) to 1.3 μM (Fig. 7). Careful repetition of this experiment confirmed that when the kinetics for acetyl-CoA was examined over the entire range of concentrations, the Lineweaver-Burk plot showed an increase in slope at high acetyl-CoA concentra-

![Fig. 1](image-url)

**Fig. 1.** Neutralization of rat kidney and brain citrate synthases by rabbit antiserum to the rat heart enzyme. The reactions were carried out in 0.1 M (final concentration) potassium phosphate buffer, pH 7.4, with varying amounts of antiserum and about 20 μg of recrystallized enzyme: kidney (O--O) and brain (O--O). Normal serum was used for the controls and no loss of activity was found. After 24 hours at 4°C, the precipitated protein was removed by centrifugation and the citrate synthase activity of the supernatant solution was determined by the DTNB method as described under "Experimental Procedure."

![Fig. 2](image-url)

**Fig. 2.** Initial velocity pattern of the kidney citrate synthase with acetyl-CoA (AcCoA) as the varied substrate. Oxalacetate concentrations: (O--O) 2.5 μM; (A--A) 5.0 μM; (W---W) 10.0 μM. Initial velocities are expressed as nanomoles of CoA-SH formed per min with 0.03 μg of enzyme protein per ml, under the conditions of assay as described in the text.

![Fig. 3](image-url)

**Fig. 3.** Initial velocity pattern of the kidney citrate synthase with oxalacetate (OAA) as the varied substrate. Acetyl-CoA concentrations: (O--O) 2.5 μM; (A--A) 5.0 μM; (W---W) 10.0 μM. Assays were carried out and the results presented as in the legend to Fig. 2.

![Fig. 4](image-url)

**Fig. 4.** Initial velocity pattern of the reverse reaction catalyzed by the kidney enzyme with coenzyme A as the varied substrate. Citrate concentrations: (O--O) 1 mM; (A--A) 2 mM; (W---W) 4 mM, and (O--O) 10 mM. The reaction mixture contained in a total volume of 1.0 ml, 100 μmoles of Tris-CI, pH 8.1, 50 μg of malate dehydrogenase, 20 μmoles of NADH. Initial velocities are expressed as nanomoles of oxalacetate formed per min with 300 μg of enzyme protein.

![Fig. 5](image-url)

**Fig. 5.** Initial velocity pattern of the reverse reaction catalyzed by the kidney enzyme with citrate as the varied substrate. Coenzyme A concentrations: (O--O) 20 μM; (A--A) 33 μM; (W---W) 50 μM, and (O--O) 100 μM. Conditions are the same as in Fig. 4.
Acetyl-CoA concentrations: (O-O) 0.5 μM; (Δ—Δ) 0.06 μM; (■—■) 1.0 μM; (▲—▲) 5 μM; and (●—●) 50 μM. The reaction was carried out in a cell with a 10-cm light path, containing in a total volume of 5.0 ml, 500 μmoles of Tris-Cl, pH 8.1, and 0.5 μmole of DTNB. The initial velocity is expressed as nanomoles of CoA-SH formed per min per ml with 0.06 μg of enzyme protein.

Fig. 7. Double reciprocal plots of the initial velocities for the kidney enzyme at low substrate levels with acetyl-CoA (AcCoA) as the varied substrate. Acetyl-CoA concentrations: (O-O) 0.5 μM; (Δ—Δ) 0.06 μM; (■—■) 1.0 μM; (▲—▲) 5 μM; and (●—●) 5.0 μM. This is a replot of the data of Fig. 6.

Fig. 8. Double reciprocal plots of the initial velocities for the kidney enzyme at low oxalacetate concentrations and low to medium acetyl-CoA (AcCoA) concentration with acetyl-CoA as the varied substrate. Oxalacetate concentration: (O-O) 0.9 μM; (Δ—Δ) 0.06 μM; (■—■) 1.0 μM; (▲—▲) 2.0 μM; and (●—●) 5.0 μM. Conditions are the same as in Fig. 6 with the exception of 0.15 μg of enzyme protein.

**Table III**

*Kinetic constants for rat kidney citrate synthase determined at various acetyl coenzyme A concentrations*

<table>
<thead>
<tr>
<th>Acetyl coenzyme A concentration range</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; [μM]</th>
<th>K&lt;sub&gt;a&lt;/sub&gt; [μM]</th>
<th>K&lt;sub&gt;b&lt;/sub&gt; [μM]</th>
<th>K&lt;sub&gt;10&lt;/sub&gt; [μM]</th>
<th>K&lt;sub&gt;1b&lt;/sub&gt; [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (&gt;50 μM)</td>
<td>3.36 ± 0.03</td>
<td>11.8 ± 0.9</td>
<td>4.8 ± 0.3</td>
<td>10 ± 5</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Medium (5-50 μM)</td>
<td>2.9 ± 0.1</td>
<td>5.0 ± 0.4</td>
<td>4.5 ± 0.5</td>
<td>5 ± 1</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>Low (&lt;5 μM)</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>2.9 ± 0.3</td>
</tr>
</tbody>
</table>

*Identifying subscripts: a, acetyl coenzyme A; b, oxalacetate, V<sub>max</sub> corrected for enzyme concentration.

* Nanomoles per 10<sup>−4</sup> g per min.
1/V versus 1/acetyl-CoA curved upward as if substrate inhibition by acetyl-CoA was occurring.

**Dissociation Constant for Oxalacetate**—When oxalacetate was used to protect against urea-unfolding of the enzyme then a plot of log $k_0 - k_1/k_2$ versus log oxalacetate yielded a straight line with a slope of 1 and an intercept (where log $k_0 - k_1/k_2 = 0$) of 5 μM (Fig. 14).

**DISCUSSION**

The form of the Lineweaver-Burk plots for the substrates of the citrate synthase reaction eliminates a ping-pong mechanism since the lines are all intersecting and indicates that we are dealing with a bireactant sequential mechanism. The most general mechanism one can write must include the following steps using the diagrammatic method of Cleland (18).

One can arbitrarily distinguish a number of bireactant sequential mechanisms depending upon the relative values of the dissociation constants involved. The fact that oxalacetate has little effect on the $K_m$ of acetyl-CoA and that acetyl-CoA has no effect on the $K_m$ for oxalacetate and that neither CoA nor citrate affect the $K_m$ of the other in the reverse reaction eliminates all sequential reactions except the Theorell-Chance and the random mechanisms (19). Fromm has shown in addition that in this situation a Theorell-Chance mechanism also has $V_{max} = V_{rand}$. Since the maximum velocities in the forward and reverse reaction are quite different, these substrate kinetic data indicate a random mechanism. A greatly simplified rate equation is obtained from the random mechanism if rapid equilibrium is assumed to exist for all steps except the interconversion of the ternary complexes. The rate equation is then

$$V = \frac{K_{A}K_{B} + K_{A} + K_{B} + K_{A}K_{B} + K_{A}K_{B} + K_{A}K_{B} + K_{A}K_{B} + K_{A}K_{B}}{K_{A}K_{B} + K_{A} + K_{B} + K_{A}K_{B} + K_{A}K_{B} + K_{A}K_{B} + K_{A}K_{B} + K_{A}K_{B}}$$

All Lineweaver-Burk plots would be linear and it is possible to calculate the dissociation constants for each of the binary complexes. In the special case where the lines intersect on the x axis then the Michaelis constant equals the dissociation constant and $K_m = K_{ei}$. Thus in the special case for citrate synthase the equilibrium constant for each substrate reacting with free enzyme is the same as the equilibrium constant for the substrate reacting with its binary complex (i.e. $K_{eb} = K_{si}$; $K_{ei} \cong K_e$; $K_{ei} \cong K_e$) (Table IV).

When we measured the effect of increasing acetyl-CoA concentration over a range of 0.1 $K_m$ (apparent) to 100 $K_m$ (apparent), we found that both $K_m$ (apparent) and $V_{max}$ (apparent) increased. It was not possible to study the entire range with one set of concentrations so that three ranges were studied 0.5 to 5 μM, 5 to 50 μM, and 50 to 500 μM. Each range gave apparently linear line segments, and in each region when oxalacetate was varied, lines in Lineweaver-Burk plots intersected on the x axis. When oxalacetate was varied over a similarly large range of concentrations, only a slight variation of its apparent $K_m$ was calculated.

Abrupt changes in the Lineweaver-Burk plots for several other enzymes have been reported (20-25). In a recent paper Engel and Ferdinand (26) have analyzed the general rate equation for a multisite enzyme to see what relation among constants must exist in order to generate abrupt transitions. Using a model in which both negative and positive cooperativity exists they were able to generate Lineweaver-Burk plots containing abrupt transition points in the slope. It is possible here in the case of rat
Fig. 13. a, Double reciprocal plots of the initial velocities of the reverse reaction for the kidney enzyme with CoA as the varied substrate, citrate as the fixed substrate (1 mM), and acetyl-CoA as the inhibitor. Acetyl-CoA concentrations: (O—O) 20 μM; (■—■) 10 μM; (▲—▲) 5 μM; and (●—●) no addition. The assay medium contained 100 mM imidazole acetate, pH 6.1, 20 μM NADH, 60 μg of malate dehydrogenase, 1 mM potassium citrate, 25 μg of citrate synthase (total volume 1 ml), and the indicated amounts of acetyl-CoA and CoA. b, Double reciprocal plots of the initial velocities of the reverse reaction for the kidney enzyme with citrate as the varied substrate, CoA as the fixed substrate (100 μM) and acetyl-CoA as the inhibitor. Acetyl-CoA concentrations: (O—O) 20 μM; (■—■) 10 μM; (▲—▲) 5 μM; and (●—●) no addition. The assay was carried out as described in a, except 100 μM CoA was used and citrate concentrations were varied.

TABLE IV

<table>
<thead>
<tr>
<th>Kinetic constant</th>
<th>Initial velocity</th>
<th>Product inhibition</th>
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<tbody>
<tr>
<td></td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>$K_a$</td>
<td>5.0 ± 0.4</td>
<td>5.3 ± 0.1$^b$</td>
</tr>
<tr>
<td>$K_{in}$</td>
<td>5 ± 1</td>
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</tr>
<tr>
<td>$K_b$</td>
<td>4.5 ± 0.5</td>
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<tr>
<td>$K_{in}$</td>
<td>4.5 ± 0.7</td>
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</tr>
<tr>
<td>$K_s$</td>
<td>30 ± 4</td>
<td>24.9 ± 1.6</td>
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<tr>
<td>$K_r$</td>
<td>50 ± 9</td>
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<tr>
<td>$K_d$</td>
<td>3000 ± 360</td>
<td>3758 ± 182</td>
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<tr>
<td>$K_{id}$</td>
<td>4300 ± 600</td>
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$^a$ Identifying subscripts: a, acetyl coenzyme A; b, oxalacetate; c, coenzyme A; d, citrate.
$^b$ All constants in Tris-HCl, pH 8.1, except the $K_i$ for acetyl-CoA which was determined at pH 6.1 in imidazole acetate.

TABLE V

<table>
<thead>
<tr>
<th>Source</th>
<th>$K_{ia}$ and $K_s$</th>
<th>$K_{ib}$ and $K_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig heart</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Mouse heart</td>
<td>4.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Hamster heart</td>
<td>3.3</td>
<td>3.3</td>
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</table>

$^a$ Identifying subscripts: a, acetyl coenzyme A; b, oxalacetate.
The computer program was not used in these determinations.

and Gulbinsky and Cleland (31) state that computer simulation of random mechanisms where ternary complex interconversion is not rate-limiting still give plots which are linear within experimental error, fitting Equation 4. In order to see clearly the nonlinear reciprocal plots Gulbinsky and Cleland (31) report that the unimolecular rate constants for release of substrates from the enzyme must be much smaller than the turnover number. Cleland (18) has pointed out that random mechanisms which yield curved Lineweaver-Burk plots are expected to become linear if the fixed substrate concentration is made saturating. When acetyl-CoA was varied at oxalacetate concentrations of 500 μM (100 × $K_m$) we observed linearity over the whole range. It is therefore possible that the nonlinearity of the acetyl-CoA plots is due to the fact that the rate constant for acetyl-CoA binding is too slow to be rate-limiting at the saturating level of the second substrate. The product inhibition pattern is different from this in that inhibition is present at

Segal et al. (28) and Dalziel (29) have pointed out that nonlinear plots are expected in a random mechanism under conditions where the rate-determining step is the rate of binary complex formation rather than the rate of ternary complex interconversion. Such a mechanism could account for both the apparent substrate activation with the rat enzyme and substrate inhibition with the pig heart enzyme. On the other hand, Schwert (30) citrate synthase which shows an abrupt change in slope for acetyl-CoA and also for the inhibition of acetyl-CoA by ATP (27) that such a mechanism with interacting sites may be operative. There is no other evidence concerning interacting sites on animal citrate synthase. Considering the similarity in the size and behavior of animal enzymes it is strange that the kinetic behavior of the pig heart enzyme is so different in this particular respect.

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Inhibition of citrate binding at the oxalacetate-citrate site and (b) the inhibition of citrate binding at the acetyl-CoA site. When $K_{SO_4}$ is used as an ionic strength control when citrate is tested as a product inhibitor, then noncompetitive kinetics was still observed but the intersection point is now on the $x$ axis. The $K_f$ for citrate against oxalacetate is 3.7 mM while the $K_m$ for citrate at the same pH is 3.2 mM and the $K_m$ and $K_f$ values for other substrates are in fair agreement ($K_m$ acetyl-CoA = 5 mM, $K_f$ acetyl-CoA = 5.3 µM; $K_m$ CoA = 39 µM, $K_f$ = 25 µM).

If we are dealing with a sequential mechanism, random or ordered, where $K_a = K_{ia}$, then $K_a$ should equal the $K_{diss}$ for each substrate. We have measured by an independent means the $K_{diss}$ of oxalacetate for rat kidney citrate synthase and found it to be 5 µM in good agreement with the kinetic prediction. In addition when $K_a$ for acetyl-CoA is measured in the reverse reaction in the range of 5 to 20 µM, a value of 5.3 is obtained in good agreement with the $K_m$ obtained in the forward direction. $K_m$ of CoA and $K_f$ for CoA are similarly in good agreement when the same concentration range is studied, i.e. 30 µM in the 10 to 50 µM range.

The kinetic data reported here are in agreement with the partial kinetics for citrate synthase reported by Shepherd and Garland (4) for a partially pure rat liver enzyme and that reported by Moriyama and Srere (1) for crystalline rat liver and rat brain citrate synthases. Similar kinetic data for acetyl-CoA and oxalacetate have been reported by Smith and Williamson (5) for a crystalline beef heart citrate synthase. These latter workers however found that CoA exhibited a mixed inhibition against acetyl-CoA and noncompetitive inhibition against oxalacetate while citrate was competitive with oxalacetate and non-competitive against acetyl-CoA. It is interesting to note that pig heart citrate synthase when tested at high acetyl-CoA and low oxalacetate concentrations yields a curved Lineweaver-Burk plot which could be interpreted as "substrate inhibition." With pig heart citrate synthase saturating concentrations of oxalacetate eliminate the "substrate inhibition." There is no need to postulate different or interacting enzyme sites in this case.

We have tested the Haldane relationship for the rat citrate synthase which takes the form here

$$K_{eq} = \frac{V_i K_i K_{id}}{V_i K_{ia} K_b}$$

and equals $1.8 \times 10^8$ at pH 8.1. Guynn et al. (33) have determined $K_{eq}$ for citrate synthase to be $1.1 \times 10^8$ at pH 7.0 which would correspond to $1.1 \times 10^7$ at pH 8. These values are in remarkable agreement and lend credence to the kinetic constants reported here.

The present data strongly support a random mechanism for animal citrate synthases in which the rate of acetyl-CoA binding to the enzyme (or binary complex) is less than the rate for interconversion of the ternary complexes or in which interacting sites occur. Studies on dead end inhibitors support the general mechanism but a final decision should await experiments testing the exchange rates at equilibrium.

Recently Weidman and Drysdale (34) reported that oxalacetate affects the binding of a spin label analog of acetyl-CoA to pig heart citrate synthase. Their results strongly suggest an ordered mechanism for citrate synthase. One difference between our experiments is that their experiments are performed at higher enzyme concentrations than used here, but it does not seem likely that these differences are due to that factor.

1 Y. Matsuoka and P. A. Srere, unpublished results.
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