Radiometric Assay of Citrate Condensing Enzyme (Citrate Synthetase) in Liver Tissue and Mitochondria

Padminak K. Dixit and Raul Cuestas

Citrate synthetase activity was determined in samples of commercially available citrate condensing enzyme. We measured the amount of radioactive citrate formed from the condensation of carbon-14 labeled acetyl CoA and oxaloacetate. The labeled citrate was oxidized by oxidative bromination; $^{14}\text{CO}_2$ evolved was collected and represented the amount of citrate synthesized. Enzyme activity as low as $10^{-4}$ $\mu$mol/min was measured reproducibly with this procedure. Results obtained with this assay compared closely with those of a widely used spectrophotometric assay in which Elman's SH-coupling reagent [5,5-dithio-bis-(2-nitrobenzoate)] is coupled with CoA released during the reaction. Accuracy of labeled citrate determination in the presence of labeled acetate or pyruvate was excellent. The method can be used to determine citrate synthetase activity in skeletal muscle, liver, and isolated liver mitochondria, or to study the fate of labeled citrate injected into animals.

Additional Keyphrases: diagnosis of diabetes, rickets • citrate metabolism • acetyl CoA

Activity of citrate synthesizing enzyme (citrate synthetase, citrate condensing enzyme)\textsuperscript{1} has been assayed by several techniques (1). Assay methods depend on the disappearance of acetyl CoA or oxaloacetate, or the formation of citrate or coenzyme A.

Here we describe a radiometric assay method in which labeled citrate formed during the enzymatic reaction is determined quantitatively. Because it has been documented that citrate synthetase activity is altered in animal tissues after the induction of diabetes (2, 3) and rickets (4, 5), measurement of the enzyme activity may be a useful diagnostic indicator.

\textsuperscript{1}Citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7.

Materials and Methods

Reagents

\[1,5^{-14}\text{C}]\text{Citric acid} \text{ (New England Nuclear Corp.; sp. act. about 6 Ci/mol).} \]

Carrier citric acid solution, prepared by dissolving 1 mg of anhydrous sodium citrate in 1 ml of 4.5 mol/liter sulfuric acid.

\textit{Potassium bromide} (KBr), 2 mol/liter.

\textit{Potassium permanganate} (KMnO$_4$), saturated solution.

\textit{Hydrogen peroxide} (H$_2$O$_2$), 6% solution.


\textit{Scintillation fluid}, “Omnifluor” (New England Nuclear Corp., 575 Albany St., Boston, Mass. 02118), 4.0 g in 1 liter of toluene.

\textit{Citrate condensing enzyme} (Calbiochem, 10933 N. Torrey Pines Road, La Jolla, Calif. 92037).

\[1^{-14}\text{C}]\text{Acetyl CoA} \text{ (New England Nuclear Corp.; sp. act. about 50 Ci/mol), diluted so that the solution contained 10 mCi/liter.} \]

Carrier acetyl CoA solution, 5.26 mmol/liter (Nutritional Biochemicals Corp., 26201 Miles Road, Cleveland, Ohio 44128).

\textit{Oxaloacetic acid}, 0.1 mol/liter.

\textit{Phosphate buffer}, pH 7.0, 0.1 mol/liter.

Principle of the Assay

The assay is based on the ability of citrate condensing enzyme to synthesize labeled citrate by combining oxaloacetate with \[1^{-14}\text{C}]\text{acetyl CoA;} the citrate thus formed is labeled at the C-1 position as shown:


\[
\begin{align*}
\text{O} & \quad \text{COOH} \\
\text{CH}_3\text{--}*\text{S}\text{--CoA} + \text{CH}_2\text{COOH} & \quad \text{C}=\text{O} + \text{H}_2\text{O} \leftrightarrow \\
\text{[1-^{14}C]acetyl CoA} & \quad \text{oxaloacetate} \\
\text{CH}_2 & \quad \text{OH} \quad \text{COO} + \text{CoASH} + \text{H}^+ \\
\text{citric acid} & \quad \text{[1-^{14}C]citrate}
\end{align*}
\]

The labeled citrate is then determined by oxidation to pentabromacetone in an acid medium as described by Ettinger et al. (6). The labeled carboxyl group, liberated as \(^{14}\text{CO}_2\), is collected and counted. It is believed that the oxidation of the citric acid to pentabromacetone occurs as follows (7).

\[
\begin{align*}
\text{O} & \quad \text{COOH} \\
\text{CH}_2 & \quad \text{COOH} \\
\text{OH} \quad \text{COO} + \text{CoASH} + \text{H}^+ & \quad \text{[1-^{14}C]citrate}
\end{align*}
\]

Results

Oxidation and Quantitative Recovery of Labeled Citrate

Because the radioassay of citrate synthetase depends on the quantitative determination of labeled citrate formed during incubation of the enzyme with appropriate substrates such as labeled acetate, acetyl CoA, or pyruvate, we considered it necessary to determine the percentage recovery of labeled citrate in the presence of various substrates. Known amounts of \([1,5-^{14}\text{C}]\text{citrate}\) were added to specially designed incubation vials that have a side arm, as described earlier (2). The amounts of radioactive citrate added to the vials ranged between 0.01 and 0.10 \(\mu\text{Ci}\). Carrier citric acid solution, 0.5 ml, was added to the vial, followed by 0.50 ml of 4.5 mol/liter \(\text{H}_2\text{SO}_4\) and 0.50 ml of 2 mol/liter \(\text{KBr}\). The side arm of the vial was closed by a constriction rubber stopper, and the mouth of the vial was closed by a solid rubber stopper containing a center hole that was sealed by another constriction rubber stopper. A small beaker was suspended from the solid rubber stopper by steel wires. The whole assembly is shown in Figure 1.

A partial vacuum was created in the vial by evacuating some air with a syringe and needle. This step was needed because, during the subsequent steps involving oxidation, considerable pressure is likely to be built up, which may blow out the stoppers. Hyamine, 0.5 ml, was delivered into the suspended beaker with a syringe and needle, and 0.75 ml of \(\text{KMnO}_4\) solution was added through the side arm into the contents of the vial. All the vials were incubated at 10 °C in a Dubnoff metabolic shaking incubator at 30 oscillations per minute for 10 min. The contents of the vial were decolorized by adding 6% \(\text{H}_2\text{O}_2\), about 0.6 ml, through the side arm, and the mixture was incubated for 30 min in the shaker bath. The carbon dioxide evolved was collected by the Hyamine solution, which was then transferred quantitatively with scintillation fluid to a counting vial and its radioactivity measured in a counter (Nuclear Chicago, Unilux II model) at about 65% efficiency.

Known amounts of radioactive citrate were added directly to separate counting vials, and the total radioactivity was measured. Recovery of citrate after
oxidation was calculated as follows: % recovery = radioactivity (cpm) collected as $^{14}$CO$_2$ after oxidation of known amounts of citrate × 100/total radioactivity (cpm) present in the same amount of citrate. As shown in Table 1, recovery of $^{14}$CO$_2$ from the added 1,5-14C-labeled citrate was between 85 and 99%.

Citrate is synthesized in the tissues by condensation of oxaloacetate with acetyl CoA formed from either acetate or pyruvate. Where acetate or pyruvate are used, the assay is based on the colorimetric determination of citrate as pentabromacetone (2, 4, 5). In the radiometric assay procedure it is essential to determine whether the presence of labeled acetate or pyruvate would affect the quantitative recovery of $^{14}$CO$_2$ from the labeled citrate formed. Known amounts of sodium salts of [1-14C]acetate or [2-14C]pyruvate were therefore added to the vials containing radioactive citrate and oxidation was carried out as described above.

| Table 1. Recovery of $^{14}$CO$_2$ from the Oxidation of [1,5-14C]Citrate |
|------------------|------------------|------------------|------------------|
| Citrate, μCl    | No. samples      | cpm added        | No. samples      | cpm recovered   | Recovery, %   |
| 0.01            | (8)              | 13151 ± 737d     | (10)             | 12969 ± 868d    | 99            |
| 0.02            | (12)             | 24299 ± 1787     | (12)             | 23000 ± 969     | 95            |
| 0.05            | (10)             | 69563 ± 1933     | (7)              | 65024 ± 2206    | 94            |
| 0.08            | (11)             | 101524 ± 1791    | (10)             | 95906 ± 4501    | 95            |
| 0.10            | (12)             | 136678 ± 1070    | (10)             | 117031 ± 3805   | 85            |

Additions of labeled and carrier citrate are described in the text. Appropriate blanks were determined and subtracted.

The results (Table 2) show that the presence of [1-14C]acetate did not greatly affect the recovery of carbon-14 from the radioactive citrate. About 3% of labeled acetate carbon was collected as $^{14}$CO$_2$. After subtracting the acetate blanks, the recovery of citrate ranged between 93 and 101%. In the presence of [14C]pyruvate, net citrate recovery was 80 to 96%; about 7% of the [14C] from pyruvate was collected as $^{14}$CO$_2$. When labeled α-ketoglutarate (an intermediate compound in the Krebs cycle formed from the oxidation of citrate) was added to labeled citrate, recovery of CO$_2$ from [14C]citrate was 101–113%. About 75% of the labeled α-ketoglutarate was oxidized to $^{14}$CO$_2$ and collected in the blanks. In the presence of this compound therefore, small amounts of citrate cannot be satisfactorily recovered. In the radio assay of citrate synthetase, radiolabeled acetyl CoA is first incorporated into labeled citrate that on subsequent oxidation gives rise to labeled α-ketoglutarate. The $^{14}$CO$_2$ that is evolved during the enzyme reaction thus comes from citrate and (or) α-ketoglutarate and can be considered as a true measure of labeled citrate synthesis. Therefore, α-ketoglutaric acid formed from glutamic acid would not influence the radioassay of citrate synthetase.

Radiometric Determination of Citrate Condensing Enzyme Activity

The following solutions were added to the incubation vessel: 0.10 ml of carrier acetyl CoA, 0.10 ml of oxaloacetic acid, 0.30 ml of phosphate buffer, 20 μl of [1-14C]acetyl CoA, and variable amounts of citrate condensing enzyme. These additions were made in the order described, always keeping the reagents and incubation vessels on crushed ice. After the enzyme was added, the vessels were swirled rapidly and incubated in the Dubnoff shaker bath at 27 °C for 15 min. The enzymatic reaction was stopped by the addition of 0.5 ml of 4.5 mol/liter H$_2$SO$_4$, and the radioactive citrate formed was oxidized and $^{14}$CO$_2$ was collected and counted as described above. As a blank, a vial containing no enzyme was similarly treated. The radioactivity of the labeled acetyl CoA was determined in vials to which known amounts of this substrate were added.

The citrate condensing enzyme activity was determined in rat skeletal muscle and liver homogenates and also in liver mitochondria. For all these experiments, normal white rats fed ad libitum were killed.
by decapitation and pieces of gastrocnemius muscle and liver were excised rapidly, weighed, and placed in chilled ground-glass homogenizers. The homogenates were prepared in ice-cold isotonic saline solution to a final concentration of 30 mg of fresh weight of tissue per milliliter. Various volumes of homogenates were added to incubation flasks containing 175 nmol of unlabeled acetyl CoA, 2 nmol (0.1 μCi) of radioactive acetyl CoA, 10 μmol of oxaloacetic acid, and enough phosphate buffer to make a final volume of 1.0 ml. The final osmolality of the mixture was about 40 mmol. The flasks were incubated for 15 min, the reaction was stopped, and the labeled citric acid that had formed was determined. The blank vials, which contained no tissue homogenate, were treated likewise and the radioactivity collected was subtracted from the counts for those containing tissue homogenates. Table 3 shows the activity of the enzyme in the samples of rat liver and skeletal muscle tissues. The liver mitochondria from fed rats were isolated by a procedure in which 0.25 molar sucrose solution is used (8) and washed twice with the sucrose solution by suspension and recentrifugation. They were then resuspended in a known volume of the sucrose solution for radioassay of citrate synthetase determination.

Calculations

Enzyme activity was calculated as follows:

\[
\text{Enzyme activity (μmol/min) = } \frac{\text{cpm recovered}}{\text{specific activity} \times \text{incubation time (min)}}
\]

Specific activity (cpm/μmol) =

\[
\text{cpm added/μmol [14C]-acetyl CoA + μmol carrier acetyl CoA}
\]

The radiometric assay described here was compared with a well-known colorimetric assay for condensing enzyme activity (9), in which Ellman’s reagent [5,5'-dithio-bis-(2-nitrobenzoate) (DTNB)] is used. The radiometric assay and the colorimetric DTNB procedure were compared by using a series of concentrations of the citrate condensing enzyme ranging from 138 to 500 μg/liter. Figure 2 shows a close correlation between the results obtained with the two methods.

A similar close relationship between the two methods was also noted when mitochondrial suspensions were used. Figure 3 depicts the results of a typical experiment.

Use of the Citrate Oxidation Method to Study
the Fate of Injected Citrate in vivo

Four normal male rats weighing about 200 g were anesthetized with sodium pentobarbital and injected intravenously with [1,5-14C]citric acid (50 μCi/kg body weight in 0.2 ml solution in saline, pH 4.5). Blood was collected from the animals by cardiac puncture before sacrifice at 5, 15, 30, and 60 min and the liver, kidney, and heart tissues were removed.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fresh tissue, mg</th>
<th>14CO2 cpm/min of incubation</th>
<th>Activity, μmol/min</th>
<th>Specific activity, (μmol/min per mg fresh tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal</td>
<td>0.60</td>
<td>4260</td>
<td>0.00312</td>
<td>0.0052</td>
</tr>
<tr>
<td>muscle</td>
<td>1.20</td>
<td>7440</td>
<td>0.00544</td>
<td>0.0046</td>
</tr>
<tr>
<td>Liver</td>
<td>0.60</td>
<td>3270</td>
<td>0.00239</td>
<td>0.0040</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>6830</td>
<td>0.00500</td>
<td>0.0042</td>
</tr>
</tbody>
</table>

Samples of the tissues were quickly frozen in liquid nitrogen, weighed on an analytical balance while still frozen, and homogenized with 4.5 mol/liter H2SO4 (1 g of tissue per 2.5 ml of acid). Replicate 0.5-ml samples of each tissue homogenate were placed in incubation vials for citrate oxidation as described earlier. Likewise, the radioactive citrate contained in 100 μl of blood and serum was determined.

The amount of radioactivity contained in 1 g of tissues or 1 ml of the biological fluids was determined (Table 4). At the various time periods mentioned, most of the radioactivity in blood appeared to be in the serum. In contrast, kidney contained 64% of the radioactivity present in blood while liver and heart contained only about 10-11%. At 15 min
the radioactivity present in the kidney was appreciably greater than in blood (153%) and was threefold at 30 and 60 min after the injection—not surprising in view of the excretory role of the kidneys. Radioactivity in the liver and heart never exceeded that present in the whole blood, although the relative amounts were increased at 15, 30, and 60 min after the injection.

Discussion

The method most commonly used to determine citrate condensing enzyme activity is that described by Srere et al. (9), in which acetyl CoA and oxaloacetate are added as substrates for the synthesis of citrate. The CoA released during this reaction immediately forms a yellow product with DTNB. The assay is based on the rate at which the colored product is formed.

The radiometric assay is based on the condensation of oxaloacetate with labeled acetyl CoA. The labeled citrated formed is then oxidized to the pentabromoacetone and the labeled carbon dioxide collected. Thus, this method combines the enzymatic procedure of Srere et al. (9) with the oxidation method of Ettinger et al. (6). Whereas the colorimetric method requires recording spectrophotometers to study the rate of the enzymatic reactions, the radiometric assay can be carried out in laboratories where liquid scintillation counters are available.

Determination of citrate synthetase activity by using added acetyl CoA may not necessarily furnish a true status of the citrate-condensing capacity of the tissues. As shown in Figure 4, formation of acetyl CoA from its precursors (i.e., pyruvate or acetate) requires the presence in the tissues of many cofactors (e.g., thiamine pyrophosphate, lipoic acid, and coenzyme A) and enzymes (e.g., pyruvic oxidase, acetyl kinase, and transacetilase). Use of acetyl CoA in the reaction mixture, therefore, bypasses the earlier steps and the results will only reflect the status of tissue condensing enzyme activity, not the overall ability of the tissue to synthesize citrate from glucose metabolite(s). In the tibial epiphysis cartilage of rachitic rats (4) and dogs (5), appreciably less citrate is formed from oxaloacetate and acetate than in cartilage obtained from vitamin D-treated rachitic animals. However, it is likely that citrate synthetase activity of the rachitic cartilage may not really be lessened. In the absence of vitamin D, the rachitic cartilage may be deficient in the cofactor(s) or enzyme(s) other than citrate synthetase. Use of [1-14C]acetate or [2-14C]pyruvate would perhaps prove more useful in clarifying this point.

It was recently reported that citrate synthetase activity (as determined by the colorimetric assay procedure in which acetyl CoA is used) of the muscle mitochondria from exercised rats is twice that of sedentary animals (10), indicating a significant increase in the capacity of the exercised muscles to generate anerobically the high-energy compound adenosine triphosphate (ATP). Whether this increased citrate synthetase activity causes a commensurate increase in the overall citrate-synthesizing ability of the tissue is worthwhile investigating by using labeled pyruvate or acetate combined with the radiometric assay procedure.

Our radiometric assay is easy, and the results compare favorably with those of the time-honored colorimetric method of Srere et al. (9). With the availability of labeled substrates of higher specific activity, combined with longer periods of incubation, the sensitivity of the assay may be further improved.

Because the radiometric assay is based on measurement of the labeled citrate formed, another possible use for this enzymatic procedure is to study the distribution and fate of injected citrate in the various tissues and body fluids in disease conditions such as diabetes mellitus. That such a study is feasible is demonstrated in Table 4, which depicts the distribution of labeled citrate in animal tissues at various times after injecting it. Although the radioactivity that was recovered as 14CO2 in this experiment was

---

**Table 4. Distribution of 1,5-14C-Labeled Citrate in Rat Tissues after Intravenous Injection**

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Time after Injection, min</th>
<th>Counts per minute per gram fresh weight tissue (per ml of biological fluid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>5</td>
<td>113,890, 37,420, 7,540, 4,340</td>
</tr>
<tr>
<td>Serum</td>
<td>15</td>
<td>251,690, 65,870, 15,940, 6,480</td>
</tr>
<tr>
<td>Liver</td>
<td>30</td>
<td>13,095, 10,665, 5,230, 2,610</td>
</tr>
<tr>
<td>Kidney</td>
<td>30</td>
<td>73,130, 57,380, 23,360, 13,425</td>
</tr>
<tr>
<td>Heart</td>
<td>60</td>
<td>12,010, 6,370, 3,135, 2,670</td>
</tr>
</tbody>
</table>

*Note:* Dose injected = 0.05 μCi/g body weight.

**Fig. 4.** The enzymatic reactions leading to the formation of acetyl CoA and citric acid from acetate or pyruvate. [Note: Formation of acetyl phosphate occurs only in bacteria]
most probably derived from unaltered citrate, it may be that a portion of the label came from a metabolite of citrate.

This investigation was supported by Research and Training Grants AM-6517 and AM-5127, from the NIAMD, NIH U.S.P.H.S. We thank Mrs. Gayle Walz for technical assistance.

References