Purification and Properties of Citraconase*

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SUMMARY

The enzyme citraconase (citramalate hydro-lyase, citraconate hydratase, EC 4.2.1) which catalyses the hydration of citraconate to levorotatory citramalate has been purified about 25-fold from an initial specific activity of 0.2 to a final specific activity of 5.0.

The enzyme requires activation by a sulfhydryl compound (such as reduced glutathione, cysteine, thioglycollate, or mercaptoethanol) and ferrous ions for maximal activity; ascorbate has no activating effect.

The activation is carried out by preincubation of the enzyme and cofactors anaerobically at room temperature. Only ferrous ions, among the metallic ions tested, activate citraconase in conjunction with glutathione. Cupric and mercuric ions inhibit the enzyme activity under similar conditions to the extent of about 80%.

The optimum pH for citraconase activity is 7.5 and the activity falls off markedly on both sides of the optimum.

The partially purified citraconase preparation does not act on other substrates such as mesaconate, maleate, fumarate, cis- and trans-aconitate. It does not contain any other related enzymic activity except very low isocitrate dehydrogenase activity.

The $K_m$ for citraconate at pH 7.5 and 30° is 1.1 mm.

Metal-complexing agents (1 mm) such as pyrophosphate, ethylenediaminetetraacetate, o-phenanthroline, 8-hydroxyquinoline and $\alpha,\alpha'$-bipyridyl have a pronounced (99%) inhibitory effect on the enzyme (in the absence of reduced glutathione). In the presence of 0.5 mm reduced glutathione the inhibition by these agents is of the order of 74% (indicating that glutathione perhaps supplies Fe$^{++}$).

Sulfhydryl reagents such as iodoacetate and $p$-hydroxymercuribenzoate markedly inhibit citraconase activity (98%).

It has been reported earlier that the enzyme citraconase which catalyzes the hydration of citraconate to levorotatory citramalate is present in a pseudomonad isolated by enrichment on citraconate (1). The enzyme is distinct from mesaconase of Clostridium tetanomorphum (2) and Pseudomonas fluorescens (3).

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Mesaconase which catalyzes the formation of (+)-citramalate from mesaconate has been considerably purified and its properties studied (4). The present communication describes the partial purification of citraconase and some of its properties.

EXPERIMENTAL PROCEDURE

Materials

The different chemicals were obtained from sources mentioned: $p$-hydroxymercuribenzoate from Sigma; streptomycin sulfate from the Hindusthan Antibiotics Ltd., Pimpri; DEAE-cellulose (Cellex-D, 0.62 meq per g) from Bio-Rad Laboratories; cysteine from E. Merck, Darmstadt; ascorbic acid was prepared in this laboratory; EDTA and o-phenanthroline were from the British Drug Houses, Ltd.

Calcium phosphate gel was prepared according to the method of Swingle and Tiselius (5). Glass-distilled water was used for the preparation of all solutions required for enzyme work. Citraconic and mesaconic acids were prepared from citric acid according to the procedure of Shriner, Ford, and Roll (6).

Methods

Growth of Cells—The medium for the growth of cells and maintenance of the culture contained the following components per liter: citraconic acid adjusted to pH 7 with KOH, 5 g; ammonium nitrate, 2 g; KH$_2$PO$_4$, 10 g; MgSO$_4$.7H$_2$O, 0.5 g; and FeCl$_3$.6H$_2$O, 10 mg. The phosphate solution was adjusted to pH 7 with KOH, sterilized separately, and added to the rest of the solution just before inoculation. Sterilization was carried out by autoclaving at 121° for 15 min. The inoculum was built up as follows. The cells from an agar slant (not more than 15 days old) were inoculated into 125 ml of the medium in a 500-ml Erlenmeyer flask which was then incubated at 30° on a rotary shaker. After 18 to 20 hours of growth, 1.5-ml aliquots were inoculated into 125 ml of medium in each of several flasks and growth was allowed to proceed as before for 12 to 15 hours corresponding to the end of the log phase. The fermented broth was then pooled and chilled. The cells were harvested by centrifugation in a Sharples supercentrifuge and washed with ice-cold distilled water. Cells were used fresh or stored at −15° until required.

Preparation of Cell-free Extracts—Cell-free extracts were prepared by subjecting a suspension of 5 g of packed cells, wet weight (equivalent to 1 g, dry weight), in 40 ml of cold 0.02 M potassium phosphate buffer (pH 7) to sonic oscillations in a...
Raytheon sonic oscillator (200 watts, 10 kr) for 15 min at 5°.
The sonic extract was centrifuged at 0-5° and 10,000 x g for 20
min. The supernatant solution, containing about 10 to 12 mg
of protein per ml, was worked up for purification.

**Analytical Methods**—Protein in crude unfractionated cell-free
extracts was determined with the Folin phenol reagent (7) with
crystalline serum albumin as the standard. The optical method
(8) was used to determine the protein content of purified frac-
tions. Measurements of pH were made with the glass electrode
on a Beckman, model G, pH meter or a Toshniwal (Polymetron)
pH meter. For spectrophotometric readings, 3-ml cuvettes with
1-cm light path were used. The sedimentation pattern of
enzyme fractions was determined in the Spinco, model E, ultra
centrifuge.

**Citraconase Assay**—Citraconase activity was measured by
incubating for 10 min at 30°, a reaction mixture (volume, 1 ml)
containing the activated enzyme (the activation procedure is
described later), 100 pmoles of Tris-HCl (pH 7.5), and 5 /Imoles
of potassium citraconate. The reaction was stopped by addition
of 1cm light path were used. The sedimentation pattern of
protein per ml, was worked up for purification.

**Material and Methods**—Purification of Citraconase

### Table I

<table>
<thead>
<tr>
<th>Age of extract</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before activation</td>
</tr>
<tr>
<td>Fresh sonic extract</td>
<td>0.20</td>
</tr>
<tr>
<td>Sonic extract after storage for 96 hours at -15°</td>
<td>0.07</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Oxygen relationship</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabolic (evacuated)</td>
<td>0.90</td>
</tr>
<tr>
<td>Aerobic (not evacuated)</td>
<td>0.33</td>
</tr>
<tr>
<td>Zero time control</td>
<td>0.15</td>
</tr>
</tbody>
</table>

The sonic extract was centrifuged at 0-5° and 10,000 x g for 20
min. The supernatant solution, containing about 10 to 12 mg
of protein per ml, was worked up for purification.

Three reaction mixtures of identical composition were used.
The reaction mixture (1 ml) contained 0.1 x Tris-HCl (pH 7.5),
0.2 ml of (2.25 mg of protein) partially purified enzyme stored at
-15°, 10 mm reduced glutathione, and 1 mm FeSO₄. Thunberg
tube containing the reaction mixture was evacuated for 3 min at
a water pump after addition of all components while a similar
tube was left exposed to air. The third tube was a zero time
control. The components were incubated for 1 hour at 30°, and
the enzyme activity was assayed in an aliquot of 0.05 ml as de-
scribed in the text.

### Results

**Activation of Citraconase**

While the present work was in progress, Blair and Barker (4)
reported that apparently inactive or feebly active mesaconase
preparations were, under certain conditions, activated by prior
anaerobic incubation in the presence of a sulfhydryl compound
and iron ions. The activation was dependent upon anaero-
biosis and the period and temperature of incubation. Since
citraconase preparations lost about 70% of their activity on
storage for 4 days, experiments were carried out to determine
whether citraconase could also be activated under similar
conditions.

The effect of activation on the age of the enzyme extract was
first determined with samples of both fresh sonic extract and one
stored for 4 days at -15°. In the experiment reported in Table
I, it is observed that the activity diminishes with the age of the
preparation but is restored by activation under the conditions
described here.

The necessity for anaerobic conditions for the activation of the
citraconase is illustrated by the experiment reported in Table II.
The results show that under anaerobic conditions there is a
3-fold increase in activity.

The effect of temperature on the activation process was inves-
tigated. The activation was carried out at two temperatures, 0°
in an ice bath) and at 30° (in a thermostat bath) under anaero-
bic conditions. The specific activity was 0.38 and 0.90 after
incubation for 1 hour at 0° and 30° respectively.

The influence of the period of incubation on the activation of
the enzyme is shown in Fig. 1. The specific activity of the
enzyme was 0.9 after 1 hour of anaerobic incubation at 30°, and
it did not increase further after longer periods of incubation.

On the basis of the above results, citraconase preparations
were routinely activated prior to assay by anaerobic incubation
for 1 hour at 30° in the presence of 100 mm Tris-HCl (pH 7.5),
10 mm GSH, and 1 mm ferrous sulfate. The amount of enzyme
used for activity determinations contained 0.1 unit or less after
activation.
Purification of Citraconase

All operations were conducted at 0-5°C. The procedures for large scale growth of cells and for preparation of cell-free extracts have already been described under "Experimental Procedure."

Treatment with Streptomycin Sulfate—The crude cell-free extract was diluted with 0.02 M potassium phosphate buffer (pH 7) to a protein concentration of 10 mg per ml. To the diluted extract, solid streptomycin sulfate was added such that the final concentration of streptomycin sulfate was 1%. Streptomycin sulfate was added in small quantities while the enzyme solution was stirred mechanically. The solution was stirred for an additional 10 min after the addition of streptomycin sulfate and the mixture was allowed to stand for 30 min. It was then centrifuged for 30 min at 12,000 × g and the precipitate was discarded. Preliminary experiments showed that much of the nucleic acid in the crude extract is removed at 1% streptomycin sulfate concentration with a loss of about 10% of citraconase activity.

Ammonium Sulfate Fractionation—Powdered ammonium sulfate (22.52 g) was added slowly with mechanical stirring during the course of 15 to 20 min to every 100 ml of streptomycin sulfate extract to give a 0.40 ammonium sulfate saturation. A pH of about 7 was maintained during this operation by periodic dropwise addition of 2 N NH₄OH. The solution was stirred for an additional 10 min and allowed to stand for 30 min before being centrifuged for 30 min at 12,000 × g. The precipitate was discarded. Then 18.65 g of ammonium sulfate were added to every 100 ml of the supernatant solution to bring the concentration to about 0.70 saturation. The precipitate which contained most of the enzyme was separated by centrifugation at 12,000 × g for 30 min and dissolved in 10 to 15 ml of 0.02 M potassium phosphate buffer (pH 7). This fraction had a specific activity of 0.4.

Calcium Phosphate Gel Treatment—To the dialyzed solution of 0.40 to 0.70 saturated ammonium sulfate fraction, a sufficient quantity of a suspension (70 mg, dry weight, per ml) of calcium phosphate gel was added to give a ratio of 5 mg of gel per mg of protein and the total volume was made up with water to twice the volume of ammonium sulfate fraction. The mixture was gently stirred for 5 min, allowed to stand for 15 min, and then centrifuged. Preliminary experiments showed that the gel does not adsorb the enzyme under the conditions tested. However, some inert proteins are removed by this procedure and the specific activity increases from 0.4 to about 0.55.

Refractionation with Ammonium Sulfate—The gel supernatant obtained from the previous step was next refractionated into 0 to 0.4, 0.4 to 0.5, 0.5 to 0.7, and 0.7 and 0.9 saturation fractions at pH 7 by successive addition of solid powdered ammonium sulfate. In the 0.5 to 0.7 saturation fraction (specific activity 0.9), 50 to 55% of the original activity was present. This fraction (16 ml) was dialyzed for 8 to 10 hours with stirring against 200 volumes of 0.01 M potassium phosphate buffer (pH 7) with three changes of the buffer.

DEAE-cellulose Column Chromatography—A DEAE-cellulose column was prepared as described by Peterson and Sober (15). The dialyzed enzyme was applied to a column (28 × 2 cm) of DEAE-cellulose which had been equilibrated against 0.01 M potassium phosphate buffer (pH 7) under slight (1 to 2 p.s.i.) pressure. Then, 2 ml of 0.01 M potassium phosphate buffer (pH 7) were passed twice through the column after the adsorption of the enzyme. The enzyme was then eluted from DEAE-cellulose by convex exponential gradient elution (10) with 250 ml of 0.01 M potassium phosphate buffer (pH 7) in the mixing chamber and 250 ml of 0.5 M phosphate buffer (pH 7) in a separatory funnel. The rate of addition of strong buffer and rate of elution were the same. Fractions of 4 ml were collected at 10-min intervals. The absorbance at 280 nm and the citraconase activity were determined in the effluent. Fractions 25 to 35 which contained citraconase activity were pooled and the enzyme was precipitated by the addition of powdered ammonium sulfate to 0.9 saturation. The precipitate was spun down at 12,000 × g for 30 min and dissolved in a small volume of 0.05 M potassium phosphate buffer, pH 7.0. The specific activity after DEAE-cellulose chromatography is about 3.

Fractionation with Ammonium Sulfate—To the enzyme solution from the previous step, a solution of neutral ammonium sulfate was added dropwise with stirring. The fraction precipitating between 0.5 and 0.65 saturation was collected and, dissolved in a small volume of 0.01 M potassium phosphate buffer, pH 7. The specific activity of this fraction was about 4.6 to 5.0.

Table III summarizes the results of the purification experiments and shows that the enzyme has been purified 23- to 25-fold in 22% yield. The final fraction (Fraction 7 in Table III) was examined in the Spinco, model E, analytical ultracentrifuge and it was observed that the enzyme preparation was not homogeneous and contained two other components.

Properties of Partially Purified Citraconase

The properties of citraconase were studied with the enzyme preparation of specific activity 4.6 (Fraction 7 in Table III).

Effect of pH on Enzyme Activity—The enzyme has maximum activity at pH 7.5 (Fig. 2). The activity falls off markedly on both sides of the optimum pH; there is only 43% and 29%

![Fig. 1. Effect of period of activation on citraconase activity. The reaction mixtures contained in 1 ml: Tris-HCl buffer (pH 7.5), 100 μmoles; enzyme, 0.2 ml (2.25 mg of protein); GSH, 10 μmoles; and ferrous sulfate, 1 μ mole. Thunberg t tubes containing the reaction mixtures were evacuated for 3 min, incubated for different time intervals as indicated and aliquots (0.05 ml) were removed for determination of citraconase activity as described in the text.](https://journals.aps.org/jbc/abstract/10.1073/jbc.239.8.2369)
activity at pH 6.5 and 8.5, respectively, as compared to that at pH 7.5.

**Substrate Specificity**—The enzyme preparation was tested for activities toward mesaconate, maleate, fumarate, cis-aconitate, and trans-aconitate. The activities were tested as described under "Methods." It is observed (Table IV) that the partially purified citraconase is essentially free from the other activities which were present in the original extract. The enzyme preparation was tested for the presence of lactate dehydrogenase, NADH oxidase, L-malate dehydrogenase, NADP-isocitrate dehydrogenase, and citrase as described under "Methods." Lactate dehydrogenase, NADH oxidase, malate dehydrogenase, and citrase were not detectable. Isocitrate dehydrogenase (specific activity 0.01) was present in low concentration.

**Effect of Substrate Concentration on Enzyme Activity**—The saturation of the enzyme by the substrate was investigated in the range of 0.5 to 10 mM (Fig. 3). The maximal activity is attained at 5 mM citraconate concentration. Citraconate concentrations of the order of 20 mM are not inhibitory but high concentrations such as 0.1 to 1 M are inhibitory. The \( K_m \) at pH 7.5 and 30° is \( 1.1 \times 10^{-3} \) M.

Attempts to show a reverse reaction (formation of citraconate from levorotatory citramalate) were not successful under a variety of conditions.

**Cofactor Concentration and Enzyme Activity**—The effect of cofactor concentration on enzyme activation was studied first by varying GSH concentrations (1 to 40 mM) at constant ferrous ion concentration (1 mM), and later at different ferrous ion concentrations (0.1 to 1.5 mM) at a constant GSH concentration (10 mM). The rate of activation was not studied under these conditions. But the enzyme activity was determined as described under "Methods" without further addition of cofactors to the assay system. The concentrations at which half-maximum

### Table III

**Purification of citraconase**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total activity (units)</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell-free extract</td>
<td>120</td>
<td>235</td>
<td>1200</td>
<td>0.2</td>
<td>100</td>
</tr>
<tr>
<td>2. Cell-free extract after streptomycin sulfate treatment</td>
<td>120</td>
<td>210</td>
<td>780</td>
<td>0.25</td>
<td>88</td>
</tr>
<tr>
<td>3. 0.4 to 0.7 ammonium sulfate fraction</td>
<td>16.5</td>
<td>165</td>
<td>413</td>
<td>0.40</td>
<td>70</td>
</tr>
<tr>
<td>4. Calcium phosphate gel-treated eluate</td>
<td>33.0</td>
<td>160</td>
<td>290</td>
<td>0.56</td>
<td>68</td>
</tr>
<tr>
<td>5. 0.5 to 0.7 ammonium sulfate fraction</td>
<td>16.0</td>
<td>130</td>
<td>140</td>
<td>0.93</td>
<td>55</td>
</tr>
<tr>
<td>6. DEAE-cellulose column</td>
<td>3</td>
<td>90</td>
<td>30</td>
<td>3.0</td>
<td>38</td>
</tr>
<tr>
<td>7. 0.5 to 0.65 ammonium sulfate fraction</td>
<td>3</td>
<td>52</td>
<td>10.5</td>
<td>5.0</td>
<td>22</td>
</tr>
</tbody>
</table>

* From 15 g of packed cells, wet weight.

### Table IV

**Substrate specificity of citraconase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Original extract</th>
<th>Purified preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Original activity</td>
</tr>
<tr>
<td>Citraconate</td>
<td>0.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Mesaconate</td>
<td>0.18</td>
<td>1.1</td>
</tr>
<tr>
<td>Maleate</td>
<td>0.10</td>
<td>2.0</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.12</td>
<td>1.7</td>
</tr>
<tr>
<td>cis-Aconitate</td>
<td>0.10</td>
<td>2.0</td>
</tr>
<tr>
<td>trans-Aconitate</td>
<td>0.0</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* Ratio of citraconase activity to the other activity.

![Fig. 2. The effect of pH on the activity of citraconase. The assay system contained 0.1 M of acetate (5.5), phosphate (6.2, 6.8, and 7.0), Tris-HCl (7.5, 7.8, 8.2, and 8.7) or glycine-NaOH (pH 10.0) buffers. Then 0.05 ml of the activated enzyme was added to the buffer and 5 moles of potassium citraconate added. Volume was 1 ml, incubation was for 10 min at 30°. Details of assay are given in the text. The pH values determined at the end of the 10-min incubation period were not significantly different from those of the buffers used.](image_url)

![Fig. 3. Lineweaver-Burk plot for the relation between activity and citraconate concentration. The ordinate is the reciprocal of the activity (micromoles of citraconate disappearing or hydrated per min) and the abscissa is the reciprocal of millimolar concentration of citraconate. Experimental details are described in the text.](image_url)
velocities are observed are 3.1 mm and 0.12 mm (in the activation mixture) for GSH and ferrous sulfate, respectively. $V_{\text{max}}$ is observed in 10 mm and 1 mm (in the activation mixture) GSH and ferrous ions, respectively. The concentration of the cofactors in the activation mixture was critical while that in the final assay system did not significantly affect the activity.

Effect of Reducing and Sulfhydryl compounds—The effect of some reducing and sulfhydryl compounds on the activation of citraconase was studied. GSH, cysteine, mercaptoethanol, thioglycollic acid, and ascorbic acid were used at a final concentration of 10 mm in these experiments. The results in Table V show that all the sulfhydryl compounds tested enhanced the activity of citraconase in the presence of ferrous ion. The extent of activation with all the sulfhydryl compounds is about the same except for mercaptoethanol which is only about 80% as effective as the other compounds. Ascorbate does not activate citraconase. Although there is an increase (100%) in the same except for mercaptoethanol which is only about 80% extent of activation with all the sulfhydryl compounds is about 80(%) even though an excess of glutathione is present.

Effect of Metal Ions on Enzyme Activity—Citraconase is activated by ferrous ions only in conjunction with glutathione as shown by results in Table VI. Cupric and mercuric ions inhibit the enzyme activity under these conditions to the extent of 98% even though an excess of glutathione is present.

Effect of Some Metal-complexing Agents and Enzyme Inhibitors on Citraconase—The enzyme (specific activity 4.6) was anaerobically activated with or without cofactors. The metal-complexing agents and inhibitors were then added and incubated for 15 min at 30°. Substrate (5 μmoles) was then added. The total volume was 1 ml. Metal-complexing agents such as pyrophosphate, EDTA, α-phenanthroline, 8-hydroxyquinolino, and α,α'-bipyridyl, all at 1 mm, have a pronounced (90%) inhibitory effect on the enzyme (in the absence of reduced glutathione). In the presence of 0.5 mm GSH in the reaction mixture, the inhibition by these reagents is only of the order of 74% (indicating that glutathione perhaps supplies Fe++). As can be expected the sulfhydryl reagents such as iodoacetate (1 mm) and p-hydroxymercuribenzoate (0.1 mm) also inhibit 98% citraconase activity.

## TABLE V

**Effect of reducing and sulfhydryl compounds**

Solutions of glutathione, cysteine, thioglycollic acid, and ascorbic acid were neutralized just before addition to the activation mixture. The compounds were added in 10 mm concentration and were incubated for 60 min with the 0.15 ml of enzyme preparation (0.5 mg of protein per ml) with and without the addition of ferrous ion (1 mm) in a total volume of 0.5 ml. The reaction mixtures were activated as usual. Aliquots (0.05 ml) of the activation mixtures were tested for citraconase activity as described.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme alone</td>
<td>0.78</td>
</tr>
<tr>
<td>+ Fe⁺⁺</td>
<td>0.80</td>
</tr>
<tr>
<td>+ GSH</td>
<td>1.70</td>
</tr>
<tr>
<td>+ GSH + Fe⁺⁺</td>
<td>4.00</td>
</tr>
<tr>
<td>+ Cysteine</td>
<td>1.7</td>
</tr>
<tr>
<td>+ Cysteine + Fe⁺⁺</td>
<td>4.6</td>
</tr>
<tr>
<td>+ Mercaptoethanol</td>
<td>1.4</td>
</tr>
<tr>
<td>+ Mercaptoethanol + Fe⁺⁺</td>
<td>3.7</td>
</tr>
<tr>
<td>+ Thioglycollate</td>
<td>1.6</td>
</tr>
<tr>
<td>+ Thioglycollate + Fe⁺⁺</td>
<td>4.3</td>
</tr>
<tr>
<td>+ Ascorbate</td>
<td>0.8</td>
</tr>
<tr>
<td>+ Ascorbate + Fe⁺⁺</td>
<td>0.85</td>
</tr>
</tbody>
</table>

## TABLE VI

**Effect of metal ions on enzyme activity**

Metallic chlorides were used in most cases except for zinc, copper, and ferrous iron which were added as sulfates. The salts of the metals to be tested were added to the activation mixture at 1 mm concentration along with 10 mm GSH. They were incubated anaerobically with the enzyme for 1 hour. The composition of the activation mixture and assay of citraconase activity were the same as described in Table V.

<table>
<thead>
<tr>
<th>Cation added</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>Fe⁺⁺</td>
<td>4.6</td>
</tr>
<tr>
<td>Mn⁺⁺</td>
<td>1.6</td>
</tr>
<tr>
<td>Cu⁺⁺</td>
<td>0.32</td>
</tr>
<tr>
<td>Hg⁺⁺</td>
<td>0.31</td>
</tr>
</tbody>
</table>

-Mg²⁺, Zn²⁺, Co²⁺, Ni²⁺, NH₄⁺, and ammonium molybdate also gave similar (within ±5%) activities.

**DISCUSSION**

There is a great similarity among the different hydratases and dehydratases (such as citraconase, mesaconase (3, 4), L-tartaric dehydratase (17),aconitase (18), and so on) in their cofactor requirements. All of them require ferrous ions for full activity. No other cation seems to be effective. There is a broad specificity in the requirement for a sulfhydryl compound for citraconase, mesaconase of C. tetanomorphum (4) and L-tartaric dehydratase of pseudomonads (17). Sulfhydryl compounds such as glutathione, cysteine, mercaptoethanol, and thioglycollate activate these enzymes in conjunction with ferrous ions, but ascorbate does not activate these enzymes indicating that an external supply of free sulfhydryl groups is necessary. Mesaconase of Pseudomonas fluorescens (3) is atypical in this respect. It does not require any added sulfhydryl compound but, more surprisingly, is inhibited by cysteine. The aged and apparently inactive preparations of some of these enzymes can be activated by anaerobic incubation with the cofactors.

The studies on the stereochemistry of the hydration reactions catalyzed by citraconase of the pseudomonad used here and by mesaconase of C. tetanomorphum have shown that both catalyze trans addition of water (19, 20). These enzymes have also been used for the preparation of the optical isomers of citramalic acid from citraconate and mesaconate (21, 22).

**REFERENCES**

8. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941).