The Purification and Properties of Yeast Isocitric Lyase*†

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Isocitric lyase, or isocitrate lyase, the enzyme which reversibly cleaves L (+)-isocitric acid to glyoxylic and succinic acids, has been identified in extracts of a number of microorganisms (1–6), and partially purified from Pseudomonas aeruginosa (7). Interest in this enzyme has been stimulated by its possible involvement in three important metabolic pathways: (a) glyoxylate formation from acetate (1, 2, 6), (b) glycine synthesis (1, 2, 6), and (c) the net production of succinate from acetate (8). In order that cleavage might be conveniently studied, it was of interest to purify the enzyme from a readily available source material. The present paper deals with the identification of the enzyme in crude extracts of the mold Penicillium chrysogenum Q176 and in other microorganisms, and the partial purification and characterization of the enzyme from baker’s yeast.

EXPERIMENTAL

Chemicals—DL-isocitric lactone (allo-free) and sodium isocitrate (DL + allo) were products of the Sigma Chemical Company. Sodium DL-isocitrate was prepared from the lactone by boiling with 3 equivalents of NaOH for 15 minutes and acidifying to pH 7.6. The dimethyl ester of D (+)-isocitric lactone was a generous gift of Dr. C. B. Anfinsen. Its optical purity was established by formation of the molybdate complex (observed [ε]²⁰⁰ = −761°, reported [ε]³⁰⁰ = −745° (10)). Glyoxylic acid was prepared in 70 per cent yield by refluxing dichloroacetic acid with sodium benzoate in neutral solution (11), and was crystallized as the sodium salt monohydrate.

Succinic acid, semicarbazide hydrochloride, 2,4-dinitrophenylhydrazine, and other chemicals were crystalline commercial products which were used without further purification.

Growth of Organisms—The molds Penicillium chrysogenum Q176, Aspergillus niger, and Rhizopus sp. were grown on Difco malt agar or a maize-asparagine-glycerol medium (12).

The spore suspensions (10 ml.) of these organisms in water were added to shake flasks containing 100 ml. of the following sterile medium at pH 5.4 to 5.7: 0.6 per cent ammonium acetate, 0.6 per cent (NH₄)₂SO₄, 0.3 per cent KH₂PO₄, 0.2 per cent glucose, 0.025 per cent MgSO₄·7H₂O, 0.02 per cent FeSO₄·7H₂O, 0.00025 per cent CuSO₄·5H₂O, 0.002 per cent ZnSO₄·7H₂O, 0.002 per cent MnSO₄·H₂O, 0.005 per cent CaCl₂·2H₂O, 0.05 per cent Na₂SO₄, and 0.1 per cent yeast extract. After 24 hours on a rotary shaker at 24°, the starter cultures were transferred to 10-l. laboratory fermentors (13) containing 5 l. of fresh media, and they were allowed to grow until the pH rose to 6.3 to 6.6 (about 17 to 24 hours). At the time of harvest, the mycelium was fermented and growing vigorously with a dry weight of 0.20 to 0.40 per cent. The mold was filtered, washed with 0.01 m potassium phosphate (pH 7.4), and either used immediately or frozen at −20°. The enzyme in the frozen mycelium was stable indefinitely.

Enzyme Preparations—Cell-free preparations of molds and yeast were obtained by the press as described by Hughes (14) at −10° in the absence of abrasive. The crushed cell mass was suspended in an equal volume of 0.1 m potassium phosphate buffer, pH 7.4, and centrifuged at 15,000 × g for 30 minutes in the cold. The slightly turbid supernatant solution contained the enzyme. Active yeast preparations were also obtained after autolysis with toluene, as described later.

Assay Method I—Crude enzyme extracts of the microorganism or tissue (0.1 ml. containing 2 to 5 mg. of protein) were incubated with 0.7 ml. of freshly neutralized 0.015 m semicarbazide and 0.2 ml. of either 0.06 m sodium citrate or sodium DL-isocitrate for 60 minutes at 25°. The reaction was stopped with 5 ml. of 2 per cent tungstic acid reagent, and the total keto acid concentration of the supernatant solution was determined by the ethyl acetate extraction method of Friedemann and Haugen (15). In the presence of semicarbazide the reaction did not go to completion, and thus a correction factor of 1.6 was used. After readings were taken at 460 μm, the solutions were heated at 100° for exactly 100 seconds, cooled quickly, and read again. Under these conditions, glyoxylic hydrate is 80 per cent decolorized, pyruvic hydrazine is 30 per cent destroyed, and α-ketoglutaric hydrate is practically unaffected. A plot of the percentage of glyoxylic hydrate against the ratio of final (heated) to initial readings is given in Fig. 1. Since the amount of glyoxylic in an unknown mixture can be determined within 15 per cent accuracy when the total amount of glyoxylic exceeds 50 per cent of the mixture, this method proved to be a rapid and useful index of glyoxylic production in crude systems.

Assay Method II—0.2 ml. of 0.03 m cysteine hydrochloride, 0.5 ml. of 0.12 m semicarbazide hydrochloride, 0.3 ml. of 0.05 m MgSO₄·7H₂O, 1.0 ml. of 0.20 m potassium phosphate buffer (pH 6.0), and 0.9 ml. of 0.074 m NaOH were mixed together. The pH of the reaction mixture was 6.0. Thereafter, 0.01 to 0.05 ml. of enzyme solution was added, and the reaction was started by the addition of 0.1 ml. of 0.2 m DT-sodium isocitrate (allo-free). Readings were taken every minute at 252 μm, the absorption
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Fig. 1. Determination of glyoxylic hydrazone by heat decolorization. Left line, the percentage of glyoxylic acid in glyoxylate-pyruvate mixtures; right line, percentage of the acid in glyoxylate-α-ketoglutarate mixtures; middle line, percentage of the acid in mixtures of all three hydrazones.

Fig. 2. The relation between the change in optical density at 252 nm and enzyme concentration (Assay Method II). The enzyme preparation used was 10-G, with a specific activity of 2.1.

maximum for glyoxylic semicarbazone, in a Beckman model DU spectrophotometer maintained at 28° with Thermospacers. After a lag phase of 2 to 6 minutes, the rate became linear. The relation between enzyme concentration and optical density at 252 nm is shown in Fig. 2. Since the molecular extinction coefficient of glyoxylic semicarbazone is 12,400, division of the optical density by 4.13 yields the number of μmoles of glyoxylic acid formed. 1 unit of enzyme was defined as the amount of enzyme which formed 1 μmole of glyoxylic semicarbazone during a 5-minute period at 28°. Specific activities are expressed as units of activity per mg. of protein.

Other Methods—Protein was determined by the biuret reaction (16) or by spectrophotometric measurement at 280 μm (17). Citrate was measured by the method of Taylor (18), succinate by a heart succinoxidase preparation (19), glutamate by carrot glutamic decarboxylase (20), and carbon dioxide by Warburg manometry. DEAE-cellulose was prepared according to the directions of Sober and Peterson (21).

RESULTS

Identification of Products—In crude, aged extracts of acetate-grown Penicillium chrysogenum Q176 supplemented with TPN, citrate was rapidly metabolized, carbon dioxide was evolved, and α-ketoglutarate and glutamate were accumulated (Table I). When TPN was omitted, no carbon dioxide was produced in dialyzed or aged preparations (4 hours, 0°), but some citrate still disappeared and a keto acid formed.

The keto acid was identified as glyoxylic acid by the spectrum, melting point (189-192°), and chromatographic behavior (RF = 0.49; 0.71 in N-butanol-ammonia (22)) of its 2,4-dinitrophenyl-hydrazone. No depression in mixed melting point was observed.

Ether extraction of the acidified reaction mixture yielded another product which was identified as succinic acid in the following manner: (a) the RF of the product was the same as that of succinic acid in three solvent systems, N-butanol-propionic acid-water, phenol-NH₂OH, and ether-acetic acid-water, and (b) the product was oxidized stoichiometrically by a heart succinoxidase preparation. Even in crude preparations, equal quantities of glyoxylate and succinate were formed.

Distribution in Nature—Aged extracts of a number of molds and baker’s yeast were able to convert isocitrate to glyoxylate, as shown in Table II. Higher enzyme titers were found when molds were grown on simple acetate-salt media than on complex nutrient broths. Extracts of glycerol-grown Acetobacter suboxydans and homogenates of rat liver and muscle were completely inactive. A number of bacteria are known to contain the enzyme (3), however, and its apparent absence in A. suboxydans is probably the result of the use of a glycerol medium for growth (7).

Purification of Isocitric Lyase from Yeast

Autolysis and Extraction—1 pound of baker’s yeast is mixed thoroughly with 30 ml. of toluene and incubated at 37° for 6
Heat Treatment—The cold supernatant solution is enclosed in a large dialysis casing, submerged in 3 to 4 l. of distilled water initially at 62°, left at 55 ± 1° for exactly 90 minutes, and then quickly iced. All further operations are carried out in a cold room at 3-5°. The solution (Fraction B) is not centrifuged.

First Ammonium Sulfate Fractionation—To each 100 ml. of Fraction B, 19.4 gm. of analytical grade ammonium sulfate (0.35 saturation) are added slowly with stirring. The precipitate is removed by centrifugation and 19.5 gm. of ammonium sulfate (0.65 saturation) are added to each 100 ml of supernatant solution CWS. The precipitate (CWS-1) is packed well by centrifugation and then is dissolved in 400 ml. of 0.02 M potassium phosphate buffer, pH 6.0.

Protamine Sulfate Treatment—1 gm. of protamine sulfate in 28.4 ml. of 0.1 M potassium phosphate buffer, pH 7.4, is added to Fraction CWS-1, and the precipitate is centrifuged off.

Second Ammonium Sulfate Fractionation—To each 100 ml. of the clear, amber supernatant solution, Fraction D, 27.2 gm. of ammonium sulfate (0.48 saturation) are added, the precipitate is centrifuged off, and 8.5 gm. of ammonium sulfate (0.62 saturation) are added to each 100 ml of the resulting supernatant (E&). After centrifugation the precipitate (E&-1) is dissolved in 50 ml of 0.02 M potassium phosphate buffer, pH 5.7, and dialyzed overnight against 2 l. of the same buffer containing about 10 mg of cysteine. The solution is centrifuged in the cold.

Ethanol Fractionation—Dialyzed supernatant Fraction E&-2 is chilled in an ethanol-Dry ice bath until ice forms. Then 95 per cent ethanol at −70° (12 ml./100 ml of enzyme solution) is added dropwise with stirring. After centrifugation at −10° the supernatant solution F10 is cooled until some ice forms and is centrifuged down at −10°, dissolved in 20 ml of cold 0.01 M potassium phosphate buffer, pH 7.4, and dialyzed for 2 to 3 hours against 3 to 4 l. of the same buffer containing 10 mg of cysteine, 100 mg of sodium ethylenediaminetetraacetate. The light precipitate which forms is centrifuged off.

Chromatographic Separation on DEAE-cellulose Column—A column (20 × 1 cm. diameter) of 2 gm. of DEAE-cellulose (21) is prepared and washed extensively with 0.01 M potassium phosphate, pH 7.4, in the cold. Dialyzed Fraction F10 is placed on the column and washed in with 1 to 2 ml. of buffer. The initial eluate is discarded. Thereafter, a gradient elution device is used with 200 ml. of 0.01 M potassium phosphate, pH 7.4, in the mixing chamber, and 100 ml. of 0.5 M potassium phosphate, pH 7.4, in the attached separatory funnel. The eluate is collected at a rate of 12 ml per hour in 3-ml fractions and assayed for protein and enzyme activity. A typical assay is given in Fig. 3. These results were highly reproducible both with respect to the peak enzyme fraction as well as to recovery. The same columns could be used again after they were washed overnight with 0.01 M potassium phosphate, pH 7.4.
TABLE III

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Step</th>
<th>Volume</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Crude extract</td>
<td>740</td>
<td>1600 (1000)*</td>
<td>7.6</td>
<td>0.21 (0.13)*</td>
</tr>
<tr>
<td>B</td>
<td>Heated extract</td>
<td>780</td>
<td>1185 (1000)*</td>
<td>3.9</td>
<td>0.30 (0.25)*</td>
</tr>
<tr>
<td>C14-44</td>
<td>First (NH₄)₂SO₄ fraction</td>
<td>415</td>
<td>969</td>
<td>2.3</td>
<td>0.41</td>
</tr>
<tr>
<td>D</td>
<td>Protamine treatment</td>
<td>455</td>
<td>950</td>
<td>2.1</td>
<td>0.45</td>
</tr>
<tr>
<td>E8-62</td>
<td>Second (NH₄)₂SO₄ fraction</td>
<td>58</td>
<td>841</td>
<td>1.05</td>
<td>0.81</td>
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<tr>
<td>E8-60</td>
<td>Dialysis</td>
<td>60</td>
<td>693</td>
<td>0.83</td>
<td>0.84</td>
</tr>
<tr>
<td>F18-21</td>
<td>Ethanol addition</td>
<td>21</td>
<td>544</td>
<td>0.16</td>
<td>3.4</td>
</tr>
<tr>
<td>G18-22</td>
<td>DEAE-cellulose column</td>
<td>12</td>
<td>255</td>
<td>0.034</td>
<td>7.6†</td>
</tr>
</tbody>
</table>

* Activity values are corrected for the formation of α-ketoglutarate and pyruvate in crude extracts. Net glyoxylate values are given in parentheses.
† F18-21 was stored for a week before chromatography, during which 30 per cent of its activity was lost. The specific activity of tube 20 was 8.7 (Fig. 3).

TABLE IV

<table>
<thead>
<tr>
<th>Reactants</th>
<th>Michaelis constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>nL-isocitrate</td>
<td>2.4 × 10⁻³</td>
</tr>
<tr>
<td>L-isocitrate</td>
<td>1.2 × 10⁻³</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1 × 10⁻⁴</td>
</tr>
<tr>
<td>Cysteine</td>
<td>4 × 10⁻⁴</td>
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</table>

![INHIBITION OF ISOCITRIC LYASE](image)

**Fig. 4.** Inhibition of isocitric lyase. Enzyme and inhibitor were incubated for 5 minutes before the addition of nL-isocitrate. Assay Method II was used.

The over-all purification was about 70-fold, with a recovery of 25 to 50 per cent of the original activity. The specific activity of some chromatographic fractions (Fraction G), however, was as high as 90.

Although the described assay methods gave highly reproducible results, 2- to 3-fold higher activities were observed at lower phosphate concentrations. Thus, a maximal specific activity of about 25 was observed, corresponding to a turnover number of 500 moles of glyoxylate formed per minute for 100,000 gm of enzyme at 98° and pH 6.0. The calculated turnover number for the purified *Pseudomonas* enzyme (7) is roughly the same, namely 350 at 30° and pH 7.6. These values cannot be directly compared, however, in view of the different assay methods employed.

The purity of the final enzyme preparation is unknown. Electrophoresis of Fraction F18-21 at pH 7.4 indicated the presence of at least three components. Changes in the specific activities of chromatographic Fractions G18-22 also suggest some contamination with inert protein.

**Enzyme Stability**—Whether the purified enzyme was stored in the frozen state or in glycerol-water mixtures (1:2; 2:1) at −20°, a 10 to 20 per cent loss of activity resulted per week. 10 per cent of the activity is lost per day at 5°, and 20 per cent at 25°. The addition of magnesium, cysteine, or both did not improve stability at 25° or at higher temperatures. The enzyme is relatively stable between pH 5.5 and 8.5, but loses activity very rapidly beyond these pH limits. The presence of ammonium sulfate during ethanol fractionation leads to rapid and irreversible enzyme inactivation.

**Reaction Requirements**—The yeast enzyme, like that obtained from *Pseudomonas* (2, 7), requires a divalent metal and a sulfhydryl compound for maximal activity. The Michaelis constants for nL- and L-isocitric acid, magnesium, and cysteine are given in Table IV. The Lineweaver-Burk plots (23) for magnesium and isocitrate are linear. Cysteine, on the other hand, does not stimulate activity at low concentrations (0 to 2 × 10⁻⁵ M), then allows a rapid increase to a maximum (2 to 20 × 10⁻⁵ M), and inhibits at higher concentrations. The Michaelis constant for cysteine was estimated graphically.

nL-Isocitric acid, at twice the concentration of L-isocitrate (i.e. the equivalent concentration of L-isocitrate) gives the same activities. The nL-1 allo-isocitrate, however, at 2 times the L-isocitrate concentration, is only 20 per cent as active. nL-Isocitric lactone and citrate are completely inert.

Magnesium is the most effective metal activator. Manganous chloride (10⁻³ M) is about 40 per cent as active, but cadmium, zinc, and copper inhibit the enzyme, even in the presence of magnesium. p-Chloromercuribenzoate (10⁻³ M) and o-iodosobenzoate (10⁻⁴ M) reduced enzyme activity by 50 per cent, as
shown in Fig. 4. *p*-Chloromercuribenzoate inhibition was released 80 per cent by excess glutathione.

The purified yeast enzyme is similar in many respects to the enzyme isolated from *Pseudomonas* (7). Both require magnesium and cysteine, and possess activity with manganese ions and glutathione as well. The Michaelis constants for isocitrate and activators are about the same. On the other hand, Saz and Hillary (4) found the \( \Delta^{+} \)-isomer 5 times as active as the \( \Delta^{2} \) compound, whereas in the present study, inhibition was observed only when the allo forms were present.

**Reversibility**—The formation of citrate from succinate and glyoxylate, observed in extracts of *Pseudomonas aeruginosa* (6) and in *P. chrysogenum* (1), indicated that the lyase reaction was reversible.

In the presence of purified yeast enzyme, glyoxylate formation increases rapidly to a maximum when isocitrate is added to an enzyme solution and then slowly declines (Fig. 5). The addition of succinate causes a rapid fall in glyoxylate to a new level, and further isocitrate addition again gives high glyoxylate concentration. Nonenzymatic condensation of glyoxylate with cysteine was responsible for the observed decline in glyoxylate concentration following the initial maximum, and hence the calculated equilibrium constants were higher than reported values (7).

**DISCUSSION**

Many early studies on the products of mold fermentations indicated that glyoxylic and oxalic acids were most often found when the growth medium contained either acetate or citric acids (24, 25). On the basis of these observations, the following metabolic sequence was proposed:

acetate \( \rightarrow \) glycolate \( \rightarrow \) glyoxylate \( \rightarrow \) oxalate.

Although other evidence has been given to support this pathway (26), attempts to demonstrate unambiguously the direct conversion of acetate to glycolate have not been successful. With the finding that isocitrate can be enzymatically cleaved to glyoxylate and succinate, however, the net production of glyoxylate from acetate in microorganisms can be readily explained by means of the citric acid cycle and the lyase enzyme. The over-all reaction may be summarized as follows:

\[
\text{acetate + ATP + 2H}_2\text{O} \rightarrow \\
\text{glyoxylate + AMP + pyrophosphate + 4H}^+ + 4e^-.
\]

The fact that isocitric lyase and Krebs-cycle enzymes are widely found in plants and microorganisms supports this pathway as the predominant metabolic route for glyoxylate synthesis.

The suggestion has been made that isocitric lyase may be involved in the formation of glycine from tricarboxylic acids (6). Crude extracts of *P. chrysogenum* are capable of converting citrate to glycine in low yield (1), and glyoxylate acid transaminates readily with several amino acids to form glycine in animal tissues (27) and in bacterial extracts (28). On the other hand, in crude *Penicillium* extracts supplemented with TPN, citrate was almost quantitatively converted to \( \alpha \)-ketoglutarate and glutamate, rather than to glyoxylate and glycine. Furthermore, when 2-\( \text{C}^4 \)-acetate was incubated with intact mycelia, over 50 per cent of the labeled carbon of the metabolized acetate was found in glutamate, whereas glycine was only slightly labeled \(^1\). Thus, although the formation of glycine from citrate and other amino acids via isocitric lyase can occur, this pathway is probably not of major importance for glycine synthesis in *P. chrysogenum*. Our inability and that of others (7, 29), to detect

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Reversibility of the isocitric lyase reaction. The reaction mixture contained 15 \( \mu \)moles of \( \Delta^{2} \)-sodium isocitrate, 15 \( \mu \)moles of \( \text{MgSO}_4 \), and 9 \( \mu \)moles of cysteine in 2.8 ml of 0.02 \( \mu \) potassium phosphate buffer, pH 7.5. At zero time 0.20 ml of enzyme (11-Fa.to, specific activity = 2.1) was added, and at intervals 0.30-ml aliquots were inactivated with 0.10 ml of \( \times \text{H}_2\text{SO}_4 \). 0.16 ml of this solution was pipetted into 2.84 ml of 0.16 \( \mu \) potassium phosphate, pH 5, and of 0.16 \( \mu \) potassium phosphate-0.024 \( \mu \) semicarbazide, pH 5. The difference between readings taken after 5 minutes was a measure of the glyoxylate present. At the arrow, 0.2 ml of 0.1 \( \mu \) sodium succinate \((S)\) and 0.2 ml of 0.1 \( \mu \) \( \Delta^{2} \)-sodium isocitrate \((IC)\) were added as indicated.

isocitric lyase activity in several mammalian tissues seems to preclude as well the possibility that glyoxylate is an important intermediate for glycine synthesis in mammalian tissues.

Isocitric lyase, together with malic synthetase (36), seems to play a major role in the net conversion of acetate to malate (8). The fact that extracts of *P. chrysogenum* and of bacteria (7) grown on simple acetate-salt media contain more enzyme than preparations from microorganisms grown on complex nutrients supports this hypothesis.

The reaction catalyzed by isocitric lyase is an aldol condensation. Nonenzymatic reactions of this type may be catalyzed by base, acid, or, in some instances, by specific amines. These reactions are reversible, and the generally accepted mechanism for the base-catalyzed cleavage reaction involves the removal of a proton from the \( \alpha \)-hydroxyl group followed by a series of electron shifts, as indicated in Diagram 1. Thus any agent which displaces the proton of the hydroxyl group might also aid in catalyzing the cleavage reaction. Of particular interest in this regard is the finding that magnesium, copper, and other
Hence it seems likely that the metal not only reacts with the copper—carboxyl interaction alone. In preliminary studies on chelation complexes of isocitric acid, an additional proton was released when isocitric acid was titrated in the presence of copper. Although a stable 1:1 complex did not form, spectral changes were observed during titration which could not be accounted for by copper—carboxyl interaction alone. Hence it seems likely that the metal not only reacts with the three carboxyl groups of isocitrate, but also displaces a proton from the hydroxyl group during titration.

Magnesium may function in a similar manner in the enzyme-catalyzed reaction, as depicted in Fig. 6.

After displacement of the proton from the hydroxyl group through the action of magnesium and possibly sulfhydryl or amino groups on the enzyme, electron rearrangement and cleavage might proceed in a manner analogous to the base-catalyzed reaction.

This suggestion is similar in some respects to the proposed mechanism for muscle aldolase action (34, 39). Metal ions are not required, however, for that reaction.

**SUMMARY**

1. Isocitric lyase, which catalyzes the reversible cleavage of \( \text{L}-\text{isocitrate} \) to glyoxylate and succinate, has been identified in a number of molds, and has been purified 70-fold from yeast autolysates.

2. The purified yeast enzyme acts only on \( \text{L}-\text{isocitric acid} \) and requires magnesium and cysteine for activity. Manganese ions are 40 per cent as active as magnesium, and reduced glutathione can replace cysteine. The enzyme is inhibited by low concentrations of copper, cadmium, zinc, \( p \)-chloromercuribenzoate, and \( o \)-iodosobenzoate.

3. The biological function of isocitric lyase is discussed, and a possible mechanism for the cleavage of isocitrinate involving chelation with magnesium is presented.

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**REFERENCES**


* At pH 1 there is no spectral evidence for chelation. With increasing pH, an enhancement of the 800 m\( \mu \) cupric ion peak and a hypsochromic shift to 750 m\( \mu \) occurred as well as a marked increase in ultraviolet absorption at 254 m\( \mu \). The effects observed in the visible region are characteristic of simple copper—carboxyl interactions, whereas the bathochromic intensification in the ultraviolet region is much greater with copper isocitrate than with copper acetate. Analysis of the system at pH 3.5 and 7 at 360 and 750 m\( \mu \) by the method of continuous variations (33) gave no suggestion of a stable complex with Cu-isocitrate ratios from 1:4 to 1:8.1.