The Purification and Properties of Yeast Isocitric Lyase*†

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Isocitric lyase, or isocitratase, 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) glycine synthesis (1, 2, 6), and (c) the net production of succinate from acetate (8). In order that the properties of isocitric lyase and the mechanism of isocitrate 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 L (+)-isocitric lactone was a generous gift of Dr. C. B. Anfinsen. Its optical purity was established by formation of the molybdate complex (observed $\varepsilon_270 = -761^\circ$, reported $\varepsilon_270 = -745^\circ$ (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 (NH$_4$)$_2$SO$_4$, 0.3 per cent KH$_2$PO$_4$, 0.2 per cent glucose, 0.025 per cent MgSO$_4$·7H$_2$O, 0.02 per cent FeCl$_3$·7H$_2$O, 0.00025 per cent CuSO$_4$·5H$_2$O, 0.002 per cent ZnSO$_4$·7H$_2$O, 0.002 per cent MnSO$_4$·H$_2$O, 0.005 per cent CaCl$_2$·2H$_2$O, 0.05 per cent Na$_2$CO$_3$, 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 filamentous 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 use of the press as described by Hughes (14) at -10° in the absence of an 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 m$\mu$, the solutions were heated at 100° for exactly 100 seconds, cooled quickly, and read again. Under these conditions, glyoxylate hydrazone is 80 per cent decolorized, pyruvic hydrazone is 30 per cent destroyed, and α-ketoglutaric hydrazone is practically unaffected. A plot of the percentage of glyoxylate hydrazone against the ratio of final (heated) to initial readings is given in Fig. 1. Since the amount of glyoxylate in an unknown mixture can be determined within 15 per cent accuracy when the total amount of glyoxylate exceeds 50 per cent of the mixture, this method proved to be a rapid and useful index of glyoxylate production in crude systems.

Assay Method II—0.2 ml. of 0.03 M cystine hydrochloride, 0.5 ml. of 0.12 M semicarbazide hydrochloride, 0.3 ml. of 0.05 M MgSO$_4$·7H$_2$O, 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 m$\mu$, the absorption...
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0.2 0.4 0.6 0.8 1.0

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R(ma-)

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 nm (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 Penicilium 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
hours. 600 ml. of cold 0.2 M potassium phosphate buffer, pH 7.5, are added, and after 20 minutes in the cold, the liquid mass is centrifuged at 4600 r.p.m. for 15 minutes in a refrigerated International centrifuge. The decanted supernatant solution (supernatant Fraction A) may be worked up immediately, or left overnight in the cold with little loss of activity. The precipitate is discarded.

**Heat Treatment**—The cold supernatant solution is enclosed in a large dialysis casing, submerged in 3 to 4 l. of distilled water initially at 60°, 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 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 0.01 M potassium phosphate buffer, 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 Table III. In addition to isocitric lyase, Fractions A and B contained enzymes which converted isocitrate to α-ketoglutarate and other keto acids which react in the semicarbazone assay. Chromatography of the 2,4-dinitrophenylhydrazones of the keto acids formed in these crude extracts indicated that glyoxylate accounted for only 50 to 70 per cent of the total keto acids present. Fraction C24-35 and subsequent fractions yielded only glyoxylate in the standard assay.

**Table II**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Source of extract</th>
<th>Growth medium</th>
<th>Glyoxylate formed (μmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-2</td>
<td>Penicillium chrysogenum</td>
<td>Acetate-salt</td>
<td>5.9</td>
</tr>
<tr>
<td>26-2</td>
<td>Penicillium chrysogenum</td>
<td>Cornsteep-lactose</td>
<td>0.8</td>
</tr>
<tr>
<td>24-4</td>
<td>Saccharomyces cerevisiae</td>
<td>Commercial</td>
<td>2.4</td>
</tr>
<tr>
<td>31-3</td>
<td>Aspergillus niger</td>
<td>Enriched acetate-salt</td>
<td>1.0</td>
</tr>
<tr>
<td>31-4a</td>
<td>Rhizopus sp.</td>
<td>Enriched acetate-salt</td>
<td>0.7</td>
</tr>
<tr>
<td>28-4</td>
<td>Streptomyces sp. Al01</td>
<td>Cornsteep lactose</td>
<td>Trace</td>
</tr>
<tr>
<td>29-2</td>
<td>Acetobacter suboxydans</td>
<td>Glycerol</td>
<td>0</td>
</tr>
<tr>
<td>25-4</td>
<td>Rat liver</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>21-7</td>
<td>Rat muscle</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 3.** Chromatography of a portion of dialyzed Fraction F18-13 on DEAE-cellulose at 0°. Protein was eluted by gradually increasing the molarity of potassium phosphate buffer, pH 7.4, from 0.01 M to about 0.3 M as described in the text. Protein: 74 mg. added, 56 mg. recovered, 75 per cent yield; lyase: 142 units added, 131 units recovered, 92 per cent yield.

**Maximal Observed Activity and Turnover Number**—The summary of a typical preparation is given in Table III. In addition to isocitric lyase, Fractions A and B contained enzymes which converted isocitrate to α-ketoglutarate and other keto acids which react in the semicarbazone assay. Chromatography of the 2,4-dinitrophenylhydrazones of the keto acids formed in these crude extracts indicated that glyoxylate accounted for only 50 to 70 per cent of the total keto acids present. Fraction C24-35 and subsequent fractions yielded only glyoxylate in the standard assay.
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TABLE III
Purification of isocitric lyase: Preparation No. 16
Starting material: 450 gm. of Fleischman's bakers' yeast

<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>Heat extract</td>
<td>780</td>
<td>1185 (1000)*</td>
<td>3.9</td>
<td>0.30 (0.25)*</td>
</tr>
<tr>
<td>C</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>E</td>
<td>Second (NH₄)₂SO₄ fraction</td>
<td>58</td>
<td>841</td>
<td>1.05</td>
<td>0.81</td>
</tr>
<tr>
<td>F</td>
<td>Dialysis</td>
<td>60</td>
<td>693</td>
<td>0.83</td>
<td>0.84</td>
</tr>
<tr>
<td>G</td>
<td>Ethanol addition</td>
<td>21</td>
<td>544</td>
<td>0.16</td>
<td>3.4</td>
</tr>
<tr>
<td>H</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.
† F₁₀₋₁₈ 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
Michaelis constants of reactants

<table>
<thead>
<tr>
<th>Reactant</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>
</tr>
</tbody>
</table>

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 9.0.

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 F₁₀₋₁₈ at pH 7.4 indicated the presence of at least three components. Changes in the specific activities of chromatographic Fractions G₁₇₋₂₂ 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 + allo-isocitrate, however, at 4 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. Manganese 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 (10⁻⁵ 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 \( \omega \)(+) isomer 5 times as active as the \( \omega \) compound, whereas in the present study, inhibition was observed only when the \( \omega \) 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 acetic or citric acids (24, 25). On the basis of these observations, the following metabolic sequence was proposed:

\[
\text{acetate} \rightarrow \text{glycolate} \rightarrow \text{glyoxylate} \rightarrow \text{oxalate}.
\]

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:

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

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 glyoxylic 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. 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 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 metals form complexes with a number of α-hydroxy acids (30, 31). Copper (Cu++) and citric acid, whose chelates have been extensively studied, form stable 1:1 complexes at pH 6 in which a proton has been displaced from the hydroxyl group (32). 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 sulphydryl 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, 35). Metal ions are not required, however, for that reaction.

**SUMMARY**

1. Isocitric lyase, which catalyzes the reversible cleavage of L-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 L-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 isocitrate involving chelation with magnesium is presented.

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