The four isomers of hydroxycitrate have been tested as substrates and inhibitors for citrate synthase, citrate lyase, and ATP citrate lyase. None of the isomers served as a substrate for citrate synthase and they were moderate to weak inhibitors of this reaction. Of the four isomers, only \((\text{pne})-(2S)-2\)-hydroxycitrate did not serve as a substrate for citrate lyase while \((\text{pne})-(4S)-4\)-hydroxycitrate was the only isomer which did not serve as a substrate for ATP citrate lyase. No consistent pattern of reactivity or inhibitor potency was seen with the different isomeric hydroxycitrates. It is proposed that more than one mode of binding is possible between the isomers and the three different active sites.

There are three citrate enzymes (1, 2) which catalyze the same bond-making and -breaking reaction which involves the equilibrium of citrate with oxalacetate and an acetyl moiety.

1. Citrate sythase (EC 4.1.3.7)
   \[
   \text{Citrate}^{3-} + \text{CoA} + \text{H}^+ \rightarrow \text{acetyl-CoA} + \text{oxalacetate}^{2-} + \text{H}_2\text{O}
   \]

2. Citrate lyase (EC 4.1.3.8)
   \[
   \text{Citrate}^{3-} \quad \text{M}^{2+} \quad \text{acetate}^{2-} + \text{oxalacetate}^{2-}
   \]

3. ATP citrate lyase (EC 4.1.3.8)
   \[
   \text{Citrate}^{3-} + \text{CoA} + \text{ATP}^{4-} \quad \text{M}^{2+} \quad \text{acetyl-CoA} + \text{ADP}^{3-} + \text{oxalacetate}^{2-} + \text{P}^{2-}
   \]

The citrate re-synthase, analogous to enzyme 1, is excluded from this list. For enzymes 1, 2, and 3, when citrate synthesis occurs, the methyl carbon atom of acetate attacks the si-face of oxalacetate, and an inversion of configuration occurs as one hydrogen atom of the methyl group of acetate is replaced by oxalacetate (3-7). Citrate re-synthase has the opposite stereochemistry.

The equilibrium in the reaction catalyzed by citrate synthase favors citrate formation, while the equilibria for citrate lyase (a bacterial enzyme) and ATP citrate lyase (a cytosolic enzyme found in eukaryotic cells) favor the reverse reaction, i.e. the cleavage of citrate to oxalacetate and acetate or acetyl-CoA. Citrate synthase is found in all cells (in the mitochondria of eukaryotes) and catalyzes the formation of citrate for further reaction in the Krebs cycle, while ATP citrate lyase, a cytosolic enzyme, cleaves citrate so that acetyl-CoA may be used for fatty acid and other biosyntheses.

The requirements of these three enzymes, aside from citrate, vary. Citrate synthase requires only CoA for activity (8), while ATP citrate lyase requires CoA, ATP, and a divalent metal (9) for activity. Citrate lyase also requires a divalent metal for activity but, instead of requiring CoA as a cofactor, contains a CoA-like prosthetic group (10-12).

The availability of the four isomers of hydroxycitrate, and the preparation of pure samples of each enzyme, led us to investigate the substrate and inhibitor activity of each isomer of hydroxycitrate with each of the three citrate enzymes. The work was done to compare the active sites of the three enzymes, keeping in mind the fact that analogs of substrates do not necessarily interact with the active site of an enzyme in the same way that the true substrates do. For example, it is believed that fluorocitrate does not bind to aconitase in the same way that citrate does (13). However, the ways in which
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the various hydroxycitrates interact with each enzyme will lead to general stereochemical information on its active site, and, perhaps, as a result, information on its mechanism of action.

The absolute configurations of the four hydroxycitrate analogs have been determined by chemical and x-ray crystallographic methods (14, 15). The results are given in Fig. 1. In replacing hydrogen by hydroxyl, fluorine, or methyl in the methylene groups of citrate, problems in nomenclature were encountered. In order to clarify this, a simple method has been proposed (Fig. 1) which, in contrast to the R/S system, allows us to relate the stereochemistry of the analogs directly back to that of the "parent" substrate (16).

Throughout the description of hydroxycitrates we have used this parent numbering system (16). The backbone of the parent, citrate (see Fig. 1), is numbered by the convention that the pro-R branch relative to the central carbon has lower numbering than the pro-S branch. Hydroxycitrate analogs are then designated pn (for parent number) and 2- or 4- depending on whether the pro-R ((2R)-OHcit-pn-cn) or the pro-S ((4R)-OHcit-pn-cn) methylene group of citrate is substituted by a hydroxyl group. In fact, this means that those hydroxycitrate analogs designated (2R-) (pn-cn) have a hydroxyl group substituted in the citryl methylene group derived from oxalacetate, while those designated (4R-) (pn-cn) have a hydroxyl group substituted in the citryl CH2-group derived from acetate. This gives information on the biosynthetic origin of the parent (citrate) but not necessarily for the substituted compound (hydroxycitrate). For convenience we have simplified the name (pn-cn)-(2R)-2-hydroxycitrate to (2R)-OHcit-(pn-cn), etc. so that the most important feature of the name is listed first. The configurations are all listed and illustrated in Fig. 1.

Some hydroxycitrate analogs are found to occur naturally in plants. The calyxes of the hibiscus plant (Hibiscus sabdariffa) contain (2S)-OHcit-(pn-cn) while the rinds of garcinia fruits (Garcinia cambogia Desr.) contain (4S)-OHcit-(pn-cn) (17). The latter is a potent competitive inhibitor of ATP citrate lyase. In the paper on nomenclature (16) some predictions were made on the substrate potential of certain hydroxycitratecitrate with citrate analogs. These and other predictions are tested by the experiments described in this paper.

**EXPERIMENTAL PROCEDURES**

**Materials** — All chemicals were analytical grade reagents purchased from the following commercial sources: Bio-Gel A-0.5m and A-1.5m (both 200 to 400 mesh) and Chelex D from Bio-Rad Laboratories; DEAE cellulose (DE52) from Reeve Angel; ATP, NADH, and G-200 from Pharmacia; coenzyme A from P-L Biochemicals; malate dehydrogenase-coupled procedure (21). The purification and analysis of kinetic data — The kinetic plots show experimental points and best fit lines as determined by computer using the program for linear competitive inhibition developed by Cleland (28) and for proposed by us for Dr. O. Spivey (Oklahoma State University, Stillwater, Oklahoma).

**Measurement of Radioactivity** — Radioactivity was measured in a Nuclear Chicago liquid scintillation spectrometer with Aquasol as the scintillation fluid.

**Preparation of [1,5-'4Cl]Citrate Lyase (K. aerogenes)** — The enzyme (5 mg) was mixed with a limited amount of 1,5-'4Cl]citrate (0.9 μmol) in the presence of MgCl2, and the reaction mixture was subjected to gel filtration on a Sephadex G-25 column (1.5 × 22 cm), previously equilibrated with 0.05 m potassium phosphate buffer, pH 7.4, containing 1.6 mm MgCl2. It was eluted with the same buffer. The activity of the enzyme as well as the radioactivity was measured in the column fractions. The fractions having enzyme activity were pooled and concentrated by dialysis against solid Sephadex G-200.

**Measurement of Rate Constants for Inactivation of Citrate Lyases by Citrate and Citrate Analogs** — Citrate lyases from K. aerogenes and S. dicentactilactis were treated with Chelex D until they showed an absolute requirement for divalent cations for enzyme activity. Measurement of rate constants for "reaction-inactivation" in the presence of citrate, from "single sweep" curves, have been described earlier (20). For hydroxycitratecitrate and tricarboxylate, the enzyme was incubated separately with the individual citrate analogs in a reaction mixture containing 29 μmol of Tris/HCl, pH 7.4, and 1 μmol of MgCl2 (or 0.35 μmol of ZnCl2) in a total volume of 0.1 ml. Suitable aliquots were taken at various time periods of incubations at 25° and the remaining citrate lyase activity was measured. The loss of enzyme activity followed first order kinetics and the rate constants of inactivation were calculated from these plots.

**RESULTS**

The enzyme was partially purified by us (Singh, M., Böttger, B. Stewart, C. Brookes, O. C., and Sree, P. A. (1979) Biochem. Biophys. Res. Commun. 53, 1); but it was misidentified as an acetyltransferase due to the presence of contaminating ATP and by the following procedure. (a) About 10 g of K. aerogenes, grown in the media described by O'Brien and Stern (26), was sonicated in 50 ml of 0.05 m potassium phosphate buffer (pH 7.4). After centrifugation at 20,000 × g for 30 min, the supernatant fluid was collected. (b) The supernatant fluid was filtrated with 2% protamine sulfate until all citrate lyase was precipitated. The solution was centrifuged and the resulting precipitate was discarded. (c) The ligase was precipitated by adding solid ammonium sulfate (50% saturation), and the precipitate was collected by centrifugation and dissolved in a small volume of the same buffer. (d) Further purification was obtained by gel filtration on a Bio-Gel A-0.5m column (2.5 × 50 cm) equilibrated and eluted with the same buffer. The ligase was assayed by the method of Schmellkamp and Eggerter (27). The reaction mixture consisted of 50 μmol of potassium phosphate, pH 7.4, 5μmol of MgCl2; 0.2 μmol of dithiothreitol; 0.5 μmol of ATP; 2.5 μmol of acetate; 0.6 mg of deacetyl citrate lyase; and 0.05 mg of the ligase in a total volume of 0.5 ml. The mixture was incubated at 25° and suitable aliquots were taken at several time periods and assayed for citrate lyase activity.

**Analysis of Kinetic Data** — The kinetic plots show experimental points and best fit lines as determined by computer using the program for linear competitive inhibition developed by Cleland (28) and performed for us by Dr. O. Spivey (Oklahoma State University, Stillwater, Oklahoma).

**Materials** — All chemicals were analytical grade reagents purchased from the following commercial sources: Bio-Gel A-0.5m and A-1.5m (both 200 to 400 mesh) and Chelex D from Bio-Rad Laboratories; DEAE cellulose (DE52) from Reeve Angel; ATP, NADH, and G-200 from Pharmacia; coenzyme A from P-L Biochemicals; malate dehydrogenase-coupled procedure (21). The purification and analysis of kinetic data — The kinetic plots show experimental points and best fit lines as determined by computer using the program for linear competitive inhibition developed by Cleland (28) and performed for us by Dr. O. Spivey (Oklahoma State University, Stillwater, Oklahoma).

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**Effect of Hydroxycitratecitrate on Citrate Lyases from Streptococcus dicentactilactis and Klebsiella aerogenes** — All four isomers of hydroxycitratecitrate are linear competitive inhibitors for citrate in the reaction catalyzed by the citrate lyases from S. dicentactilactis (Fig. 2) and from K. aerogenes (Fig. 3). The kinetic constants are summarized in Table I. The isomers of hydroxycitratecitrate differ in their activities with the two enzymes. For the S. dicentactilactis enzyme, (2R)-OHcit-(pn-cn) was the strongest inhibitor and (4R)-OHcit-(pn-cn) was the weakest. For the K. aerogenes enzyme, (4S)-OHcit-(pn-cn) was the strongest inhibitor and (4R)-OHcit-(pn-cn) the weakest. (2S)-OHcit-(pn-cn) caused a rapid inactivation of the enzyme from K. aerogenes which made difficult the determination of the value of Km of citrate (Table I).

Citrate lyase has been demonstrated to undergo inactivation during the course of the reaction it catalyzes (20). The rate constants for inactivation of citrate lyase from both K. aerogenes and S. dicentactilactis by citrate and citrate analogs are presented in Table II. The rate of inactivation produced acetate in the preparation of acetyl phosphopantetheine which was presumed to be the acetyl donor.
by preincubation of the enzyme with Mg²⁺ or Zn²⁺ and various citrate analogs was greater with the K. aerogenes enzyme than the S. diacetilactis enzyme. Zn²⁺ caused a slower rate of apparent inactivation than did Mg²⁺ in the presence of all citrate analogs except (2S)-OHcit-(pπ). The most potent inactivator, of all tricarboxylic acids examined with both enzymes, was (2S)-OHcit-(pπ). (4S)-OHcit-(pπ) produced the slowest rate of inactivation.

Citrate lyase contains an essential acetyl group, the loss of which may occur during the course of the reaction it normally catalyzes. This loss results in inactivation of the enzyme (10, 11). The nature of this inactivation with various citrate analogs was investigated. [¹⁴C]Acetyl citrate lyase was incubated with citrate, tricarballylate, or the hydroxycitrate isomers and the mixture then placed over a Sephadex G-25 column (Fig. 4). The control (free enzyme) gave a peak near...
Fraction 21 (Peak A) while on deacetylation a peak near Fraction 38 (Peak B) was obtained, which was shown to be free acetate (10, 22). The release of [14C]acetate from [14C]acetyl citrate lyase by citrate, hydroxycitrates, and a citrate analog is summarized in Table III. Citrate and the hydroxycitrate isomers caused a complete deacetylation of the enzyme as evidenced by the absence of any radioactivity in the fractions (Peak A) containing the enzyme. Tricarballylate produced a partial deacetylation of the enzyme.

These data suggested that the inactivation produced by these tricarboxylic acids is due to deacetylation of the enzyme. This was confirmed by incubating the inactivated citrate lyase from K. aerogenes (produced by treatment with (2S)-OHcit-(pn,1)) with purified acetate:SH-(acyl carrier protein) enzyme ligase (AMP) in the presence of acetate and ATP. A recovery of 38% citrate lyase activity was obtained after a 40-min incubation. On further incubation for a total of 6 h, 82% of the initial activity was recovered.

Effect of Hydroxycitrate Inhibition on ATP Citrate Lyase—(4S)-OHcit-(pn,1) is a very potent linear competitive inhibitor of ATP citrate lyase from rat liver, as already reported (15). (4R)-OHcit-(pn,1), the other stereoisomer with a hydroxyl group substituted on carbon 4, was a less effective inhibitor, but a more potent inhibitor than the two isomers with hydroxyl groups substituted on carbon 2. These and other kinetic constants are summarized in Table I.

Effect of Hydroxycitrates on Citrate Synthase—In general, the hydroxycitrate isomers are very weak inhibitors of citrate synthase (Table I). (4R)-OHcit-(pn,1) and (4S)-(pn,1)-OHcit, the isomers with a hydroxyl group on carbon 4, were somewhat stronger inhibitors than the isomers hydroxylated on carbon 2.

General substrate Activity of Hydroxycitrates for the Citrate Enzymes—A comparison of the kinetic constants of the stereoisomers of hydroxycitrate for the four citrate enzymes is presented in Table I. Values of $K_i/K_m$ (citrate) are listed. The extremely low value for (4S)-OHcit-(pn,1) with ATP citrate lyase and the high values for three of the citrate analogs with
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No simple pattern of activity or inhibition of the three citrate enzymes by hydroxycitrate, as listed in Tables I and IV, emerged from these studies. Therefore, we found it necessary to consider both the detailed mechanisms of each enzyme, as understood to date, and the various possible modes of binding of the hydroxycitrates to enzyme. In the hydroxycitrates there are four groups available for chelation to a metal ion, but, in an unstrained situation, only three of these four groups will take part in this chelation. The four groups under discussion are the central carboxyl group (on C(3)), the central hydroxyl group (on C(3)), the terminal hydroxyl group (on C(2) or C(4)), and the terminal carboxyl group (on C(5) or C(5)).

From the known sterochemistry of the cleavage of citrate (3, 4), it is reasonable to assume that (2R)-OHcit-(pn⇝) and (2S)-OHcit-(pn⇝) would give rise to acetate and hydroxyoxalacetate (which in turn can tautomerize to dihydroxymaleate or dihydroxyfumarate). Englard and Siegel reported that...
was incubated in the absence of divalent cations for enzyme activity. Measurements of rate constants for "reactivation-inactivation" were carried out as described in the text. 

Citrate lyases from *Klebsiella aerogenes* and *Streptococcus diaeetilactis* were treated with Chelex D until they showed absolute absence of any radioactive activity. Measurements of rate constants for "reactivation-inactivation" were carried out as described in the text. 

**TABLE II**

| Preincubation | $k_{-25}^*$ | $S$ | $D$ | $T$ |
|---------------|-------------|-----|-----|-----|
| 1. 10 mM Citrate + 10 mM Mn$^{2+}$ | 1.21 | 0.06 | 0.08 |
| 2. 10 mM Citrate + 7.5 mM Zn$^{2+}$ | 0.17 | 0.01 | 0.02 |
| 3. 10 mM Tricarballylate + 10 mM Mn$^{2+}$ | 0.06 | 0.03 | 0.04 |
| 4. 10 mM Tricarballylate + 2.5 mM Zn$^{2+}$ | 0.04 | 0.03 | 0.05 |
| 5. 10 mM (4S)-OHcit-(pn$_{2+}$) + 10 mM Mn$^{2+}$ | 0.01 | 0.004 | 0.005 |
| 6. 10 mM (4S)-OHcit-(pn$_{2+}$) + 2.5 mM Zn$^{2+}$ | 0.00 | 0.00 | 0.001 |
| 7. 10 mM (2S)-OHcit-(pn$_{2+}$) + 10 mM Mn$^{2+}$ | 0.05 | 0.05 | 0.06 |
| 8. 10 mM (2S)-OHcit-(pn$_{2+}$) + 2.5 mM Zn$^{2+}$ | 0.05 | 0.07 | 0.08 |
| 9. 10 mM (4S)-OHcit-(pn$_{2+}$) + 10 mM Mn$^{2+}$ | 0.05 | 0.01 | 0.02 |
| 10. 10 mM (4S)-OHcit-(pn$_{2+}$) + 2.5 mM Zn$^{2+}$ | 0.03 | 0.009 | 0.01 |
| 11. 0.5S mL (4S)-OHcit-(pn$_{2+}$) + 10 mM Mn$^{2+}$ | 0.15 | 0.02 | 0.03 |
| 12. 0.5S mL (4S)-OHcit-(pn$_{2+}$) + 2.5 mM Zn$^{2+}$ | 0.21 | 0.27 | 0.28 |

**FIG. 4.** Deacetylation of $[^{14}C]$Acetyl citrate lyase from *Klebsiella aerogenes* by citrate and (4S)-OHcit-(pn$_{2+}$). $[^{14}C]$Acetyl citrate lyase was obtained by reacting the enzyme with a limited amount of [1,5-$^{14}$C]citrate as described in the text. The $[^{14}C]$Acetyl enzyme was incubated with citrate or (4S)-OHcit-(pn$_{2+}$) and subjected to gel filtration on a Sephadex G-25 column (1.5 x 22 cm) and each fraction (15 drops) was counted. For the control, coreactant was added. The peak centering at Fraction 21 (void volume) contained citrate lyase, and the peak centering at Fraction 38 (inner volume) was free acetate. 

D(-)-tartrate and meso-tartrate are substrates of malate dehydrogenase (30), presumably giving rise to hydroxylacetate and thus this compound is probably substrate for the malate dehydrogenase reaction. (4S)-OHcit-(pn$_{2+}$) and (4R)-OHcit-(pn$_{2+}$) give rise to oxalacetate and glyoxylate so that if reaction occurs their assay with malate dehydrogenase presents no problems.
Therefore the anhydride, postulated as an intermediate in the citrate synthase reaction (31), is less likely to form, i.e. lactone formation (5-carboxyl to 2-hydroxyl group or 1-carboxyl to 4-hydroxyl group) might take precedence over anhydride formation (5-carboxyl to 3-carboxyl). If this were the explanation it would lead us to predict that aminocitrates would also not be substrates of citrate synthase since they might also preferentially form γ-lactams.

Oxalacetate is thought to bring citrate synthase into a good conformation for reaction (32). The two isomers of hydroxycitrate that are unsubstituted in the oxalacetate-derived (-2-n,-J) end of citrate (i.e. (4S)-OHcit-@n,-J and (4R)-OHcit-(pn,-J) bind best. Thus conformation changes in the oxalacetate-derived end of the parent citrate molecule are less well tolerated than those in the acetate-derived end.

Citrone Lyase — In the first part of the citrate lyase reaction, citrate, on binding, is exchanged with an acetyl group bound to a prosthetic group to give citryl enzyme and acetate (33-35). Cleavage of the citryl enzyme gives oxalacetate and regenerates acetyl enzyme. This cleavage probably involves initial ionization of the central hydroxy group of citrate (36-38), and this ionization is facilitated by metals. Indeed, divalent metal ions (magnesium) are necessary for the activity of this enzyme (and also for ATP citrate lyase).

Citrate lyase contains an acetyl group bound to a sulphydryl group of a CoA-like bound prosthetic group, so that a built-in thiol ester group (analogous to acetyl-CoA in citrate synthase) is present. Any mechanism which results in loss of the acetyl group from the enzymes leads to inactivation (10). Normally citrate binds and is cleaved, leaving an acetyl group bound to the enzyme and liberating oxalacetate. The acetyl enzyme is then available for cleavage of another citrate ion.

However, if the binding is nonproductive, the acetyl group is displaced on binding but is not replaced when the ligand leaves the active site. This nonproductive binding apparently occurs with (2S)-OHcit-(pn,-J), as described under "Results." The other three hydroxycitrates bind and react, and, as a result, leave acetyl or glycolate to bind to the enzyme. However, in these reactions some inactivation also occurs. The reaction is fastest with (2R)-OHcit-(pn,-J) which gives an acetyl group on cleavage. (4S)-OHcit-(pn,-J) and (4R)-OHcit-(pn,-J) undergo reaction at a rate which is about 9 to 23% of the rate with (2R)-OHcit-(pn,-J). The product of this cleavage is presumably glycolate rather than acetate.

However, the question arises, of the four isomers tested why does (2R)-OHcit-(pn,-J) undergo the fastest reaction in the presence of citrate lyase while (2S)-OHcit-(pn,-J) (which also gives acetate on cleavage) undergoes no reaction at all. The answer probably lies in the fact that (2S)-OHcit-(pn,-J) can bind in more than one way to the enzyme and possibly binds predominantly in the nonproductive mode. Presumably, hydroxycitrates are bound in a "citrate-like" manner when they undergo cleavage as shown diagrammatically in Fig. 5. However, an alternate mode of binding, involving the terminal hydroxyl and carboxyl groups and the central carboxyl group, which is found in crystalline rubidium fluorocitrate (13), may be nonproductive and hence result in inhibition. We assume that the magnesium binding site involves the pro-R end of citrate (the oxalacetate-derived end) since chelation to the thioester position of the acetyl-derived end is less likely. As a result we have included the (pn,-J)-1-carboxyl group but not the (pn,-J)-5-carboxyl group in the metal chelation in Figs. 5 and 6. A diagram of the proposed "fluorocitrate-like" binding is given in Fig. 6. (2R)-OHcit-(pn,-J) cannot bind in this fluorocitrate-like manner and therefore competition with the citrate-like manner of binding does not occur.

**ATP Citrate Lyase** — The ATP citrate lyase is believed to catalyze its reaction by way of the following steps. Initially,
ATP interacts with the enzyme, in the presence of magnesium ions, to give phosphorylated enzyme (39) and ADP. Citrate is then believed to react with the phosphoenzyme to give an enzyme-citryl-phosphate complex (40). It is not clear whether magnesium is necessary at this second stage or not. Phosphate is released and the citryl enzyme then interacts with CoA, without the intervention of any metal ion, to give enzyme-bound citryl-CoA which is cleaved to give oxalacetate and acetyl-CoA (41).

When citrate reacts with phosphoenzyme, phosphate is liberated. The release of phosphate may not be the result of a direct displacement when the citrate binds since it is believed that an enzyme-citryl-phosphate complex can exist as an intermediate in the reaction. The loss of phosphate is also caused by other tricarboxylic acids, including di-isocitrate, tricarboxylic acids, and cis- and trans-aconitate, but not by dicarboxylic acids (39). Since none of the four acids listed above contain central hydroxyl groups, the reaction (i.e. cleavage) cannot occur and therefore an inhibition occurs.

The four hydroxycitrates bind well and all undergo a reaction except (4S)-OHcit-(pncit) which is found to be an extremely potent binder (see Table I). No reports of other analogs, e.g. isocitrate, binding as tightly, have been made. We suggest that, again, alternate modes of binding of (4S)-OHcit-(pncit) to the enzyme occur but that, in addition, interaction of the group not involved in binding to the normal binding sites can occur in some additional way with another active site group, perhaps the phosphate binding site.

Conclusion—We suggest that hydroxycitrate isomers, with an extra hydroxyl group available for chelation, can bind in the same manner as citrate. However, alternate modes of binding are also possible, some of which compete with or replace the citrate-like binding with the result that no cleavage can occur, and inhibition occurs.

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