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CONCERTED INHIBITION OF NADP$^+$-SPECIFIC ISOCITRATE DEHYDROGENASE BY OXALACETATE AND GLYOXYLATE

I. OXALOMALATE FORMATION AND STABILITY, AND NATURE OF THE ENZYME INHIBITION

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Summary

Oxalacetate and glyoxylate are each weak inhibitors of NADP$^+$-specific isocitrate dehydrogenase (*threo*-D_S-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42). Together, however, they act in a concerted manner and strongly inhibit the enzyme. The rates of formation and dissociation of the enzyme inhibitor complex, and the rate of formation and the stability of the aldol condensation product of oxalacetate and glyoxylate, oxalomalate, were examined. The data obtained do not support the often suggested possibility that oxalomalate, per se, formed non-enzymatically in isocitrate dehydrogenase assay mixtures containing oxalacetate and glyoxylate, is responsible for the observed inhibition of the enzyme. Rather, the data presented in this communication suggest that oxalacetate binds to the enzyme first, and that the subsequent binding of glyoxylate leads to the formation of a catalytically inactive enzyme-inhibitor complex.

Introduction

NADP$^+$-dependent isocitrate dehydrogenases (*threo*-D_S-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) catalyze the NADP$^+$-specific oxidation of isocitrate to form oxalosuccinate and the subsequent decarboxylation of this compound to yield α-ketoglutarate and bicarbonate. A number of NADP$^+$-specific isocitrate dehydrogenases have been shown to be strongly

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inhibited in the presence of a mixture of oxalacetate and glyoxylate and this has been referred to as a "concerted inhibition" [1,2]. However, when either compound is present separately in assay mixtures, the enzyme is only weakly inhibited.

The concerted inhibition has been found to occur with the $NADP^+$-specific isocitrate dehydrogenases from a number of sources including *Escherichia coli* [3,4], *Brevibacterium flavum* [4,5], *Bacillus subtilis* [4], pig heart [4,6,7] and liver [8], *Crithidia fasciculata* [9], *Thiobacillus novellus* [10], *Tetrahymena pyriformis* [11], *Bacillus megaterium* [12], *Hydrogenomonas eutropha* [13], and *Acinetobacter lwoffi* [14].

Although most NAD^+-specific isocitrate dehydrogenases are not subject to concerted inhibition by oxalacetate plus glyoxylate, the inhibition has been reported to occur with the NAD^+-specific enzymes from the bacterial autotrophs, *H. eutropha* [13] and *Thiobacillus thiooxidans* [15] and from the protozoan, *C. fasciculata* [16].

Payes and Laties [17] reported that isocitrate dehydrogenase was inhibited by a non-enzymatic condensation product of glyoxylate and oxalacetate and suggested that the inhibitor was not the direct condensation product, oxalomalate, but rather γ-hydroxy-α-ketoglutarate which is formed by the subsequent spontaneous decarboxylation of oxalomalate. In more detailed studies, Ruffo et al. [18] reported that oxalomalate was stable, and could be synthesized if acid conditions were avoided. Ruffo et al. [6] subsequently reported that oxalomalate was a much stronger inhibitor of partially purified pig heart isocitrate dehydrogenase than was γ-hydroxy-α-ketoglutarate and suggested that since oxalomalate could form non-enzymatically from glyoxylate and oxalacetate under physiological conditions, it might play an important role in the cellular regulation of isocitrate metabolism.

Ozaki and Shiio [5] and Shiio and Ozaki [4] also studied the concerted inhibition employing partially purified $NADP^+$-dependent isocitrate dehydrogenase from *B. flavum* and pig heart and crude preparations from *B. subtilis* and *E. coli*. Marr and Weber [9] published the results of a similar study using partially purified enzyme from *C. fasciculata*. In these studies, the condensation product, oxalomalate, was reported to be a much weaker inhibitor of the enzyme than the same concentrations of glyoxylate plus oxalacetate. Furthermore, it was not possible to demonstrate the formation of either oxalomalate or γ-hydroxy-α-ketoglutarate from oxalacetate plus glyoxylate during the course of the enzyme assays. From their data, oxalomalate did not appear to be present at sufficiently high levels to account for the observed inhibition of the enzyme. On this basis, the authors suggested that oxalacetate and glyoxylate bind separately to the enzyme with the subsequent formation of an enzyme-concerted inhibitor complex.

Despite the efforts of numerous investigators, the mechanism of the concerted inhibition of isocitrate dehydrogenases by glyoxylate plus oxalacetate is still obscure. In an effort to clarify some of the ambiguity concerning the inhibition, we have undertaken a study to determine whether oxalacetate and glyoxylate bind independently in the formation of the enzyme-concerted inhibitor complex or whether they first condense to form either oxalomalate or γ-hydroxy-α-ketoglutarate which then inhibit the enzyme. Whereas all previous

studies have been conducted utilizing only partially purified isocitrate dehydrogenases, the studies to be reported here were carried out employing electrophoretically pure $NADP^+$-specific isocitrate dehydrogenase isolated from *E. coli.*

Experimental Procedure

Materials. Malic dehydrogenase, bovine L-glutamic dehydrogenase, bovine lactic dehydrogenase, phenylhydrazine · HCl, sodium glyoxylate, NADH, morpholinopropane sulfonic acid, morpholinoethane sulfonic acid, $NADP^+$, and monosodium α-ketoglutaric acid were purchased from Sigma. Porcine asparate aminotransferase was obtained from Worthington, and analytical reagent grade glycerol was obtained from Mallinckrodt. Sodium D,L-isocitrate (*allo*-free) and oxalacetic acid were obtained from Calbiochem. Sodium [^{14}C]bicarbonate and DL[4-^{14}C]aspartic acid were purchased from New England Nuclear. Insta-Gel was obtained from Packard. All other organic and inorganic chemicals were of high purity.

Purification of isocitrate dehydrogenase. E. coli $NADP^+$ specific isocitrate dehydrogenase was purified to electrophoretic homogeneity by a modified version (Johanson et al. [19]) of the procedure of Reeves et al. [20]. Before use in kinetic studies, the enzyme was dialyzed at 5° C against buffer containing 25 mM potassium morpholinopropane sulfonate, pH 6.5, 250 mM KCl, 2.0 mM $MnCl_2$ and 5% glycerol.

Assays. The isocitrate dehydrogenase assay was based on methods described previously by Reeves et al. [21]. The assays were carried out at 30° C in a "standard assay buffer" which contained 50 μmol potassium morpholinopropane sulfonate, pH 7.5; 100 μmol KCl; 1.0 μmol $MnCl_2$, and 0.05 ml glycerol. $NADP^+$ was added to a concentration of 0.59 mM immediately before the assay. D,L-isocitrate was used in all assays, and the concentration of the true substrate, *threo*-D_S-isocitrate, is given in all cases. The final assay volume was 1.0 ml. The increase in absorbance at 340 nm from NADPH formation was recorded on a Cary 15 recording spectrophotometer. The ϵ_{340} for NADPH is $6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$. Variations in the assay buffer, or procedure, are indicated where they were made.

Pyruvate was determined at pH 7.5 in a reaction mixture containing 0.3 μmol NADH, and 10 units of lactic dehydrogenase in a final volume of 1.0 ml. The reaction was started by the addition of the enzyme and the resulting decrease in absorbance at 340 nm was measured. The ϵ_{340} for NADH is $6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

Oxalacetate determinations were based on assay methods described previously by Reeves et al. [21] employing malic dehydrogenase and 50 mM potassium morpholinopropane sulfonate, pH 7.5. The NADH was added to a concentration of 0.3 mM followed by 1 unit of malic dehydrogenase. The decrease in absorbance at 340 nm after addition of malic dehydrogenase was measured.

Glyoxylate was assayed by the procedure of McFadden and Howes [22] as modified by Shiio and Ozaki [4].

γ-Hydroxy-α-ketoglutarate was assayed using glutamic dehydrogenase to aminate the compound reductively [23,24]. Each assay contained 50 μmol potassium morpholinopropane sulfonate, pH 7.5; 20 μmol NH_4Cl; 100 μmol

KCl; 0.05 ml glycerol; 0.3 μmol NADH, and sample in a total volume of 1.0 ml. The reaction was started by the addition of 10 units of glutamic dehydrogenase and the decrease in absorbance at 340 nm was measured.

Preparation of [4-^{14}C]oxalacetate (Jenkins et al. [25] and Krebs [26]). The reaction mixture contained 100 μmol potassium phosphate, pH 7.4; 96 μmol α-ketoglutarate, pH 7.4; 1.92 μmol DL[4-^{14}C]aspartic acid containing 4.0 μCi of ^{14}C, and 10 units of asparate aminotransferase in 4 ml total volume. The mixture was incubated for 90 min at 30°C at which time approx. 90% of the L-[4-^{14}C]asparate had been converted to [4-^{14}C]oxalacetate.

Results

Decarboxylation of oxalacetate. It has been well established that oxalacetate decarboxylates spontaneously to yield pyruvate and bicarbonate [27,28]. Since the decarboxylation of oxalacetate was an important consideration in experiments to be described later, we examined the reaction in 50 mM potassium morpholinopropane sulfonate, pH 7.5, and in 50 mM potassium morpholino ethane sulfonate, pH 6.0. The decarboxylation was catalyzed markedly by 1.0 mM Mn^{2+} and was found to be substantially the same at both pH 7.5 and pH 6.0. The decarboxylation of oxalacetate follows pseudo-first-order kinetics with a k of 0.018 min^{-1} for the Mn^{2+}-catalyzed reaction at pH 7.5, as determined by the expression $\ln([OAA]/[OAA]_o) = -kt$ where [OAA] = oxalacetic acid concentration. At pH 7.5, in the absence of Mn^{2+}, the k was 0.0014 min^{-1}.

Condensation of oxalacetate and glyoxylate. A condensation reaction occurs between 1.0 mM glyoxylate and 1.0 mm oxalacetate as shown in Fig. 1. Divalent metal ions have been reported to catalyze the condensation [17,18] and, as shown in Fig. 1, Mn^{2+} substantially increases the rate of the reaction.

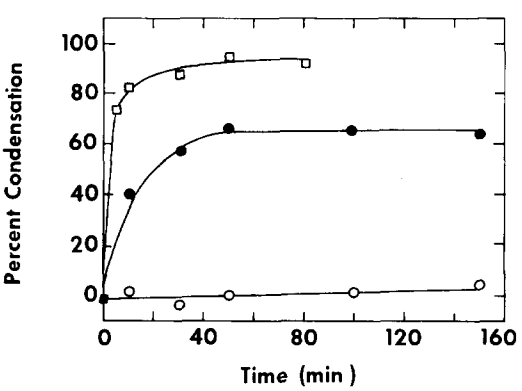


Fig. 1. Condensation of oxalacetate and glyoxylate. The rate of condensation was determined by measuring the changes in glyoxylate concentration using the assay described in Experimental Procedure. The reactions were conducted at 30°C with 1.0 mM oxalacetate and 1.0 mM glyoxylate in 50 mM potassium morpholinopropane sulfonate, pH 7.5, with 1.0 mM $MnCl_3$ (●) and without $MnCl_2$ (○). Also shown (□) is the condensation between 200 mM oxalacetate and 400 mM glyoxylate in 500 mM potassium phosphate, pH 7.0, at 40°C.

The condensation reaction which occurs between 0.1 mM oxalacetate and 0.1 mM glyoxylate in the standard assay buffer was determined. As can be seen (Fig. 2), the rate of disappearance of glyoxylate closely paralleled the rate of appearance of γ-hydroxy-α-ketoglutarate, the spontaneous decarboxylation product of oxalomalate. The oxalacetate was undergoing two reactions, a condensation reaction with glyoxylate and a spontaneous decarboxylation to pyruvate. The disappearance of oxalacetate from the reaction mixture could be accounted for by the amount of pyruvate and γ-hydroxy-α-ketoglutarate that were formed.

Stability of oxalomalate at neutral pH. In order to determine whether oxalomalate is stable at neutral pH, a reaction was carried out with 400 mM glyoxylate and 200 mM of [4-^{14}C]oxalacetate. As shown in Fig. 3, the ^{14}C was quantitatively removed from the reaction mixture as $^{14}CO_2$. The oxalacetate control, which contained no glyoxylate, showed only 30% label loss in 70 min. In a separate control experiment, which contained $NaH^{14}CO_3$ in 500 mM potassium phosphate, pH 7.0, 90% of the label was lost in 5 min.

It was not possible by the methods employed to obtain a direct measurement of the rate of $H^{14}CO_3$ loss from oxalomalate. However, it was possible to

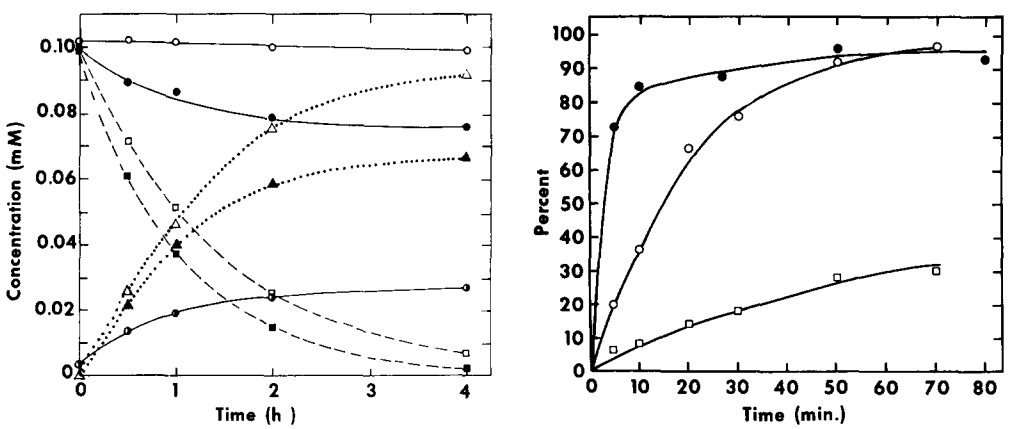


Fig. 2. Condensation of glyoxylate and oxalacetate in "standard assay buffer" at 30°C. The curves represent: (○) Control containing 0.1 mM glyoxylate; disappearance of glyoxylate. (□) Control containing 0.1 mM oxalacetate: disappearance of oxalacetate. (△) Same as □; formation of pyruvate. (●) Experimental containing 0.1 mM glyoxylate and 0.1 mM oxalacetate; disappearance of glyoxylate. (■) Same as ●; disappearance of oxalacetate. (▲) Same as ●; formation of pyruvate. (◑) Same as ●; formation of γ-hydroxy-α-ketoglutarate.

Fig. 3. Decarboxylation of oxalomalate and oxalacetate. The condensation between 200 mM oxalacetate and 400 mM glyoxylate in 500 mM potassium phosphate at pH 7.0 was determined by measuring the disappearance of glyoxylate from the reaction mixture (●). The spontaneous decarboxylation of oxalacetate was measured by determining the loss of $^{14}CO_2$ from a reaction mixture which contained 200 mM [^{14}C]-oxalacetate containing 0.5 μCi of [4-^{14}C] in 500 mM potassium phosphate, pH 7.0 (□). The decarboxylation of oxalomalate, formed from the condensation of 400 mM sodium glyoxylate and 200 mM [^{14}C]-oxalacetate containing 0.5 μCi of [4-^{14}C] in 500 mM potassium phosphate at pH 7.0, was also determined (○). All reaction mixtures were maintained at 40°C and continuously flushed with nitrogen. For radioactivity measurements, samples were withdrawn at the times indicated, added to 10.0 ml of scintillation fluid, and counted.

establish a maximum half-life of 12 min for oxalomalate under the experimental conditions employed. Furthermore, a qualitative comparison of the curves in Fig. 3 suggests that oxalomalate has a half-life of only 5—10 min under these experimental conditions.

Glutamic dehydrogenase catalyzes the reductive amination of γ-hydroxy-α-ketoglutarate and under the assay conditions employed, no measureable enzyme activity was found with glyoxylate, oxalacetate, or pyruvate as substrates. At the end of the condensation reaction between glyoxylate and [4-^{14}C]-oxalacetate described above, γ-hydroxy-α-ketoglutarate was assayed and found to be present at the expected levels.

Concerted inhibition of isocitrate dehydrogenase by oxalacetate plus glyoxylate. The concerted inhibition of *E. coli* $NADP^+$-specific isocitrate dehydrogenase was examined using "standard assay buffer" (see Experimental Procedure). The enzyme has a K_m for *threo*-D_S-isocitrate of 2 μM under these assay conditions.

The inhibition of the enzyme by oxalacetate and glyoxylate, both singly and in combination, at moderately high (10 μM) and highly saturating (150 μM) isocitrate concentrations are shown in Table I. Both oxalacetate and glyoxylate alone were found to be weak inhibitors of the enzyme. When present together, however, they act in a concerted manner and strongly inhibit enzyme activity. As shown in Table II, 10 μM oxalacetate plus 10 μM glyoxylate reduce the enzyme activity to 55% in the presence of 10 μM isocitrate. The fact that this inhibition can be completely reversed by increasing the isocitrate concentration to 150 μM strongly suggests that the inhibition may be competitive, and corroborates previous reports by other workers [4,7,9]. The inhibition of the enzyme by γ-hydroxy-α-ketoglutarate was also measured, and it was found to be a weak inhibitor of the enzyme (Table I).

Rates of enzyme-concerted inhibitor complex formation and dissociation. The rates of formation and dissociation of the enzyme-concerted inhibitor complex were determined. When oxalacetate, glyoxylate, $NADP^+$ and isocitrate were added simultaneously to an assay mixture, enzyme activity decreased

TABLE I

INHIBITION OF ISOCITRATE DEHYDROGENASE BY OXALACETATE, GLYOXYLATE, AND γ-HYDROXY-α-KETOGLUTARATE

Isocitrate dehydrogenase activity was determined as described under Experimental Procedure. The inhibitors and substrate were added to give the indicated final concentration.

Inhibitor	Concentration (μM)	Percent Activity	
		10 μM isocitrate	150 μM isocitrate
None	—	100	100
Glyoxylate	100	99	102
Oxalacetate	100	85	100
γ-Hydroxy-α-ketoglutarate	40	84	102
Oxalacetate + glyoxylate	100 + 100	4	32
Oxalacetate + glyoxylate	20 + 20	28	88
Oxalacetate + glyoxylate	10 + 10	55	98

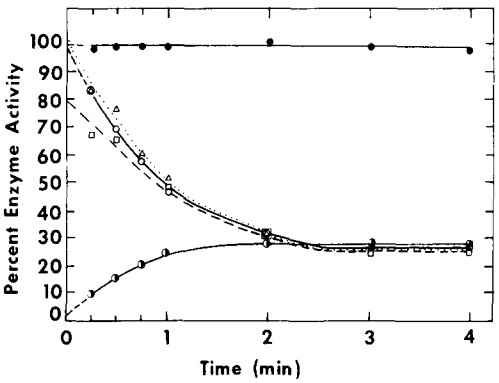


Fig. 4. Formation and dissociation of the enzyme-concerted inhibitor complex. Enzyme activity was assayed as described under Experimental Procedure. Curve • represents data obtained in control assay mixtures which contained 150 nmol of isocitrate and which were started by the addition of 0.0025 units of isocitrate dehydrogenase. Curve ○ represents the reduction of $NADP^+$ in assay mixtures which contained 150 nmol isocitrate, 100 nmol oxalacetate and 100 nmol glyoxylate. The reaction was started by the addition of 0.0025 units isocitrate dehydrogenase. Curve △ represents the reduction of $NADP^+$ in cuvettes in which 0.0025 units of enzyme were preincubated for 5 min with 100 nmol glyoxylate, and the reaction then started by the simultaneous addition of 150 nmol isocitrate and 100 nmol oxalacetate. Curve (□) represents the reduction of $NADP^+$ in cuvettes in which 0.0025 units of enzyme were preincubated for 5 min with 100 nmol oxalacetate, and the reaction then started by the simultaneous addition of 150 nmol isocitrate and 100 nmol glyoxylate. Curve (◑) represents the reduction of $NADP^+$ in cuvettes in which 0.0025 units of enzyme were preincubated for 5 min with 100 nmol each of oxalacetate and glyoxylate, and the reaction then started by the addition of 150 nmol isocitrate.

gradually and reached an equilibrium as shown in Fig. 4. In experiments in which the enzyme was preincubated for 5 min with oxalacetate, glyoxylate and $NADP^+$, and then isocitrate was added to start the reaction, enzyme activity was initially almost zero but then increased slowly and reached the same equilibrium level (see Fig. 4).

When the enzyme was preincubated with oxalacetate and $NADP^+$ for 5 min and then glyoxylate and isocitrate were added simultaneously at zero time, an initial 20% inhibition was observed and the enzyme activity decreased gradually to the same equilibrium level as before (see Fig. 4). When the enzyme was incubated for 5 min with isocitrate, $NADP^+$, and oxalacetate, the enzyme activity was not measurably inhibited by the presence of the oxalacetate and the 12 μM NADPH that was formed during the course of the incubation. This precludes the possibility that the concerted inhibition observed under our experimental conditions is due to the reoxidation of NADPH by oxalacetate in the presence of isocitrate dehydrogenase like Illingworth and Tipton found in their work [8]. When glyoxylate was added after the enzyme was preincubated for 5 min with oxalacetate, $NADP^+$, and isocitrate, the enzyme activity decreased to the same equilibrium level as before. The presence of the NADPH that was generated by the enzyme during the latter preincubation had no apparent effect on the formation of the enzyme concerted inhibitor complex. In the reciprocal experiments in which the enzyme was preincubated for 5 min with glyoxylate plus $NADP^+$ and then oxalacetate was added with isocitrate at zero time, no initial inhibition was observed.

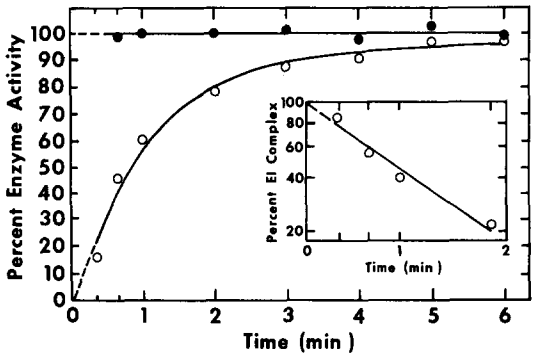


Fig. 5. Rate of dissociation of the enzyme-concerted inhibitor complex. The procedure was as described in Fig. 4. The data show the enzyme activity in a control (•) containing 3.0 mM isocitrate and 0.0023 units of isocitrate dehydrogenase, and in an identical reaction mixture in which 0.1 mM oxalacetate and 0.1 mM glyoxylate were preincubated with the enzyme for 5 min before 3.0 mM isocitrate was added (○). The inset is a $\log_{10}$ plot which represents the changes in the level of the enzyme-concerted inhibitor complex after the addition of isocitrate.

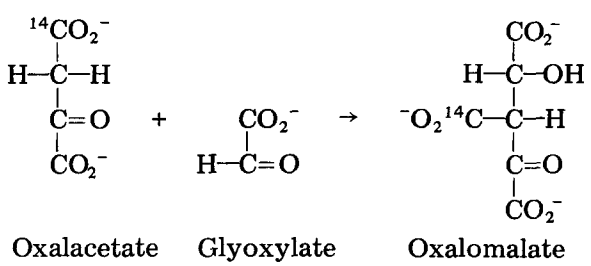
Other experiments demonstrated that lowering the Mn^{2+} concentration decreases the rates of formation and dissociation of the enzyme-concerted inhibitor complex. It was also determined that when $NADP^+$ was omitted from the reaction mixture during the 5 min preincubation with oxalacetate and glyoxylate and then added simultaneously with isocitrate to start the dehydrogenation reaction, the enzyme activity was initially 80% inhibited.

Effect of KCl on the formation and dissociation of the enzyme-concerted inhibitor complex. In other experiments (Fig. 5), the enzyme was preincubated for 5 min in the presence of oxalacetate and glyoxylate in buffer containing 250 mM KCl. After the preincubation, isocitrate was added to a highly saturating concentration of 3.0 mM and the complex dissociated gradually and enzyme activity increased to 95% of the uninhibited activity. The breakdown of the complex followed first order kinetics as shown in the inset to Fig. 5. A k of 0.8 min^{-1} was determined using the equation $\ln C/C_0 = -kt$ where C = the concentration of the enzyme-concerted inhibitor complex.

In further experiments, it was found that increasing the KCl concentration from 100 mM to 250 mM also decreased the rate of formation of the enzyme-concerted inhibitor complex and resulted in a decrease in the degree of enzyme inhibition at equilibrium.

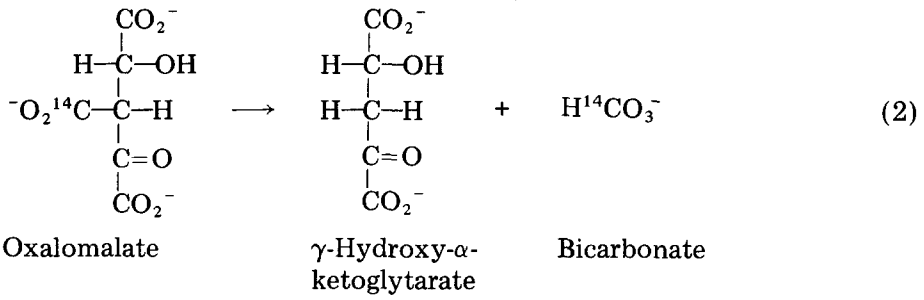
Discussion

The experiments reported in this communication demonstrate that oxalomalate, the direct condensation product of oxalacetate and glyoxylate, decarboxylates readily to form γ-hydroxy-α-ketoglutarate. The results can be visualized as follows:



(1)

Oxalacetate Glyoxylate Oxalomalate



Oxalomalate γ-Hydroxy-α-ketoglytarate Bicarbonate (2)

An aldol condensation between the acidic methylene group of oxalacetate and the aldehyde group of glyoxylate might be expected to occur on a priori grounds yielding oxalomalate, as shown in Reaction 1. Oxalomalate, which has a keto group β to the β-carboxyl group would be expected to decarboxylate a priori as shown in Reaction 2. Indeed, Payes and Laties [17] and Ruffo et al. [18] have presented good evidence that these reactions do occur. The difference between their conclusions, however, was that Ruffo et al. [6,18] suggested that oxalomalate was stable at neutral pH and decarboxylated only when the solution was acidified, while Payes and Laties [17] indicated that oxalomalate decarboxylated readily at neutral pH to yield γ-hydroxy-α-ketoglutarate. The evidence for the rapid loss of $^{14}CO_2$ from a condensation mixture obtained in the present study supports the conclusion that oxalomalate is quite unstable and readily decarboxylates even at neutral pH. The appearance at the end of the reaction of a compound that is readily reductively aminated by glutamic dehydrogenase lends further support to the conclusion that Reaction 1 and Reaction 2 occur readily at neutral pH.

Ochoa [29] has reported detailed studies on the stability of an analog of oxalomalate, oxalosuccinate. He found that in acetate buffer, pH 5.1, at 25° C, oxalosuccinate decarboxylates 8 times faster than oxalacetate. Interestingly, under our conditions oxalomalate decarboxylates 7—8 times faster than oxalacetate. Thus, the decarboxylation rates of oxalomalate and oxalosuccinate in solution appear to be closely similar.

Shiio and Ozaki [4] and Marr and Weber [9] have reported that the condensation product of oxalacetate and glyoxylate forms too slowly to account for the concerted inhibition of isocitrate dehydrogenase. As shown in Figs. 2 and 4, no more than 1% condensation occurred under our assay conditions before the inhibition reached maximal levels. This result is in agreement with the earlier studies.

In our experiments in which oxalacetate was preincubated with the enzyme and then glyoxylate and isocitrate added simultaneously, the formation of the enzyme-concerted inhibitor complex was more rapid than when both oxalacetate and glyoxylate were added simultaneously with the substrate. This result is inconsistent with the suggestion that the concerted inhibition of the enzyme results from the formation of oxalomalate in solution prior to the inhibition of the enzyme. From this, we conclude that oxalomalate is not the concerted inhibitor as has been suggested [6]. Rather, oxalacetate and glyoxylate must be binding separately to the enzyme in the formation of the enzyme-concerted inhibitor complex.

Levy [11] reported a slow formation and dissociation of an enzyme-concerted inhibitor complex in studies with the $NADP^+$-dependent isocitrate dehydrogenase from *T. pyriformis.* Charles [10] and Ruffo and Adinolfi [30] have also reported that the complex forms slowly. Evidence obtained in the present study shows that the rate of formation and dissociation are dependent on both ionic strength and Mn^{2+} concentration. This may account for the fact that other investigators have not observed the slow formation and dissociation of the complex.

Illingworth and Tipton [8] have reported studies on the reduction of oxalacetate by pig liver $NADP^+$-specific isocitrate dehydrogenase. In their studies, they found that the reduction was strongly inhibited by a slow forming complex when glyoxylate was added to the reaction mixture. It appears likely that this is the same inhibitory complex that is observed in the concerted inhibition of isocitrate dehydrogenase by oxalacetate and glyoxylate.

The enzyme-concerted inhibitor complex was found to disassociate very slowly with a k of 0.8 min^{-1}. The rate of formation of the complex was also found to be intrinsically slow. By using high substrate concentrations to inhibit the formation of the enzyme-concerted inhibitor complex, it is possible to slow the formation of the complex sufficiently so that it is similar to the rate of dissociation of the complex as shown in Fig. 4. At lower inhibitor concentrations, the level of substrate needed to compete with the concerted inhibition is lower (Table I).

In the experiments described, the binding of oxalacetate did not significantly inhibit the enzyme but a strong inhibition was seen after the addition of glyoxylate. Based on the results presented in Table I, the inhibition appears to be competitive and suggests that the glyoxylate binding site may overlap the isocitrate binding site, while it appears that the oxalacetate site does not overlap the isocitrate site. This competitive inhibition is similar to the results reported by Shiio and Ozaki [4], Marr and Weber [1], and Little and Holland [7] in their studies with the $NADP^+$-dependent isocitrate dehydrogenase from *B. flavum*, *C. fasciculata*, and pig heart, respectively. These results suggest that the binding of oxalacetate is the slow step in the formation of the enzyme-concerted inhibitor complex. When glyoxylate is also bound, an inhibitory complex is formed which dissociates slowly as shown in Fig. 5.

The results obtained when the enzyme was preincubated with oxalacetate, and then glyoxylate added together with isocitrate, suggest either that oxalacetate binds first in the formation of the enzyme-concerted inhibitor complex or that the binding occurs by a random mechanism. The data obtained are clearly inconsistent with glyoxylate binding before oxalacetate. Furthermore, the results presented suggest that the binding of glyoxylate occurs at least several times faster than oxalacetate and at a rate which was too fast to quantitate by our methods.

The results on the concerted inhibition of isocitrate dehydrogenase obtained in the present study suggest that a strong interaction occurs between oxalacetate and glyoxylate when both are bound to the enzyme. This interaction may occur by (a) conformational changes in the enzyme structure; (b) direct noncovalent interaction between oxalacetate and glyoxylate; or (c) covalent reaction between oxalacetate and glyoxylate. A clearer understanding of the exact nature of the enzyme-concerted inhibitor complex awaits further study.

Acknowledgement

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