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Mechanism of Action of Isocitrate Lyase from *Pseudomonas indigofera**

Judith O. Williams, Thomas E. Roche,† and Bruce A. McFadden‡

ABSTRACT: Steady-state kinetic analyses were carried out with highly purified isocitrate lyase from *Pseudomonas indigofera* on both the forward and reverse reactions at pH 6.8 and 7.7. The effect of the inhibitors phosphoenolpyruvate, itaconate, and maleate was also examined. At pH 7.7 the product glyoxylate was found to be a linear competitive inhibitor of isocitrate cleavage, while succinate (at concentrations as high as twice its Michaelis constant) was a linear noncompetitive inhibitor, and itaconate was a linear uncompetitive inhibitor. Phosphoenolpyruvate, itaconate, and maleate were all linear competitive inhibitors with respect to succinate and linear uncompetitive inhibitors with respect to glyoxylate. Kinetic analysis of the data suggests that the binding of glyoxylate and succinate to the enzyme is preferentially ordered with the binding of glyoxylate greatly enhancing succinate binding. Phosphoenolpyruvate, itaconate, and maleate are bound to the succinate-specific site, and require prior binding of glyoxylate.

The equilibrium constant for the condensation reaction at 30° and pH 7.7 was found to be 430 M^{-1} and is in reasonable accord with the value of 630 M^{-1} that was calculated by the Haldane equation.

The mechanism of action and regulation of activity of isocitrate lyase is of particular interest because it is the key enzyme in the anaplerotic glyoxylate cycle, which functions in numerous microorganisms and fatty seedlings (Kornberg and Elsden, 1961; Beevers, 1961). In bacteria its activity is probably important in regulating the intracellular concentrations of tricarboxylic acid cycle intermediates.

Kinetic studies of condensation of succinate and glyoxylate were carried out by Daron *et al.* (1966) on isocitrate lyase from *Pseudomonas aeruginosa*, and they concluded that the reaction proceeded either by a strictly ordered addition of substrates or by random binding of substrates in rapid equilib-

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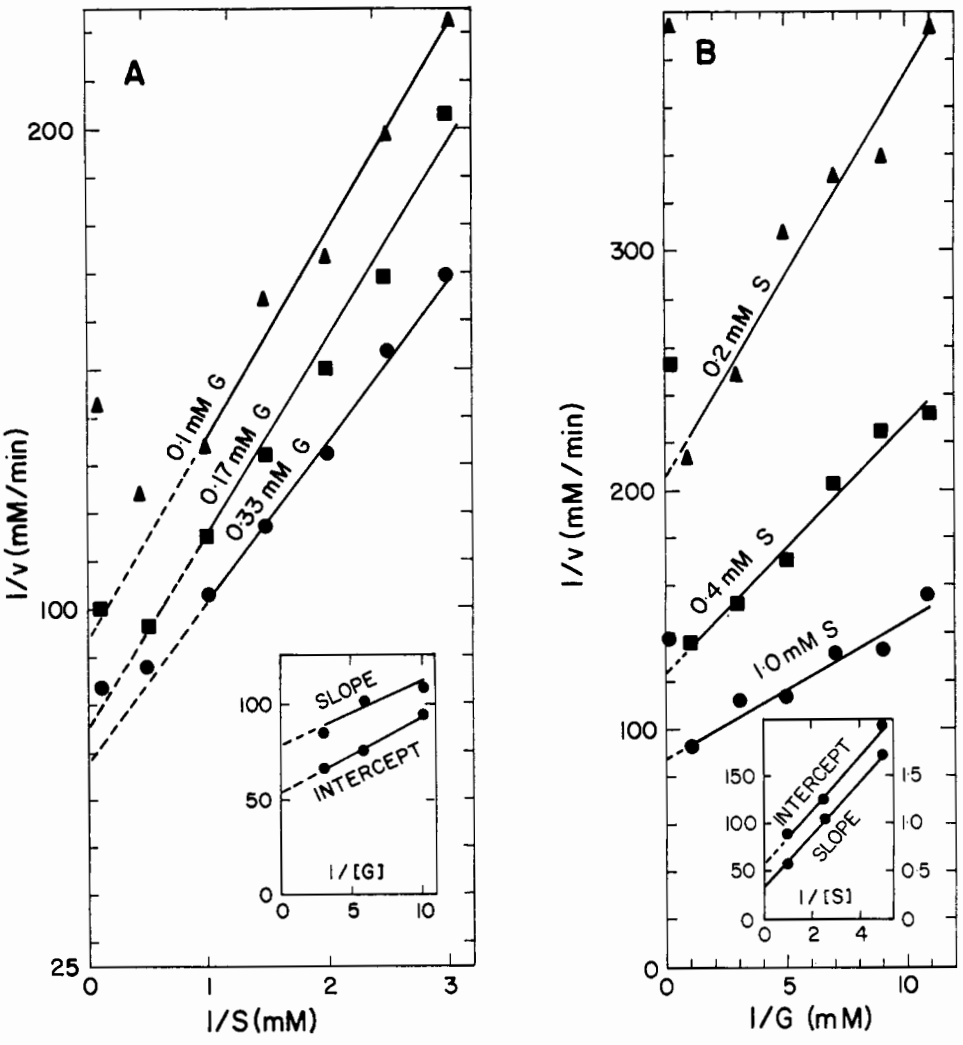


FIGURE 1: Effect of varying succinate (A) and glyoxylate (B) on the condensation reaction at pH 7.7. The isocitrate lyase concentration was 0.05 unit/ml. The *insets* show replots of the slopes and intercepts.

rium with the enzyme. In this paper we present more extensive kinetic studies with isocitrate lyase from a closely related source which are in basic accord with their results. Furthermore, we have eliminated the possibility of a rapid equilibrium random addition and show that glyoxylate is the substrate which must bind the enzyme before succinate can bind.

Materials and Methods

Materials. MOPS[1] and DL-isocitrate, trisodium salt, were purchased from Calbiochem. Highly purified isocitric dehydrogenase, trisodium phosphoenolpyruvate hydrate, sodium glyoxylate monohydrate, and monosodium $NADP^+$ were purchased from Sigma Chemical Co. Sodium 2,6-dichlorophenolindophenol was purchased from Eastman Kodak Company. Itaconic acid was purchased from the Matheson Company. Succinic acid was purchased from Mallinckrodt Chemical Works.

Highly purified isocitrate lyase was prepared from *P. indigofera* M1 as described earlier (Roche *et al.*, 1970).

Reaction Buffer. The stock buffer components for reactions in both directions were 0.1 M MOPS–NaOH containing 3 mM $MgCl_2$ and 1 mM EDTA. This combination will be referred to as MME buffer. The pH was either 6.8 or 7.7 (23°) as indicated. GSH (0.0125 M) was also present in the incubation buffer except where glyoxylate was added to the reaction mixture. In these cases an enzyme concentrate was reduced at room temperature or at 30° for at least 15 min in MME containing GSH at 0.0125 M. It was then diluted into the incubation buffer so that the final concentration of GSH did not exceed 50 μM.

Reaction of Glyoxylate with Tris and Glutathione. Tris not only inhibits isocitrate cleavage at low substrate concentrations (Roche *et al.*, 1970), but also reacts with glyoxylate under our reaction conditions to form an *N*-hemiacetal or Schiffs base (Duggan *et al.*, 1964; Mahler, 1961) which cannot act as a substrate for the condensation reaction. The use of MOPS buffer rather than Tris has eliminated these problems. Glyoxylate also reacts with thiols such as GSH at concentrations regularly used in our incubation mixtures. However, these complexes with Tris and GSH decompose and give the normal color yield in the glyoxylate assay (Rao and McFadden, 1965). In studies where uncomplexed glyoxylate was desired, GSH used to reduce the enzyme was diluted as described before addition of glyoxylate.

Measurement of the Cleavage and Condensation Reactions.

[1] Abbreviations used are: MOPS, morpholinopropanesulfonic acid; GSH, glutathione.

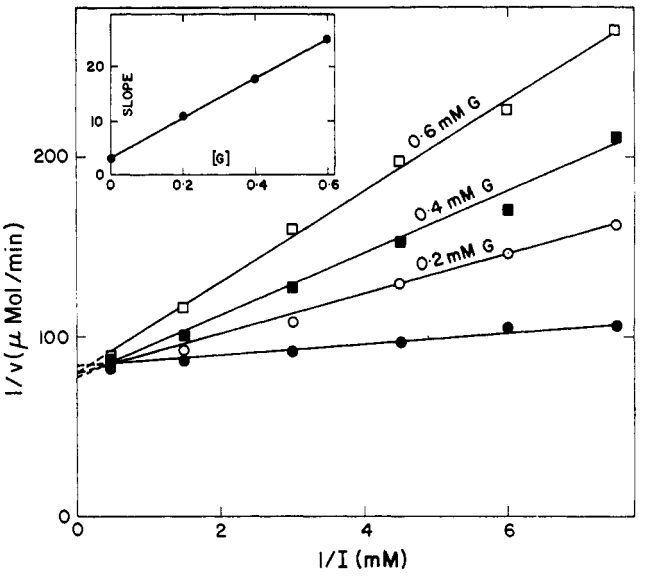


FIGURE 2: Inhibition of isocitrate cleavage by glyoxylate at pH 7.7. The incubations were carried out at 30° in 5.0 ml of MME containing 0.2 unit of isocitrate lyase; 5 μmoles of GSH was carried over with the enzyme from the reducing solution. The reaction was terminated by heat. Three time points were taken for each velocity measurement. Intercepts were not significantly different at a 0.95 confidence level. The *inset* shows a replot of the slopes.

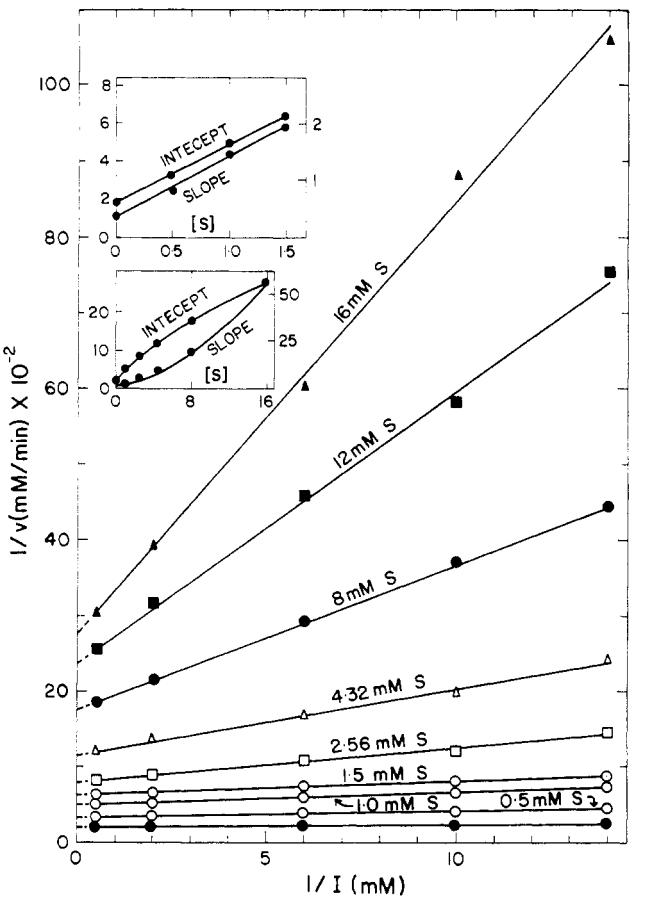


FIGURE 3: Inhibition of isocitrate cleavage by succinate at pH 7.7. The *insets* show replots of the slopes and intercepts. Not all points for (S) less than 1.5 mM are shown in the lower inset (condensed scale).

Except as noted, the cleavage reaction was initiated with isocitrate, and the incubation, termination, and measurement of glyoxylate were conducted essentially as described by Roche *et al.* (1970). The specific activity of enzyme was measured by this method at saturating DL-isocitrate (4.0 mM) using a 10-min incubation time under conditions for which there is no rate decrease in this interval. Succinate was assayed using succinic dehydrogenase (Rodgers, 1961) prepared from chicken hearts.

For the condensation reaction the amount of isocitrate formed was measured spectrophotometrically by observing the continuous reduction of $NADP^+$, which had been added to the reaction mixture along with an excess of isocitric dehydrogenase. The reactions were carried out at 30° in 0.8 ml of MME containing 0.36 μmole of $NADP^+$, 0.2 unit of isocitric dehydrogenase, 0.01–0.05 unit of isocitrate lyase, and substrates as indicated. The reaction was usually initiated by addition of glyoxylate or succinate.

Equilibrium Constant. The isocitrate present in equilibrium mixtures was assayed after heat inactivation of isocitrate lyase by adding 0.3 unit of isocitric dehydrogenase and 0.45 μmole of $NADP^+$ to 1.0 ml of a suitable dilution in MME

TABLE I: Kinetic Constants Obtained with Isocitrate Lyase at 30°.[a]

	Condensation					
	Michaelis Constants (mM)		Inhibition Constants (mM)			
pH	Succinate (K_S)	Glyoxylate (K_G)	Glyoxylate (K_{iG})	Itaconate (K_{iIt})	Phosphoenol-pyruvate	Maleate
6.8	0.07	0.05		0.0009	0.04	0.02
7.7	0.5	0.06	0.10	0.004	1.6	0.25

	Cleavage			
	Michaelis Constant[b] (mM)	Inhibition Constants (mM)		
pH	Isocitrate (K_I)	Succinate (K_{iS})	Glyoxylate (K_{iG})	Itaconate (K_{iIt})
6.8	0.006			
7.7	0.04	0.6	0.09	0.007

[a] Measurements and calculations are based on the ordered model. [b] (Roche *et al.*, 1970).

buffer. The amount of D_s-isocitrate present was calculated from the total increase in optical density at 340 mμ using standardized solutions of isocitrate (Stern, 1957).

Succinate was measured indirectly by the procedure just described for isocitrate except that 0.03 unit of reduced isocitrate lyase and excess (2.0 μmoles) glyoxylate as well as $NADP^+$ and isocitric dehydrogenase were added to the inactivated mixture. The total increase in optical density was corrected for that due to isocitrate present in the sample and was related to the amount of succinate present through the use of succinate standards.

Glyoxylate was measured colorimetrically (Roche *et al.*, 1970).

Results

Nomenclature and Kinetic Analysis. The nomenclature and general methods of kinetic analysis of Cleland (1963a–c,1967) have been used in this paper. Although isocitrate lyase requires Mg^{2+} (or certain other metal ions) for activity (Shiio *et al.*, 1965), the role of metallic ion was not investigated here. All work was done at saturating, noninhibitory concentrations of Mg^{2+}, and the system has been treated as uni-bi. The symbols, G, S, I, and It refer to glyoxylate, succinate, D_s-isocitrate, and itaconate, respectively. V_f and V_r are the maximum velocities in the directions of cleavage and condensation, respectively.

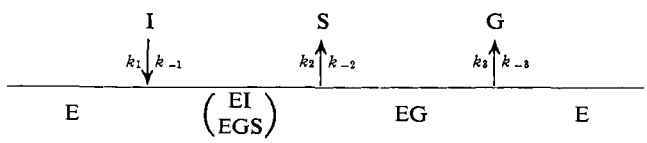
All data were analyzed using the IBM Model 360/67 computer and a program for linear least-squares analysis donated by S. R. Fink.

Kinetics and Product Inhibition. The earliest observed rate of condensation fell about 20% within 0.5 min to a constant rate. There was no obvious variation with concentrations or order of addition of substrates, with the exception that a lag of about 1 min was found when the enzyme was preincubated with high concentrations (about 10 mM) of glyoxylate. Except where otherwise indicated, the reaction rates were measured after the steady-state velocity was reached.

Figure 1 shows Lineweaver–Burk plots for data obtained at pH 7.7. At high concentrations both substrates were inhibitory. The inhibition pattern was not changed by increasing the concentration of either Mg^{2+} or isocitric dehydrogenase. Intercept replots were linear (see insets). Similar experiments were conducted at pH 6.8. Inhibition by both substrates above 1 mM was also seen at this pH.

Glyoxylate was found to be a linear competitive inhibitor of isocitrate cleavage at pH 7.7 (Figure 2), while succinate was a linear noncompetitive inhibitor (upper inset, Figure 3) at concentrations below those known to inhibit condensation. Over a larger concentration range of succinate, replots were nonlinear.

Postulated Mechanism. Based upon the preceding data, we tentatively propose the following ordered uni-bi mechanism.



The full steady-state rate equation for this mechanism is

$$v = \frac{V_f V_r([I] - [S][G]/K_{eq})}{V_r K_I + V_r[I] + \frac{K_G V_f[S]}{K_{eq}} + \frac{K_s V_f[G]}{K_{eq}} + \frac{V_f[S][G]}{K_{eq}} + \frac{V_r[I][S]}{K_{is}}}$$

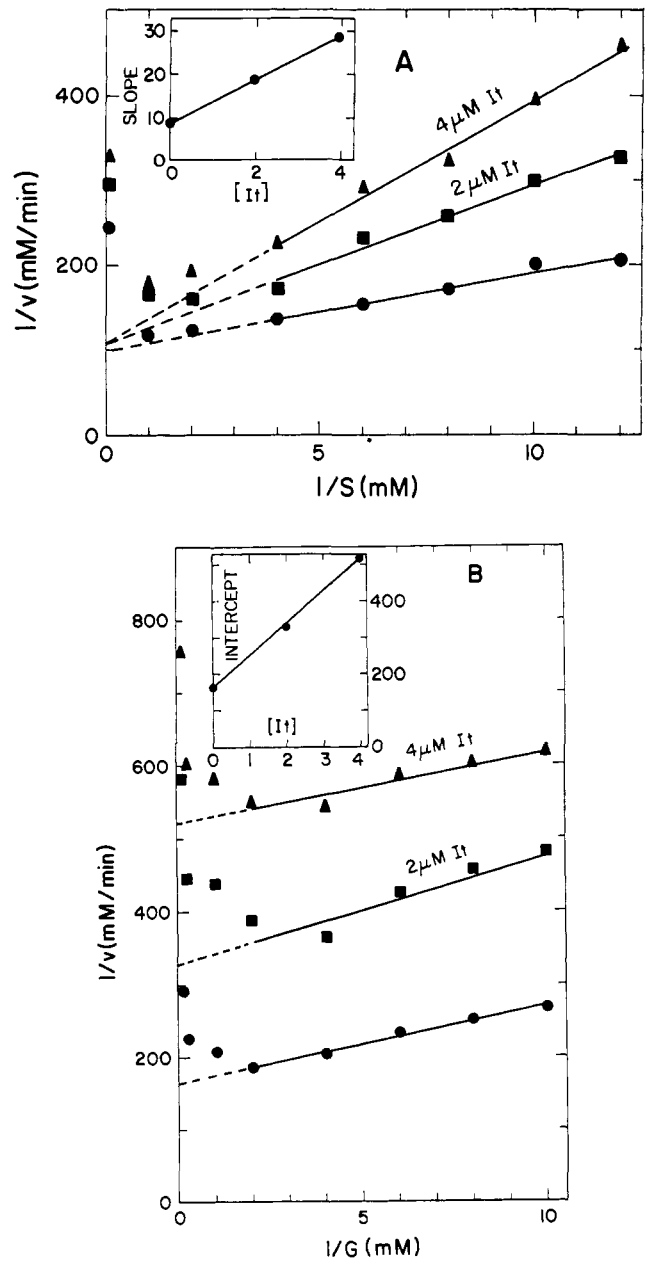


FIGURE 4: Inhibition of the condensation reaction at pH 6.8 by itaconate. The isocitrate lyase concentration was 0.04 unit/ml in (A) and 0.03 unit/ml in (B). The insets show replots of slope (A) and intercept (B). Slope differences in B were not significant at a 0.95 confidence level.

The kinetic constants are defined in terms of rate constants by Bridger and Cohen (1968).

Kinetic constants were obtained from the experiments described (Figures 1–3) and to be described assuming the ordered mechanism and are presented in Table I.

Other Inhibition Studies. The inhibitor itaconate was found to equilibrate unusually slowly with its enzyme-bound forms, and, in general, steady-state velocities of either the forward or reverse reactions were reached only after about 5 min of reaction. When itaconate was present, rate measurements were made after the steady state has been attained. Relaxation studies with itaconate will be described in another communication.

Inhibition of condensation by itaconate at pH 7.7 (Figure

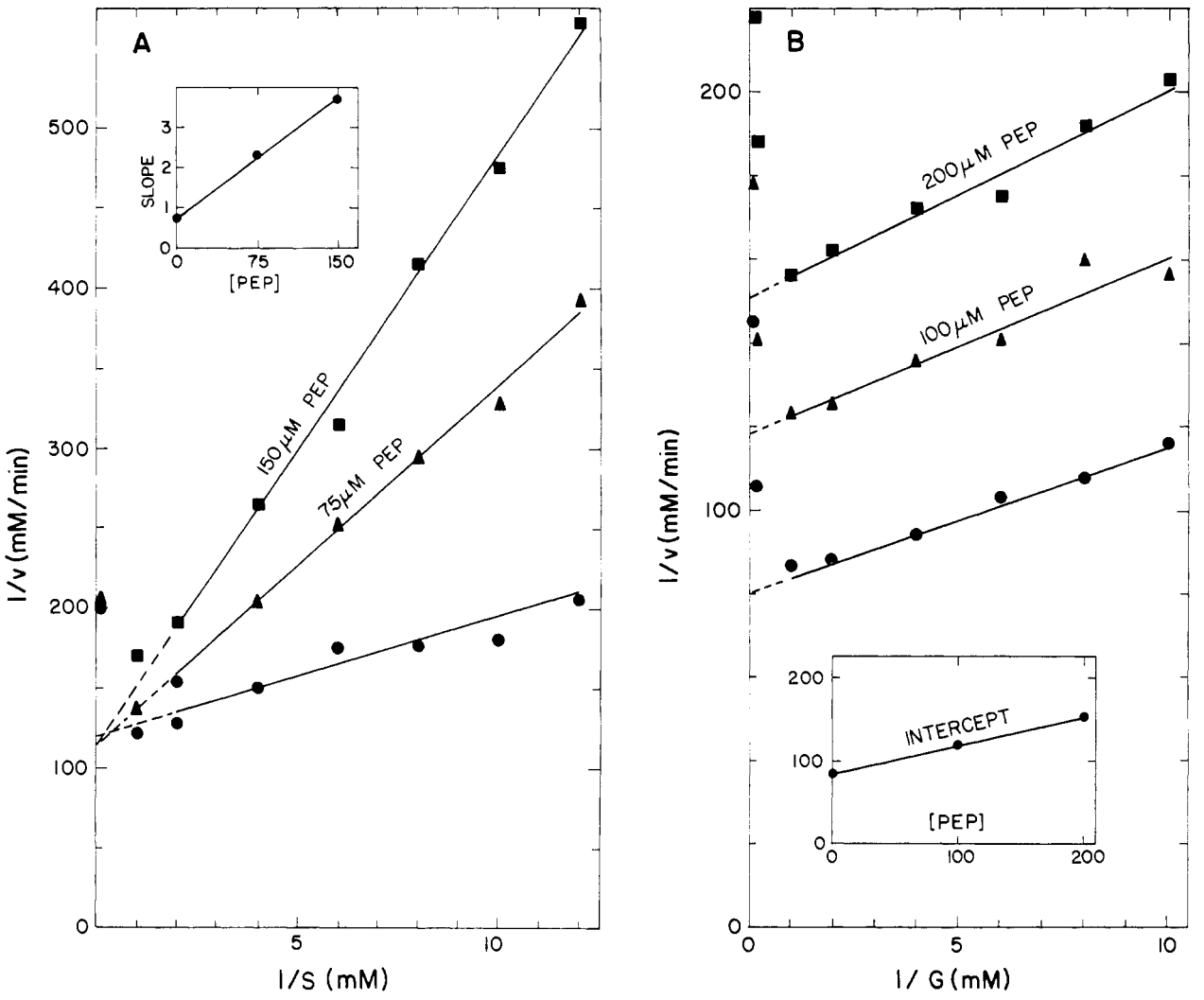


FIGURE 5: Inhibition of the condensation reaction at pH 6.8 by phosphoenolpyruvate. The isocitrate lyase concentration was 0.02 unit/ml. The insets show replots of slope (A) and intercept (B). Slope differences in B were not significant at a 0.95 confidence level.

TABLE II: Measurement of the Equilibrium Constant.[a]

Initial Reactant Concentrations (mM)			Final Reactant Concentrations (mM)			
Isocitrate	Succinate	Glyoxylate	Isocitrate	Succinate	Glyoxylate	Equilibrium Constant[b] (M^{-1})
1.0			0.18	0.73	0.74	330
1.0			0.19	0.72	0.73	360
0.5			0.065	0.36	0.43	420
	1.7	0.84	0.38	1.4	0.62	440
	1.4	0.42	0.18	1.3	0.36	380
0.30	1.4		0.12	1.4	0.19	450
0.30		0.67	0.077	0.14	0.87	630

[a] Equilibration was done at 30° for 2 hr in 4.0 ml of MME buffer at pH 7.7, containing 0.8 unit of reduced isocitrate lyase and 0.08 μmole of residual GSH plus the indicated amounts of isocitrate, succinate, and glyoxylate. Before succinate and isocitrate assays, isocitrate lyase activity was inactivated by heat. [b] K_{eq} = (isocitrate)/[(succinate)(glyoxylate)].

4) was linearly competitive with respect to succinate and linearly uncompetitive with respect to glyoxylate. This pattern indicates that it binds at the succinate-specific site and requires the prior binding of glyoxylate. The same inhibition patterns were shown by phosphoenolpyruvate (Figure 5) and maleate. Inhibition constants are in Table I.

The effect of itaconate on isocitrate cleavage was reexamined (see Rao and McFadden, 1965) in MME buffer at pH 7.7. As

expected, itaconate at concentrations of at least 0.015 mM was a linear uncompetitive inhibitor. An intercept replot gave 0.007 mM for the apparent inhibition constant $K'_{iIt} = K_{iIt}/W_{(I)\to\infty}$, where $W_{(I)\to\infty}$ equals the fraction of total enzyme which is in the EG form during the cleavage reaction when isocitrate is saturating and products are absent. Thus, $W_{(I)\to\infty} = K_{iIt}/K_{iIt}'$ was calculated to be 0.6 (pH 7.7), which indicates that the major fraction of enzyme during cleavage of saturating isocitrate is in the EG form.

Equilibrium Constant. The average equilibrium constant in the direction of condensation was 430 M^{-1} at 30°, pH 7.7, in MME (Table II).

The Haldane treatment (1930) was used to calculate the equilibrium constant for pH 7.7 at 30° as follows.

$$K_{eq} = \frac{V_r}{V_t} \times \frac{K_I}{K_S K_{iG}} = \frac{1}{1.4} \times \frac{4 \times 10^{-5}\ M}{5 \times 10^{-4}\ M \times 9 \times 10^{-5}\ M} = 630\ M^{-1}$$

Although somewhat higher, it is in reasonable agreement with the equilibrium constant obtained by direct measurement (Table II). The equilibrium constant of 34 M^{-1} obtained by Smith and Gunsalus (1957) at 27° and pH 7.6 is much less than our value obtained at a similar temperature and pH. It should be emphasized, however, that the concentration of free glyoxylate in their reaction mixtures, which contained Tris and cysteine, was lower than estimated, resulting in an erroneously low equilibrium constant.

Discussion

Preferentially Ordered Mechanism. The observed linear reciprocal plots obtained in the condensation reaction when either succinate or glyoxylate was varied at concentrations up to at least twice the respective K_m values at pH 7.7 indicate either ordered binding or a rapid equilibrium random mechanism. The latter, however, is ruled out, because the presence of a large fraction of enzyme in the EG form during isocitrate cleavage means that E and EG cannot be in rapid equilibrium. Thus, the binding is apparently ordered, and glyoxylate must be bound first (*i.e.*, to free enzyme). The linear competitive inhibition by glyoxylate and linear noncompetitive inhibition by lower concentrations of succinate further support this conclusion.

Although not conclusive, curves for slope and intercept replots representing inhibition of isocitrate cleavage by higher succinate concentrations (lower inset, Figure 3) suggest that the release of products may be technically random although highly preferential for the release of S before G (Cleland, 1963b).

Ordered Mechanism and Hydrogen Isotope Exchange. The isocitrate lyase-catalyzed incorporation of tritium (Daron *et al.*, 1966) or deuterium (Sprecher *et al.*, 1964) from the medium into succinate was glyoxylate dependent. This result can be explained by the present finding that succinate does not bind to the free enzyme except at high concentrations. Also, Sprecher *et al.* (1964) found that 1.4 moles of deuterium was incorporated per mole of succinate when isocitrate was cleaved by isocitrate lyase in the presence of excess semicarbazide. They suggested that the reason for incorporation of more than 1 mole of dueterium into succinate was that some free glyoxylate was present. However, our experiments indicate that breakdown of EG is a rate-limiting step, so that some reversal of isocitrate cleavage would be expected in the absence of free glyoxylate under the conditions used in which the reaction went essentially to completion.

Succinate Binding Site. K_S, K_I, and the inhibition constants for maleate, phosphoenolpyruvate, and itaconate, each of which acts at the succinate binding site, all decrease markedly (5- to 40-fold) when the pH decreases from 7.7 to 6.8; in contrast, K_G, K_{iG}, and the maximum velocities all decrease less than 2-fold. Presumably isocitrate binding is affected by pH at the succinate-specific portion of the active site. The pK_a's of the substrates and inhibitors are less than 6.4. Consequently, a change in the succinate-specific site on the enzyme in the pH range of 6.7 to 7.7 seems likely.

Physiological Implications. In several organisms phosphoenolpyruvate is a likely regulatory molecule for the anaplerotic glyoxylate cycle, and may act as a feedback inhibitor of isocitrate lyase, diverting metabolites through the degradative tricarboxylic acid cycle (Ashworth and Kornberg, 1963; Syrett and John, 1968; Roche *et al.*, 1970). Although they found no evidence of cooperative effects, Ashworth and Kornberg (1963) termed phosphoenolypyruvate an allosteric inhibitor of isocitrate lyase from *Escherichia coli* because it was a noncompetitive inhibitor with respect to isocitrate cleavage. Rather than allosteric (Monod *et al.*, 1965), they apparently meant that phosphoenolpyruvate was a second-site inhibitor. In a two-product system this conclusion is certainly not the only plausible one. In fact, the present work establishes that phosphoenolpyruvate inhibits by interacting with the succinate-binding site. In accord with this is the observation that phosphoenolpyruvate at lower concentrations is an uncompetitive inhibitor of isocitrate cleavage (Roche *et al.*, 1970).

Some possible advantages to the ordered release mechanism are that succinate is a good inhibitor at both low and saturating isocitrate levels, while the regulator phosphoenolpyruvate is a good inhibitor only when isocitrate is saturating. Since low isocitrate concentrations reflect depletion of intermediates in the tricarboxylic acid cycle, and isocitrate lyase replenishes these intermediates, the decreased inhibition by phosphoenolpyruvate under these conditions may be important. Thus, the preferentially ordered mechanism may have evolved from a more random one due to regulatory pressure.

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Coprecipitation of Carbonic Anhydrase by 1,1-Bis(*p*-chlorophenyl)-2,2,2-trichloroethane, 1,1-Bis(*p*-chlorophenyl)-2,2-dichloroethylene, and Dieldrin*

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ABSTRACT: The effect of 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane, (DDT), 1,1-bis(*p*-chorophenyl)-2,2-dichloroethylene (DDE), and dieldrin on the enzyme carbonic anhydrase from bovine erythrocytes and from spinach is carefully examined. Contrary to beliefs expressed in the literature, we found that these compounds are not true inhibitors of enzyme action. The esterase activity of bovine carbonic anhydrase (BCA) and the hydrase activity of spinach carbonic anhydrase (SCA) are found to be totally unaffected by any amount of DDT, DDE, or dieldrin present. However, the hydrase activity of BCA is reduced by DDT, DDE, and dieldrin; but only when these compounds are added in excess of their respective solubility limits. An amount of precipitate merely visible as a slight turbidity is sufficient to occlude from solution small amounts of enzyme (up to 7 μg/ml). It is shown that these amounts of occluded BCA represent a significant percentage of the total enzyme present in studies of hydrase activity, and furthermore that the reduction in enzymatic activity is *entirely* due to the removal of BCA from solution. We find no true inhibition whatsoever by DDT, DDE, or dieldrin.

The fact that SCA is not similarly coprecipitated is attributed to the fact that while BCA is a monomer with a molecular weight of ~30,000, SCA is probably a hexamer with a molecular weight around 180,000 and apparently does not present the proper surfaces for facile adsorption.

The growing world-wide concern for the environment has given rise to considerable interest in the effects of DDT [1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane],[1] of DDE [1,1-bis-(*p*-chlorophenyl)-2,2-dichloroethylene, a stable metabolite of DDT], and dieldrin [1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*endo,exo*-1,4:5,8-dimethononaphthalene]. One of the effects of large concentrations of DDT, DDE, and dieldrin in birds has been shown to be a decrease in egg shell thickness (Peakall, 1970). Since it is felt by some that carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) plays an important role in egg shell production,[2] one is led to look for inhibition of carbonic anhydrase activity by these compounds (Bitman *et al.*, 1969).

It was reported that small amounts of DDT can be measured very accurately with carbonic anhydrase by the inhibitory effect on the enzyme-catalyzed dehydration of HCO_3^- (Keller, 1952). Peakall found that injection of DDE into a ringdove shortly before egg laying severely depressed carbonic anhydrase activity and brought a marked decrease in the thickness of the egg shell (Peakall, 1970). Similar findings were reported (Bitman *et al.*, 1970) with Japanese quail using DDT. They observed carbonic anhydrase activity depression of 16–19% in the shell-forming gland and 22–24% in blood. Such evidence is often taken to imply that carbonic anhydrase is inhibited by DDT, DDE, and dieldrin. However, no correlation was found between shell deformability and carbonic anhydrase activity for three strains of hens (Heald *et al.*, 1968). This would tend to indicate that the effect of these compounds, in birds at least, may be more complex than a mere inhibitory action on carbonic anhydrase. Since we have been performing extensive studies on competitive and noncompetitive inhibitors of bovine carbonic anhydrase (BCA) (Pocker and Meany, 1965, 1967; Pocker and Dickerson, 1965; Pocker and Stone, 1968a,b), it was natural to investigate the inhibitory effect of

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[1] Abbreviations used are: DDT, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane; DDE, 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethylene; dieldrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*endo,exo*-1,4:5,8-dimethylnonaphthalene; BCA, bovine carbonic anhydrase.

[2] The main evidence in support of the intimate involvement of carbonic anhydrase in eggshell formation is that certain sulfonamides of the type $ArSO_2NH_2$ inhibit the calcification of eggs. These sulfonamides are known to be powerful and specific inhibitors of carbonic anhydrase.