Isocitrate Lyase. Kinetics and Substrate–Tritium Exchange Reactions*

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ABSTRACT: A kinetic analysis of the effects of substrate concentration and some substrate analogs on the isocitrate lyase reaction is presented. The data are consistent with a random reaction sequence under equilibrium conditions or an ordered sequence under steady-state conditions. Several other alternatives are eliminated. The Michaelis constants are 7.0×10^{-4} M for succinate and 1.6×10^{-4} M for glyoxylate. Other dissociation constants derived from the (Florini and Vestling, 1957) treatment of two substrate systems are: K_{GS} = (E)(S)(G)/(ESG) = 7.4×10^{-6}, K_S = (E)(S)/(ES) = 4.6×10^{-4}, K_G = (E)(G)/(EG) = 1.1×10^{-4}. The strong competitive inhibition of the isocitrate lyase reaction by maleate as compared to fumarate suggests succinate is bound to the enzyme in the *cis* form. Isocitrate lyase does not catalyze a demonstrable hydrogen exchange between succinate and the medium in the absence of glyoxylate. The enzymatically catalyzed incorporation of tritium from tritated water into succinate requires the complete reaction mixture. Pyruvate or acetaldehyde cannot be substituted for glyoxylate. A comparison of the rate of tritium incorporation into succinate with the rate of glyoxylate formation during isocitrate cleavage reveals an isotope effect of 6–15, depending on the concentration of isocitrate or glyoxylate. This suggests that the hydrogen incorporation step is rate limiting in the reaction sequence. During isocitrate formation hydrogen is incorporated into succinate at a rate which cannot be accounted for by the reverse reaction.

Enzymes which catalyze the formation of a bond between a carbon bearing an active hydrogen and a carbonyl carbon may be referred to as aldolases because of the formal similarity of their reactions with the aldol condensation. The question arises whether this similarity extends to the reaction mechanisms. By analogy with the base-catalyzed aldol condensation (Hine, 1962), *e.g.*, the enzyme might facilitate the loss of the active hydrogen from the appropriate substrate with formation of a carbanion or its enzyme-bound equivalent. Subsequent addition of this carbanion to a carbonyl acceptor, followed by proton capture, would yield the condensation product. Such a mechanism suggests the possibility of an enzyme-catalyzed hydrogen exchange between the active hydrogen compound and the medium in the absence of carbonyl acceptor. Both rabbit muscle and yeast (fructose 1,6-diphosphate) FDP[1] aldolases have been shown to catalyze a hydrogen exchange between dihydroxyacetone phosphate and water in the absence of glyceraldehyde 3-phosphate (Rose and Rieder, 1955; Bloom and Topper, 1956; Rose and Rieder, 1958; Rutter and Ling, 1958). Recent kinetic studies indicate that during the reaction sequence these enzymes form a complex with dihydroxyacetone phosphate lacking a proton on the α carbon (Rose *et al.*, 1965). In contrast with the above-mentioned aldolases, citrate synthase (condensing enzyme) does not catalyze a detectable hydrogen exchange between the medium and acetyl coenzyme A in the absence of oxaloacetate (Bove *et al.*, 1959; Marcus and Vennesland, 1958); therefore, a free or bound carbanion is not formed from acetyl coenzyme A in the absence of oxalacetate unless the proton released from acetyl coenzyme A is transferred to a nonexchangeable position on the enzymes.

The present study shows that isocitrate glyoxylate lyase (EC 4.1.3.1) from *Pseudomonas aeruginosa* does not catalyze a hydrogen exchange between the medium and succinate in the absence of glyoxylate. The catalytic pathway is further characterized by observed isotope effects and a kinetic analysis of the condensation reaction.

Materials and Methods

Chemicals. Isocitric acid prepared by the alkaline hydrolysis of D,L(−)-dimethylisocitric lactone and

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[1] Abbreviations used in this work: FDP, fructose 1,6-diphosphate; NADP, nicotinamide–adenine dinucleotide phosphate; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; and POPOP, *p*-bis-2-(5-phenyloxazole)benzene.

sodium glyoxylate monohydrate formed by the hydrolysis of dibromoacetic acid according to the procedure of Smith and Gunsalus (1957) were kindly provided by Dr. R. A. Smith. Additional glyoxylate was prepared by periodic acid oxidation of tartaric acid by the procedure of Metzler *et al.* (1954). (*Anal.* Calcd: C, 20.96; H, 2.71. Found: C, 21.06; H, 2.65.)

Succinic acid-2,3-^{14}C (13.9 mc/mmole) was purchased from Nuclear Chicago Corp. and succinic acid-2,3-^{3}H (25 mc/mmole) from New England Nuclear Corp. Before use, the latter compound was chromatographed on Dowex-1-formate. The recovered succinic acid had a specific activity of 13.6 mc/mmole. Tritiated water (1000 mc/g) was purchased from Volk Radio-Chemical Co. and New England Nuclear Corp.

Potassium pyruvate (crystalline) was prepared from freshly vacuum distilled commercial pyruvic acid by a modification of the procedure of Korkes *et al.* (1951). Various preparations were 80–95% pure as determined by the direct method of Friedemann and Haugen (1943), using freshly distilled pyruvic acid as a standard.

Fumaric acid was purified by two crystallizations from distilled water. Maleamic acid was prepared by bubbling gaseous ammonia through a solution of maleic anhydride in dry dioxane according to the procedure of Frankel *et al.* (1953). The resulting crystals were washed with dioxane, dried, and recrystallized from absolute ethanol. The melting point of the dried crystals (153–156°) was similar to that (152–153°) of Hodgman, 1957–1958. Solutions of maleic acid were prepared by the hydrolysis of commercial maleic anhydride. All other chemicals used in these studies were obtained from commercial sources and used without further purification.

Enzymes. The isocitrate dehydrogenase was prepared from acetone powder of pig heart according to Ochoa (1952) to the stage of the 0.5–0.6 saturated ammonium sulfate precipitate. Activity was measured according to Ochoa, but one unit is defined here as the amount of enzyme catalyzing the formation of 1 μmole of reduced nicotinamide–adenine dinucleotide phosphate per min, calculated for the third 15-sec period after the start of the reaction.

Isocitrate lyase from *P. aeruginosa*, ATCC 9027, was purified and assayed according to Smith and Gunsalus (1957). One unit of activity is defined as the amount of enzyme catalyzing the net cleavage of 1 μmole of D$_s$-isocitrate/min at 30° as measured by the rate of glyoxylate production, and the specific activity is expressed as units per milligram of protein. The specific activities of various preparations made by the Smith and Gunsalus procedure ranged from 5 to 10. In one preparation, elution from a calcium phosphate gel–cellulose column (Massey, 1960a,b) was substituted for the usual batch treatment with calcium phosphate gel; the specific activity of the 0.5–0.55 saturated ammonium sulfate precipitate was 13.5. McFadden and Howes (1963) report a specific activity of 46 for crystalline isocitrate lyase isolated from *P. indigofera*. The degree of homogeneity of our isocitrate lyase preparation was examined by ultracentrifugation and electrophoresis. A moving boundary electrophoretic analysis (Spinco Model H electrophoresis-diffusion instrument) showed a fast and a slow component in addition to the major component which contained all of the isocitrate lyase activity and 70% of the total protein as estimated by peak area. On ultracentrifugation (Spinco Model E ultracentrifuge) a rapidly sedimenting component and a double peak which sedimented more slowly were detected. The heavy component was again estimated from peak areas to contain about 70% of the total protein. The peaks for the heavy and light components were cleanly separated in an Yphantis–Waugh moving partition cell, and nearly all of the isocitrate lyase activity was associated with the heavy component, $s_{20,w} = 8.7$ S. This value compares with the value of 9.49 S found by Shio *et al.* (1965) for the crystalline *P. indigofera* enzyme.

Rate Studies. The velocity of the isocitrate lyase catalyzed reaction was followed at various glyoxylate and succinate concentrations, and in the presence of substrate analogs, by coupling to isocitrate dehydrogenase and measuring the reduction of NADP spectrophotometrically at 340 mμ. Isocitrate dehydrogenase is well suited to this analytical purpose because of the low K_m for isocitrate (Moyle, 1956) and a favorable equilibrium toward α-ketoglutarate (Ochoa, 1945). Isocitrate dehydrogenase was always in excess in the reaction mixtures so that either isocitric lyase or its substrates, succinate or glyoxylate, was rate limiting. The reactions were initiated by the addition of glyoxylate and carried out at 28 ± 1°. Initial velocities were calculated from the 30- to 45-sec interval, which usually gave the highest rate; however the variation between this period and the 0–30- and 45–60-sec intervals was less than 15%.

Tritium Exchange. Two types of experiments were used to detect a possible tritium exchange: (1) incorporation from tritiated water into succinate; and (2) release of tritium from succinic acid-2,3-^{3}H into the medium. The amount of tritium incorporation was determined from succinate isolated from the reaction mixture by ion exchange chromatography. Correction for losses of succinate incurred during isolation was made, either by using succinic acid-2,3-^{14}C of known specific activity as substrate, or by adding a known quantity of succinic acid-2,3-^{14}C to the reaction mixture at the end of the incubation period. In both cases the ^{14}C recovery and the ^{3}H/^{14}C ratio of the product was determined. The transfer of tritium from succinic acid-2,3-^{3}H to water was measured by quantitative transfer of the reaction mixture to a Dowex-1-formate column and elution with water. About 85% of the radioactivity appeared in the first 5-ml eluate and the remainder appeared in the second 5-ml portion. Thus, in routine experiments, the first 15 ml of water eluate was collected and the radioactivity determined.

Chromatographic Separation of Acids. Succinic, glyoxylic, and isocitric acids were separated on a Dowex-1-X8-formate column. The reaction mixture was placed on a column, 10 × 80 mm (for 5–50 μmoles of succinate) or 6.5 × 80 mm (for <5 μmoles of succinate),

and washed with 100–300 ml of water. Succinic and glyoxylic acids were eluted with 0.2 N formic acid. Isocitric acid was eluted with 2 N formic acid. A typical elution pattern for a 10 × 80 mm column is shown in Figure 1. As indicated, succinate, glyoxylate, and isocitrate were clearly resolved in fractions 39–45, 46–53, and 68–73, respectively. In the glyoxylate determination, the additional background 540 mμ absorbance (low in fractions below 55, and higher in fractions above 55) is due to the reaction of dinitrophenylhydrazine with the eluting solutions, 0.2 N and 2 N formic acid, respectively.

Determination of Radioactivity. After chromatography, those fractions containing succinic acid were combined and freeze dried. The residue was dissolved in 1 ml of water and a 0.5-ml aliquot was added to 14 ml of Kinard's solution (1957) and counted with a Packard Tri-Carb liquid scintillation spectrometer. ^{3}H and ^{14}C were determined by the screening method of Okita *et al.* (1957), but with modified equations to compensate for overlap in the radiation spectra occurring in Kinard's solution. Quenching by succinate was found to be less than 3% for the quantities present and no correction was made.

Partial Resolution of ^{3}H- and ^{14}C-Succinic Acids. A partial resolution of the isotopically different succinic acids occurred during chromatography. The ^{3}H to ^{14}C ratio decreased progressively during elution of a three times crystallized mixture of succinic acid-2,3-^{3}H and succinic acid-2,3-^{14}C. This effect could be due to a partial resolution of the isotopically different succinic acids during chromatography, or by an exchange of the tritium atoms of succinate with the solvent. The evidence supports the former possibility. Similar isotope separations by column chromatography have been reported (Piez and Eagle, 1955; Van Dyken, 1955). In the experimental analysis, therefore, the fractions of the succinate peak containing significant radioactivity were combined in order to obtain valid ratios of ^{3}H and ^{14}C.

Results

Kinetic Analysis. The effect of substrate concentration on reaction velocity is presented in Figures 2A–C, as Lineweaver–Burk plots adapted for two-substrate systems by Florini and Vestling (1957). The linear plots shown are consistent with a random reaction sequence under equilibrium conditions (Alberty, 1953) or steady-state conditions with certain restrictions (see Reiner, 1959, for discussion), or an ordered reaction sequence under steady-state conditions (Segal, 1959). The data shown are not compatible with the rate equations for mechanisms involving: (1) a trimolecular collision resulting in the formation of a ternary complex (Reiner, 1959); (2) the direct displacement of the enzyme from a binary enzyme–substrate complex by the other substrate so that no ternary complex is formed (Reiner, 1959); (3) an interaction of the substrates preceding the formation of the ternary complex (Segal *et al.*, 1952); or (4) an ordered reaction sequence under equilibrium conditions (Segal, 1959).

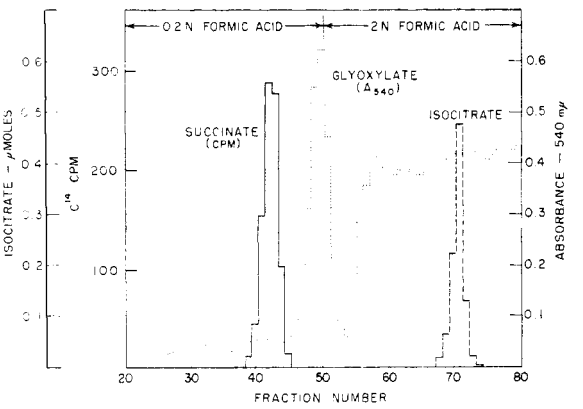


FIGURE 1: Chromatographic separation of acids. Isocitrate, glyoxylate, and succinate-2,3-^{14}C were placed on a 10 by 80 mm column and eluted by the sequential addition of 100 ml of water, 150 ml of 0.2 N formic acid, and 150 ml of 2 N formic acid. Succinate was identified by placing 0.5-ml aliquots from each 5-ml fraction on planchets, drying, and measuring radioactivity with a gas flow counter. Another 0.5-ml aliquot was treated with dinitrophenylhydrazine for the detection of glyoxylate by the procedure of Friedemann and Haugen (1943) as modified by Smith and Gunsalus (1957). This reagent gave an absorbance of 0.042 and 0.416 for 0.5-ml samples of the eluting solvents, 0.2 N formic acid and 2 N formic acid, respectively. Isocitrate was determined by assaying a 0.5-ml aliquot by the method of Ochoa (1952). The first 20 fractions (water wash) did not contain significant levels of radioactivity or absorbing materials and are not shown on the figure. The ordinate scales refer to 0.5 ml.

The following Michaelis constants were calculated from these data: for succinate, 7.0×10^{-4} M; for glyoxylate, 1.6×10^{-4} M. The dissociation constants (which are valid only if equilibrium conditions are applicable) are: $K_{GS} = (E)(S)(G)/(ESG) = 7.4 \times 10^{-6}$, $K_1 = (E)(S)/(ES) = 4.6 \times 10^{-4}$, and $K_2 = (E)(G)/(EG) = 1.1 \times 10^{-4}$.

Succinate and glyoxylate have been reported to show noncompetitive inhibition of isocitrate cleavage, the respective inhibitor constants being 7×10^{-3} M and 2×10^{-3} M (Smith and Gunsalus, 1957). These values are about an order of magnitude greater than the apparent dissociation constants. There are several possible interpretations for this data. Noncompetitive inhibition of isocitrate cleavage by its products would be observed if an ordered release of products occurs and the rate of the removal of the first product is high relative to the rate of removal of the second. Another possibility which deserves particular consideration because of its possible metabolic significance is that the enzyme has both inhibitory and substrate sites for succinate and glyoxylate.

An average V_{max} of 0.33 μmole/min is shown in Figure 2C; a value of 0.39 is calculated from the equa-

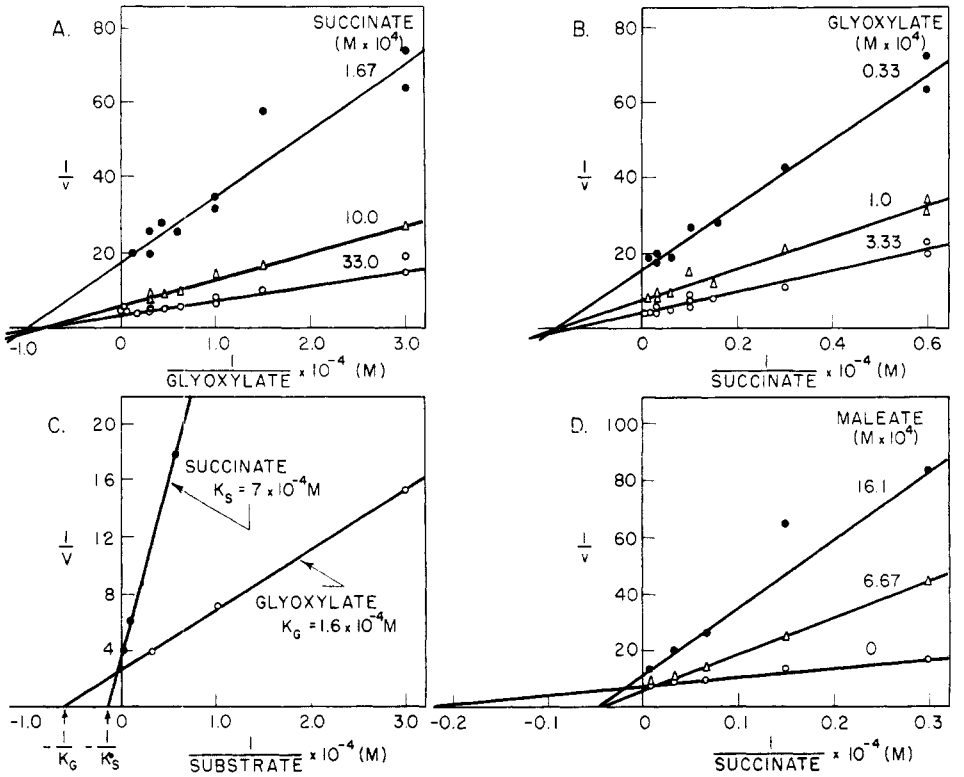


FIGURE 2: Reaction rate of isocitrate lyase, effect of substrate and inhibitor (maleate) concentration. Rates were measured as described in Methods. A 3-ml quartz cuvet (1-cm light path) contained in μmoles: Tris buffer, pH 8.0, 200; $MnCl_2$, 2; $MgCl_2$, 6; cysteine hydrochloride (not neutralized), 4; NADP, 0.54; isocitrate dehydrogenase, 0.55 unit; isocitrate lyase (specific activity = 5), 0.97 unit; succinate, glyoxylate, and maleate, as shown.

tion $V_{max} = (K_{GS} - K_G K_S)v/K_{GS}$ (Florini and Vestling, 1957) derived for the common point of intersection of lines shown in Figure 2A,B. Using the values given above and 4.5×10^{-4} M for the K_m of isocitrate (Smith and Gunsalus, 1957), an equilibrium constant of 23 in the direction of isocitrate formation was calculated from the Haldane equation; this is in satisfactory agreement with the reported value of 35 (Smith and Gunsalus, 1957).

Inhibition by Substrate Analogs. As shown in Table I, several substrate analogs affect the rate of isocitrate formation from succinate and glyoxylate. Maleate and particularly oxalate inhibit isocitrate formation at relatively low concentrations. The oxalate inhibition is partially released by an increased concentration of either succinate or glyoxylate, whereas only succinate substantially relieves maleate inhibition. Maleamate and malonate were significant inhibitors only at higher concentrations. Essentially no inhibition was observed with fumarate, D- or L-aspartate, or acetate. McFadden and Howes (1963) and more recently Rao *et al.* (1965) have reported similar findings with the *P. indigofera* enzyme.

The nature of maleate inhibition is shown in Figure 2D. Isocitrate lyase is implicated as the site of maleate inhibition in the isocitrate dehydrogenase coupled assay, because the inhibition is competitive with respect to succinate, a substrate for isocitrate lyase but not for isocitrate dehydrogenase. Maleate is a competitive inhibitor of succinate only at low concentrations (6.67×10^{-4} M), whereas, at higher concentrations (1.67×10^{-3} M), the inhibition is neither completely competitive nor noncompetitive with respect to succinate. From the data observed at the lower concentration of maleate, a K_I of 3.6×10^{-4} M was calculated. The mixed inhibition observed at the higher concentrations may result from maleate binding to both succinate and glyoxylate sites.

The strong inhibition by maleate and the lack of inhibition by fumarate suggest that succinate exists in the *cis* form, when bound to isocitrate lyase. Since replacing one free carboxyl of maleate with an amide group (maleamate) greatly reduces the inhibition, it is presumed that both carboxyl groups are involved in enzyme binding.

Tritium Exchange Reaction. As shown by the data in Table II, all the reactants and cofactors of the catalytic system are required for optimal incorporation of tritium into succinate. There is apparently a specific requirement for glyoxylate since neither pyruvate nor acetaldehyde can substitute for glyoxylate in this system. The presence of cysteine is also required, and magnesium ions are necessary for optimal activity.

Tritium could be incorporated into isocitrate by a

TABLE I: Isocitrate Lyase Inhibition by Substrate Analogs.

Inhibitor	μmoles	Activity[a] (% without Inhibitor) Substrates (μmoles) Succinate 10	Succinate 10	Succinate 30	Glyoxylate 2	Glyoxylate 10	Glyoxylate 2
Maleate	0	100					
	5	33	40	54			
	10	27					
	30	12					
Oxalate	0.5	33	62	60			
	1	21	49				
	5	4					
	10	0					
Malonate	10	104					
	30	70					
Maleamate	10	95					
	30	75					
Fumarate	10	92					
	30	90					

[a] Reaction carried out as in Figure 2 (succinate 10 μmoles/3 ml, glyoxylate 2 μmoles/3 ml) with additions of inhibitor at concentrations shown.

condensation of enzymatically produced tritiated succinate even if the reaction is stereospecific with respect to the methylene hydrogens of succinate as shown by Sprecher *et al.* (1964) since succinate containing tritium in the methylene groups could condense with glyoxylate either with concomitant expulsion of tritium ion, thus producing untritiated isocitrate, or with the elimination of a proton, producing isocitrate tritiated in the γ position. The molar specific activity of isocitrate, therefore, should always be less than that of succinate. In the experiment reported in Table II, the isocitrate isolated from the reaction mixture had a specific activity less than half that of succinate ($^3H/^{14}C$ = 2.2 and 5.2 for isocitrate and succinate, respectively; see line 1 and footnote Table II).

As shown in Table II, there was considerable variation in the recovery of succinate (^{14}C recovery) in these experiments. The incorporation of tritium into succinate is therefore expressed as the $^3H/^{14}C$ ratio, which is unaffected by losses incurred during isolation. The $^3H/^{14}C$ ratios were always greater than 0 in these experiments; *i.e.*, there was significant incorporation of isotope into succinate, even in the absence of enzyme (lines 3, 10, 11, and 12, Table II). This radioactivity was apparently in the methylene groups of succinate since it could not be reduced by omitting carrier succinate during the isolation procedure or by washing the isolated succinate several times after freeze drying. The variability of this background level of incorporation

TABLE II: Isocitrate Lyase Reaction in ^3H_2O. Tritium Incorporation into Succinate.

Expt No.	Reaction Mixture	dpm × 10^{-3} ^3H	dpm × 10^{-3} ^{14}C	^{14}C Recovery[a] (%)	$^3H/^{14}C$
1	Complete	96.0	18.6	69[b]	5.2
2	Complete	50.6	11.9	38	4.3
1	Minus enzyme	7.1	24.9	80	0.28
2	Minus Mg^{2+}	12.4	14.4	46	0.86
2	Minus cysteine	7.4	13.6	44	0.54
1	Minus glyoxylate	12.7	24.3	78	0.52
2	Minus glyoxylate	5.6	14.0	45	0.40
3	Minus glyoxylate + acetaldehyde	3.9	22.7	73	0.17
3	Minus glyoxylate + pyruvate	2.1	14.4	46	0.15
1	Minus glyoxylate and enzyme	7.6	20.7	66	0.37
3	Minus glyoxylate and enzyme + acetaldehyde	4.7	20.6	66	0.23
3	Minus glyoxylate and enzyme + pyruvate	0.7	9.4	30	0.07

[a] 100% = 31.2 × 10^3 dpm. [b] This value is the total ^{14}C recovery and includes 3.0 × 10^3 dpm recovered as isocitrate, which also contained 6.7 × 10^3 dpm ^3H ($^3H/^{14}C$ = 2.2). The complete reaction mixture contained in μmoles/0.5 ml: Tris buffer, pH 8.0, 50; $MgCl_2$, 1; cysteine hydrochloride, 0.8; succinic acid-2,3-^{14}C, 5 (31.2 × 10^3 dpm); glyoxylate, 5; acetaldehyde and pyruvate (where applicable), 5; and isocitrate lyase, 0.83 unit (specific activity = 3; original specific activity = 8). The water contained approximately 4.7 × 10^{10} dpm ^3H. Reaction mixtures were incubated at 30° under nitrogen for 20 min and stopped by the addition of 0.05 ml of 80% TCA (trichloroacetic acid). Carrier succinate, 45 μmoles, was added in experiments 1 and 3, and carrier isocitrate, 20 μmoles, to the complete reaction mixture in experiment 1 prior to chromatography. Succinic and isocitric acids were isolated as described in the text and identified by radioactivity. Following recovery from the column effluent and freeze drying, they were methylated with diazomethane (Arndt, 1943), transferred to scintillator solution (3 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP (*p*-bis-2-(5-phenyloxazole)benzene) in 1 l. of toluene), and counted as described in the text.

TABLE III: Tritium Incorporation into Succinate during Isocitrate Formation.

Incubation Period (min)	Isocitrate Lyase (units)	Reactants (μmoles)		Rates		Isocitrate Formed per Hydrogen Incorpd (μmoles/μg-atom)
		Succinate	Glyoxylate	Hydrogen Incorporation[a] (μg-atoms/min/unit enzyme)	Isocitrate Formation[b] (μmoles/min/unit enzyme)	
1.5	0.57	50	4	0.023	0.42	18.3
3	0.57	50	4	0.033	0.47	14.2
10	25	5	0	1.5×10^{-5}		

[a] Calculated from ^3H incorporation into succinate assuming no isotope effect and corrected for incorporation in absence of enzyme. [b] Determined from reaction mixture identical with that for ^3H incorporation except that radioisotopes were excluded. The reaction mixture contained in μmoles/0.5 ml: Tris buffer, pH 8.0, 50; $MgCl_2$, 1; cysteine hydrochloride, 0.8; succinate, glyoxylate, and isocitrate lyase (specific activity = 11), as indicated. The 3-min reaction mixture contained 4.25×10^{10} dpm ^3H_2O and the other two reaction mixtures contained 8.5×10^{10} dpm ^3H_2O. After 5-min temperature equilibration, the reaction was started by the addition of glyoxylate (succinate, when glyoxylate absent), carried out at 30° for the times specified, stopped by the addition of 0.05 ml of 80% TCA, and 2.13×10^4 dpm succinic acid-2,3-^{14}C (13.3 μc/μmole) added. Succinic acid was isolated and its ^3H and ^{14}C radioactivity measured as described in the text.

(see, *e.g.*, the apparent effect of pyruvate and acetaldehyde in depressing the level of tritium incorporation in the presence or absence of enzyme; lines 8, 9, 11, and 12, Table II) under different experimental conditions made it difficult to ascertain whether small increases in $^3H/^{14}C$ ratio observed in the absence of glyoxylate (lines 6 and 7, Table II) represented a real enzyme-catalyzed exchange reaction. Efforts were made to increase the resolution of the system and to determine the magnitude of the disparity between incorporation into succinate in the presence and absence of glyoxylate. In the experiment reported in Table III, no significant incorporation into succinate in the absence of glyoxylate was observed, even during longer incubation periods with large quantities of enzyme. A completely valid comparison of the exchange rates in the presence and absence of glyoxylate is not possible from this experiment because the succinate concentrations were not identical; still, the lowest concentration employed (5×10^{-3} M) is considerably larger than the K_m for succinate (7×10^{-4} M) or the dissociation constant for succinate (5×10^{-4} M), as determined kinetically. If it is assumed that both concentrations are adequate to saturate the enzyme, then the difference in the rates with and without glyoxylate is of the order of 2000.

In another experiment succinate-2,3-^3H (13.6 μc/μmole), 5 μmoles/ml, was incubated at 30° with magnesium ions, cysteine, and isocitrate lyase, 5 units/ml. At the end of 2 hr, during which two-thirds of the enzymatic activity was lost, the amount of ^3H in the water was 10^5 dpm/ml greater than that found in a similar reaction mixture lacking enzyme. This corresponds to an average rate of hydrogen exchange (assuming no isotope effect) of 1.1×10^{-4} μg-atom/min, which is about twice the rate per unit enzyme found for tritium incorporation (line 3 of Table III). Other experiments showed that nonenzymatic detritiation was quite variable and could be stimulated by bovine serum albumin or heat-denatured isocitrate lyase. In no case was there clear evidence for an enzymatically catalyzed tritiation or detritiation of succinate in the absence of glyoxylate. Thus, the values presented ($\sim 10^{-5}$ μg-atoms/min/unit) represent maximum limits, not absolute values, for the rate of hydrogen exchange in the absence of glyoxylate.

Tritium Incorporation into Succinate during Isocitrate Cleavage and Formation. The incorporation of tritium into succinate in the presence of glyoxylate may be a result of reversal of the over-all reaction in which there is an obligatory incorporation of hydrogen, or it could be due to an exchange at one of the earlier steps in the reaction sequence. If it were the former, the rate of isocitrate formation should be much faster than hydrogen incorporation; if the latter, the rate of hydrogen incorporation might assume sizable proportions depending on the relative rates of the steps leading to isotope incorporations compared to those leading to isocitrate formations. In the experiments reported in Table III, the total hydrogen incorporation was estimated from tritium incorporation, assuming no selectivity of ^3H or ^1H in the reaction (*i.e.*, no isotope effect), and shows that the rate of isocitrate formation was 14–19 times faster than hydrogen incorporation. This difference is not a measure of an isotope effect because a one to one relationship is not demanded between hydrogen incorporation and product formation. An isotope effect would, however, influence the magnitude of this difference; thus if there were an isotope effect of 6–15 (*vide infra*), the rate of isocitrate formation would be only 1–3 times greater than the actual rate of hydrogen incorporation (exchange). Calculations based on the amount of isocitrate formed, which also take into

TABLE IV: Effect of Concentration of Reactants on Tritium Incorporation into Succinate during Isocitrate Cleavage.

No. Determinations	Reactants (μmoles)			Rates[a]		
	Isocitrate	Glyoxylate	$MgCl_2$	Hydrogen Incorpn[b] (μg-atom/min/unit enzyme)	Glyoxylate Formation[c] (μmoles/min/unit enzyme)	Isotope Effect
5[d,e]	9.6	0	1	0.068 (0.061–0.076)	0.95 (0.82–1.10)	13.9 (12.0–15.5)
5[e,f]	19–20	0	1	0.148 (0.127–0.167)	0.94 (0.79–1.01)	6.47 (5.45–7.73)
1	9.6	0.2	1	0.190	1.00	5.26
1	9.6	1.0	1	0.160	1.12	7.00
1	20	0	2	0.158	0.72	4.55
2	20	0	10	0.100 (0.096–0.104)	0.84 (0.66–1.01)	8.39 (6.28–10.5)
1	20	0	20	0.057	0.55	9.57

[a] Average value is given with range in parentheses. [b] Calculated from ^3H incorporation into succinate, and corrected for ^3H incorporation in absence of enzyme. [c] Determined in a reaction mixture identical with that used for ^3H incorporation except that radioisotopes were excluded. [d] Two values using enzyme concentrations of 0.23 and 0.29 unit/ml. One value using 4.25×10^{10} dpm ^3H_2O. [e] One value using 5.61×10^4 dpm succinic acid-2,3-^{14}C. [f] Two values from 10-min reaction period. The reaction was carried out in 0.5 ml containing, in μmoles: Tris buffer, pH 8.0, 50; cysteine hydrochloride, 0.8; isocitrate, glyoxylate, and $MgCl_2$, as above; and isocitrate lyase (specific activity = 11), 0.057 unit (except as noted). The reaction mixture contained 8.5×10^{10} dpm ^3H_2O and was incubated at 30° for 5 min (except as noted). The reaction was stopped by adding 0.05 ml of 80% TCA and 2.13×10^4 dpm of succinic acid-2,3-^{14}C (13.3 mc/mmole) (except as noted). Succinic acid was isolated and ^3H and ^{14}C radioactivity measured as described in text.

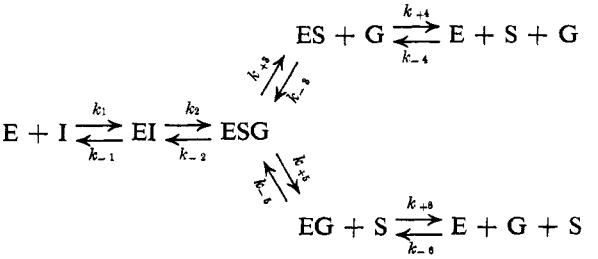
account the noncompetitive inhibition of isocitrate cleavage by succinate, indicate that only about 10% of the observed hydrogen incorporation can be accounted for by the reverse reaction. This suggests that a step in the reaction sequence prior to that which yields isocitrate is primarily responsible for the observed hydrogen incorporation.

The possible occurrence of an isotope effect was investigated by carrying out the isocitrate cleavage reaction in tritiated water of known specific activity. The stoichiometry of this reaction demands that for each mole of isocitrate cleaved, one mole each of succinate and glyoxylate are formed and one g-atom of hydrogen is incorporated into succinate. An isotope effect could therefore be detected as a discrepancy in the rate of hydrogen incorporation when measured by the rate of product formation and when calculated from the rate of tritium incorporation, taking into account only the specific activity of ^3H_2O. The data presented in Table IV were obtained under conditions where the rate of product formation was constant; a significant contribution of the back reaction is not likely with the high initial isocitrate concentrations and in the time periods employed. An isotope effect occurs during the cleavage of isocitrate by the enzyme. The magnitude of the effect varies somewhat according to the concentration of components of the system. Doubling the concentration of isocitrate reduces the isotope effect by a factor of about 2. (Compare line 1 with line 2.) The increase in isocitrate concentration did not affect the over-all reaction rate (glyoxylate produced), but did increase the relative incorporation of tritium (hence the observed decrease in the isotope effect). This concentration effect cannot be a result of complexing magnesium ions by isocitrate, since a 20-fold increase in the magnesium ion concentration does not restore the isotope effect found at the lower isocitrate concentration.

Addition of glyoxylate to the initial reaction mixture also lowered the isotope effect (compare line 1 with lines 3 and 4). These concentrations of glyoxylate did not influence the rate of glyoxylate (succinate) formation, but increased the tritium incorporation thus decreasing the isotope effect.

Discussion

A possible reaction sequence for the isocitrate lyase reaction is as follows



Where E = enzyme, I = isocitrate, G = glyoxylate, and S = succinate.

The experimental work presented here allows certain amplifications and restrictions on this formulation. The kinetic data were consistent with a random reaction

sequence in which reaction 2 is rate limiting (equilibrium conditions), or an ordered reaction sequence under steady-state conditions (reactions 1–4 or 1, 2, 5, 6). Several other kinetic sequences have been eliminated.

Rose *et al.* (1965) have shown that the FDP aldolase reaction is an ordered sequence (analogous to reactions 1–4), in which an enzyme–dihydroxyacetone phosphate complex (lacking a proton on the α carbon) is an obligatory intermediate. The hydrogen exchange between the medium and dihydroxyacetone phosphate is an important feature of this reaction. The present experiments have shown that isocitrate lyase does not catalyze an analogous hydrogen exchange between water and succinate in the absence of glyoxylate. This fact has been recently confirmed by Sprecher *et al.* (1964). If such an exchange occurs at all, it is very slow and cannot be considered an important feature of the isocitrate lyase catalyzed reaction. This eliminates mechanisms involving the reversible formation of a succinate carbanion (or its equivalent). Reaction 4, if a part of the reaction sequence, can be involved in proton removal only if the proton is transferred to a nonexchangeable position on the enzyme. This seems unlikely since the isotope effect (up to 15) approximates the maximum value (15.1) which can be expected at this temperature according to current theory (Wiberg, 1955) which assumes no bonding to the hydrogen in the activated complex. Reactions 2, 3, and 5 remain as possible sites of hydrogen incorporation without similar restrictions. Because of the observed isotope effect, it can be presumed that hydrogen incorporation is a feature of the rate-limiting step of the reaction sequence. The magnitude of the isotope effect was shown to be dependent to some degree on the concentrations of isocitrate and glyoxylate. One explanation for this observation is that changes in the substrate concentration influence the configuration of the active site thus altering the structure of the rate-limiting transition state. It is also possible that several reaction pathways with different transition states are involved and that their relative contribution to the over-all reaction is influenced by the substrate concentration. Under conditions where the back reaction should be negligible, the rate of hydrogen incorporation is at least 5–7% that of isocitrate formation. Thus, there are probably reversible steps in the reaction pathway prior to the formation of isocitrate which allow exchange of hydrogen. A more thorough study of this possibility is warranted.

A few postulates concerning the structure of substrates, intermediates, and the general features of the catalytic process can be made at this time. The kinetic experiments using analogs of succinate (see Table I), which suggest a *cis* form for enzyme-bound succinate, and the absolute requirement for a metal ion are consistent with the postulate that succinate is bound to the enzyme as a metal chelate complex. The formation of a succinate–magnesium chelate might also reduce the charge normally associated with the carboxylate group, thereby increasing the lability of the methylene proton; however, the removal of a proton from one of the methylene groups of succinate apparently requires the combined action of enzyme and glyoxylate. The recent studies of Sprecher *et al.* (1964) indicate that the stereochemical course of isocitrate cleavage involves protonation with inversion of configuration of C-3 of isocitrate-forming succinate. Although rare, electrophilic substitutions on saturated carbon atoms leading to inversion of configuration have been reported (see ref 30, 31, and 32 of Sprecher *et al.*, 1964). The kinetic data reported here are consistent with such a displacement if the reacting species are bound to the enzyme, but eliminate a displacement mechanism where free glyoxylate is the electrophile and free isocitrate is formed. A more likely alternative is that an enzyme-bound intermediate enediol is formed, similar to the enol or enamine postulated for other aldolases (Rutter, 1964). Such a two-step mechanism could result in either retention or inversion of configuration, as discussed by Lienhard and Rose (1964) for the reactions catalyzed by glucose 6-phosphate and isocitrate dehydrogenases. A mechanism of this type could account for the tritium incorporation which occurs during isocitrate formation, if the rates of protonation and glyoxylate addition of the postulated enediol are not greatly different.

From its cofactor requirements and the general reaction type, isocitrate lyase appears to be a member of Class II (metal-requiring) aldolases (Rutter, 1964, 1965); however, it apparently has a number of distinctive catalytic differences from yeast FDP aldolase, the prototype for this class. In one aspect it resembles citrate synthase, a Class I (nonmetal-requiring aldolase), since it does not catalyze a quantitatively significant exchange reaction under optimal conditions for the over-all reaction. A more detailed study of these citrate and isocitrate aldolases will hopefully elucidate the basic similarities and differences in their structure and catalytic mechanisms, and define the degree of relationship which exists with other aldolases.

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