Stereochemistry of the Interconversions of Citrate and Acetate Catalyzed by Citrate Synthase, Adenosine Triphosphate Citrate Lyase, and Citrate Lyase*

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ABSTRACT: The preparation of citrate, stereospecifically labeled in the (*pro-S*)-acetate portion of the molecule, was carried out with aconitase and aconitate isomerase, an enzyme which activates the (*pro-S*)-hydrogen at carbon-4 of *cis*-aconitate. The steric course of the cleavage of specifically tritiated citrate in D_2O by ATP citrate lyase (EC 4.1.3.8) from rat liver and citrate lyase from *Aerobacter aerogenes* was determined by the conversion of the chiral-labeled products, acetyl-CoA or acetate, into chiral-labeled malate with malate synthase, and the analysis of tritium in malate with fumarase. Samples of stereospecifically labeled acetates were prepared by the oxidative decarboxylation of pyruvate obtained from (*E*)- and (*Z*)-[3,3-^{2}H,^{3}H]phosphoenolpyruvates with pyruvate kinase. The steric course of citrate formation, catalyzed by citrate synthase from pig heart, was determined by the conversion of these acetates to chiral-labeled citrate, which was analyzed for tritium with aconitase and aconitate isomerase. The aldol and reverse aldol reactions, catalyzed by the three citrate enzymes examined, have been shown to proceed by inversion.

There is a large class of enzymes which catalyzes aldol-type cleavages, characterized by the reversible formation of a C–H bond from a C–C bond. In this paper, we have studied three enzymes, which catalyze either an aldol- or reverse aldol-type cleavage of citrate, in order to determine the stereochemical relationship between the C–C- and C–H-bond-making steps. The three reactions studied were as shown in Scheme I. As illustrated in Figure 1, the incorporation of hydrogen into the methyl group of acetate derived from citrate can occur by inversion or retention. The steric course of ATP citrate lyase and citrate lyase can be determined by the use of citrate, stereospecifically labeled at C-4,[1] to generate chiral-labeled acetate. In order to determine the steric course of citrate synthase, one needs to know the chirality[2] of the labeled acetyl-CoA used for the synthesis and the predominant chirality of the labeled C-4 methylene group in the citrate formed.

SCHEME I

$$\text{acetyl-CoA} + \text{oxaloacetate} \xrightarrow[\text{(EC 4.1.3.7)}]{\text{citrate synthase (}si\text{ specific)}} \text{citrate} + \text{CoA}$$

$$\text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{P}_i \xleftarrow[\text{(EC 4.1.3.8)}]{\text{ATP citrate lyase}} \text{citrate} + \text{ATP} + \text{CoA}$$

$$\text{acetate} + \text{oxaloacetate} \underset{\text{(EC 4.1.3.6)}}{\overset{\text{citrate lyase}}{\rightleftharpoons}} \text{citrate}$$

The chemical preparation of H,D,T acetates of known chirality has been described recently by Cornforth *et al.* (1969) and Lüthy *et al.* (1969). In their studies of malate synthase, the condensation of acetyl-CoA[3] and glyoxylate to form malate with malate synthase was shown to proceed by inversion at C-2 of acetate.

In our studies, citrate was stereospecifically tritiated at C-4 with aconitate isomerase; as described in the preceding paper (Klinman and Rose, 1971b), aconitate isomerase activates the (*pro-S*)-hydrogen at C-4 of *cis*-aconitate. The hydration of such tritiated *cis*-aconitate with aconitase gives citrate labeled at C-4 (in the (*pro-S*)-acetate portion of the molecule).

The cleavage of (3*S*,4*S*)-[4-^{3}H]citrate[4] by ATP citrate lyase from rat liver to form acetyl-CoA, or by citrate lyase from *Aerobacter aerogenes* to give acetate was carried out in D_2O. The chirality of the [2,2-^{2}H,^{3}H]acetates so obtained was determined by conversion of these acetates to malate with malate synthase, and the subsequent dehydration of malate with fumarase, an enzyme which catalyzes the removal of a (*pro-R*)-hydrogen at C-3 of malate (Anet, 1960; Gawron *et al.*, 1961). This is illustrated in Figure 2.

The steric course of the condensation of acetyl-CoA and oxaloacetate with citrate synthase from pig heart was determined with chiral-labeled acetates prepared from deuterated, tritiated phosphoenolpyruvates of known chirality (Cohn *et al.*, 1970), and pyruvate kinase, an enzyme which has been shown to catalyze the addition of a proton to the *si* face

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[1] The carbon skeleton of citrate is numbered in such a way that dehydration of citrate by aconitase occurs at C-2,C-3, *i.e.*, in the (*pro-R*)-acetate portion of the molecule (Hanson, 1966).

[2] A molecule is chiral or "handed" if it cannot be brought into coincidence with its mirror image.

[3] Abbreviations used are: CoA, coenzyme A; PEP, phosphoenolpyruvate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DTT, dithiothreitol.

[4] The *R*/*S* nomenclature follows the convention of Cahn *et al.* (1966).

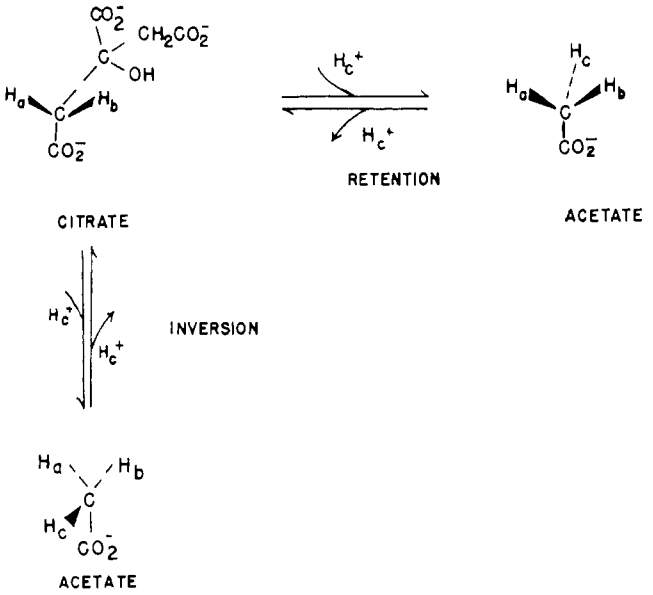


FIGURE 1: Stereochemical pathways for the interconversion of citrate and acetate.

of phosphoenolpyruvate (Rose, 1970a). The C-4 chirality of citrates produced by the condensation of these acetates with oxaloacetate was determined by the conversion of citrate into *cis*-aconitate with aconitase, and the subsequent detritiation of *cis*-aconitate with aconitate isomerase. This is outlined in Figure 3.

Materials and Methods

Preparation of (4S)-cis-[4-^{3}H]Aconitate. In 1.1 ml of tritiated water–glycerol (80:20, v/v), 100 μmoles of imidazole chloride (pH 7.5), 75 μmoles of K_3PO_4 (pH 7), 3 μmoles of cysteine, 100 μmoles of *cis*-aconitate, and 3.5 units of aconitate isomerase were equilibrated for 5 hr. At the end of this time the mixture was freeze-dried. The residue was deproteinized with perchloric acid and freeze-dried a second time. The separation of tritiated *cis*- and *trans*-aconitate was carried out by silicic acid column chromatography (Bulen *et al.*, 1952). (4*S*)-*cis*-[4-^{3}H]Aconitate (specific activity ≅ 18,000 cpm/μmole) was obtained by elution with 20% chloroform–butanol.

Preparation of (3S,4S)-[4-^{3}H]Citrate. In 1 ml, 100 μmoles of Tris-chloride (pH 8), 2.5 μmoles of (4*S*)-*cis*-[4-^{3}H]aconitate (45,000 cpm), and 0.2 unit of aconitase were incubated until equilibrium was reached. The disappearance of *cis*-aconitate was monitored at 280 nm. The reaction mixture was deproteinized with $HClO_4$. At equilibrium *cis*-aconitate is converted with aconitase to a mixture which is 90% citrate, 6% isocitrate, and 4% *cis*-aconitate (Krebs and Eggleston, 1943). Isocitrate was converted to glutamate with isocitrate dehydrogenase and glutamate dehydrogenase. Carrier citrate (10 μmoles) was added and citrate (specific activity ≅ 3000 cpm/μmole) was separated from *cis*-aconitate on a Dowex 1 (formate) column (1 × 20 cm) by elution with 3 N formic acid.

Preparation of (2S)-[2,2-^{2}H,^{3}H]Acetate and (2R)-[2,2-^{2}H,-^{3}H]Acetate. The preparation of phosphoenolpyruvate labeled stereospecifically with isotopes of hydrogen by the use of glycolytic enzymes has been described, and the absolute stereochemistry of PEP prepared in this manner established by nuclear magnetic resonance studies (Cohn *et al.*, 1970). The conversion of (*Z*)-[3,3-^{2}H,^{3}H]PEP and (*E*)-[3,3-^{2}H,^{3}H]PEP[5] to pyruvate with pyruvate kinase has been shown to generate (3*S*)-[3,3-^{2}H,^{3}H]pyruvate and (3*R*)-[3,3-^{2}H,^{3}H]pyruvate, respectively (Rose, 1970a). The conversion of 0.25 μmole of (3*S*)-[3,3-^{2}H,^{3}H]pyruvate (specific activity 1 × 10^7 cpm/μmole) to (2*S*)-[2,2-^{2}H,^{3}H]acetate was carried out (pH 6.5) in the presence of 0.6% H_2O_2 for 0.5 hr. Acetate was obtained from a Dowex 1 (chloride) column by elution with 5 mM HCl. The conversion of 1 μmole of (3*R*)-[3,3-^{2}H, ^{3}H]pyruvate (specific activity 70,000 cpm/μmole) to (2*R*)-[2,2-^{2}H,^{3}H]-acetate was carried out in a similar manner. The acetates so obtained were further purified by distillation from acid and dried at neutral pH.

All materials were obtained commercially unless otherwise noted. ATP citrate lyase from rat liver was a gift of Dr. Paul Srere. Citrate lyase from *Aerobacter aerogenes* and citrate synthase from pig heart were obtained from Boehringer Mannheim Corp., as were acetate kinase (EC 2.7.2.1), phosphotransacetylase (EC 2.3.1.8), malate dehydrogenase (EC 1.1.1.37), and glutamate dehydrogenase (EC 1.4.1.2). Malate synthase (EC 4.1.3.2) from yeast was prepared according to the method of Dixon and Kornberg (1962). Aconitase from pig heart was prepared according to the method of Morrison (1954), and was assayed with *cis*-aconitate by the change in absorbance at 260 nm. Aconitate isomerase was prepared and assayed as described (Klinman and Rose, 1970a).

ATP Citrate Lyase: Conversion of (3S,4S)-[4-^{3}H]Citrate into L-[3-^{3}H]Malate. In 1 ml of 96.5% D_2O, 100 μmoles of TES-chloride (pH 7.1), 10 μmoles of $MgCl_2$, 5 μmoles of ATP, 1.3 μmoles of (3*S*,4*S*)-[4-^{3}H]citrate (specific activity ≅ 3000 units cpm/μmole), 5 μmoles of CoA, 5 μmoles of DTT, 2 μmoles of DPNH, 0.06 unit of ATP citrate lyase, and 36 units of malate dehydrogenase were reacted at 25° for 1 hr, until the absorbance change at 340 nm indicated that the reaction was complete. The pH was then brought to 8 with triethanolamine, and 5 μmoles of glyoxylate and 0.5 unit of malate synthase were added to convert [2,2-^{2}H,^{3}H]acetyl-CoA into malate. After incubation for 1 hr at 25° the solution was deproteinized by heat and added to a Dowex 1 (chloride) column (0.9 × 4 cm). Malate (2900 cpm) was eluted with 5 mM HCl following treatment of the column with 1 mM HCl.

Citrate Lyase: Conversion of (3S,4S)-[4-^{3}H]Citrate into L-[3-^{3}H]Malate. In 1 ml of 96.5% D_2O, 100 μmoles of TES-chloride (pH 7.1), 10 μmoles of $ZnCl_2$, 1.2 μmoles of (3*S*,4*S*)-[4-^{3}H]citrate (specific activity ≅ 3000), 2 μmoles of DPNH, 36 units of malate dehydrogenase, and 3 units of citrate lyase were incubated. When the absorbance change at 340 nm indicated that the reaction was complete, the reaction mixture was deproteinized by heat, acidified with HCl, and distilled *in vacuo*. The [2,2-^{2}H,^{3}H]acetate obtained from the distillate was adjusted to pH 7.4 with triethanolamine and subsequently converted into malate in a system containing 70 μmoles of KCl, 6.4 μmoles of ATP, 10 μmoles of $MgCl_2$, 1 μmole of CoA, 6.6 μmoles of DTT, 3 μmoles of glyoxylate, 0.5 unit of malate synthase, 2.5 units of phosphotransacetylase, and 1.7 units of acetate kinase in a final volume of 1.6 ml. The reaction mixture was incubated for 1 hr at 25°. After deproteinization by heat the sample was added to a Dowex 1 (chloride) column (0.4 × 9 cm) and malate (1400 cpm) was eluted with 5 mM HCl.

Citrate Synthase: Conversion of (2S)-[2,2-^{2}H,^{3}H]Acetate (I)

[5] The *E*/*Z* nomenclature follows the convention of Blackwood *et al.* (1968).

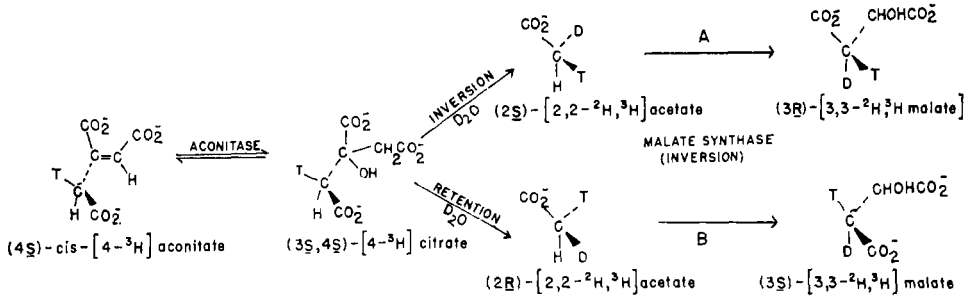


FIGURE 2: Stereochemistry of citrate lyase. This figure illustrates the idealized case ($k_H/k_D = \infty$) where only the C–H bond is broken in the conversion of acetate into malate ($k_H/k_{D(obsd)} \simeq 2$).

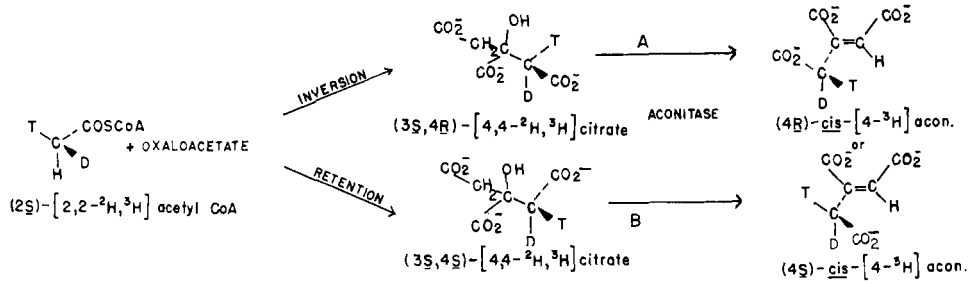


FIGURE 3: Stereochemistry of citrate synthase. This figure illustrates the idealized case ($k_H/k_D = \infty$) where only the C–H bond is broken in the condensation of acetyl-CoA and oxaloacetate ($k_H/k_{D(obsd)} \simeq 3$).

and (2R)-[2,2-²H,³H]Acetate (II) into [4-³H]Citrate (I′) and [4-³H]Citrate (II′). A 1-ml solution, containing 5×10^{-3} μmole of I (50,000 cpm), 1 μmole of carrier sodium acetate, 100 μmoles of Tris-chloride (pH 8), 10 μmoles of $MgCl_2$, 4 μmoles of DTT, 5 μmoles of ATP, 5 μmoles of CoA, 10 μmoles of oxaloacetate, 20 μmoles of KCl, 3.4 units of acetate kinase, 5 units of phosphotransacetylase, and 28 units of citrate synthase, was reached for 2 hr at 25°. Following the removal of unreacted acetate with 1 N formic acid, citrate (I′) (43,200 cpm) was eluted from a Dowex 1 (formate) column (0.9 × 8 cm) with 3 N formic acid, representing 95% yield. The conversion of 0.14 μmole of II (9500 cpm) was identical to the method described for I, with the exception that only 0.85 μmole of carrier acetate was added to the reaction mixture. Citrate (II′) (4152 cpm) was recovered from a Dowex 1 (formate) as described above in 61% yield.

Determination of Chirality of Labeled Malate. L-[3-³H]-Malate was incubated in phosphate buffer (0.1 M, pH 7) with fumarase under conditions where (3*R*)-L-[3-³H]malate was shown to detritiate completely. The water from these samples was obtained by sublimation and both the volatile fraction and residue were counted by liquid scintillation spectrometry.

Determination of Chirality of Labeled Citrate. [4-³H]-Citrate was incubated in a 20% glycerol, 50 mM Tris-chloride (pH 8) solution containing DTT, aconitase, and aconitate isomerase, under conditions where citrate randomly labeled at C-4 was found to be detritiated to the extent of 50%. Water was obtained by sublimation and both the volatile fraction and residue were counted by liquid scintillation spectrometry.

Results

The stereochemistry of the C–C-bond-breaking C–H-bond-making step for ATP citrate lyase and citrate lyase was determined as outlined in Figure 2. Citrate specifically labeled at C-4, (3*S*,4*S*)-[4-³H]citrate, was cleaved in D_2O to acetate, which was subsequently converted to malate *via* malate synthase. For both enzymes examined, the malates obtained were enriched at carbon three with tritium in the *pro-R* position as determined with fumarase (Table I). The malate derived from acetyl-CoA formed with ATP citrate lyase was a mixture of (3*R*)- and (3*S*)-L-[3-³H]malate in the ratio 63:37, indicating an enrichment of tritium in the *pro-R* position of malate of 26%. Similarly, malate derived from acetate formed with citrate lyase was 32% enriched in the *pro-R* position. The extent to which the malates obtained were a diastereomeric mixture is expected since, for example, in the production of malate from (3*S*,4*S*)-[4-³H]citrate with ATP citrate lyase, 16.5% of the total counts were in water. Twenty per cent of these counts arise in the conversion of citrate to acetyl-CoA, presumably from the enzymatic enolization of acetyl-CoA prior to its release from the enzyme. Since in these studies only molecules containing tritium were examined, the loss of tritium does not, in itself, represent racemization. However, depending on the isotope effect, k_H/k_T, for every tritium lost there are k_H/k_T protons exchanged with deuterium, and the exchange of hydrogen with deuterium gives racemic acetyl-CoA. The remaining 13.5% of the volatile counts arise in the C–H-bond-breaking step in the condensation of acetyl-CoA and glyoxylate to give malate. The conversion of acetyl-CoA to stereospecific tritiated malate with malate synthase depends on an isotope effect of sufficient magnitude such that the cleavage of a C–H bond occurs preferential to C–D bond cleavage. Cornforth *et al.* (1969) and Lüthy *et al.* (1969) have reported intramolecular isotope effects, k_H/k_D, for the malate synthase reaction of 2.2 and 4.5, respectively. From our observation that 13.5% of the tritium is made volatile in the course of this condensation and using the Swain equation to convert the tritium isotope effect into a deuterium

TABLE I: Stereochemistry of ATP Citrate Lyase and Citrate Lyase. Detritiation of L-[3-^{3}H]Malate Derived from (3*S*,4*S*)-[4-^{3}H]-Citrate.[a]

Enzyme	% Volatile Counts (after Fumarase)		% Enrichment of Tritium in the *pro-R* Position of [3-^{3}H]Malate
	[3-^{3}H]Malate	[3-^{3}H]Malate + (3*R*)-[3-^{3}H]Malate	
ATP citrate lyase	594/953 = 63	14,400/15,100 = 96	26
Citrate lyase	326/493 = 66	14,100/14,300 = 99	32

[a] Incubations were carried out with 0.2–0.8 mM solutions of [3-^{3}H]malate in 165 mM K_3PO_4 (pH 7) with 7–18 units of fumarase for 1 hr at 25°. Water was removed *in vacuo* and both the volatile fraction and residue were counted for 2 min. The (3*R*)-[3-^{3}H]malate present in the control incubations had a specific activity of 1.5×10^5 cpm/μmole.

TABLE II: Stereochemistry of Citrate Synthase. Detritiation of (3*S*)-[4-^{3}H]Citrate Derived from (2*R*)-[2,2-^{2}H,^{3}H]Acetate and (2*S*)-[2,2-^{2}H,^{3}H]Acetate.[a]

Acetate	% Volatile Counts (after Aconitase and Isomerase)		% Enrichment of Stereospecific Tritium in [4-^{3}H]Citrate
	[4-^{3}H]Citrate	[4-^{3}H]Citrate + Citrate Randomly Labeled in (*pro-S*)-Acetate Portion	
(2*S*)-[2,2-^{2}H,^{3}H]Acetate	1045/3,120 = 34	4,173/8,000 = 52	*pro-R:* 32
(2*R*)-[2,2-^{2}H,^{3}H]Acetate	741/1,135 = 65	1,610/3,143 = 51	*pro-S:* 30

[a] Incubations of 0.3 mM solutions of [4-^{3}H]citrate in 20% glycerol containing approximately 100 mM Tris-chloride (pH 8) and 6 mM DTT were carried out with 0.8 unit of aconitase and 3 units of aconitate isomerase overnight at 25°. Water was removed *in vacuo*, and both the volatile fraction and residue were counted for 1 min. In the controls containing tritiated citrate randomly labeled in the (*pro-S*)-acetate portion of the molecule (specific activity 3.4×10^6 cpm/μmole), the counts refer to the randomly labeled citrate.

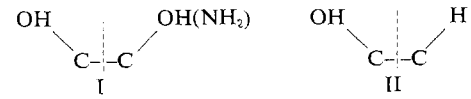
isotope effect (Swain *et al.*, 1958), we obtain a value of $k_H/k_D = 1.9$.

The stereochemistry of the C–H-bond-breaking, C–C-bond-making step of citrate synthase was determined by analyzing the volatile counts with aconitase and aconitate isomerase in [4-^{3}H]citrate formed from chiral-labeled acetates. The data are summarized in Table II. A mixture of (3*S*,4*R*)- and (3*S*,4*S*)-[4-^{3}H]citrate was obtained. Citrate obtained from (2*S*)-[2,2-^{2}H,^{3}H]acetate was enriched 32% in tritium at the *pro-R* position of C-4. Citrate from (2*R*)-[2,2-^{2}H,^{3}H]-acetate was enriched 30% at the *pro-S* position. The presence of diastereomeric mixtures in these reactions arises in the condensation of acetyl-CoA and oxaloacetate. Experiments with tritiated acetate under the conditions described in the Experimental Section for the conversion of acetate into citrate indicate an intramolecular isotope effect, k_H/k_T, of 4.4 for citrate-condensing enzyme; previously Kosicki and Srere (1961) reported a value of $k_H/k_D = 1.4$ for the net conversion of acetyl-CoA as compared to deuterioacetyl-CoA. An intramolecular tritium isotope effect of 4.4, which is equivalent to a deuterium isotope effect of 3.0 (Swain *et al.*, 1958), indicates that in the condensation C–H would be broken 75% of the time, and one predicts an enrichment of 50% of tritium in one of the positions at C-4 of citrate. The observation of either 30 or 32% enrichment implies additional modes giving rise to racemization at C-4 of citrate.

Discussion

From the data presented in Tables I and II, one concludes that the stereochemical course for the three enzymes studied is inversion. ATP citrate lyase and citrate lyase catalyze the conversion of (3*S*,4*S*)-[4-^{3}H]citrate to (3*R*)-L-[3-^{3}H]malate (Figure 2, path A) and citrate synthase catalyzes the conversion of (2*S*)-[2,2-^{2}H,^{3}H]acetyl-CoA into (3*S*,4*R*)-[4-^{3}H]-citrate (Figure 3, path A).

The finding that for the enzyme systems examined there is a stereochemical constancy, might imply that there is a mechanistic relevance to inversion. However, from model systems, the stereochemical path of electrophylic replacement reactions has been found to depend primarily on the dielectric constant and proton-donating ability of the solvent. In solvents of high dielectric constant (*e.g.*, ethylene glycol), net inversion plus racemization is observed, whereas in solvents such as *tert*-butyl alcohol net retention plus racemization is observed (Cram, 1965). In studying the effects of structure of the reacting molecule on the stereochemical course of these reactions, it has been shown that cleavage of bonds of type I as opposed to those of type II gives net



I II

retention even in inversion-producing solvents (Cram, 1965). The implication drawn from this is that the additional hydroxyl or amino group serves as an internal source of protons. This distinction may be applicable to enzymes; for example, yeast and muscle aldolase (Rose, 1958; Rose and Rieder, 1958), rhamnose-1-P aldolase (Chiu and Feingold, 1959), and serine aldolase (Akhtar and Jordan, 1969) catalyze cleavage by retention—these aldolases all catalyze the cleavage of type I bonds. Isocitrate lyase (Sprecher *et al.*, 1969; Hanson, 1965; Daron *et al.*, 1966), malate synthase (Cornforth *et al.*, 1969; Lüthy *et al.*, 1969), and the three citrate enzymes studied in the present investigation catalyze the cleavage of a type II bond—all of these enzymes show inversion. It will be of interest to examine aldolases catalyzing the cleavage of type II bonds, such as 2-keto-3-deoxy-6-phosphogluconate aldolase (Meloche and Wood, 1964), α-keto-β-deoxyl-6-glutarate aldolase (Fish and Blumenthal, 1966), and 2-keto-4-hydroxyglutarate aldolase (Rosso and Adams, 1967).

The observation of a stereochemical constancy for a series of enzymes catalyzing similar reactions might be an indication of a constancy in the substrate binding sites. One of the authors (Rose, 1970b) has discussed the stereochemistry of proton-replacement reactions in terms of whether or not a cosubstrate is required for the enzyme-catalyzed proton activation step. In the case of the aldolases, which go by retention, proton activation, and replacement occur separately. In the case of isocitrate lyase, malate synthase, and citrate synthase, enzymes requiring a cosubstrate for proton activation, the steric course is inversion, suggesting that the different sites on these enzymes, involved in either proton or carbonyl addition to the enolate intermediate, prefer to be placed at opposite faces of the plane of the enolate.

Eggerer (1965) and Bové *et al.* (1959) discuss the requirement for cosubstrate to obtain proton activation of acetyl-CoA with citrate synthase in terms of the role of the β-carboxylate ion of oxaloacetate as a general base. However, this mechanism requires retention in the reaction, which is ruled out by the present study. It must be supposed that the oxaloacetate probably plays a secondary role in the proton activation as was proposed by Srere (1967).

Citrate synthase utilizes fluoroacetyl-CoA as a substrate (Peters, 1954), and the enzymatic condensation of fluoroacetyl-CoA and oxaloacetate has been shown to occur in a stereospecific manner (Fanshier *et al.*, 1964). Recent crystallographic studies on the rubidium ammonium salt of fluorocitrate suggest that the absolute configuration of the enzymatically formed isomer of fluorocitrate is 2*R*,3*R* (Carrell *et al.*, 1970). On the assumption that the steric course of the condensation of fluoroacetyl-CoA is the same as that for acetyl-CoA, *i.e.*, inversion, it is concluded that the (*pro-S*)-hydrogen of fluoroacetyl-CoA is activated in the enzymatic condensation.

After the completion of this study, the stereochemistry of the citrate enzymes as determined by two other independent methods was reported. Eggerer *et al.* (1970) determined the stereochemistry of the three citrate enzymes described in this paper and an additional enzyme, citrate synthase (*re* specific) (EC 4.1.3.7) from *Clostridium acidi urici*. Citrate synthase (*re* specific) catalyzes a condensation of oxaloacetate and acetyl-CoA to form citrate in which the (*pro-S*)-acetic acid chain originates from oxaloacetate; this is of opposite stereochemistry to that of citrate synthase (*si* specific). The stereochemistry of citrate lyase was determined with citrate synthesized from either (3*R*)- or (3*S*)-[3-^{3}H]malate and acetyl-CoA with citrate synthase (*re* specific). The cleavages of these citrates in D_2O with citrate lyase gave chiral acetates, which were converted into malate with malate synthase and analyzed for tritium with fumarase. The stereochemistry of the remaining three citrate enzymes was determined in a sequential fashion from the determination of the steric course of citrate lyase.

Rétey *et al.* (1970) determined the stereochemistry of citrate synthase by the degradation of citrates, formed from (*R*)- and (*S*)-[2,2-^{2}H,^{3}H]acetyl-CoA's and oxaloacetate with citrate synthase, to succinate and an analysis of the tritium in succinate with succinate dehydrogenase.

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Studies on the Allosteric Modification of Nucleoside Diphosphatase Activity by Magnesium Nucleoside Triphosphates and Inosine Diphosphate*

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ABSTRACT: A kinetic study has been made of the effects of various nucleotide species as modifiers of the reaction catalyzed by nucleoside diphosphatase when magnesium–inosine diphosphate is used as the substrate. The results showed that the magnesium complexes of both nucleoside and deoxynucleoside triphosphates, as well as free inosine diphosphate, are capable of activating the enzyme at lower concentrations of substrate. The experimental data have been analyzed in terms of the mechanism previously proposed for the reaction (*Biochemistry 8*, 3821 (1969)) and values obtained for the various kinetic constants. These indicate that the structure of the modifier determines the strength of its binding to free enzyme and that the resulting enzyme–modifier complexes have different abilities to combine with the substrate. Certain nucleotide species were also able to significantly lower the maximum velocity of the reaction.

Nucleoside diphosphatase (EC 3.6.1.6) catalyzes the hydrolysis of a wide range of nucleoside diphosphates and hence the reaction can be written in general form as

$$\text{nucleoside diphosphate} \xrightarrow{M^{2+}} \text{nucleoside monophosphate} + P_i$$

where M^{2+} represents an essential divalent metal ion which may be Mg^{2+}, Mn^{2+}, or Ca^{2+}. Characteristic features of the enzyme freshly prepared from rat liver are that it does not exhibit Michaelis–Menten kinetics and can be activated by $MgATP^{2-}$ which functions as an allosteric modifier (Yamazaki and Hayaishi, 1965, 1968; Schramm and Morrison, 1968). Detailed kinetic investigations of the reaction using $MgIDP^-$ as the substrate in the presence and absence of $MgATP^{2-}$ (Schramm and Morrison, 1969) have led to the proposal that the reaction proceeds *via* a rapid equilibrium, random mechanism that allows for (a) the interdependent reaction of two molecules of substrate at two identical catalytic sites, (b) differences in the rate of product formation from enzyme forms containing one or two molecules of substrate, and (c) combination of a modifier at a distinct site on the enzyme which can affect both the binding of substrate and rate of product formation.

Since various magnesium nucleoside and deoxynucleoside triphosphate complexes, as well as IDP^{3-}, are capable of activating the enzyme, it was of interest to determine the effect of these compounds relative to that of $MgATP^{2-}$. The results of such experiments have been found to be in accord with the mechanism previously proposed and quantitative analysis of the data in terms of the initial rate equation for the mechanism has shown the different nucleotide species exhibit varying abilities to combine with the enzyme and to affect the maximum velocity of the reaction.

Materials and Methods

All nucleoside and deoxynucleoside triphosphates were products of P-L Biochemicals. The deoxynucleoside triphosphates were not further purified as they were purchased just prior to use and did not contain significant amounts of the corresponding deoxynucleoside diphosphates. On the other hand, ITP, UTP, GTP, and CTP, which had been stored as the crystalline sodium salts at $-10°$ for about 2 years, contained 6–10% of the corresponding diphosphates. ITP was not purified since allowance could be made for the presence of IDP when calculating the total amount of IDP required to give particular concentrations of $MgIDP^-$ which was used as the substrate. The removal of CDP from solutions of CTP was also unnecessary as CDP is a poor substrate (Schramm and Morrison, 1968) and the amounts introduced with CTP would have no significant effect on the reaction velocity. Because $MgUDP^-$ and $MgGDP^-$ are good substrates for nucleoside diphosphatase, it was essential to purify the samples of UTP

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