α-Methyl-*cis*-aconitic Acid. Aconitase Substrate. II. Substrate Properties and Aconitase Mechanism*

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ABSTRACT: α-Methyl-*cis*-aconitate behaves as a typical substrate for aconitase, two hydroxy acids, α-methylisocitric acid and α-methylcitric acid, resulting from the enzyme-catalyzed hydration. Enzyme-catalyzed hydration to yield α-methylisocitric acid is *trans* and, presumably, optical configurations of the two α-methylhydroxy acids are identical with those of D-*threo*-isocitric acid and citric acid.

K_M at pH 7.5, 28°, is 6.0×10^{-4} M and V_{max}, relative to *cis*-aconitate as 1.00, is 0.38. At equilibrium (pH 7.5, 28°), 27.1% of α-methyl-*cis*-aconitic acid and approximately equal amounts of each of the two hydroxy acids are present. The results do not permit a conclusion as to the possibility of *cis*-aconitate occupying the active site in two ways. A hypothetical enzyme mechanism for unidirectional addition of H or OH to a given carbon atom of the double bond is presented.

Aconitase (EC 4.2.1.3 *cis*-aconitase, aconitate hydratase, citrate (isocitrate) hydro-lyase) catalyzes by reversible hydration the equilibration of *cis*-aconitic acid, D-*threo*-isocitric acid, and citric acid. The stereochemical configurations of the components of the system are such that, as depicted in Figure 1, frontal addition of H and OH to the α carbon of *cis*-aconitic acid and addition from the rear of OH and H to the β carbon is required for the equilibration (Gawron *et al.*, 1961; Hanson and Rose, 1963). This unidirectional addition of H and OH to a given carbon atom suggests that either the enzyme is mechanistically quite versatile or that *cis*-aconitic acid can occupy the active site in two ways, the position of the γ-carboxyl group not varying albeit the α and β carboxyls exchange positions, and therefore only H or OH is enzymatically added to a given carbon atom (Figure 2). The latter concept of aconitase action (Gawron *et al.*, 1961) requires the aconitate conformation depicted in Figure 2. Since this conformation would be expected to be less favored in α-methyl-*cis*-aconitic acid, determination of substrate properties of the methyl analog became of interest. Presumably, slight alteration in the values of K_M and V_{max} would favor a versatile mechanism while a decided change in their values and in the products formed would favor the "flip-over" concept.

Experimental Section

α-Methyl-cis-aconitic Acid. A 1.00×10^{-2} M stock solution of this acid at pH 7.5 was prepared by dissolving the requisite quantity of α-methyl-*cis*-aconitic anhydride (Gawron and Mahajan, 1965b) in 0.2 M phosphate buffer, pH 7.5, readjusting the pH to 7.5 with 1.0 M potassium hydroxide, and diluting to volume with buffer. In a similar manner a 2.00×10^{-2} M stock solution of *cis*-aconitic acid was prepared. Both solutions were prepared as required and not used for more than 1 day.

Aconitase Preparation. Aconitase was prepared from horse heart using the purification procedure of Buchanan and Anfinsen (1949) with modifications introduced by Morrison (1954a). From 200 g of frozen horse heart an initial extract (610 ml) containing 50,000 enzyme units with a specific activity of 10 was obtained. Two ethanol fractionations yielded 22,400 enzyme units (15 ml of a clear amber-red solution, 45% recovery) of specific activity 66, a 6.6-fold enrichment being thereby attained. This enzyme solution was used without further purification and without addition of ferrous ion and cysteine, the latter interfering with the spectrophotometric assay employed (Herr *et al.*, 1956). The enzyme was unstable on storage at −20° and although activity could be restored, as previously noted (Dickman and Cloutier, 1951; Morrison, 1954a), by the addition of ferrous ion and cysteine, the enzyme was prepared fresh, as needed. For this work a unit of aconitase activity was defined as that quantity of enzyme converting 1 μmole of substrate (*cis*-aconitic acid, initial concentration 1.45×10^{-2} M) in 15 min at 28°, pH 7.5. For calculation of specific activity, units per milligram of protein, protein was estimated spectrophotometrically, the absorbance at 280 mμ for 1.00 mg of protein/ml of solution was taken to be 1.6 (Anfinsen, 1955).

Spectrophotometric Assay. The assay was conducted in 1-cm cuvets in the thermostated (28°) compartment of a Beckman DB spectrophotometer with an attached recorder. The reference cuvet contained

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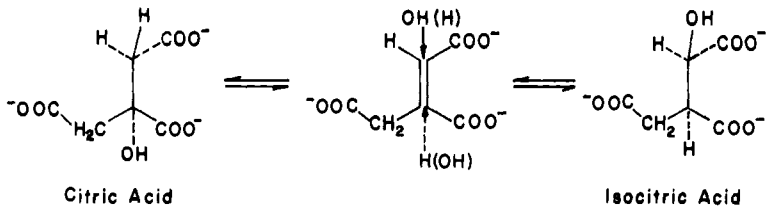


FIGURE 1: Unidirectional addition of H and OH to a given carbon of the double bond to yield D-*threo*-isocitric acid and citric acid.

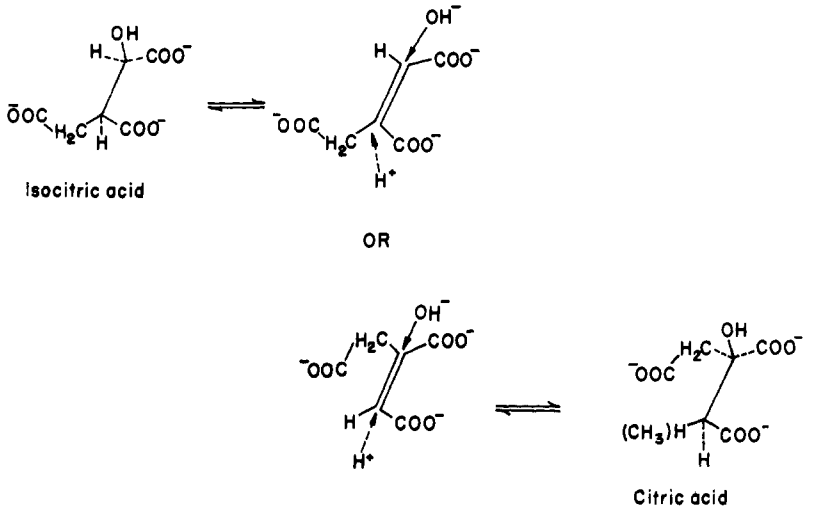


FIGURE 2: Unidirectional addition of H to one carbon and OH to the other, the substrate occupying the active site in two ways, yielding in one case D-*threo*-isocitric acid and in the other citric acid.

2.90 ml of 0.2 M phosphate buffer, pH 7.5, and 0.10 ml of enzyme solution. The reaction cuvet contained 2.90 ml of 1.5×10^{-2} M *cis*-aconitic acid solution, pH 7.5, in 0.2 M phosphate, and reaction was initiated after temperature equilibration by the addition of 0.10 ml of enzyme solution. Absorbance at 280 mμ was monitored, the molar extinction coefficient at this wavelength being 80.0.

Kinetics. Initial rates as a function of initial substrate concentration were obtained at pH 7.5, 28°, as described above, the rate being measured over the first 2 min after mixing, the initial rate being taken from the initial straight-line portion of the rate curve. Initial substrate concentrations of *cis*-aconitic acid varied from 1.0×10^{-4} to 8.00×10^{-3} M and rates were monitored at 240 mμ (ϵ 4.10×10^3), 250 (ϵ 2.03×10^3), 260 (ϵ 0.70×10^3), 270 (ϵ 2.75×10^2), and 280 mμ (ϵ 80.0). For α-methyl-*cis*-aconitic acid initial substrate concentrations varied from 9.67×10^{-5} to 5.80×10^{-3} M and reactions were monitored at 240 mμ (ϵ 4.50×150^3), 250 (ϵ 2.70×10^3), 260 (ϵ 6.28×10^2), and 270 (ϵ 1.56×10^2).

Position of Equilibrium. To 4.2 ml of substrate solution in 0.2 M phosphate, pH 7.5, 0.1 ml (150 units) of enzyme solution was added and the mixture was incubated at 28° for 3 hr. During this time an additional 0.2 ml of enzyme solution was added. Equilibrium, as evidenced by no further change in absorbance on addition of 0.05 ml of enzyme solution, was attained during the 3 hr of incubation. At the end of the incubation period absorbance at a suitable wavelength was measured and the equilibrium concentration of unsaturated acid was calculated. With three different starting concentrations of α-methyl-*cis*-aconitate, 18.6×10^{-4}, 9.3×10^{-4}, and 1.86×10^{-4} M, 27.1 ± 0.2% of α-methyl-*cis*-aconitate was present at equilibrium. With *cis*-aconitate 4.35 ± 0.1% of unsaturated acid was present at equilibrium, the starting concentrations being 5.6×10^{-3}, 9.3×10^{-4}, and 4.7×10^{-4} M.

Thin Layer Chromatography of Tricarboxylic Acids. A column procedure previously employed (Gawron *et al.*, 1958) for the separation of tricarboxylic acids by partition chromatography was adapted to thin layer chromatography. Glass plates (20 × 20 cm) after thorough cleaning and rinsing with distilled water, were rinsed with alcohol, and then air dried. The dry plates were coated (0.2 mm thickness) with a slurry made from 15 g of silica gel G (Research Specialties Co.) and 30 ml of 0.17 N sulfuric acid. After coating, the plates were stored and exposed to air in a horizontal

rack for 24 hr at room temperature. For chromatography, *ca.* 1-μg samples containing 20–50 μg of tricarboxylic acid were spotted on a line 2 cm from an edge. Spotting was performed with a capillary pulled from a melting point tube and six to eight spots were applied on one plate. The plates were then hung in an all-glass chromatography jar (Kensco thin layer chromatography apparatus) and allowed to equilibrate for 15 min prior to immersion in the developing solvent—600 ml of chloroform–1-butanol–ethanol (75:20:5). After equilibration, the plates were lowered into the solvent to a depth of 1 cm. Development was allowed to proceed for 15 cm (90–110 min). The plates were then dried overnight at 60–70° in an oven equipped with a hot-air blower. After cooling, the plates were sprayed with either alkaline permanganate (white spots with a purple background) or with dilute ammonium hydroxide followed by bromocresol green (yellow spots with a blue background).

Thin Layer Chromatography of Hydration Products. A solution of 85 mg of α-methyl-*cis*-aconitic anhydride in 60 ml of 0.2 M phosphate buffer, pH adjusted to 7.5, was incubated at 28° for 4 hr with 5 ml (7000 units) of enzyme solution. Enzyme was then inactivated by heating at 95° for 0.5 hr. After cooling and acidifying with 50% formic acid to pH 2, coagulated protein was removed by filtration and the filtrate was lyophilized. The residue thus obtained was extracted (Soxhlet) with ether. Removal of ether on the flash evaporator left a gummy residue from which traces of formic acid were removed by storage *in vacuo* over potassium hydroxide. The gum was then dissolved in 3.0 ml of anhydrous ether and the resulting solution was used for thin layer chromatography as described above. A control solution of α-methyl-*cis*-aconitic anhydride was also carried through the isolation procedure.

Isolation by Column Chromatography of Hydration Products. A relatively large-scale aconitase-catalyzed hydration of α-methyl-*cis*-aconitic acid was run as follows. A solution of 3.2 g of α-methyl-*cis*-aconitic anhydride in 600 ml of 0.2 M phosphate buffer, pH 7.5, at 28° was treated with 30 ml (44,000 units) of an aconitase preparation (sp act. 63), the enzyme being added over 5 hr. After an additional hour of incubation, enzyme was inactivated by heating at 90° for 1 hr. The reaction mixture was then cooled and adjusted to pH 1 with 6 N hydrochloric acid and precipitated protein was removed by filtration. The filtrate was lyophilized and the lyophilization residue was extracted (Soxhlet) with ether. Removal of ether by distillation at 40° left 6.7 g of moist gum which contained phosphoric acid. This gum was dissolved in a mixture of 5 ml of alcohol and 5 ml of solvent I (below) and the solution was placed on the chromatography column. The column, 3.2 cm in diameter, contained a stationary phase of 14.0 ml of 0.5 N hydrochloric acid thoroughly dispersed on a mixture of 35 g of silica gel G and 35 g of Celite (535, Johns-Manville) and an initial mobile phase, solvent I. Elution (1 ml/min, 10-ml fractions) was carried out with solvent mixtures of varying proportions of chloroform–1-butanol–ethanol: I, 130 ml, 90:9:1; II, 200 ml, 85:13:2; III, 200 ml, 80:16:4; IV, 330 ml, 75:20:5; V, 300 ml, 70:23:7; VI, 240 ml, 65:25:10; and VII, 300 ml, 60:28:12. Individual fractions were characterized by titration (Figure 5) and composite fractions by thin layer chromatography. Composite fraction I (tubes 13–46) contained α-methyl-*trans*-aconitic acid; II (56–67), α-methyl-*cis*-aconitic acid; III (96–114), α-methylisocitric acid; IV (115–138), α-methylisocitric acid plus α-methylcitric acid; and V (139–160), α-methylcitric acid. Fractions IV and V also contained phosphoric acid.

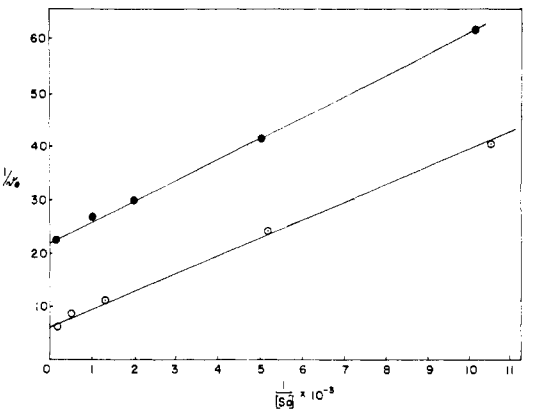


FIGURE 3: $1/v_0$ *vs.* $1/[S_0]$ plots at pH 7.5, 28°, for *cis*-aconitate (upper line) and for α-methyl-*cis*-aconitate (lower line), 150 units of enzyme employed. The ordinate is to be multiplied by 10^{+2} for *cis*-aconitate and by 10^{+3} for α-methyl-*cis*-aconitate.

Fractions IV and V were freed of phosphoric acid as follows. Solvent was removed under reduced pressure and the residue was dissolved in 15 ml of water. The pH of this solution was brought to 5 with 1 N sodium hydroxide and then adjusted to pH 1.8 with 50% formic acid. The residue after removal of water by lyophilization was extracted (Soxhlet) with ether. Removal of ether on a flash evaporation yielded a residue free of phosphoric acid. Fraction IV after removal of phosphoric acid was rechromatographed with the results shown in Figure 6.

Tri-p-phenylphenacyl Ester of α-Methylisocitric Acid. Fraction III, 60 mg after removal of solvent, was dissolved in 5 ml of 50% ethanol and neutralized (pH 7.0) with 1 N sodium hydroxide. To this solution 200 mg of *p*-phenylphenacyl bromide in 10 ml of 90% ethanol was added and the mixture was refluxed for 4 hr. After refluxing, the solution was concentrated to one-half of the original volume. On cooling, colorless crystals of the ester separated. The crystals were filtered off and recrystallized two times from anhydrous ethanol to yield 40 mg of the pure triester, mp 119–120°.

Anal. Calcd for $C_{49}H_{40}O_{10}$: C, 74.62; H, 5.07. Found: C, 74.96; H, 5.35.

Tri-p-phenylphenacyl Ester of α-Methylcitric Acid. The residue (50 mg) from fraction V after removal of

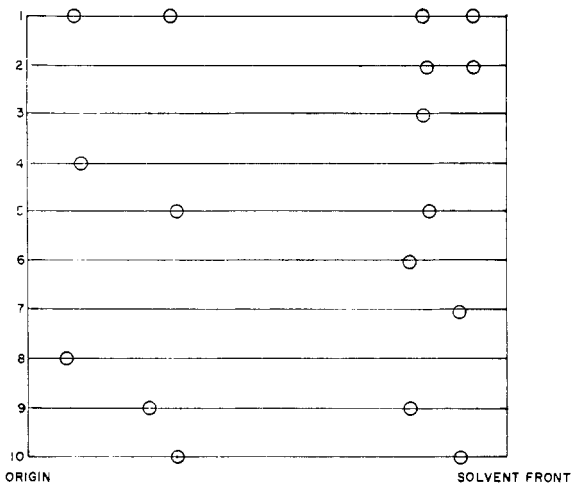


FIGURE 4: Thin layer chromatography of hydration products and of related tricarboxylic acids. (1) Enzymic hydration of α-methyl-*cis*-aconitate; (2) control; (3) α-methyl-*cis*-aconitic acid; (4) α-methylcitric acid; (5) α-methylisocitric acid (lactone); (6) *cis*-aconitic acid; (7) *trans*-aconitic acid; (8) citric acid; (9) DL-isocitric lactone; and (10) DL-*allo*-isocitric lactone.

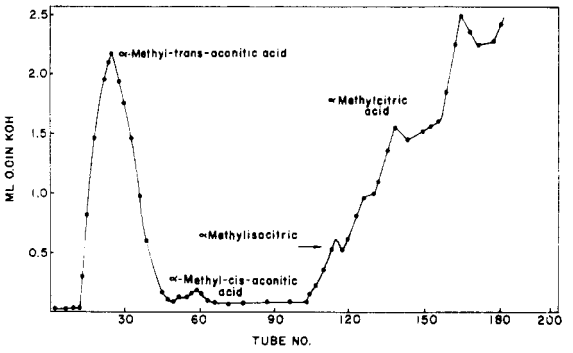


FIGURE 5: Column chromatography of enzymic hydration products from α-methyl-*cis*-aconitic acid. Experimental details given in the test.

phosphoric acid was treated in the same way as that from fraction III. Once recrystallized (50% ethanol, 30 mg) triester, mp 85–86°, was obtained.

Anal. Calcd for $C_{49}H_{40}O_{10}$: C, 74.62; H, 5.07. Found: C, 74.13; H, 4.84.

Results

Figure 3 presents $1/v_0$ *vs.* $1/[S_0]$ plots for α-methyl-*cis*-aconitate and for *cis*-aconitate, the data being obtained at pH 7.5 and 28° for both acids. K_M and V_{max} values calculated from the intercepts and slopes of these plots are 6.0×10^{-4} M and 1.7×10^{-4} mole $l.^{-1}$ min^{-1} for α-methyl-*cis*-aconitate and 1.9×10^{-4} M and 4.5×10^{-4} mole $l.^{-1}$ min^{-1} for *cis*-aconitate. The K_M value, 1.9×10^{-4} M (0.2 M phosphate, pH 7.5, 28°), reported herein for *cis*-aconitate is of the same magnitude, albeit somewhat higher, than that reported, 1.2×10^{-4} M, for 0.05 M phosphate, pH 7.7, 22° (Morrison, 1954b).

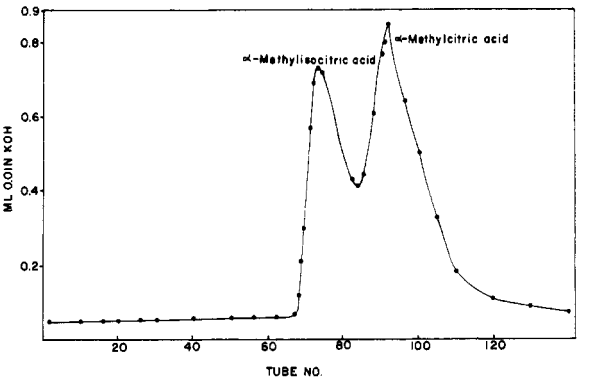


FIGURE 6: Rechromatography of mixed hydroxy acids after removal of phosphoric acid. Experimental details given in the text.

The percentage of unreacted α-methyl-*cis*-aconitate, 27.1%, at equilibrium is considerably higher than that, 4.35%, found for *cis*-aconitate under the present conditions. The latter value is the same as that reported by Eggleston and Krebs (1949) at 38°, pH 6.8 (0.05 M phosphate), but somewhat higher than that, 2.9%, reported (Krebs, 1953) at 25°, pH 7.4.

The products of the enzyme-catalyzed hydration were initially investigated by thin layer chromatography. Figure 4 shows the chromatographic behavior of acids isolated from an enzyme run and a control run, and of related acids. It is to be noted that in the enzyme run and the control (items 1 and 2) a fast moving acid (R_F 0.92) in addition to α-methyl-*cis*-aconitic acid (item 3, R_F 0.82) appears. By comparison with the behavior of *cis*-aconitic acid (item 6, R_F 0.78) and *trans*-aconitic acid (item 7, R_F 0.88), the new fast moving acid, R_F 0.92, appearing in both the control and enzyme runs, may be identified as α-methyl-*trans*-aconitic acid. In addition to α-methyl-*trans*-aconitic acid and α-methyl-*cis*-aconitic acid, the enzyme run (item 1) shows two other acids, R_F 0.10 and 0.30. The R_F values of these acids are similar to those for citric acid (item 8, R_F 0.08) and DL-isocitric lactone (item 9, R_F 0.25). On the basis on this comparison, the acids of R_F 0.10 and R_F 0.30 were tentatively identified as α-methylcitric acid and α-methylisocitric acid (lactone), respectively. This identification was made definite by the thin layer chromatographic behavior of the individual acids isolated by column chromatography (Figures 5 and 6) following the enzyme-catalyzed hydration. One of the two acids (item 4) yielded one spot, R_F 0.10, while the other (item 5) yielded two spots, R_F 0.30 and 0.82, the latter being readily identified as α-methyl-*cis*-aconitic acid. This chromatographic pattern is completely analogous to that of citric acid (item 8, R_F 0.08) and DL-isocitric lactone (item 9, R_F 0.25 and 0.78), the lactone undergoing partial trans-

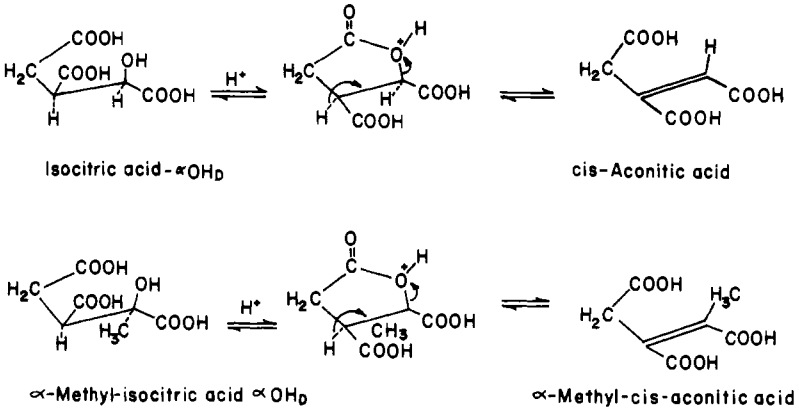


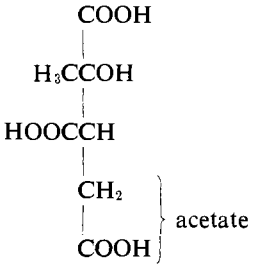
FIGURE 7: Thin layer chromatography dehydrations *via* the lactone of D-*threo*-isocitric acid and α-methylisocitric acid.

formation (dehydration) to *cis*-aconitic acid (item 6, R_F 0.78). Accordingly, the acid yielding one spot, R_F 0.10, is α-methylcitric acid, and that yielding two spots, R_F 0.30 and 0.82, is α-methylisocitric acid lactone. The stereochemical configuration of α-methylisocitric acid is also to be deduced from its behavior on thin layer chromatography. Since this acid yielded a *cis*-aconitic acid on dehydration the configurational relationships of the H and OH involved must be identical with that of DL-isocitric acid, the DL-*allo*-isocitric lactone (item 10, R_F 0.28 and 0.88) yielding *trans*-aconitic acid (item 7, R_F 0.88) on chromatography. By analogy with enzyme-catalyzed formation of D-*threo*-isocitric acid from *cis*-aconitic acid, it is expected that the isolated α-methylisocitric acid is the D-*threo*-α-methylisocitric acid.

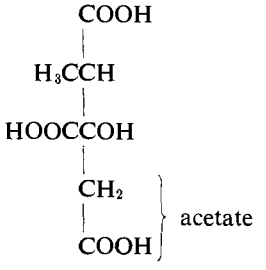
Separation on a preparative scale of the hydroxy acids resulting from aconitase-catalyzed hydration of α-methyl-*cis*-aconitate was accomplished by partition chromatography (Figures 5 and 6). The isolated acids, obtained in small quantities, could not be induced to crystallize and were therefore converted to tri-*p*-phenylphenacyl esters for analytical purposes. Satisfactory agreement of the analytical values for C and H with the calculated values corroborated the assigned structures. The hydroxyl group of α-methylcitric acid gave a positive hexanitratocerate test (Duke and Smith, 1940) while that of the isolated α-methylisocitric acid did not respond. Since DL-isocitric lactone also gave a negative test, the isolated α-methylisocitric acid is in the lactone form.

Discussion

It is clear from the results that the aconitase-catalyzed hydration of α-methyl-*cis*-aconitate is similar to the aconitase-catalyzed hydration of *cis*-aconitate, the products being the analogous α-methylcitrate and α-methylisocitrate. It is to be expected that the stereochemistry of these acids is identical with that of enzymatically formed citrate and isocitrate, and in the case of α-methylisocitrate evidence from dehydration (*via* the lactone, Figure 7) during thin layer chromatography demonstrates the configurational relationships of the enzyme added H and OH to be the same as that in D-*threo*-isocitrate, the natural isomer. It can readily be seen that the acid-catalyzed dehydrations of Figure 7 occur in a *trans* fashion, DL-isocitric lactone giving rise to *cis*-aconitic acid and DL-*allo*-isocitric lactone yielding *trans*-aconitic acid. Accordingly, α-methylisocitric acid, yielding on dehydration α-methyl-*cis*-aconitic acid, must have the same optical configuration as either D- or L-*threo*-isocitric acid. It is, of course, to be expected that the D-enantiomorph is enzymatically produced and, also, that α-methylcitrate will be stereochemically equivalent to citrate, the stereochemistry of the latter acid also being known (Gawron *et al.*, 1961; Hanson and Rose, 1963). The two acids on this basis may be depicted by the following Fischer projections.



α-methyl-D-*threo*-isocitric acid

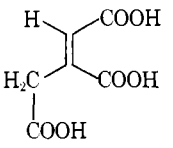


α-methylcitric acid

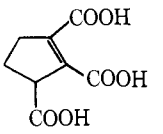
In addition to similarity of products, substrate parameters of α-methyl-*cis*-aconitate and *cis*-aconitate are comparable, the respective K_M values being 6.0 $\times 10^{-4}$ and 1.9 $\times 10^{-4}$ M and the ratio of the respective V_{max} values being 0.38. The similarity in value of these parameters indicates the two substrates are essentially kinetically equivalent with respect to the enzyme.

At equilibrium, 27.1% of α-methyl-*cis*-aconitate remains unreacted under the present experimental conditions. Of the 72.9% reacted, it would appear from the chromatographic and isolation data that approximately one-half has been converted to each of the two hydration products. For *cis*-aconitate, 4.35% remains unreacted (this study), and using values obtained under somewhat different conditions, 89.1% citrate and 6.6% isocitrate are present at equilibrium (Eggleston and Krebs, 1949). It is apparent that α-methyl-*cis*-aconitate and α-methylisocitrate are thermodynamically more stable than the corresponding aconitate and isocitrate while α-methylcitrate is less stable than citrate. In this connection changes in the population of ground-state conformers induced by substitution of methyl for H may be significant.

While the results clearly show that the active site can accommodate an α-methyl group in place of an α-H atom, the conformation of the acetate side chain in the reactive complex cannot be deduced, conformation of the acetate side chain being of importance to "mapping" the active site and to an understanding of the enzyme-catalyzed reaction. If the conformation of the acetate side chain in the reactive complex is that shown in Figure 2, then the enzyme would be capable of handling substrate in the two ways depicted in Figure 2. On the other hand, if the conformation of the acetate side chain is that shown below, it would be difficult to conceive of substrate being handled in two ways, all three carboxyl groups being required



for substrate activity[1] and "flipping" over radically altering the position of the γ carboxyl with respect to the active site. While the α-methyl substituent does not permit attainment[2] of the conformation shown in Figure 2, it does permit a close approach to this conformation and, therefore, as the results indicate, α-methyl-*cis*-aconitate is not sufficiently different to bear on this question. A compound such as 1,2,3-tricarboxycyclopentene-1, in which the conformation of the acetate side chain is fixed, would seem to be more suitable for investigation of the conformation of the acetate side chain in the reactive complex.



It is of interest to note that mechanisms of enzyme-catalyzed hydrolyses involving two histidine residues at the active site may with slight modification be used to construct a hypothetical mechanism for aconitase-catalyzed hydration, including unidirectional addition of H or OH to a given carbon atom. The mechanisms alluded to are those for chymotrypsin (Hartley, 1964) and for ribonuclease (Findlay *et al.*, 1962) and may be applied in the manner shown in Scheme I and provide an alternative to the "flip-over" hypothesis. The two histidines at the active site may be considered to exist as a protonated–unprotonated pair (a or d) or a protonated–hydrated pair (b or c), each member of a pair having an alternate arrangement and all pair forms being in equilibrium. For the hydration of *cis*-aconitate forms b and c are used, the substrate occupying the enzyme site in the same position in both cases, with isocitrate arising from b and citrate from form c. The mechanism, as written, is a concerted attack[3] of incipient H^+ and OH^- on the double bond of *cis*-aconitate and on reversal a concerted attack of protonated acid on the α- or β-hydroxyl group and base on the β- or α-H. On further consideration of the dehydration, it is noted the nonhydrated enzymic pair, d and a, is used and the dehydration leads to the hydrated pair, b and c. If the hydrates (b and c) are slow to exchange with water of the medium relative to equilibration of b with c then the elements of water may be transferred from one hydroxy acid to the other, the extent of the transfer being dependent on the relative rates. In a qualitative sense, this aspect of the mechanism is in keeping with the relative incorporation from a deuterium oxide medium of deuterium into citrate by enzyme attack on *cis*-aconitate in one case and on isocitrate in the other case. With *cis*-aconitate as citrate precursor, deuterium incorporation reflects the deuterium content of the medium while with isocitrate deuterium incorporation is decidedly lower (Speyer and Dickman, 1956; Rose *et al.*, 1962).

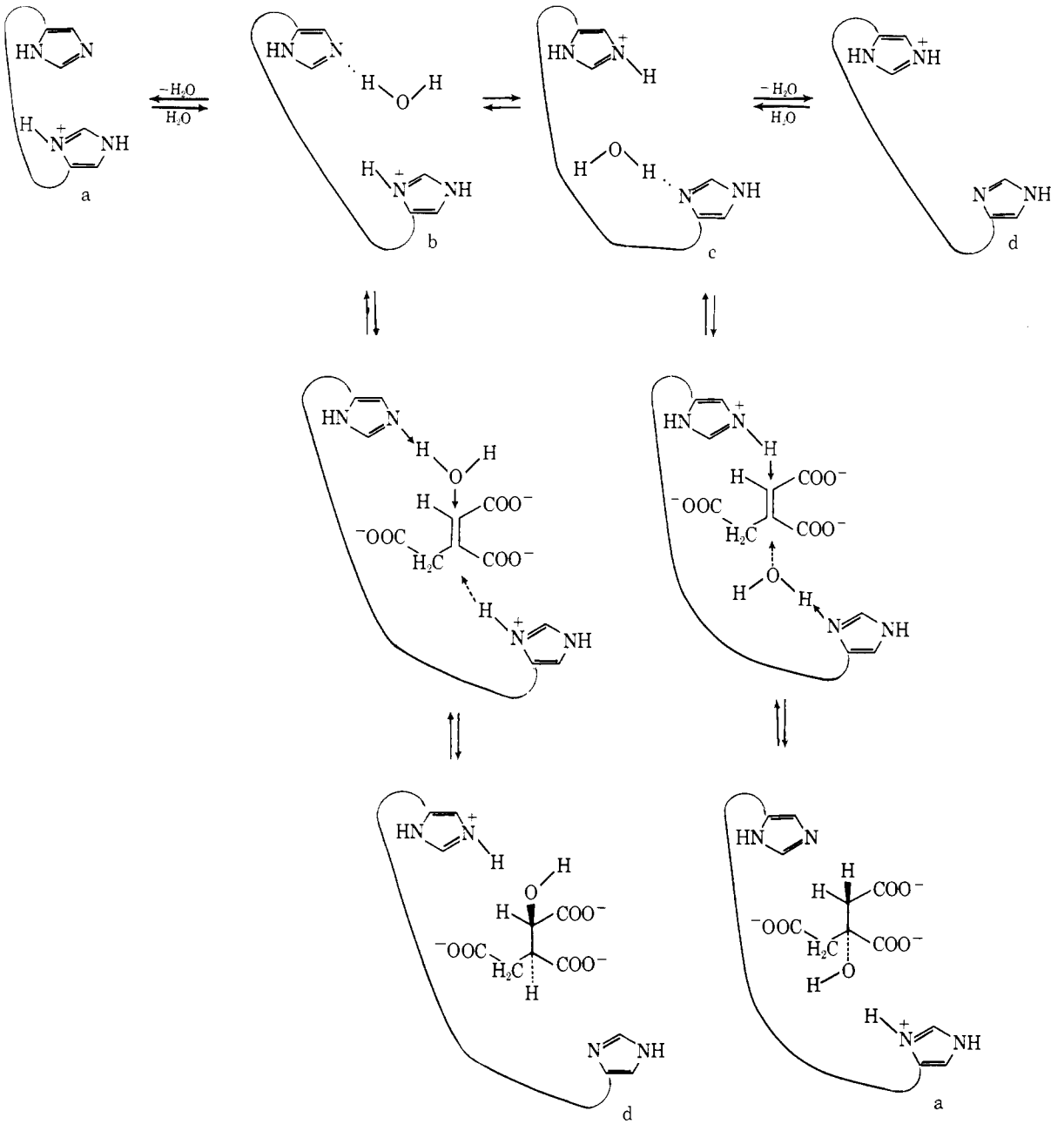
Related to aconitase is the reported enzyme, citrate dehydratase (EC 4.2.1.4 aconitic hydrase, citrate hydrolyase) (Neilson, 1956, 1962), catalyzing the reversible hydration of *cis*-aconitate to citrate but not to isocitrate. On the basis of the present concepts, *cis*-aconitate would occupy the active site of this enzyme in only one way and mechanistically only H could be added to the α carbon and OH to the β carbon. Unfortunately, the enzyme has resisted isolation in this laboratory

[1] All three carboxyl groups are required for substrate activity, the three monomethyl esters of *cis*-aconitic acid being inactive (Dickman and Speyer, 1954).

[2] By inspection of Fisher–Taylor–Hirschfelder models and Godfrey molecular models.

[3] Enzymatic-catalyzed additions to a double bond may be *trans* because the enzyme groups concerned are located on opposite sides of the active site.

SCHEME I: Hypothetical Enzyme Mechanism for Unidirectional Addition of H and OH to a Given Carbon Atom of the Double Bond.



(O. Gawron and K. P. Mahajan, unpublished observations).

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Spinach Ribulose Diphosphate Carboxylase. I. Purification and Properties of the Enzyme*

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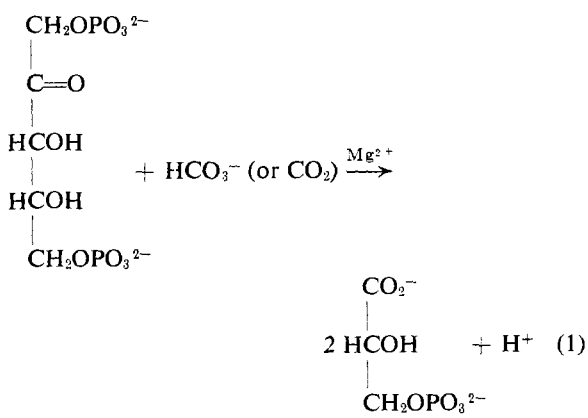
ABSTRACT: Spinach leaf ribulose diphosphate carboxylase has been purified to a homogeneous state free of 5-phosphoriboisomerase and 5-phosphoribulokinase activities.

The carboxylase has a sedimentation coefficient ($s^0_{20,w}$) of 21.0 S and molecular weight by sedimentation equilibrium of 557,000. The pure enzyme catalyzes the carboxylation of 1300 moles of D-ribulose 1,5-diphosphate/min per mole of enzyme at pH 7.9 and 30°. K_m and V values for substrates and metal activators are reported. Orthophosphate (K_i = 4.2 mM) and sulfate (K_i = 8.1 mM) inhibit the carboxylation reaction competitively with respect to ribulose diphosphate. 3-Phosphoglycerate appears to inhibit competitively with respect to HCO_3^- and noncompetitively with respect to ribulose diphosphate.

The isolation and partial characterization of ribulose diphosphate carboxylase[1] (3-phospho-D-glycerate carboxy-lyase (dimerizing) EC 4.1.1.f; Report of the Commission on Enzymes, 1961; Dixon and Webb, 1964a) from spinach leaves has been reported by a number of investigators (Weissbach *et al.*, 1956; Racker, 1957; Jakoby *et al.*, 1956; Trown, 1965). Convincing evidence has been presented by Trown (1965) and Thornber *et al.* (1965) that the principal component of leaf "fraction I protein" (Wildman and Bonner, 1947) is, in fact, RuDP carboxylase. While the stoichiometry of the reaction (reaction 1) catalyzed, as well as many properties of the carboxylase, have been known for nearly a decade, information on the carboxylation mechanism or structural characteristics of the enzyme is meager. Only recently, Müllhoffer and Rose (1965) unequivocally proved that C–C bond



(1)

cleavage occurs at the C-2–C-3 bond of RuDP during the carboxylation reaction. Carboxylation of RuDP in the presence of D_2O resulted in deuterium incorporation into 3-PGA at the carbon atom originating from the C-2 position of RuDP. Trown and Rabin (1964) have obtained evidence for an interaction between RuDP carboxylase sulfhydryl groups and RuDP. The nature of this interaction is still uncertain.

The initial objective of this investigation was to devise a procedure for the preparation of homogeneous RuDP carboxylase in sufficient quantity for structural characterization and mechanism studies, which will be reported subsequently as part of this series. In addition to describing such a purification procedure, the molec-

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[1] Abbreviations used: RuDP, D-ribulose 1,5-diphosphate; 3-PGA, 3-phospho-D-glyceric acid; R 5-P, D-ribose 5-phosphate; Ru 5-P, D-ribulose 5-phosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2(5-phenyloxazolyl)benzene; ATP, adenosine triphosphate; GSH, glutathione.