Mechanism of Aconitate Action

I. THE HYDROGEN TRANSFER REACTION*

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SUMMARY

The following scheme is proposed to represent the path of tritium in the aconitate (aconitate hydratase) reaction.

\[ E + 2\text{-T-citrate} \rightleftharpoons E\text{-cis-aconitate} \rightleftharpoons E + 3\text{-T-isocitrate} \]

Five experiments that support this scheme are presented.

1. The conversion of 3-T-isocitrate to citrate at early times gives T-citrate with no loss of tritium.

2. When the conversion of 2-T-citrate to isocitrate is brought to completion by trapping the isocitrate with isocitrate dehydrogenase, and the \( \alpha \)-ketoglutarate formed with glutamate dehydrogenase, the amount of tritium found in the glutamate agrees with the prediction based on the relative initial rates of formation of isocitrate and cis-aconitate from citrate.

3. The transfer of deuterium from citrate to isocitrate in the absence of high concentrations of cis-aconitate is shown to be largely intramolecular.

4. At high concentrations of cis-aconitate some of the tritium of 2-T-citrate that is normally found in the water is diverted to isocitrate.

5. This conservation of tritium is due to an intermolecular transfer since in the presence of high cis-aconitate the tritiated 2-methyl hydroxy acids give rise to tritiated isocitrate.

Aconitase or cis-aconitate hydratase (E.C. 4.2.1.3) from pig heart is thought to be a single protein species (1) that catalyzes the interconversion of citrate, isocitrate, and cis-aconitate. cis-Aconitate is converted at about equal rate to each hydroxy acid. If the conversion of citrate to isocitrate is observed continuously with isocitrate dehydrogenase and triphosphopyridine nucleotide reduction there is no delay, as though a critical concentration of cis-aconitate does not have to develop before isocitrate formation begins. The cis-aconitate being formed reaches its maximum concentration of about 3% of the citrate and thereafter remains at equilibrium with the falling citrate concentration, contributing to the production of isocitrate which is being removed by the dehydrogenase. A mechanism in which an intermediate enzyme complex is common to all three interconversions is suggested by the kinetics, but not unambiguously (2). Isotopic evidence for a direct interconversion of the hydroxy acids, not involving cis-aconitate, was obtained by Speyer and Dickman (3). As seen from the scheme, which includes the equilibrium percentages (4), a medium proton, \( H_M \), is stereospecifically placed (5) in the alternate hydration of cis-aconitate.

\[
\text{citric acid (91\%)} \quad \text{H}_2\text{C-CO}_2\text{H} + E + \text{H}_2\text{O} \quad \text{isocitric acid (6\%)} \quad \text{HO}_2\text{C-CO}_2\text{H} + \text{E} + \text{H}^+ + \text{OH}^- \quad \text{cis-aconitic acid (3\%)} \quad \text{HOC-CH}_2\text{C-CO}_2\text{H} + \text{E} + \text{H}^+ + \text{OH}^- \]

It was observed (3) that in a medium of 10% D\(_2\)O, the citric acid formed from isocitrate contained less deuterium than the citric acid formed from cis-aconitate. The citrate formed from cis-aconitate had a deuterium content considerably less than 10% of the one position that was to be expected, making a quantitative interpretation of the data difficult. The inference that hydrogen from the C-3 position of isocitrate together with the same carbon skeleton proceeds through an enzyme intermediate to citrate, would exclude cis-aconitate as a necessary intermediate. The conclusion that hydrogen retention occurs in the interconversion of the hydroxy acids was confirmed in an earlier communication from this laboratory (6).

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In the mechanism proposed by Speyer and Dickman (3) to explain hydrogen retention, a bound carbonium ion is formed by abstraction of hydroxyl ion in an iron-enzyme complex of the hydroxy acid. The carbonium ion could then either rearrange by hydride transfer and with subsequent hydroxylation form the hydroxy acid product, or by elimination of H+ would form the dehydrated product. The formation of an olefinic product is characteristic of the carbonium ion mechanism of chemical solvolysis at a tertiary aliphatic carbon atom. An alternative to the direct transfer of the hydrogen as an hydride is the abstraction of the proton by the enzyme followed by either transfer to the neighboring carbon of bound cis-aconitate or dissociation of the complex. Gawron, Ghaid, and Fondy (7) pointed out that the stereochemistry of the substrates required that the alternative approaches of the proton must be from opposite sides of the cis-aconitate plane and suggested that the bound cis-aconitate could flip over and be accommodated in the alternative manner in being hydrated. No stereochemical restriction is applicable to the hydride transfer mechanism since the conformation of the carbonium ion is not limited by a double bond.

A major objective of the present investigation has been to distinguish between the hydride and proton mechanisms. The proton transfer mechanism, if it is found to be correct, has promise of being relevant to the path of hydrogen in the dehydration reaction due to aconitase and perhaps other enzymes as well. In particular it would provide the first direct evidence for the role of the enzyme as base in the proton abstraction step of α-β elimination reactions.

**MATERIALS AND METHODS**

Aconitase was prepared from pig heart by the method of Morrison (8). The enzyme, at the last step of purification, was stimulated greatly (10- to 400-fold) upon preliminary incubation with Fe(NH4)2(SO4)3 (5 mM) and cysteine (10 mM) at pH 7.4 (trithanolamine, 60 mM) for 30 min at 4°C according to Morrison (9). Upon activation the purified enzyme had a specific activity of 2 units per mg. The enzyme was stored in the frozen state after the last (NH4)2SO4 fractionation in 4 mM citrate, pH 5.7, in which it had been dialyzed. Unless otherwise stated the enzyme was used without dialysis since the citrate introduced into the incubation with the enzyme was quantitatively undetectable. In all cases the enzyme was activated before use. The enzyme was assayed at 25°C with isocitrate dehydrogenase and citrate by measuring the reduction of TPN at 340 mμ in aqueous solution containing per ml: trithanolamine-Cit-, pH 8.0 (0.1 M); MgCl₂ (1 mM); TPN (1 mM); Na₂ citrate (1 mM); and isocitrate dehydrogenase (1 unit). Succinate dehydrogenase was prepared from pig heart according to Keilin and Hartree (10).

Other enzymes used, isocitrate dehydrogenase, 1.8 units per mg and glutamate dehydrogenase, 3 units per mg, were obtained commercially from C. F. Boehringer und Soehne, and were substantially free of aconitase. Enzyme units of activity are defined as the rate in micromoles per min under standard conditions in all cases.

**Chemicals—α-Methyl-cis-aconitate** was prepared by condensation of diethylmethylxaloacetate and ethyl cyanoacetate according to Gawron and Mahajan (11), and purified after ester hydrolysis by ether extraction and silica column chromatography with elution by 20% butanol in HCCl₃. The product was judged to be 70% pure on the basis of the extent of reaction with aconitase as judged by the change in absorbance at 240 mμ (12).

Three-n, isocitrate was a gift of Dr. H. Vickery, cis-Aconitate anhydride was prepared from commercial trans-aconitic acid (13).

**Isotopically Labeled Compounds—3-T-Ketoglutarate** randomly labeled in position 3 was prepared by chemical exchange (14). Its position of labeling has been established (15). Tritiated glutamate was converted to α-ketoglutarate for Fig. 1 by reaction of glutamate dehydrogenase (1 unit) with 3-acetylpyruvyl-ADP (1 mM) at pH 8.0. The α-ketoglutarate (for Fig. 1) was isolated on Dowex 1 (Cl⁻) by elution with 0.1 N HCl. Randomly tritiated cis-aconitate was obtained by incubation of 0.5 m M cis-aconitic acid in tritiated water for 20 hours. This was used for the preparation of 4-T-α-ketoglutarate by the aconitase-isocitrate dehydrogenase reaction for Fig. 1.

Tritiated citrate and isocitrate were prepared by incubation of cis-aconitate (1 μmole) in tritiated water (10⁴ cpm per μmole of H) with 6 units of aconitase at pH 7.4. Samples were removed to determine the amount of isocitrate formed. At 6 hours there were 170 μmoles of isocitrate and the enzyme was inactivated by heating after sublimation of the water. The combined radioactive acids containing 5 × 10⁶ cpm were isolated from Dowex 1-formate by elution prior to cis-aconitic acid with a 0 to 3.5 N formic acid gradient (16). The radioactive peak was dried in a vacuum repeatedly with dry acetone; the isocitric acid present (160 μmoles) was then partially lactonized in 25 ml of trifluoroacetic anhydride-acetic-acetone (4:1, v/v) at 25°C for 1 hour, after which the solvents were removed in a vacuum. The dry residue was dissolved in acetic acid, neutralized, and chromatographed again on Dowex 1-formate. The isocitrate lactone is recovered after the citrate-isocitrate peak (0 to 3.5 N formic acid) by use of 6 N HCl (17). The lactone was hydrolyzed and the isocitric acid was recovered on the same column (34 μmoles with 535,000 cpm per μmole). The citrate content of this 3-T-isocitrate preparation was 0.9% as determined by the isolation of citrate after addition of carrier.

The 2-T-citrate¹ was isolated from the first radioactive peak after the removal of formic acid by evaporation, conversion of isocitric acid to the lactone, as above, separation on a silica gel column of the citric acid from the isocitric lactone (19), and removal of the trace of isocitrate with isocitrate dehydrogenase and glutamate dehydrogenase. The citrate (50 μmoles) finally recovered from Dowex 1-formate had a specific activity of 670,000 cpm per μmole.

Isocitrate labeled with 18O in the C-2 hydroxyl group was prepared by the reaction of cis-aconitate (6000 μmole) at pH 7.3 with 10 units of aconitase in 15 ml of H₂O (10 atom % excess) for 160 min. At this time 1700 μmoles of isocitrate had been formed. The isolation, as above, gave 300 μmoles of isocitric acid lactone which, when hydrolyzed, contained 1.25 atom % excess of 18O among the 7 oxygen atoms. The contamination with citrate was 0.3%.

Deuterated cis-aconitate was prepared by incubating 10 g of free acid and 0.6 M HCl in 100 ml of 95% D₂O for 3 days at 75°C. The nonvolatile residue, primarily trans-aconitate, was con-

¹ The designation of the carbon atoms of citrate used in this paper is contrary to the rule of Hirschmann (18) according to which the position of citrate labeled for hydrogen exchange is the C-4 (P) position (5). The present special numbering is chosen to correspond to that of isocitrate and cis-aconitate so that the carbons involved in the elimination are C-2 and C-3 for all substrates.
converted to cis-aconitic anhydride in 99% acetic anhydride (12). The crystalline anhydride was pure as judged by enzymatic assay after hydrolysis to cis-aconitate. The content of deuterium was determined only for the methylene position since the analysis involved the conversion of carbon atoms 3, 4, 5, and 6 to succinic acid and mass analysis of succinic anhydride (below). About 50% of the methylene group contained 1 deuterium and 20% had 2. Subsequent studies by nuclear magnetic resonance have indicated that cis-aconitic acid undergoes an acid-catalyzed exchange of the methylene hydrogens under milder conditions. The rate and mechanism of this exchange are to be considered in a subsequent report. No exchange is observed at neutrality.

Deuterated citrate was made either by conversion of the above 4-D-cis-aconitate to 4-D-citrate or of cis-aconitate in D2O to 2-D-citrate by means of aconitase. The citrates were isolated after equilibrium had been established and isocitrate had been converted enzymatically to glutamic acid and CO2. The acids were separated on Dowex 1-acetate, glutamic acid by elution with 0.5 N acetic acid and citric acid with 5 N acetic acid-O.5 M sodium acetate. The citrates were isolated by elution with 0.5 N acetic acid. It was convenient in the cis-aconitate enzymatically was first isolated on Dowex 1-acetate by virtue of their earlier elution with 0.5 N acetic acid. The crystalline anhydride was pure as judged by enzymatic assay after hydrolysis to cis-aconitate.

RESULTS

The first objective of this study is to correlate the amount transfer of isotopic hydrogen with the partition of products rived from an hydroxy acid substrate.

Conversion of Aconitate to Citrate—It can be established as has been reported also with the aconitase of beef liver that the formation of citrate from isocitrate occurs with a stant initial rate, indicating that cis-aconitate is not requ to accumulate appreciably before citrate begins to appear. failure to observe a substantial lag suggests, but does not esh (2), a direct path between isocitrate and citrate. The tive initial rates of formation of citrate and cis-aconitate isocitrate with the heart enzyme were 4:1 at pH 8 at 25°. would be expected from these initial rates and the equilib constants among the three pairs of reactants, when the reactants are isolated from citrate there will be a period of increase of aconitate followed by a "steady state" value that is deter by the isocitrate-aconitate equilibrium and which slowly to 3% of the total acids as final equilibrium is reached. Du the early part of this curve it would be expected that the cit formed is primarily derived from the direct route, not pas through free cis-aconitate. Thus a comparison of the spe activities of early citrate and initial 2-tritiated isocitrate w indicate whether, in the direct conversion, there is any tritium whatsoever.

To test this, tritiated isocitrate (2 mM, 103,000 cpm per ml in 0.1 M P1 buffer, pH 7.6, was allowed to react to the exter about 11% total products formed with activated acclain in 1 ml. The citrate was isolated after addition of carrier citrate, showed that there were 3,540 cpm present in citrate initially. At 11% reaction there were 16,000 cpm in citrate. Thus 12,460 cpm were present in the 0.10 µmole of citrate forml The specific activity, 124,600, is larger than that of the in isocitrate. Clearly, then, by the criterion of hydrogen tran
there is direct conversion of isocitrate to citrate as concluded by Speyer and Dickman (3) and, barring some unusual isotope effect, retention of all of the hydrogen during the direct conversion phase is indicated. In a second experiment, as shown in Table VI, which will be considered in detail later, when 57% of the isocitrate had been converted to products, the citrate had 48% the specific activity of the average isocitrate. This experiment was terminated at a time when the cis-aconitate-citrate ratio was not 4, as expected from initial rates, but 0.6, indicating that much of the citrate had been formed from cis-aconitate.

Conversion of 2-Tritiated Citrate to Isocitrate—In the formation of isocitrate from 2-T-citrate with aconitase it is necessary to trap the isocitrate in order to promote the reaction beyond its unfavorable equilibrium point as well as to prevent the loss of the tritium of isocitrate through its conversion to cis-aconitate by aconitase. In the isocitrate dehydrogenase reaction the C-3 position of isocitrate becomes the C-3 of α-ketoglutarate. The hydrogens of this position are somewhat labile at neutral pH, showing an exchange rate of 10% per 40 min at 60° (23). Hence, it is usually desirable that both TPN-isocitrate dehydrogenase and glutamate dehydrogenase plus (NH₄)⁺ be present so that glutamate can be isolated instead of α-ketoglutarate.

\[
\begin{align*}
\text{CO}_2\text{H} & \quad \text{H}^+ \quad \text{C} - \quad \text{T} \\
\text{H}_2\text{O}_2\text{C} - \quad \text{C} - \quad \text{OH} & \quad \text{T} \quad \text{N} \quad \text{H}^+ \\
\text{CH}_2 & \quad \text{H}^+ \quad \text{C} - \quad \text{OH} \\
\text{CO}_2\text{H} & \quad \text{T} \quad \text{N} \quad \text{H}^+ \\
\end{align*}
\]

As shown, the three enzymatic reactions result in the retention of the C-4 hydrogens of citrate as the C-4 methylene of glutamate. One of the C-2 protons (H₂) of citrate is found in the TPN as a result of the opposite side specificities of isocitrate and glutamate dehydrogenases for the C-4 hydrogens of the pyridine ring of TPNH (24), and the other C-2 proton (T) would be found either in water or in the C-3 methylene of glutamate, depending on the path taken for the formation of isocitrate.

An example of such an experiment is the following: 2-T-citrate (0.175 μmole, 165,000 cpm), labeled by incubation of cis-aconitate in tritiated water with aconitase, was incubated in 1 ml containing triethanolamine-Cl, pH 8.0, 100 μmole; TPN, 0.5 μmole; isocitrate dehydrogenase, 0.2 unit; and aconitase, 0.04 unit. The reaction was completed in 10 min and at 20 min were added 100 μmoles of ammonium acetate and 0.45 unit of glutamate dehydrogenase in 0.10 ml. After complete oxidation of the TPNH the water of the reaction mixture was obtained by sublimation (130,000 cpm) and glutamate from the residue was isolated on Dowex 1-acetate and contained 24,700 cpm in 0.136 μmole. The glutamate had 19% of the specific activity of the citrate. The fact that most of the remaining counts are in the water indicates that this value is not low because of a very large kinetic discrimination against tritium. Crystallization of the glutamate with added carrier led to the expected radioactivity in the crystals.

It is important that the trapping system be considerably more active than aconitase in reacting with the isocitrate formed. This is especially true because the reaction rate of aconitase with isocitrate is much higher than with citrate so that as the citrate level decreases there will be a greater tendency for the aconitase to react with any steady state level of isocitrate and hence promote exchange of its tritium with the medium. The results of Table I with differing ratios of isocitrate dehydrogenase

<table>
<thead>
<tr>
<th>Isocitrate dehydrogenase</th>
<th>Ratio of dehydrogenase to aconitase</th>
<th>Rate of TPN reduction</th>
<th>Specific activity ratio of glutamate to citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>unit/ml</td>
<td>(μmoles/min) × 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.064</td>
<td>14.3</td>
<td>5</td>
<td>0.175</td>
</tr>
<tr>
<td>0.021</td>
<td>4.6</td>
<td>4</td>
<td>0.155</td>
</tr>
<tr>
<td>0.005</td>
<td>1.1</td>
<td>1</td>
<td>0.074</td>
</tr>
<tr>
<td>0.0009</td>
<td>0.2</td>
<td>0.6</td>
<td>0.003</td>
</tr>
</tbody>
</table>

![Fig. 1. Labilization of tritium of α-ketoglutarate.](http://www.jbc.org/)

Fig. 1. Labilization of tritium of α-ketoglutarate. Glutamates that had been derived from 2-T-citrate by reaction as in Table I ( ), or from isocitrate made from cis-aconitate in tritiated water ( ), or from α-ketoglutarate labeled by alkaline exchange in tritiated water ( ), or from 4-T-cis-aconitate ( ) were converted to α-ketoglutarate by glutamic dehydrogenase reaction. Samples of the α-ketoglutarates, isolated by ion exchange, were heated at 100° in 0.05 M phosphate buffer, pH 7.5, and were sublimed after different intervals to determine nonvolatile counts.
The results of Table 1 suggest that the counts finally present in glutamate are in a position of isocitrate that is labilized during the reaction. This is consistent with the expectation that the isocitrate formed is permitted to return, in part, to an intermediate capable of conversion to cis-aconitate. The tritium in glutamate is in the C-4 methylene position, was acted upon by aconitase in the presence of any carbonium ion complex would be consistent with these data.

**Table II**

*Effect of cis-aconitate concentration on tritium transfer*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>cis-Aconitate range (mM)</th>
<th>Total radioactivity in</th>
<th>Radioactivity ratio of glutamate to water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Water</td>
<td>Glutamate</td>
</tr>
<tr>
<td>1a</td>
<td>0.01</td>
<td>223,000</td>
<td>45,800</td>
</tr>
<tr>
<td>1b</td>
<td>0.02</td>
<td>175,000</td>
<td>34,500</td>
</tr>
<tr>
<td>2a</td>
<td>0.01</td>
<td>145,000</td>
<td>28,800</td>
</tr>
<tr>
<td>2b</td>
<td>0.02</td>
<td>120,000</td>
<td>25,600</td>
</tr>
</tbody>
</table>

The results of Table I suggest that the counts finally present in glutamate are in a position of isocitrate that is labilized during the reaction. This is consistent with the expectation that the isocitrate formed is permitted to return, in part, to an intermediate capable of conversion to cis-aconitate. The tritium in glutamate is in the C-4 methylene position, was acted upon by aconitase in the presence of any carbonium ion complex would be consistent with these data.

The preferred mechanism, abbreviated in Scheme 1, favors hydrogen transfer between adjacent carbon atoms of the same molecule. Direct evidence for intramolecular transfer was sought with the following experiment. A mixture of citrate labeled with deuterium at the aconitate-specific C-2 position, and citrate labeled with deuterium in the C-4 methylene position, was acted upon by aconitase in the presence of the isocitrate dehydrogenase-glutamate dehydrogenase trapping system. The resulting glutamate was degraded to succinate under conditions that do not labilize any of the methane hydrogens (see "Materials and Methods").

The resulting succinic anhydride was examined directly in the mass spectrometer to analyze the (CH$_3$CH$_2$CO)H$_2$O fragments for the multiply deuterated species expected from an intramolecular reaction. The two forms of deuterated citrate were separately converted to succinate in this system to provide the information about their individual contributions to the ratio of peak heights. In these experiments (Table III) the occurrence of 22% deuterium in the succinate derived from 2-D citrate is in good agreement with the 19.3% tritium transfer observed in glutamate in a parallel experiment with 2-T-citrate. Analysis of succinate derived from the separate aconitase conversion with each of the two citrates allows prediction of the relative peak heights that would be obtained with the mixture for either a purely intramolecular or intramolecular mechanism (Table III).
obtained with a 1:1 mixture of the citrates fall much closer to the intramolecular route. Evidence of the precision of mass analysis was obtained by comparing independent replicate experiments starting with 4-D-cis-aconitate, and the results obtained from the citrate prepared from this cis-aconitate. A parallel incubation with 2-T-citric acid resulted in a distribution of radioactivity: water to glutamate = 80:19:3.

Intramolecular character of proton transfer

Three incubations were set up each of 2.0 ml containing: citrate (50 μmoles), Tris (100 μmoles, pH 8), MnSO₄ (1 μmole), TPN (1 μmole), ammonium acetate (250 μmoles), isocitrate dehydrogenase (9 units), glutamate dehydrogenase (6 units), and aconitase (0.6 unit). The citrate present contained deuterium in the aconitase activated C-2 position, or the unactivated C-4 position, or was made up of an equal mixture of these two. After 140 min the glutamates were isolated by ion exchange and converted to succinic anhydride which was analyzed in the mass spectrometer for the normal and deuterated species: m/e 56 (no deuterium), 57 (1 deuterium), 58 (2 deuteriums), and 59 (3 deuteriums). A parallel incubation with 2-T-citric acid resulted in a distribution of radioactivity: water to glutamate = 80:19:3.

<table>
<thead>
<tr>
<th>Peak position</th>
<th>Peak heights found at location of deuterium in citrate used</th>
<th>Peak heights expected for reaction mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>C-4 C-2 + C-4 (1:1)</td>
<td>Intra-</td>
</tr>
<tr>
<td>m/e</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>56</td>
<td>77.4</td>
<td>31.0</td>
</tr>
<tr>
<td>57</td>
<td>22.2</td>
<td>48.2</td>
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<tr>
<td>58</td>
<td>0.46</td>
<td>20.8</td>
</tr>
<tr>
<td>59</td>
<td>0</td>
<td>0.24</td>
</tr>
</tbody>
</table>

* Each peak (m/e) is corrected for 14C by subtracting 3% of each (m/e - 1) peak and is presented as the percentage of the sum of the corrected peak heights.

* Calculated as the arithmetic mean due to 1:1 mixing: thus for peak 57, (22.2 + 48.2)/2 (2).

* Calculated for a random sorting of donor and acceptor species: thus peak 57 is the sum of the probabilities that a proton transfers to 4-(DH)-citric acid and that a deuteron transfers to 4-(HH) -citric acid, [(1.00 + 0.774)/2 - (0.482)/2 + (1.00 + 0.31)/2 - (0.222)/2].

Effect of Very High cis-Aconitate on Retention of Tritium—Scheme 1 indicates that in the reaction of tritiated citrate the loss of tritium occurs during the dissociation of cis-aconitate from the ET-cis-aconitate complex. If, however, the cis-aconitate dissociates first, it might be possible to capture the tritium from ET before its dissociation by performing the reaction in the presence of a high concentration of cis-aconitate in accordance with Scheme 2.

When the conversion of citrate to isocitrate is carried out in the presence of large amounts of cis-aconitate the rate of reaction of the citrate is greatly depressed and most of the isocitrate, or glutamate is derived from the cis-aconitate, tending to reduce its concentration preferentially. Hence these experiments must be terminated when only a small percentage of the added cis-aconitate has been entirely depleted, as judged from the absorbance at 240 mμ. The experiments without added cis-aconitate were terminated at much earlier times to act as suitable controls. All reactions were stopped with acid. The counts that passed through the anion exchange column and were also volatile were determined, and the glutamate was isolated on Dowex 1-acetate.

TABLE III: Effect of high cis-aconitate on tritium transfer

<table>
<thead>
<tr>
<th>Initial</th>
<th>Final</th>
<th>Volatile</th>
<th>Glutamate</th>
<th>Counts mobilized</th>
<th>Counts mobilized transferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/e</td>
<td>cpm</td>
<td>%</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.02</td>
<td>3432</td>
<td>520</td>
<td>5.0</td>
<td>13</td>
</tr>
<tr>
<td>0</td>
<td>0.02</td>
<td>6948</td>
<td>1126</td>
<td>10.0</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>1170</td>
<td>438</td>
<td>2.0</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>996</td>
<td>628</td>
<td>2.1</td>
<td>39</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>2850</td>
<td>1394</td>
<td>5.4</td>
<td>33</td>
</tr>
</tbody>
</table>

* The percentage of the total counts that are either volatile or in glutamate.

* The percentage of the mobilized counts that are in glutamate.
was collected from the effluent of the Spinco amino acid analyzer, disappeared. Water was isolated by sublimation. Glutamate and aconitate in tritiated water with aconitase ("Materials and Methods"). cis-Aconitate (10 rmols) was present as noted. Methods). The reactions were started with 0.08 unit of activated aconitase and terminated at 60 min when about half the cis-aconitate had disappeared. Water was isolated by sublimation. Glutamate was collected from the effluent of the Spinco amino acid analyzer 60 cm, AA15 cation exchange column eluted with 0.065 M sodium citrate buffer, pH 3.28. Carrier glutamate, 5 rmols, was added to the control.

An experiment to test the occurrence of such an intermolecular transfer of tritium could be planned with the knowledge that 2-methyl-cis-aconitate was an effective substrate of aconitase. Gawron and Mahajan (12) have reported that replacement of the vinyl hydrogen with methyl gives a substrate of K, and V, quite comparable with that of high concentrations of cis-aconitate can be explained in terms of a hydride transfer mechanism instead of Scheme 2. Here, it could be supposed that the elimination of cis-aconitate itself from the enzyme carbocation complex might be inhibited by large amounts of cis-aconitate and hence provide a larger proportion of reaction by the hydride transfer path. A clear experimental distinction between the proton and hydride mechanisms of transfer can be made. It depends on whether the hydrogen transfer that is enhanced in the presence of high concentrations of cis-aconitate can be shown to be intermolecular or intramolecular. A hydride transfer between neighboring carbon atoms could never be made intermolecular without a drastic modification of the simple carbocation ion mechanism. The conditions of this experiment were the same as those of Table V except that the combined tritiated methyl hydroxy acids used (2.0 mM, 511,000 cpm) were free of methyl-cis-aconitate. The three experiments were performed at different times with approximately 0.05 unit of activated aconitase. In each case in which cis-aconitate was the present the absorbance at 240 nm was monitored, with a cuvette with a 1- or 2-mm light path. cis-Aconitate was added when the absorbance due to cis-aconitate fell by 10% or so. The experiments were terminated at 30 min and water and glutamate isolated as for Table V.

### Table V

**Test of intermolecular tritium transfer**

<table>
<thead>
<tr>
<th>cis-Aconitate added (mM)</th>
<th>Total radioactivity in Water (cpm)</th>
<th>Glutamate (pmoles)</th>
<th>Counts mobilized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55,000</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>6,600</td>
<td>1,060</td>
<td>2</td>
</tr>
</tbody>
</table>

*The percentage of total counts found in water and glutamate.*

### Table VI

**Effectiveness of cis-aconitate and citrate in intermolecular tritium capture**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>cis-Aconitate (mM)</th>
<th>Citrate</th>
<th>Total radioactivity in Water (pmoles)</th>
<th>Glutamate (pmoles)</th>
<th>Counts mobilized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>56,365</td>
<td>20</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>68,465</td>
<td>000</td>
<td>0.34</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>26,265</td>
<td>1656</td>
<td>5.47</td>
<td>~100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>10,065</td>
<td>1700</td>
<td>14.45</td>
<td>~20</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>90,365</td>
<td>2</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.0-0.6</td>
<td>7,705</td>
<td>338</td>
<td>4.2</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>50,360</td>
<td>100</td>
<td>0.2</td>
<td>55</td>
</tr>
<tr>
<td>0.75</td>
<td>16,640</td>
<td>830</td>
<td>4.75</td>
<td>~10</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>15,800</td>
<td>950</td>
<td>5.70</td>
<td>~10</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>11,600</td>
<td>619</td>
<td>5.05</td>
<td>~7</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>2.0</td>
<td>8,840</td>
<td>6.85</td>
<td>~5</td>
<td></td>
</tr>
</tbody>
</table>

*Relative to the incubation with neither cis-aconitate nor citrate added.*
TABLE VII

\( ^{18}O \)-Isocitrate to Citrate

Sodium isocitrate (33 mM, pH 7.5), containing \( ^{18}O \) in the 2-OH position and tritium at the tertiary carbon, was incubated with 3 units of activated aconitase in 6 ml. Samples were removed to determine disappearance of isocitrate and the reaction was stopped with acid when 57% of the isocitrate had been removed and 55% of the tritium had been made volatile. The isocitrate remaining was converted to glutamate to determine specific activity and the citric acid was isolated by silicic acid column chromatography for \( ^{18}O \) and tritium determination.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocitrate</td>
<td>200 ( \mu )moles</td>
<td>84 ( \mu )moles</td>
</tr>
<tr>
<td>Isocitrate-(^{18}O)</td>
<td>1.048 cpm/( \mu )mole</td>
<td>840 cpm/( \mu )mole</td>
</tr>
<tr>
<td>Isocitrate-(^{3}H)</td>
<td>0.54 atom % excess</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>0.4 ( \mu )mole</td>
<td>73 ( \mu )mole</td>
</tr>
<tr>
<td>Citrate-(^{18}O)</td>
<td>1.08 atom % excess</td>
<td>450 cpm/( \mu )mole</td>
</tr>
<tr>
<td>Citrate-(^{3}H)</td>
<td></td>
<td>0.018 atom % excess</td>
</tr>
</tbody>
</table>

aconitate greater than 0.50 mM and appears to require a threshold value above which capture is roughly proportional to cis-aconitate. Citrate initially at 2 mM is much less effective than 0.5 mM cis-aconitate in leading to tritiated glutamate. The 0.2% of the mobilized tritium that was captured with citrate may be attributed to the cis-aconitate, up to 0.06 mM, formed from the citrate. This failure of citrate to promote the incorporation of tritium into isocitrate from either \( \alpha \)-methyl citrate or \( \alpha \)-methyl isocitrate is consistent with the results of Table III in showing the lack of transfer between the two forms of citrate. The results of Experiment 3, Table VI are intended to examine the possibility that citrate and cis-aconitate compete for the ET intermediate. Although Experiment 2 suggests that such an ET-citrate complex could not give rise to tritiated isocitrate, the alternative that it could lead to dissociation of \( T^+ \) and hence a lower percentage of capture by cis-aconitate could be tested.

As seen from Table VI, 2 mM citrate, while having a noticeable inhibitory effect on the total counts mobilized, did not lower the percentage of the mobilized counts that were captured by cis-aconitate. This result suggests that either ET-citrate does not form under these conditions or, if it does form, it is only able to dissociate to ET plus citrate and hence it would not alter the ratio of counts transferred to cis-aconitate relative to those found in water.

Estimate of Hydroxyl Transfer from \( ^{2}H \)-Isocitrate to Citrate—

Isocitrate containing \( ^{18}O \) at C-2 and tritium at C-3 was incubated with aconitase (Table VII). When 55% of the tritium had been labledized, the reaction was stopped. The citrate was isolated and found to contain about 45% of the specific radioactivity, indicating that this amount of direct conversion had occurred. The \( ^{18}O \) content of the citrate was 0.018%, which when corrected for the known amount of \( ^{18}O \)-citrate contaminating the initial isocitrate indicates that no more than 5% of the citrate derived directly from isocitrate retained the original hydroxyl. Since the determined value of \( ^{18}O \) falls very close to the natural abundance value, 0.204 atom %, it seems likely that no retention of hydroxyl oxygen occurs.

DISCUSSION

Stereochemical studies establishing the absolute configuration of isocitrate (27-30) and citrate (31, 5) demand that the hydration of cis-aconitate be trans- in the formation of both products and, furthermore, that the alternative attack on carbon 2 and carbon 3 by \( H^+ \) cannot be from the same side of the plane made by C-1, C-2, and C-3 (7, 12). This has led to alternative hypotheses that the cis-aconitate fits the active site in both a heads or tails fashion (7) or that the protonating and hydroxylating groups of the enzyme be interchangeable (12). In keeping with the complete retention of the substrate hydrogen in the direct interconversion of the hydroxy acids, such a turning over of the intermediate bound cis-aconitate seems more readily accommodated than the intramolecular proton migration. A scheme which incorporates the former hypothesis with the present studies might be Scheme 3.

According to this scheme hydrogen exchange occurs only after cis-aconitate dissociation whereas hydroxyl group exchange must
occur prior to dissociation as required by the intramolecular nature of H transfer and the lack of hydroxyl group transfer. The fact that the labeled proton can be conserved in part during the dissociation and reassociation of cis-aconitate, if the latter is made sufficiently rapid, means that it certainly can be retained during the turnover of cis-aconitate within the enzyme. The distinction between the inside and outside of the enzyme in solution, in this case, is based on whether mixing occurs between the intermediate cis-aconitate and the dissolved cis-aconitate.

If one postulates a space within the enzyme at the active site, continuous with the outside but which will hold only 1 molecule of cis-aconitate, it is only necessary that newly formed cis-aconitate rotate freely within the space and reassociate with the protein about as frequently as it dissociates into the substrate-containing medium. A refinement of this picture was introduced in the initial proposal of Gawron et al. (7) in which the “turnover” occurs with the acetate group attached to the enzyme as a pivot. Were it not for the fact that cis-aconitate is a substrate capable of producing alternate products, the single site hypothesis, which requires that the enzyme accommodate the a-H and β-acetate group equally well at the proton-donating site, would seem rather unlikely. Other examples of such selection specificities at a single site of an enzyme have been proposed on kinetic grounds for glutamate-aspartate (32) and glutamate-alanine (33) transaminases although when alternate substrates are grossly different glutamate-aspartate (32) and glutamate-alanine (33) transaminases although when alternate substrates are grossly different a two-site mechanism may be required as for glutamate-tyrosine transaminase (34). The observation that α-methyl-cis-aconitate is a substrate for aconitase (12) indicates that —CH₃ can be accommodated as well as —H in producing either hydroxy acid.

The fact that only one in five citrates going to isocitrates transfers its tritium might have been explained by a mechanism in which the tritium is diluted by protons of the enzyme base (—NH₂) which with an isotope effect would transfer tritium only one-fifth of the time. However, since there is no dilution of tritium in the direct conversion of isocitrate to citrate and since the dilution observed in the citrate to isocitrate direction can be adequately explained by the occurrence of the inevitable indirect path through free cis-aconitate, it is possible to exclude any dilution of the substrate proton by hydrogens of the enzyme. In this latter respect the mechanism resembles both Δ¹-3 keto steroid isomerase (35) and phosphoglucone isomerase (36). The stereochemical arguments indicating a reorientation of the bound cis-aconitate in the intermediate stages between the two hydroxy acids further requires that such a dilution not be possible during this time period. This makes it seem very unlikely that there are equivalent conjugate acid hydrogens with which mixing could occur and hence this would exclude —NH₂, but not —S—, —COO⁻, or imidazole as possible functioning bases. On the other hand if the conjugate acid of such groups were involved and exposed to water, the exchange rate would be too rapid and make intramolecular transfer according to Scheme 3 undetectable. The turnover rate of aconitase, assuming Morrison’s (8) estimate of purity to be reasonable and assuming a molecular weight no lower than 100,000, would be very low, approximately 15 per sec. For a bound proton to show almost no exchange in 70 msec in an aqueous environment means that it cannot simply be the conjugate acid of one of the basic amino acid residues (37). One is forced to consider stabilization by hydrogen bonding or the complete exclusion of water from a site that is readily accessible to cis-aconitate, which seems unlikely. It is clear from the failure to observe hydroxyl group transfer under conditions of proton transfer that water must be accessible to the active site. A hydrogen bond which depends on the conformational structure of the protein for its stability is consistent with these speculations. The surprising stability of the reactive proton in the form of the enzyme EH that reacts with cis-aconitate suggests that the rate-determining step in the conversion of citrate to cis-aconitate or the hydration of cis-aconitate might involve the dissociation or association of the proton. However, the rates of formation of cis-aconitate and isocitrate from normal and 2-D-citrate were found to be the same. On the other hand, Speyer and Dickman (3) observed substantially inhibited rates in D₂O. Similar results have been reported with the Fe⁺⁺-dependent aconitase of beef liver (22) which can also be shown to catalyze the intermolecular tritium transfer from methyl citrate to cis-aconitate. Thus the process EH ≡ E + H⁺ may be limited by a slow conformational change dependent on the rupture of hydrogen bonds which is followed by the dissociation of the proton. French and Hammes (38) have observed an apparently slow dissociation of an imidazolium group of native ribonuclease that is limited by rearrangement of the protein.

It is observed that the amounts of cis-aconitate required to enhance the retention of tritium in the experiments of Tables IV and VI are much higher than the Kₘ value of cis-aconitate observed in initial rate studies, 0.12 mM (25). This suggests that although the rate of interconversion of EH and E is not exceedingly fast it is probably faster than a subsequent rate-limiting step in the formation of products from cis-aconitate. Consider the situation:

\[
\begin{align*}
\text{EH} & \xrightarrow{A} \text{EH} \cdots \text{A} \\
\text{E} & \xrightleftharpoons[\text{slow}]{(H^+)} \text{C} \\
\end{align*}
\]

Under the conditions of the measurement of Kₘ, 0.12 mM and 1.2 mM cis-aconitate reduce the concentration of free enzyme to 50% and 10% of the total enzyme and shift it to forms that precede the slow catalytic step. These values are a measure of the amount of cis-aconitate necessary to shift the steady state in this way. However, steady state considerations do not indicate the rate at which isotope exchange might occur between H⁺ and [EH...A] through a dissociation of EH that might be rapid compared with the formation of product, C or I. The concentration of A necessary to suppress this dissociation and hence lead to the conservation of tritium in the experiments of Tables IV and VI depends on both the concentration of EH and the first order rate constant for dissociation. The larger the rate constant the more the concentration of EH will have to be decreased by higher A in order to suppress dissociation. It is indeed fortunate that the conservation of tritium can be demonstrated at concentrations of cis-aconitate that do not fully suppress the reaction of hydroxy acid with the enzyme. An additional observation suggests that the reaction, E + H⁺ ≡ EH, may not be orders of magnitude faster than the rate-determining step in the hydration of cis-aconitate. When citrate is formed from cis-aconitate in tritiated water it is observed to have a specific activity that is 60% less than that of the water hydrogen. An isotopic discrimination of similar magnitude was observed by Speyer and Dickman (3) in partially deuterated medium and can be similarly interpreted.
It would be incorrect to conclude on the basis of the present study that the abstraction of hydroxide precedes that of $H^+$ in the path of dehydration. The sequence of bond breaking cannot be established from data about the occurrence or rates of exchange since these are a consequence of the stabilities of enzyme-bound $H^+$ and possibly also of bound hydroxyl ion. These stabilities are unrelated to the sequence of bond cleavage in the original elimination mechanism. The schemes that have been considered in the present paper are therefore not intended to relate to this question and there is no basis, at the present writing, for favoring a carbanion, carbonium ion, or concerted mechanism.

Theaconitase reaction is unique among elimination reactions in that it provides in the alternate product a potential trap for both the $-H^+$ and hydroxyl groups being activated. In this case, as with the isomerases, both the retention or loss of the groups have significance. The demonstration of hydrogen transfer in the isomerization of the tricarboxylic hydroxy acids and the demonstration of intermolecular hydrogen transfer to cis-aconitate provides strong evidence for the function of the enzyme as a base in promoting the abstraction of the substrate hydroxyl. The apparent lack of hydroxyl group transfer suggests that the hydroxyl group is lost either directly to the medium or in the rearrangement of the enzyme-bound intermediate. A further possibility is that a different hydroxide-donating site is involved in forming each of the hydroxy acids. A distinction between these possibilities should be possible by studying exchange rates (39).

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Irwin A. Rose and Edward L. O'Connell


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