Mechanism of Aconitase Action

I. THE HYDROGEN TRANSFER REACTION

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SUMMARY

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

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

Five experiments that support this scheme are presented.

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

2. When the conversion of 2\text{-}T\text{-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\text{-}T\text{-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.

These data support a mechanism of base-catalyzed proton abstraction from hydroxy acid substrate. The conjugate acid group so formed on the enzyme is slow to dissociate and either is transferred to bound cis-aconitate before the latter dissociates, or, if the cis-aconitate is released, the conjugate acid group dissociates to regenerate the enzyme base.

In the interconversion of the hydroxy acids there is little or no transfer of \(\text{\text{H}}\text{O}\text{-}\text{labelled hydroxyl group.}\)

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Aconitase or cis-aconitate hydratase (EC 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 iso-citrate 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.

\[ E + H_2C=CH-CO_2H \rightleftharpoons HOC=CH-COH + E \]

It was observed (3) that in a medium of 10\% D\textsubscript{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).
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 hydroyx acid. The carbonium ion could then either rearrange by hydride transfer and with subsequent hydroxylation form the hydroyx 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-aconitase 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 substrate after the last (NH₄)₂SO₄ fractionation in 4 mM citrate, with Fe(NH₄)₂(SO₄) (5 mM) and cysteine (10 mM) at pH 7.4, was 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 diethyldimethylacetate and ethyl cyanacetate 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 nm (19).

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.

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°. 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 D$_2$O 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 CO$_2$. The acids were separated on Dowex 1-acetate, glutamic acid by elution after equilibrium had been established and isocitrate had been converted to cis-aconitate enzymatically was first isolated on Dowex 1-acetate by elution with 0.5 N acetic acid and citric acid with 5 N acetic acid-0.5 M sodium acetate. The citric acid was then isolated by silic gel chromatography (19).

$\alpha$ Methyl cis-aconitate (170 $\mu$moles) was converted to an equilibrium mixture containing the two tritiated $\alpha$-methyl hydroxy acids by reaction with well dialyzed aconitase (10 units) in tritiated water for 4 hours. Volatile tritium was removed by repeated drying of the sample which retained 2 x 10$^{-6}$ cpm, equivalent to the hydration of 100 $\mu$moles of substrate. Reincubation with aconitase resulted in the labilizations of all the radioactivity. This equilibrium mixture was used in the experiments of Table V. The mixed $\alpha$-methyl hydroxy acids were separated from the methyl-cis-aconitate by virtue of their earlier elution together from Dowex 1-formate with 3 N formic acid.

Conversion of Glutamate to Succinic Anhydride-In these experiments, glutamate which had been derived from citrate or cis-aconitate enzymatically was first isolated on Dowex 1-acetate by elution with 0.5 N acetic acid. It was convenient in the deuterium experiments to include a trace of 1,5-3HC-citrate to facilitate the detection of product during the isolation steps. The acetic acid was removed by two successive evaporations in a vacuum and the glutamate (about 30 $\mu$moles) was oxidatively deaminobylated in 4 ml containing 1 mmole of sodium acetate buffer, pH 5.0, 0.5 ml of Br$_2$-saturated water, and 100 mg of ninhydrin. The mixture was shaken at 40$^\circ$ for 90 min, at which time half of the $^3$C had been released as CO$_2$. The purpose of including Br$_2$ in the reaction was to obviate the isolation of succinic semialdehyde since it could be shown that much tritium of 3-T-glutamate was found in water due to the lability of this position in succinic semialdehyde. However, it was found that instead of succinate, the product of the reaction behaved as though it had aromatic character in the sense that it chromatographed with a strong ultraviolet absorbance and was readily absorbed on charcoal even in the presence of a large amount of succinate. On the assumption that the succinic acid was in amide linkage with a ninhydrin derivative the material was treated with strong HCl and chromatographed. Succinate could then be isolated in good yield as indicated by assay with succinate dehydrogenase. The procedure used after the ninhydrin-bromine reaction was as follows. The solution was adjusted to pH 8 with NaOH and extracted five times with equal volumes of ether. The extracted solution was applied to a Dowex 1-acetate column, 0.8 x 8 cm, and any remaining glutamate was eluted with 0.5 N acetic acid. The radioactive product of reaction was eluted with 3 N acetic acid, taken to dryness, diluted with 9 ml of 1.5 N HCl. After heating at 100$^\circ$ for 15 min the HCl was removed in a vacuum and the succinic acid was recovered from Dowex 1-acetate by elution with 3 N acetic acid. The dry samples of succinic acid were converted to anhydride by heating for 2 min at 90$^\circ$ with 1 ml of freshly tilled acetyl chloride which was then removed in a stream of dry N$_2$ at 25$^\circ$. After remaining overnight over P$_2$O$_5$ with vacuum, crystals were usually found. Crystal growth was a by allowing 1 ml of distilled ether to evaporate slowly from sample in a desiccator containing paraffin and NaOH pel (no vacuum). The mass analysis did not give evidence for taminating material.

Assays-Glutamate was assayed either with aconitase and citrate dehydrogenase or colorimetrically (20). cis-Aconitate was also assayed enzymatically or by absorbance at 240 with an internal standard. Glutamate was assayed with succinate dehydrogenase and 3-acetylpyridine-DPN (21). Methyl-cis-aconitate was determined with the published sorbance coefficients (11) and the change in absorbance with equilbrium with aconitase had been reached, making use of fact that 27.1% remains (12). The mass analyses of the succinic anhydride were made with the Hitachi RMU-6D instrument.

RESULTS

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

Conversion of Trifluoril Sisocitate to Citrate-It can be estabilsh as has been reported also with the aconitate of beef liver t that the formation of citrate from isocitrate occurs with a stant initial rate, indicating that cis-aconitate is not requi to accumulate appreciable before citrate begins to appear. failure to observe a substantial lag suggests, but does not es in (2), a direct path between isocitrate and citrate. The tive initial rates of formation of citrate and cis-aconitate f isocitate with the heart enzyme were 4:1 at pH 8 at 25$^\circ$. would be expected from these initial rates and the equilib constants among the three pairs of reactants, when the reac starts from isocitrate there will be a period of increase of acionate 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 is tritium whatsoever.

To test this, trifluorinated isocitrate (2 mm, 103,000 cpm per $\mu$ml in 0.1 M P$_1$ buffer, pH 7.6, was allowed to react to the exter about 11% total products formed with activated aconitase ml. The citrate was isolated after addition of carrier citrat silicate acid gel chromatography and compared with a zero control. The control, due to a 0.9% contamination with ticated citrate, showed that there were 3,540 cpm present in cit initially. At 11% reaction there were 16,000 cpm in cit Thus 12,460 cpm were present in the 0.10 $\mu$mole of citrate for 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.

![Chemical Reaction](image)

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 (H⁺) 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 μmoles; 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.

**Figure 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 (Δ), or from 4-T-cis-aconitate (■) were converted to α-ketoglutarate by glumatic 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.

**Table I**

<table>
<thead>
<tr>
<th>Isocitrate dehydrogenase</th>
<th>Ratio of dehydrogenase to aconitase</th>
<th>Rate of TPN reduction (μmoles/min) × 10⁴</th>
<th>Specific activity ratio of glutamate to citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>unit/ml</td>
<td></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>

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...
to aconitate show that a limit of about 18% exists in the retention of tritium in the reaction when aconitate is well trapped.

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 expectation if 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 C-2 position, and citrate labeled with deuterium was 3.6 and 3.8, which corresponds to 21 to 22% of the citrate going to isocitrate "directly." This is to be compared with a ratio of 3.0 found by Morrison (25) for similar conditions. Thus, the percentage of the tritium of citrate that is found in glutamate was 0.197.

In Experiment 2b, four additions of cis-aconitate were made to maintain its concentration at about 0.25 mM. After 180 min or 70 min (Experiment 1 or 2) the reactions were stopped with acid, the final cis-aconitate was determined, and the water and glutamic acid were recovered.

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TABLE III

Intramolecular character of proton transfer

Three incubations were set up each of 2.0 ml containing: citrate (30 μmoles), Tris (100 μmoles, pH 8), MnSO4 (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 aconitate 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>
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</thead>
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<tr>
<td>m/e</td>
<td>C-2</td>
<td>C-4</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 (26) 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).
* 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 deuterons transfers to 4-(HH)-citric acid, [(1.00 + 0.774)/2] - [0.482/2] + [(1.00 + 0.31)/2] - [0.222/2].

TABLE IV

Effect of high cis-aconitate on tritium transfer

Each incubation contained in 1 ml: 2-T-citric acid (1 mM, 78,000 cpm), Tris-Cl (0.1 M, pH 8.0), TPN (0.2 mM), MnSO4 (1 mM), NH4+ (50 mM), isocitrate dehydrogenase (1.8 units), glutamate dehydrogenase (1.5 units), activated aconitase (0.015 unit) and the noted additions of cis-aconitate. The incubations at 25°C were terminated before the added cis-aconitate had been entirely depleted, as judged from the absorbance at 240 μm. 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>
<thead>
<tr>
<th>cis-Aconitate</th>
<th>Counts at time stopped</th>
<th>Counts mobilized*</th>
<th>Counts transferred*</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/e</td>
<td>Initial</td>
<td>Final</td>
<td>Volatile</td>
</tr>
<tr>
<td>0</td>
<td>0.02</td>
<td>3432</td>
<td>520</td>
</tr>
<tr>
<td>0</td>
<td>0.02</td>
<td>6948</td>
<td>1128</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>1170</td>
<td>438</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>996</td>
<td>628</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>2850</td>
<td>1394</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.

A standard deviation of 1 to 2% of the mean in only four samples indicates a low experimental error. Therefore the chances are good that the close agreement of the mixed citrates experiment with the calculated peak height for an intramolecular transfer mechanism justifies the conclusion that this is the predominant or only route of hydrogen transfer.

Effect of Very High cis-Aconitate on Retention of Tritium—Scheme I 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 iso-citrate, 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 citrate has been allowed to react and the comparison that can be made is of the counts found in glutamate with the counts in water as a function of the presence of cis-aconitate. Results were obtained such as those of Table IV which indicate that a high ratio of cis-aconitate to citrate leads to a conservation of tritium. The comparisons are made with low percentages of reaction in the absence of added cis-aconitate in order to provide a control for any contribution by contaminants in the citrate. The counts attributed to glutamate are fully dependent upon the addition of NH4+ to the incubation mixture and are fully retained in the glutamic acid recovered by crystallization from 75% ethanol with added carrier.

These data lend support to the expanded mechanism (Scheme 2) if it can also be shown that the ratio of initial rates of cis-aconitate and isocitrate formation from citrate is not altered in the presence of large amounts of cis-aconitate. Such a study.
The percentage of total counts found in water and glutamate.

cannot be done spectrophotometrically because of the presence and reaction of the cis-aconitate. It might be done with C-citrate, though this presents problems due to the extensive dilution of the isotope by cis-aconitate and the difficulty of separating accurately the cis-aconitate radioactivity from that of citrate and from small amounts of decomposition products of the citrate. If indeed cis-aconitate were allosterically altering the kinetics of breakdown of the intermediate it might be possible to cause a similar effect with trans-aconitate. However, no change in the fraction of counts transferred was seen with the use of 10 mM trans-aconitate with 1 mM tritiated citrate.

The enhancement of the transfer reaction by large amounts 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-carbonyl ion 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 carbonyl ion mechanism.

**Intermolecular Tritium Transfer from 2-Methyl Citrate and 2-Methyl Isocitrate to cis-Aconitate**—A definitive experiment to test the occurrence of such an intermolecular transfer of tritium could be planned with the knowledge that 2-methyl-cis-aconitate is an effective substrate of aconitase. Gawron and Mahajan (12) have reported that replacement of the vinyl hydrogen with methyl gives a substrate of $k_m$ and $V_{max}$ quite comparable with cis-aconitate. If methyl-cis-aconitate were acted upon by aconitase in tritiated water, the equilibrated products, methyl citrate and methyl isocitrate, could provide the source of tritium for an intermolecular transfer to cis-aconitate. The trapped isocitrate could be isolated as glutamate and any radioactivity would be the result of transfer by way of the tritiated free enzyme. The results of such an experiment are shown in Table V. When the tritiated methyl citrate-methyl isocitrate mixture (1.6 µmoles) was incubated with aconitase in the absence of cis-aconitate, negligible counts were found in glutamate, whereas with cis-aconitate, initially 10 mM, the counts in glutamate were one-sixth of those in the water. The glutamate failed to lose counts upon crystallization with carrier. The reaction resulted in only 2.6% of the tritium being found in the water plus glutamate, but in a separate experiment it was shown that all of the counts of the methyl citrate-methyl isocitrate stock were exchangeable with water in the presence of aconitase.

A measure of the effectiveness of cis-aconitate in capturing the ET species before dissociation of tritium was obtained in an experiment in which the concentration of cis-aconitate was maintained close to the initial value by adding cis-aconitate periodically to correct for the loss of 240 µM absorbance. In this experiment the combined tritiated methyl hydroxy acids free of methyl-cis-aconitate were used. It will be noted (Table VI) that when a low level of cis-aconitate, 0.5 mM, is present, such that little or no inhibition of the labilization of tritium is seen, the amount of tritium captured in glutamate is small, only 0.8% of that labilized. Such amounts would escape notice in the experiments of Table II and may have made a small intermolecular contribution to the experiment of Table III. Effective intermolecular tritium transfer becomes important with cis-
TABLE VII

\[ ^{18}O\text{-isocitrate to citrate} \]

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 µmoles</td>
<td>84 µmoles</td>
</tr>
<tr>
<td>Isocitrate-(^{18}O)</td>
<td>1048 cpm/µmole</td>
<td>840 cpm/µmole</td>
</tr>
<tr>
<td>Isocitrate-(^{1}H)</td>
<td>0.54 atom % excess</td>
<td>0.4 µmole</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.4 µmole</td>
<td>73 µmole</td>
</tr>
<tr>
<td>Citrate-(^{18}O)</td>
<td>1.08 atom % excess</td>
<td>450 cpm/µmole</td>
</tr>
<tr>
<td>Citrate-(^{1}H)</td>
<td></td>
<td>0.018 atom % excess</td>
</tr>
</tbody>
</table>

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 \(^{2}^{18}O\)-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 labilized, 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 α-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 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 Δ₃-3 keto steroid isomerase (35) and phosphoglucoisomerase (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 ribonucleic acid 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 exceeding two orders of magnitude faster than the rate-determining step in the formation of products from cis-aconitate. Consider the situation:

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 isotopic 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 preceeds that of $\mathrm{H}^+$ 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 $\mathrm{H}^+$ 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.

The aconitase reaction is unique among elimination reactions in that it provides in the alternate product a potential trap for both the $-\mathrm{H}^+$ 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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