dency for tetrabenzoylpropane to exist in the enolic form.

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Synthetic Evidence for the Stereochemistry of Isocitric Acid and Alloisocitric Acid Mechanism of *cis*-Aconitase Action

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The configuration of the asymmetric carbon atoms of isocitric acid and alloisocitric acid has been elucidated by stereospecific synthesis of the two acids. Synthesis of DL-isocitric acid and DL-alloisocitric acid was achieved by malonate anion attack on the oxide rings of DL-*trans*-dicarboxyethylene oxide dimethyl ester and *cis*-dicarboxy-ethylene oxide dimethyl ester, respectively, followed by acid saponification, decarboxylation and chromatographic isolation of the acids as their respective lactones. The synthetic evidence plus the known *trans* opening of oxide rings by malonate anion leads to the conclusion that *d*-isocitric acid, the naturally occurring isomer, has the configuration α_{Ls}-β_{Ds}. This configuration, α_{Ls}-β_{Ds}, for *d*-isocitric acid indicates a *trans* mechanism to be operative in the *cis*-aconitase system.

In continuation of work on the stereochemistry of isocitric acid and alloisocitric acid,[2] we wish to report in this paper a sterospecific synthesis of DL-isocitric acid and DL-alloisocitric acid. The synthesis (Figs. 1 and 2) proceeds *via* malonate opening of the oxide ring of the dimethyl ester of dicarboxyethylene oxide, followed by acid saponification, decarboxylation and lactonization. Utilizing DL-*trans*-dicarbomethoxy-ethylene oxide dimethyl ester (I), DL-isocitric acid (IV), as the DL-lactone III, is obtained as the initial product of the synthesis and utilizing *cis*-dicarbomethoxy-ethylene oxide dimethyl ester (V), DL-alloisocitric acid (VIII), as the DL-lactone VII, is obtained.

Since malonate opening of oxide rings occurs by a *trans* mechanism,[3] assignment of relative configuration to the two asymmetric carbon atoms of DL-isocitric lactone (III) may be made on the basis of the configuration of the *trans*-oxide I and the relative configuration of the asymmetric carbons of DL-alloisocitric lactone (VII) may be deduced from the configuration of the *cis*-oxide V. Accordingly, the relative configurations of the two asymmetric carbons of DL-isocitric lactone (III) and of DL-alloisocitric lactone (VII) are such that the two free carboxyl groups are *cis* and *trans* in the respective lactones. This assignment of relative configuration is in agreement with that we have previously proposed[4] on the basis of pK_A values for the free carboxyl groups of the two lactones.

Experimental

DL-*trans*-Dicarboxy-ethylene Oxide.—The acid, isolated as the barium salt, was synthesized according to the procedure of Kuhn and Ebel.[5] The free acid was obtained from the barium salt by ion exchange with a sulfonic acid resin[6] in the (H^+) form, as follows. To a well-stirred suspension of 20.0 g. of the barium salt in 100 ml. of water, 80.0 g. of moist resin was added slowly and stirring was continued until the barium salt had gone into solution. The slightly cloudy supernatant liquid then was decanted and the resin was washed two times with 35-ml. portions of water. The combined supernatants were filtered rapidly and vacuum concentrated at 35°, and the residue from the vacuum concentration was dried *in vacuo* over sodium hydroxide and phosphorus pentoxide to yield 8.0 g. (70%) of crude DL-*trans*-dicarboxy-ethylene oxide. The product was purified by extraction in a Soxhlet apparatus with anhydrous ether, the pure acid, m.p. 212–213°,[7] crystallizing in the boiler during the extraction.

cis-Dicarboxy-ethylene Oxide.—The acid, isolated as the barium salt, was prepared by hydrogen peroxide oxidation of hydroquinone,[8] using the procedure of Weitz, *et al.* In a one-l. beaker, 11.0 g. (0.01 mole) of hydroquinone and 90 ml. of 30% hydrogen peroxide[9] were stirred and heated to 80°. The heat source was then removed and with continued stirring 135–140 ml. of 2 *N* potassium hydroxide was dropped in over a period of 15–20 minutes. The alkali was added at a rate that maintained the reaction temperature at 80–85° (initially the temperature may rise to 100°) and that does not permit the reaction *p*H to become too alkaline. After addition of alkali, the reaction mixture was stirred for an additional 5–10 minutes, cooled at the tap, and the *p*H was adjusted to 6 with acetic acid. After the addition of 30 g. of barium chloride dihydrate and stirring for 30 minutes, the mixture was cooled to 5° and the dihydrate of the barium salt of the acid crystallized from solution, yield 16–20 g. (53–66%). The free acid was obtained by treating a suspension of the barium salt in moist ether with the calculated quantity of concentrated sulfuric acid.[5,8] The acid after recrystallization from anhydrous ether melted at 145–146°, literature value[8] 149°.

Dimethyl Ester of DL-*trans*-Dicarboxy-ethylene Oxide (I).—To 8.0 g. (0.066 mole) of DL-*trans*-dicarboxy-ethylene oxide suspended in 50 ml. of anhydrous ether, a cold ether solution of diazomethane was added dropwise. The addition of diazomethane was continued until all the acid went into solution and a faint yellow color persisted in the solution. The solution was then concentrated, the dimethyl ester crystallizing as the volume of solution was reduced. After filtering, the ester was recrystallized from ether, yield 9.6 g. (91%), m.p. 75–76°.

Anal.[10] Calcd. for $C_6H_8O_5$: C, 45.0; H, 5.00; sapon. equiv., 80.0. Found: C, 45.1; H, 4.91; sapon. equiv., 80.5.

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(1) Abstracted in part from the Masters' Thesis of Senophia Gary.

(2) O. Gawron and A. J. Glaid, III, THIS JOURNAL, **77**, 6638 (1955).

(3) S. Winstein and R. B. Henderson in R. C. Elderfield's "Heterocyclic Compounds," John Wiley and Sons, Inc., Vol. I, New York, N. Y., 1950, p. 31.

(4) Ref. 2. A preliminary account of the synthetic approach is also given in this reference.

(5) R. Kuhn and F. Ebel, *Ber.*, **58**, 919 (1925).

(6) Amberlite IR-120 (H^+), reagent grade. Prior to use 100 grams of resin was treated four times with 300-ml. portions of 6 *N* hydrochloric acid followed by thorough water washing to neutrality.

(7) Literature m.p. 209°, ref. 5.

(8) E. Weitz, H. Schobbert and H. Seibert, *Ber.*, **68B**, 1163 (1935).

(9) Stabilized with sodium stannate.

(10) Analyses by Drs. G. Weiler and F. B. Strauss.

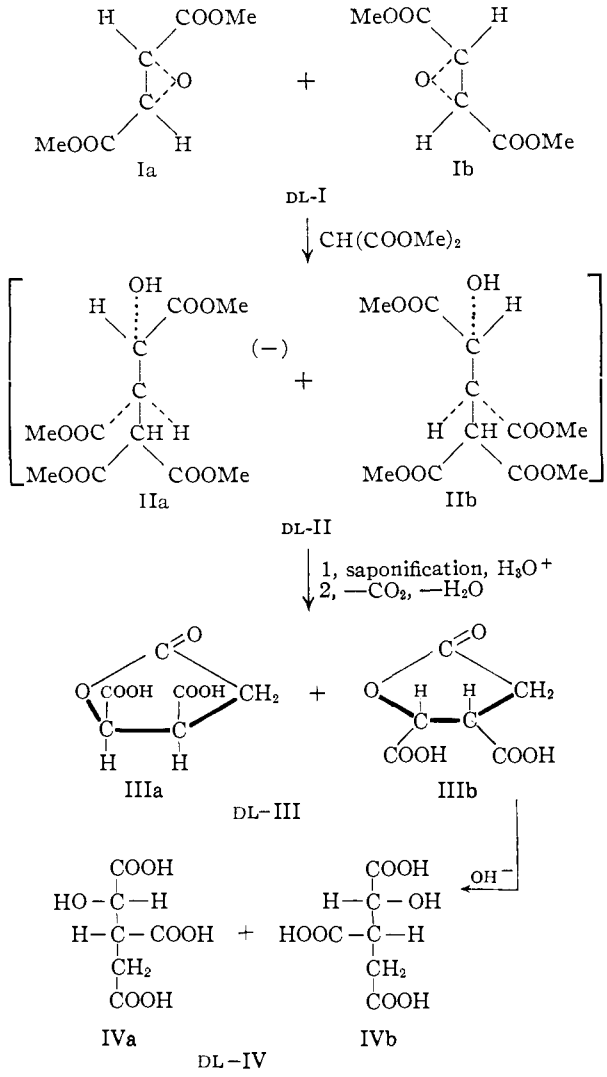


Fig. 1.—Route to DL-isocitric acid (IV) from DL-*trans*-dicarboxy-ethylene oxide dimethyl ester (I).

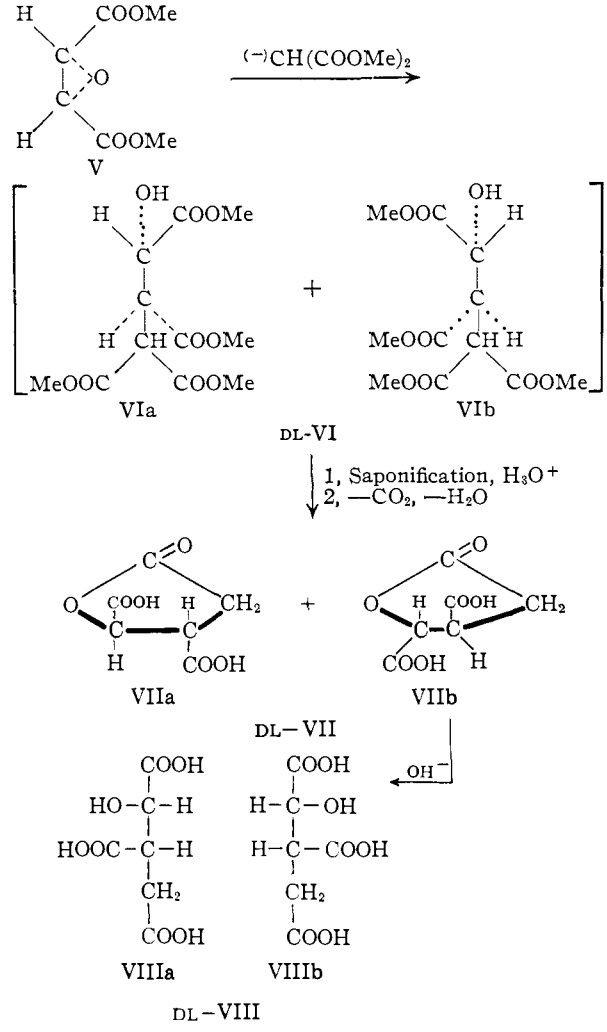


Fig. 2.—Route to DL-alloisocitric acid (VIII) from *cis*-dicarboxyethylene oxide dimethyl ester (V).

Dimethyl Ester of *cis*-Dicarboxy-ethylene Oxide (V).—The acid was esterified with diazomethane utilizing the above procedure and the ester after removal of ether on the water-bath was purified by distillation. From 3.5 g. (0.0265 mole) of *cis*-dicarboxy-ethylene oxide, there was obtained 3.7 g. (88%) of dimethyl ester, b.p. 240–244° (760 mm.).

Anal. Calcd. for $C_6H_8O_5$: C, 45.0; H, 5.00. Found: C, 45.2; H, 5.19.

Synthetic Procedure.—All synthetic runs were performed in the same manner except for variations in mole ratios of dimethyl malonate to sodium methoxide and for variations in time allowed for synthesis. These variables were investigated for their effect on the ratio of isocitric lactone to alloisocitric lactone in the synthetic product. The mole ratio of sodium methoxide to ethylene oxide diester was held constant at a ratio of 1:1 in all runs. A typical synthetic run was carried out as follows. To one ml. of 2.0 *M* sodium methoxide (2.0 mmoles) in methanol, 0.795 g. (6.0 mmoles) of dimethyl malonate and 0.320 g. (2.0 mmoles) of *cis*-dicarboxy-ethylene oxide dimethyl ester were added with gentle swirling. The reaction mixture was allowed to stand at room temperature for 15 hours and then 5 ml. of concentrated hydrochloric acid was added. After standing overnight at room temperature, sodium chloride was filtered off and the filtrate vacuum concentrated. The residue was then taken up in 10 ml. of 3 *N* hydrochloric acid and after refluxing for 3 hours, the solution was vacuum concentrated. The residue from the vacuum concentration was heated *in vacuo* at 100° for 1 hr. and then dried in a vacuum desiccator over sodium hydroxide and phosphorus pentoxide for 24 hours. The product was then analyzed by the chromatographic procedure given below.

Chromatographic Procedure.—The procedure finally adopted was a modification of that used by Lorber and Cook.[11] Five grams of Celite[12] and 2.5 ml. of 0.5 *N* sulfuric acid were thoroughly mixed and then slurried with 100 ml. of chloroform–butanol, 9:1. The slurry was introduced into a glass chromatography column, recycling the excess chloroform–butanol as required to bring all the Celite into the column and to completely pack the column. The column of Celite (1.0 × 40 cm.) was then washed with 60 ml. of chloroform–butanol at a rate of 1 drop per 2 seconds and then was ready for use.

Both chloroform and butanol were distilled prior to use. After mixing, the chloroform–butanol, 9:1, was saturated with 0.5 *N* sulfuric acid by shaking in a separatory funnel. The two layers were separated after 24 hours standing, the chloroform–butanol layer being filtered through a cotton plug. After filtering one ml. of 95% ethanol was added to each 100 ml. of chloroform–butanol.

The chromatographic sample, dissolved in 2.0 ml. of chloroform–butanol in a small beaker, was run into the column at a slow rate and was followed by a 2.0-ml. wash. Chromatography was then carried out at a rate of one drop every 2–3 seconds, 3.0-ml. fractions being collected with an automatic fraction collector and titrated with dilute base. A synthetic mixture of DL-alloisocitric lactone, malonic

(11) V. Lorber and M. Cook, *J. Biol. Chem.*, **215**, 823 (1955).
(12) Number 535, Johns-Manville.

acid, DL-isocitric lactone, DL-*trans*-chloromalic acid[13] and DL-*meso*-chloromalic acid[14] gave the results shown in Fig. 3.

Enzymatic Assay for Isocitric Acid.—A number of products were analyzed for *d*-isocitric acid by the method of Meister and Baker.[15] In these instances, the product was titrated with base to phenolphthalein at room temperature and the lactone ring was opened by continuing the titration at 100°. Results obtained by the chromatographic method and the enzymatic method were in agreement.

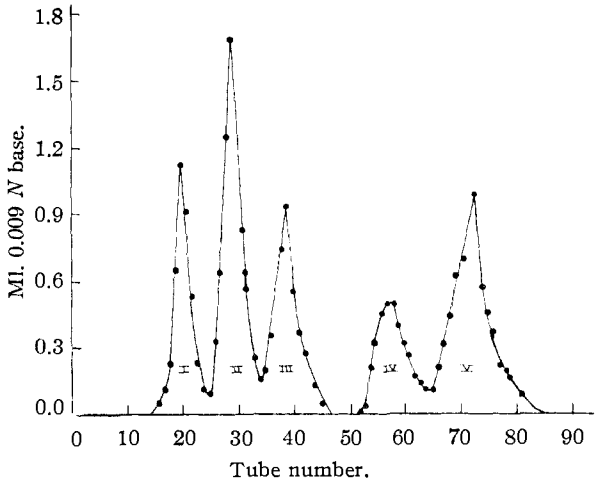


Fig. 3.—Chromatography of a mixture of 4.0 mg. of DL-alloisocitric lactone, 4.0 mg. of malonic acid, 4.2 mg. of DL-isocitric lactone, 4.0 mg. of DL-*trans*-chloromalic acid and 7.6 mg. of DL-*meso*-chloromalic acid. Peaks in order of emergence and recoveries: I, DL-alloisocitric lactone (90%); II, malonic acid (93%); III, DL-isocitric acid (95%); IV, DL-*trans*-chloromalic acid (82%); V, DL-*meso*-chloromalic acid (83%).

Results and Discussion

The results (Fig. 4) of a 15 hour synthetic run with *cis*-dicarboxy-ethylene oxide dimethyl ester (V) and employing a 3:1 mole ratio of dimethyl malonate to sodium methoxide indicate that DL-alloisocitric lactone (VII) is the main product (ratio of alloisocitric lactone to isocitric lactone, 17:1). This, of course, is the product expected on the basis of the assigned *trans* configuration to the carboxyl groups of DL-alloisocitric lactone and on the basis of the configuration of *cis*-dicarboxy-ethylene oxide. This result also indicates that little racemization of the tetra-ester VI or of *cis*-dicarboxy-ethylene oxide dimethyl ester (V) occurs during the course of the reaction. This latter point is also borne out by the fact that a small amount of DL-*trans*-chloromalic acid (IX), the expected product of the reaction of hydrochloric acid with *cis*-dicarboxy-ethylene oxide, appears in the synthetic mixture.

A similar experiment (15 hour syntheses time, 3:1 dimethyl malonate, sodium methoxide) with DL-*trans*-dicarboxy-ethylene oxide as the starting material yielded the results shown in Fig. 5. The ratio of DL-isocitric lactone (III) to DL-alloisocitric lactone (VII), 0.76:1.0, indicates, in conjunction

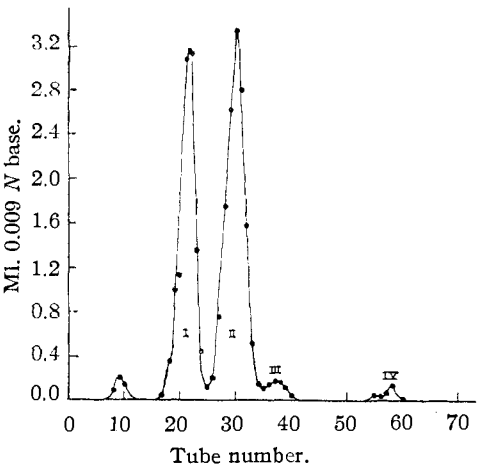
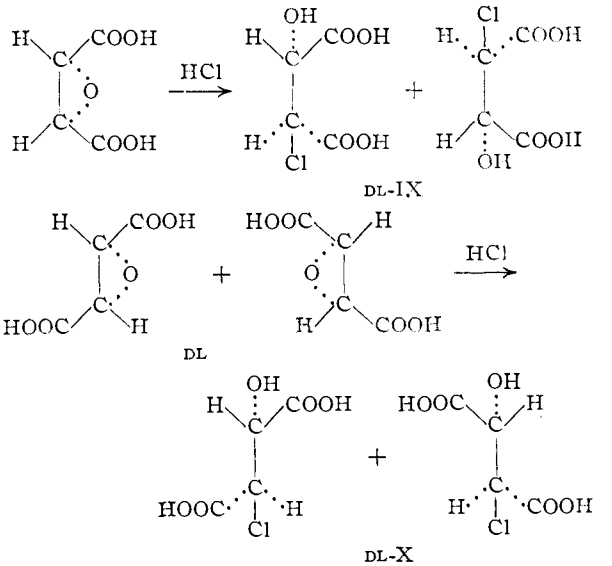


Fig. 4.—Chromatography of 23.2 mg. of synthetic product from 15-hour condensation of dimethyl malonate with *cis*-dicarboxy-ethylene oxide dimethyl ester, 3:1 mole ratio of dimethyl malonate to methoxide: I, DL-alloisocitric lactone; II, malonic acid; III, DL-isocitric lactone; IV, DL-*trans*-chloromalic acid; initial peak not identified.

with the results of the above experiment, that DL-isocitric lactone is the product obtained from DL-*trans*-dicarboxy-ethylene oxide dimethyl ester (I), albeit that the tetraester II racemizes during the course of the experiment to yield DL-alloisocitric lactone and/or that DL-*trans*-dicarboxy-ethylene oxide dimethyl ester (I) racemizes to *cis*-dicarboxy-ethylene oxide dimethyl ester (V) which subsequently yields DL-alloisocitric lactone. To substantiate these conclusions, two sets of experiments were carried out.



In the first set of experiments (Table I), the effect of varying acidity, considering dimethyl malonate and malonate anion as the acid and the base of a buffer pair, of the condensation medium on the ratio of DL-isocitric lactone to DL-alloisocitric lactone formed from DL-*trans*-dicarboxy-ethylene oxide dimethyl ester was investigated. These experiments were undertaken since base-catalyzed racemization of optically active α-esters possessing

(13) Prepared from *cis*-dicarboxy-ethylene oxide, ref. 5.

(14) Prepared from DL-*trans*-dicarboxy-ethylene oxide by the procedure of R. Kuhn and R. Zell, *Ber.*, **59**, 2514 (1926), or by treatment of fumaric acid in aqueous solution with chlorine.

(15) A. Meister and C. G. Baker, *Arch. Biochem. Biophys.*, **31**, 460 (1951).

an α-H atom proceeds *via*[16] (1) and an increase in BH

$$R{-}CHX{-}COOR' + B^{(-)} \rightleftharpoons R{-}\overset{(-)}{C}X{-}COOR' + BH \quad (1)$$

concentration should tend to diminish the tendency of reaction 1 to go to the right, thereby diminishing the extent of racemization and increasing the yield of initial product. The results (Table I) are in agreement with this concept since

TABLE I

EFFECT OF MOLE RATIO OF DIMETHYL MALONATE TO METHOXIDE ON RELATIVE YIELDS OF DL-ISOCITRIC LACTONE AND DL-ALLOISOCITRIC LACTONE[a]

Diethyl malonate / methoxide	DL-Isocitric lactone / DL-Alloisocitric lactone
1	0.29
2	.62
3	.76

[a] From DL-*trans*-dicarboxy-ethylene oxide dimethyl ester after a 15 hour condensation period.

an increase in the ratio of DL-isocitric lactone to DL-alloisocitric lactone in the product was found with an increasing dimethyl malonate to methoxide ratio and since DL-isocitric lactone is the product expected[17] from the *trans*-oxide employed.

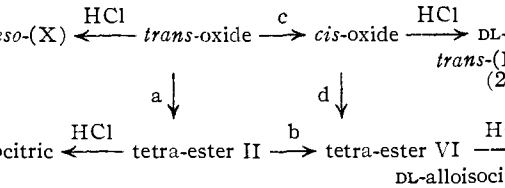
In the second set of experiments (Table II), the product ratio of DL-isocitric lactone to DL-alloisocitric lactone was investigated as a function of time of condensation, DL-*trans*-dicarboxy-ethylene oxide dimethyl ester and a 3:1 mole ratio of dimethyl malonate to methoxide being employed for the reaction. The results (Table II) show a decrease in the ratio of DL-isocitric lactone to DL-alloisocitric lactone with time and thus also indicate that DL-isocitric lactone (or rather the tetra-ester II) is the initial product of the synthesis from DL-*trans*-dicarboxy-ethylene oxide dimethyl ester (I). Total yields of product were the same for the 10 hour and 15 hour samples indicating complete or close to complete utilization of oxide[18] by 10 hours and that decrease in the ratio of DL-isocitric lactone to DL-alloisocitric lactone over this period was therefore due to racemization of tetra-ester II to tetra-ester VI. Prior to this time, an alternate pathway (cd) is available for formation of DL-alloisocitric lactone through tetra-ester VI and the extent to which each pathway is responsible for formation of tetra-ester VI would depend on the relative values of the four rate constants involved. Both the 1.5 and 4 hour products contained DL-*meso*-chloromalic acid (X) but no DL-*trans*-chloromalic acid (IX), the 1.5 hour sample containing some 10 times the quantity of DL-*meso*-chloromalic acid as did the 4 hour sample. The total yield[19] of

TABLE II

RELATIVE YIELDS OF DL-ISOCITRIC LACTONE AND DL-ALLOISOCITRIC LACTONE AS A FUNCTION OF TIME OF CONDENSATION[a]

Hours	DL-Isocitric lactone / DL-Alloisocitric lactone
1.5	5.4
4.0	4.3
10.0	2.3
15.0	0.76

[a] From DL-*trans*-dicarboxy-ethylene oxide dimethyl ester, employing a 3:1 mole ratio of dimethyl malonate to methoxide.



lactones and DL-*meso*-chloromalic acid for the 1.5 hour sample amounted to 70% of starting DL-*trans*-dicarboxy-ethylene oxide, indicating that over this period tetra-ester VI arises by pathway ab and provided that reaction d is not faster than reaction c, that little racemization of the *trans* oxide has occurred over this period.

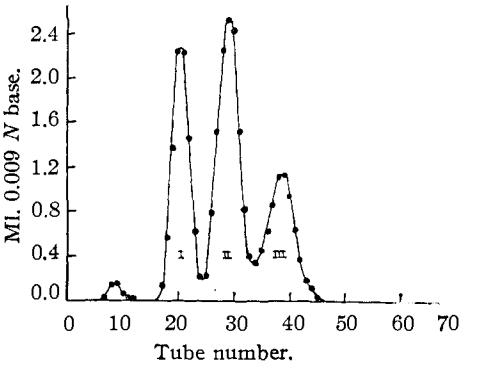


Fig. 5.—Chromatography of 25.2 mg. of synthetic product from 15 hour condensation of dimethyl malonate with *trans*-dicarboxy-ethylene oxide dimethyl ester, 3:1 mole ratio of dimethyl malonate to methoxide. Peaks: I, DL-alloisocitric lactone; II, malonic acid; III, DL-isocitric lactone; initial peak not identified.

The accumulated synthetic evidence thus indicates that DL-isocitric lactone and DL-alloisocitric lactone arise from DL-*trans*-dicarboxy-ethylene oxide and *cis*-dicarboxy-ethylene oxide, respectively. On the basis of *trans* opening of oxide rings by malonate anion, structures III and VII can be assigned to DL-isocitric lactone and DL-alloisocitric lactone and structures IV and VIII can be assigned to DL-isocitric acid and DL-alloisocitric acid. Since *d*-isocitric acid, the naturally occurring isomer, has been shown to possess the α_{Ls} configuration,[20] the complete structure of *d*-isocitric acid is IVa, the configuration of the β-carbon being β_{Ds}. The enantiomorph IVb of *d*-isocitric acid may be then designated as α_{Ds}-β_{Ls}-isocitric acid and the two enantiamorphs of alloisocitric acid may be then designated as α_{Ls}-β_{Ls}-alloisocitric acid (VIIIa) and α_{Ds}-β_{Ds}-alloisocitric acid (VIIIb).[21] These

(16) G. W. Wheland, "Advanced Organic Chemistry," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1949, p. 255.

(17) In two instances, employing the *trans*-dicarboxy-ethylene oxide, a 2:1 mole ratio of dimethyl malonate to methoxide and a 15 hour condensation period, only DL-isocitric lactone, DL-*meso*-chloromalic and malonic acid were found in the product.

(18) Chloromalic acids were not found in the products.

(19) Destruction of chloromalic acid occurs during the reflux period with 3 *N* hydrochloric acid.

(20) M. Winitz, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **77**, 716 (1955).

(21) See refs. 20 and 2 for nomenclature.

assignments of configuration on the basis of the synthetic evidence are the same as those previously made on the basis of physical evidence for the configuration of the two diastereoisomeric lactones and also corroborate the suggested[2] *trans* mechanism for the *cis*-aconitase catalyzed hydration of *cis*-aconitic acid and dehydration of isocitric acid.

A *trans* mechanism for the reversible dehydration of *d*-isocitric acid by *cis*-aconitase also implies a *trans* mechanism for the reversible dehydration of citric acid and leads to the conclusion that one of the α-H atoms (H*, Fig. 6) of citric acid (XI), as

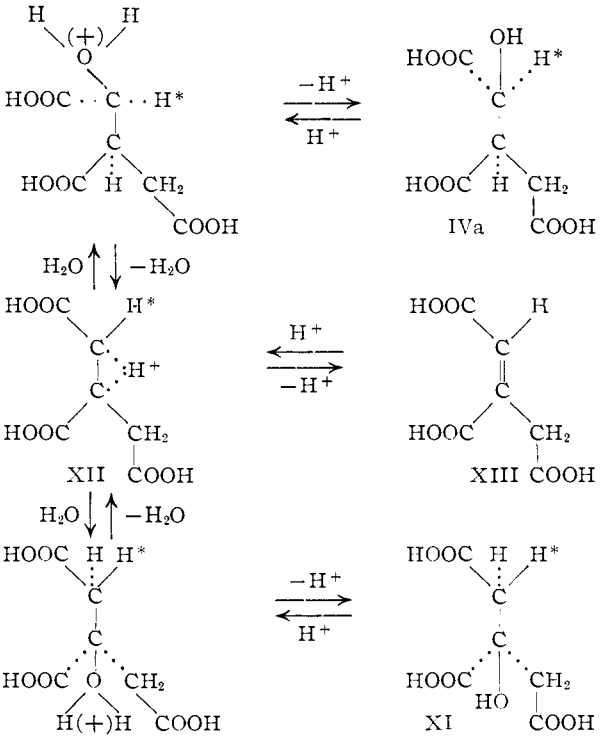


Fig. 6.—Mechanism for *cis*-aconitase action involving *trans* hydrations and dehydrations.

well as the α-H atom of isocitric acid, does not participate in the reversible dehydration catalyzed by *cis*-aconitase. This α-H atom may be described as $H(\alpha_{D_S})$ and is the H atom which is transferred to TPN in the isocitric dehydrogenase reaction even if this reaction is carried out in the presence of *cis*-aconitase and D_2O.[22] A *trans* mechanism for the reversible dehydrations is also consistent with the scheme proposed by Speyer and Dickman[23] for the *cis*-aconitase reaction and the mechanism (Fig. 6) suggested is a modification of that scheme to take into account the *trans* nature of the reactions. In view of the *cis*-nature of the fumarase system,[24] the finding of a *trans*-mechanism for the *cis*-aconitase system might be considered unexpected were it not for the considerable differences in the two enzyme systems. In this mechanism, the common intermediate is considered to be the protonated *cis*-aconitic acid (XII). This intermediate can react with a water molecule to give either *d*-isocitric acid (IVa) or citric acid (XI), regenerating H^+ during the process, or can lose the proton to regenerate *cis*-aconitic acid (XIII). The mechanism is then a reversible acid-catalyzed hydration of a double bond, the proton possibly coming from the molecule of water which is coördinated to Fe^{++} in the substrate–Fe^{++}–aconitase complex visualized by Speyer and Dickman.[23]

It is of interest to compare the stereochemistry of the isocitritase catalyzed[25] condensation of succinic acid and glyoxylic acid yielding *d*-isocitric acid with other enzymatically catalyzed condensations. Both aldolase and transaldolase[26] catalyzed equilibrations also require the L_S, D_S configuration for the carbon atoms participating in bond formation and breakage. Similarly, threonine aldolase catalyzing the equilibration of threonine, acetaldehyde and glycine is specific for the *trans*-configuration, albeit a protein also has been found with allothreonine aldolase activity.[27] However, it is doubtful that allothreonine is the natural substrate for this enzyme.[27] Transketolase catalyzed equilibrations[28] require the L_S, D_S configuration albeit the D_S carbon atom is one carbon removed from the site of bond formation and breaking. Since *trans*-isomers are thermodynamically more stable than *cis*-isomers, particularly of geometrically isomeric pairs, it would seem, at least in the above examples, that the enzyme–substrate complex with its several points of attachment confers sufficient rigidity on the substrate so that *trans*-isomers, presumably more stable than *cis*-isomers in the complex, are formed.

PITTSBURGH, PENNA.

(22) S. Englard and S. P. Colowick, *J. Biol. Chem.*, **226**, 1047 (1957).
(23) J. F. Speyer and S. R. Dickman, *ibid.*, **220**, 193 (1956).
(24) T. C. Farrar, H. S. Gutowsky, R. A. Alberty and W. G. Miller, THIS JOURNAL, **79**, 3978 (1957).
(25) R. A. Smith, J. R. Stamer and I. C. Gunsalas, *Biochim. Biophys. Acta*, **19**, 567 (1956).
(26) B. L. Horecker and P. Z. Smyrniotis, *J. Biol. Chem.*, **212**, 811 (1955).
(27) M. A. Karasek and D. M. Greenberg, *ibid.*, **227**, 191 (1957).
(28) B. L. Horecker, J. Hurwitz and P. Z. Smyrniotis, THIS JOURNAL, **78**, 692 (1956).