Synthesis and Absolute Configuration of the Isomers of Homoisocitric Acid (1-Hydroxy-1,2,4-butanetricarboxylic Acid) and the Stereochemistry of Lysine Biosynthesis*

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ABSTRACT: The four isomers of homoisocitric acid, an intermediate in the α-aminoadipic acid pathway of lysine biosynthesis, have been synthesized and their absolute configurations determined. Diels–Alder condensations of 1-acetoxy-1,3-butadiene and methyl acrylate yields, after gas chromatographic separation and saponification, the two racemic forms of 2-hydroxy-3-cyclohexenecarboxylic acid. Each racemate was resolved. The four optically active isomers were then reduced to the saturated 2-hydroxy-3-cyclohexanecarboxylic acids, the absolute configurations of which are known. The four isomers of homoisocitric acid were obtained by ozonization of the four methyl-2-acetoxy-3-cyclohexenecarboxylates. Bioassay of the products, using homoaconitase, established that (+)-*trans*-2-hydroxy-3-cyclohexenecarboxylic acid yields natural homoisocitric acid. Thus, the absolute configuration of the natural compound can be designated as *threo*-D_s-homoisocitrate (1-(*R*)-hydroxy-2-(*S*)-1,2,4-butanetricarboxylic acid) and the enzymatic conversion of homocitrate into homoisocitrate is shown to be stereochemically analogous to the enzymatic conversion of citrate into isocitrate.

Yeast and certain fungi are known to synthesize lysine by a sequence of reactions different from the well-known diaminopimelic acid pathway found in bacteria and higher plants. Initial evidence for this came from studies with mutants of *Neurospora crassa* which could utilize α-aminoadipic acid in place of lysine as a growth requirement but could not substitute diaminopimelic acid (Mitchell and Houlahan, 1948). Strassman and Weinhouse (1953) proposed, based on their studies of [^{14}C]acetate incorporation into lysine in yeast, that the first step in the biosynthesis of lysine which could give rise to α-aminoadipic acid as an intermediate is the condensation of acetate with α-ketoglutarate to yield homocitrate. The enzymatic conversion of acetate and α-ketoglutarate into homocitrate in yeast (Strassman and Ceci, 1964; Weber *et al.*, 1964) and in *N. crassa* lysine auxotrophs (Hogg and Broquist, 1968), the reversible conversion of *cis*-homoaconitate into homoisocitrate (Strassman and Ceci, 1966), and finally the enzymatic conversion of homoisocitrate into α-ketoadipate (Strassman and Ceci, 1965) have been clearly demonstrated. In addition, Bhattacharjee *et al.* (1967) and Bhattacharjee and Strassman (1967) have recently reported the accumulation of homocitric acid, *cis*-homoaconitic acid, and homoisocitric acid in the growth medium of a lysine-requiring yeast mutant, Ly_{12}.

These intermediates in the α-aminoadipic acid pathway and their interconversions, shown in Figure 1, are of particular interest because of their analogy with the condensation of acetyl-CoA and oxalacetate to form citrate and the subsequent conversion of citrate into isocitrate and α-ketoglutarate. As a first step in the elucidation of the stereochemistry and mechanism of the conversion of homocitrate into homoisocitrate, Thomas *et al.* (1966) determined the absolute configuration of natural homocitrate by synthesis of one enantiomorph from naturally occurring (−)-quinic acid. A comparison of this isomer with homocitrate isolated by Maragoudakis and Strassman (1966) from a lysine-requiring yeast mutant showed that natural homocitric acid may be designated (*R*)-2-hydroxy-1,2,4-butanetricarboxylic acid (Figure 5, I). Strassman and Ceci (1966) have shown that natural homoaconitic acid has a *cis* configuration.

In the present work, the absolute configuration of each of the four isomers of homoisocitric acid has been established by a synthesis from the corresponding 2-hydroxy-3-cyclohexenecarboxylic acids through the sequence of reactions shown in Figure 2. The absolute configuration of each isomer of 2-hydroxy-3-cyclohexenecarboxylic acid was determined by reduction to the respective saturated 2-hydroxy-3-cyclohexanecarboxylic acid of known configuration (Febrer *et al.*, 1964). The four isomers of homoisocitric acid, obtained by ozonolysis of the four methyl 2-acetoxy-3-cyclohexenecarboxylates, were compared with natural homoisocitrate in their ability to serve as a substrate for *cis*-homoaconitase. In addition, each isomer of homoisocitric acid was reduced to the corresponding β-carboxyadipic acid. The absolute configuration of the enantiomers of β-carboxyadipic acid have been determined by Freudenberg and Geiger (1952).

Experimental Results and Discussion

All melting points were taken on a Fischer-Johns melting point apparatus and are uncorrected.

Methyl 2-Acetoxy-3-cyclohexenecarboxylates. 1-Acetoxy-1,3-butadiene was synthesized from isopropenyl acetate and crotonaldehyde according to the procedure of Bailey and Bar-

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clay (1956). The average yield of product was 101 g (39% calculated for crotonaldehyde): bp 43° (19–20 mm), lit. (Bailey and Barclay, 1956) bp 56.8–58° (37 mm) and (Wichterle and Hudlicky, 1947) bp 42–43° (16 mm).

The Diels–Alder condensation of 1-acetoxy-1,3-butadiene and methyl acrylate was carried out as described by Bailey and Baylouny (1959). The resulting dark brown liquid was distilled under vacuum (bp 60–70° (0.1–.5 mm), lit. (Bailey and Baylouny, 1959) bp 82–83° (0.7 mm)) yielding a mixture of methyl *cis*- and methyl *trans*-2-acetoxy-3-cyclohexenecarboxylate. The *trans* isomer has not been isolated previously; however, gas chromatographic analysis showed that our condensation product contained approximately 20% of this isomer. The *cis* isomer did not crystallize as described by Bailey and Baylouny. Therefore, *cis*-2-acetoxy-3-cyclohexenecarboxylic acid, prepared by the method of Alder and Schumacher (1949), was methylated with diazomethane in ether to obtain crystals of methyl *cis*-2-acetoxy-3-cyclohexenecarboxylate. The mixture of the *cis* and *trans* isomers was seeded with the crystalline methyl *cis*-acetoxy ester and the *cis* isomer which crystallized immediately was removed by filtration. Recrystallization from petroleum ether (bp 30–40°) gave white crystals of methyl-*cis*-2-acetoxy-3-cyclohexenecarboxylate: mp 43–44°, lit. (Bailey and Baylouny, 1959) mp 44.5–46.5°.

The mother liquor was successively seeded with crystals of the *cis* isomer and this selective crystallization was continued at room temperature and at 0° until no more of the *cis* isomer crystallized from the mixture. The final liquid contained approximately 30% *cis* isomer and 70% *trans* and was subsequently separated by preparative gas–liquid partition chromatography to obtain pure methyl *trans*-2-acetoxy-3-cyclohexenecarboxylate.

Preparative Gas Chromatography. Separation of methyl *cis*- and *trans*-2-acetoxy-3-cyclohexenecarboxylate was achieved with an Aerograph Autoprep A-700. The following experimental conditions gave the best separation: $^3/_8 \times 10$ ft glass column with 15% FFAP on 60–80 Chromosorb P (acid washed) purchased from Varian Aerograph; helium flow rate 300 cc/min; column temperature 160 ± 3°, injector temperature 210°; detector temperature 220°; sample size injected, 0.35 ml of a 1:10 dilution in hexane. Two major peaks were obtained with a retention time of 60 min for peak I and 64 min for peak II. As a means of identification, material from each peak was converted into the free hydroxycarboxylic acid by saponification and the *cis*- and *trans*-2-hydroxy-3-cyclohexenecarboxylic acids obtained were hydrogenated to the corresponding saturated acids. All products were recrystallized from ethyl acetate–ligroin (1:10, v/v).

PEAK I. Saponification of material from peak I yielded beige crystals, mp 103–104°. *Anal.* Calcd for $C_7H_{10}O_3$ (142.15): C, 59.14; H, 7.09. Found: C, 59.00; H, 6.97.

Catalytic hydrogenation of this compound produced a white crystalline product: mp 74–76°; lit. for *cis*-2-hydroxy-3-cyclohexanecarboxylic acid (Alder and Schumacher, 1949), mp 82–83°; (Pistor and Plieninger, 1946), mp 75–77°; (Real and Pascual, 1953), mp 79–80°. *Anal.* Calcd for $C_7H_{12}O_3$ (144.166): C, 58.31; H, 8.32. Found: C, 58.40; H, 8.50.

PEAK II. Saponification of material from peak II yielded beige crystals, mp 82–84°. *Anal.* Calcd for $C_7H_{10}O_3$ (142.15): C, 59.14; H, 7.09. Found: C, 59.41; H, 7.25.

The melting point of the saponification of peak II was variable, occasional preparations of crystalline hydroxycarboxylic acid having a melting point of 91–93°. However, nuclear magnetic resonance spectra, R_F (thin-layer chromatography, ethyl acetate–formic acid–H_2O, 100:20:20, v/v), neutralization equivalent, and elemental analysis were essentially identical with preparations having mp 82-84°.

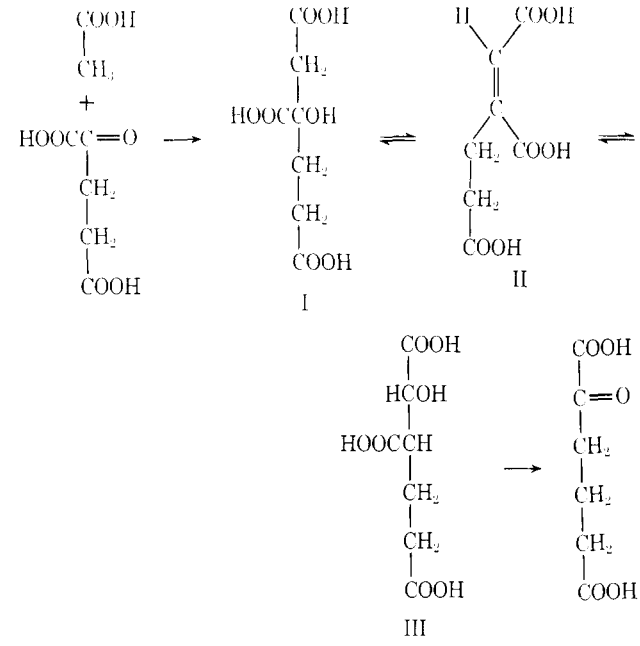


FIGURE 1: The α-aminoadipic acid pathway of lysine biosynthesis has been shown to involve the following sequence: α-ketoglutarate + acetyl-CoA → homocitric acid (I) → homoaconitic acid (II) → homoisocitric acid (III) → α-ketoadipic acid.

Catalytic hydrogenation produced a white crystalline product: mp 108–109°; lit. for *trans*-2-hydroxy-3-cyclohexanecarboxylic acid (Alder and Schumacher, 1949), mp 108°; (Bailey and Baylouny, 1959), mp 108–109°; (Pascual *et al.*, 1949), mp 111°. *Anal.* Calcd for $C_7H_{12}O_3$ (144.166): C, 58.31; H, 8.32. Found: C, 58.13; H, 8.49. A higher yield of *trans*-2-hydroxy-3-cyclohexanecarboxylic acid was obtained if the unsaturated acetoxy methyl ester was hydrogenated, followed by saponification.

The nuclear magnetic resonance spectra of the methyl *cis*- and *trans*-cyclohexenecarboxylates were determined in $CDCl_3$ solution. Since the near coincidence of vinyl proton resonances with those of the proton α to the acetoxy group prevented unequivocal assignment of configuration, the nuclear magnetic resonance spectra of the free hydroxy acids were also examined. The usual upfield shift of *ca.* 0.8 ppm (Bhacca and Williams, 1964a) accompanying HC–OAc → HC–OH saponification was observed, confirming the assignment of a one proton doublet (J_{app} = 9 cps) at τ 5.58 to this proton in the *trans*-hydroxy acid and a one proton broad resonance (width at half-maximum = 8 cps) at τ 5.60 to the corresponding proton in the *cis* isomer. The configurational assignments arise from the following considerations. In the *trans* isomer, the carboxyl and hydroxyl groups may be expected to occupy equatorial positions in the most abundant conformer. The proton α to the hydroxyl group is thus axial and *trans* to the axial proton α to the carboxyl group. Thus, a large coupling constant in the range 8–14 cps is expected (Bhacca and Williams, 1964b). In the *cis* isomer, the similarly situated

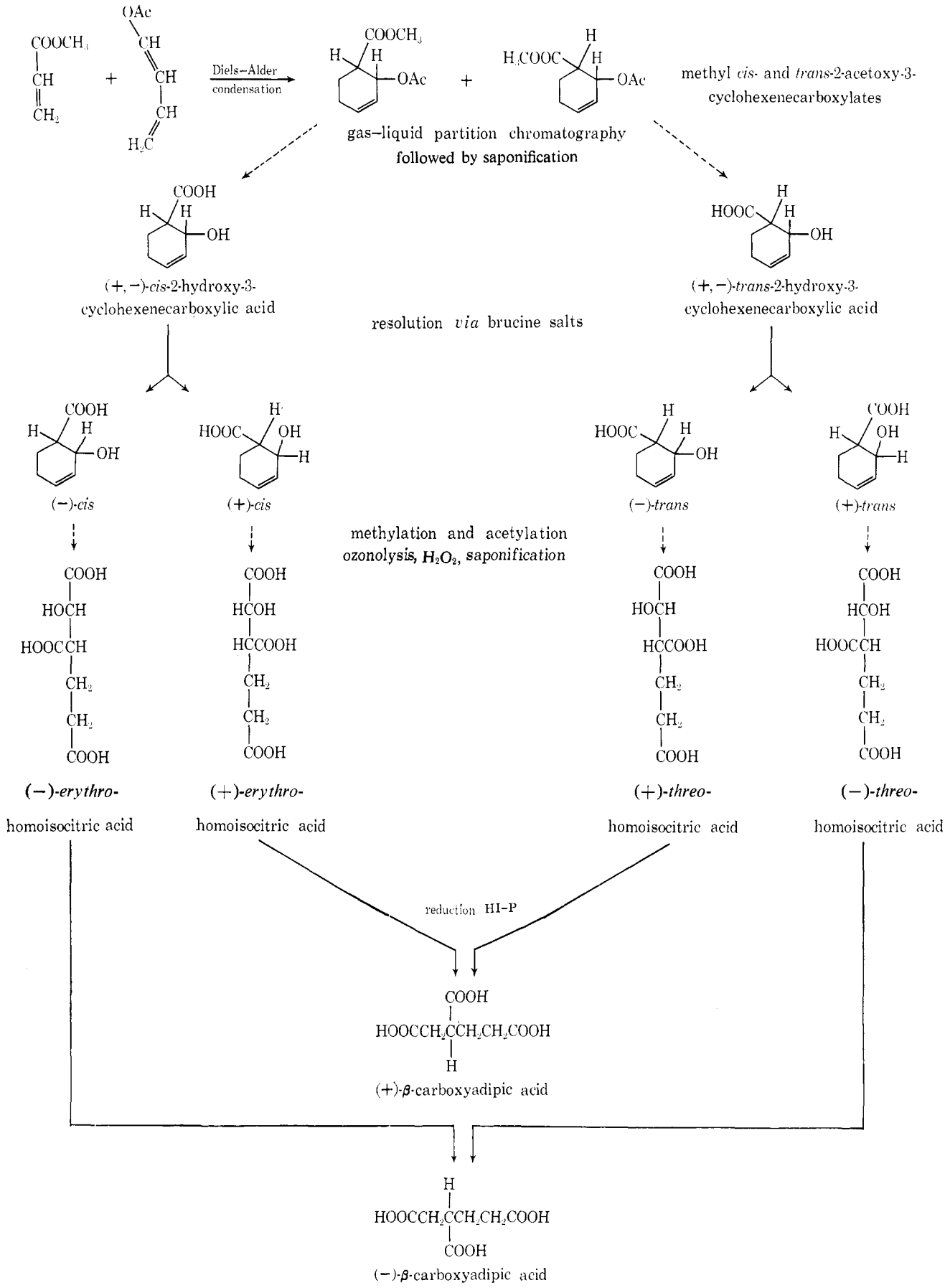


FIGURE 2: Synthesis and proof of absolute configuration of the optical isomers of homoisocitric acid.

TABLE I: Properties of the 2-Hydroxy-3-cyclohexenecarboxylic Acids.

Properties	Optical Isomers			
	(−)-*cis*	(+)-*cis*	(−)-*trans*	(+)-*trans*
Brucine salts				
$[\alpha]_{589}^{23}$ (deg) (*c* 0.60, H_2O)	−95	+35	−56	−5
Free acids				
$[\alpha]_{589}^{23}$ (deg) (*c* 0.40, H_2O)	−305	+312	−70	+77
Mp (°C)	62–64	62–64	78–80	79–81
Anal. Calcd for $C_7H_{10}O_3$				
C, 59.14	C, 59.17	C, 59.94	C, 58.93	C, 59.36
H, 7.09	H, 7.05	H, 6.91	H, 6.99	H, 7.05
Products of hydrogenation[a]				
$[\alpha]_{589}^{23}$ (deg) (in $CHCl_3$)	−24.3 (*c* 2.016) (lit. $[\alpha]_{589}$ −34.7, *c* not given)	+28.4 (*c* 0.738) (lit. $[\alpha]_{589}^{20.5}$ +30.7, *c* not given)	−32 (*c* 0.342) (lit. $[\alpha]_{589}^{17.0}$ −53.6, *c* 4.18)	+37.9 (*c* 1.24) (lit. $[\alpha]_{589}^{17.5}$ +53, *c* 3.64)
Mp (°C)	Oil (lit. 76–76.5)	Oil (lit. oil)	98–100 (lit. 110–111)	99–101 (lit. 110–111)
Anal. Calcd for $C_7H_{12}O_3$				
C, 58.31	C, 58.00	C, 58.40	C, 58.13	C, 58.26
H, 8.32	H, 8.20	H, 8.26	H, 8.40	H, 8.43
O, 33.37	O, 33.63	O, 33.40	O, 33.43	O, 33.23

[a] Literature values are reported by Real and Pascual (1953) and Febrer *et al.* (1964).

proton would be expected to be equatorial, since the preference for a conformation with equatorial carboxyl and axial hydroxyl will be significant (Hanack, 1965). In an equatorial position, this proton will be subject to weak coupling from adjacent protons on both sides, resulting in a broad resonance. Other resonances in the spectra of both isomers are consistent with their assignments.

Optical Isomers of 2-Hydroxy-3-cyclohexenecarboxylic Acid. The procedure for resolution of racemic *cis*- and *trans*-2-hydroxy-3-cyclohexenecarboxylic acids was patterned after that for the saturated compounds, described by Real and Pascual (1953); 0.025 mole of brucine (10 g) and 0.025 mole of 2-hydroxy-3-cyclohexenecarboxylic acid (3.5 g) were refluxed in acetone or ethyl acetate for 1–3 hr. The brucine salts were carefully and repeatedly recrystallized until an essentially constant rotation was achieved. To obtain the free hydroxycarboxylic acids, the salts were hydrolyzed with ten volumes of 2 N NaOH and the brucine which separated was removed by filtration. The filtrate was acidified with 2 N H_2SO_4, saturated with ammonium sulfate, and extracted with ether. The ether extract was evaporated, yielding a crystalline product. Recrystallization of the optically active 2-hydroxy-3-cyclohexenecarboxylic acids from ethyl acetate–ligroin (1:5, v/v) and/or from ether was performed until constant rotation was achieved. The final yield of each isomer of 2-hydroxy-3-cyclohexenecarboxylic acid varied from 0.5 to 0.75 g. Optical rotations were determined on a Durrum-JASCO optical rotatory dispersion spectrophotometer at a wavelength of 589 mμ and a temperature of 23 ± 1°. The properties of the brucine salts and of the free hydroxycarboxylic acids obtained are summarized in Table I.

Each optically active 2-hydroxy-3-cyclohexenecarboxylic acid was reduced to the corresponding saturated acid. 2-Hydroxy-3-cyclohexenecarboxylic acid (0.20 g) in 25 ml of glacial acetic acid was hydrogenated in the presence of platinum oxide at room temperature and atmospheric pressure until hydrogen consumption ceased (4–8 hr). The platinum was removed by filtration, the glacial acetic acid was evaporated, and the residue (about 0.20 g) was purified on a column of silicic acid according to the general method of Marvel and Rands (1950). The column was developed with a stepwise gradient of 1–15% 1-butanol in water-saturated chloroform. The yield of purified product varied from 50 to 125 mg. Table I includes the properties of the 2-hydroxy-3-cyclohexanecarboxylic acids obtained from the unsaturated compounds. The rotations measured for the four isomers of 2-hydroxycyclohexanecarboxylic acid suggest that the corresponding isomeric 2-hydroxycyclohexenecarboxylic acids are not completely resolved. However, on the basis of the direction of rotation of the hydrogenation products, the four isomers of 2-hydroxy-3-cyclohexenecarboxylic acid may be assigned the absolute configurations shown in Figure 2.

Racemic erythro-Homoisocitric Acid Lactone. This compound was prepared initially by stepwise oxidation of *cis*-2-acetoxy-3-cyclohexenecarboxylic acid.

Osmium tetroxide oxidation to the diol was accomplished by a procedure similar to that of McCrindle *et al.* (1960). A solution of 3.8 g of *cis*-2-acetoxy-3-cyclohexenecarboxylic acid in 125 ml of anhydrous ether was added to a solution of 5 g of osmium tetroxide in 125 ml of ether and 30 ml of pyridine. The mixture was allowed to stand in the dark at room temperature for 3 days.

The dark brown hygroscopic precipitate was collected and dissolved immediately in methanol. (The filtrate was saved for

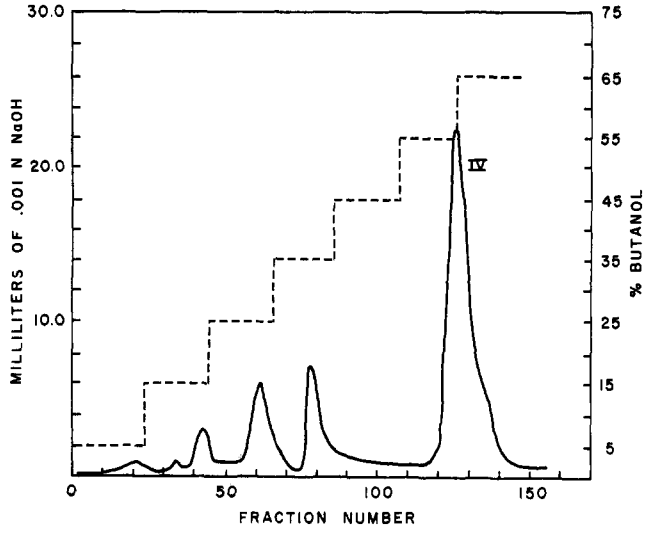


FIGURE 3: Purification of ozonolysis products on silicic acid. From 0.2 to 0.3 g of ozonolysis residue was layered onto the column of silicic acid (30 g). The column was developed with a stepwise gradient of 5–65% butanol in chloroform. Fractions (4 ml) were collected and 0.5 ml from each fraction was titrated. Peak IV corresponds to homoisocitric acid.

later work-up.) The methanolic solution was saturated with H_2S, allowed to stand overnight, and the resulting precipitate was removed by filtration and washed with methanol. The filtrate and washings were evaporated to dryness *in vacuo* at 35°, the residue was dissolved in water, a small amount of insoluble material was removed, and the solution was again evaporated to dryness. On standing, the product solidified to a crystalline mass, which was dissolved in hot ethyl acetate, decolorized with carbon, and crystallized by addition of ligroin, yield 3.0 g.

The original filtrate from the OsO_4 oxidation was evaporated, the residue was dissolved in methanol, and the resulting solution was treated with H_2S and worked up exactly as above, yield 0.25 g.

The two crops of crystalline product were combined, dissolved in water, decolorized with carbon, and evaporated to dryness. The product was recrystallized twice from ethyl acetate–ligroin yielding 2.6 g, mp 138–139°, resolidifying and remelting at 146–147°. *Anal.* Calcd for $C_9H_{14}O_6$ (218.2): C, 49.54; H, 6.47; acetyl, 19.72. Found: C, 49.80, 49.70; H, 6.58, 6.67; acetyl, 21.63, 20.06.

This compound (2-acetoxy-3,4-dihydroxycyclohexanecarboxylic acid) was subjected to periodate oxidation, followed by bromine oxidation, and saponification of the product.

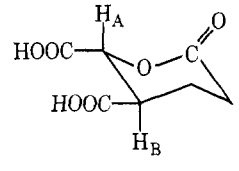
To a solution of 2.25 g of the acetoxydiol in 30 ml of water was added 22 ml of 0.5 M aqueous periodic acid. The reaction mixture was kept in the dark at room temperature for 2 hr. A saturated solution of silver acetate was then added until the supernatant was only slightly acid to congo red and contained a slight excess of Ag^+. The precipitate was removed, washed repeatedly with water, and combined filtrate and washings were evaporated to 50 ml *in vacuo*. The solution was then passed through a column of Amberlite IR-120 (H^+ form), the column was washed until all acidic material was removed, and the combined effluents were evaporated to a volume of 100 ml. To this solution was added 2.7 ml of bromine and the mixture was kept at room temperature for 4 days. The excess bromine was removed by bubbling air through the solution. Bromide ion was removed and the filtrate and washings were evaporated to 50 ml. Passage through Amberlite IR-120 (H^+ form) as before and evaporation of the effluent yielded 2.0 g of syrupy prodcuct.

This product was saponified by solution in 60 ml of absolute methanol, addition of 2.1 g of potassium hydroxide in 50 ml of methanol and 2 ml of water. The mixture was heated on a boiling-water bath for 1 hr, evaporated *in vacuo* to a small volume, and diluted with 50 ml of water. Potassium ion was removed by passage through Dowex 50W-X8 (H^+ form, 50–100 mesh). The effluent was evaporated to dryness, the resulting syrup was dissolved in ethyl acetate, treated with carbon, and again evaporated. This product crystallized only when seeded with a small crystal of material obtained from purification on a silicic acid column. Recrystallization from ethyl acetate–ligroin yielded 1.66 g, mp 173–175°. *Anal.* Calcd for $C_7H_8O_6$ (188.13): C, 44.70; H, 4.26. Found: C, 44.59, 44.51; H, 4.33, 4.40.

Analytical values are in excellent agreement with those calculated for the *lactone* of *erythro*-homoisocitrate. Additional evidence in support of this structure is provided by determination of the neutralization equivalent. Direct titration with alkali yielded values of 92.6 and 95.2 as compared with a calculated value for the lactone of 94.07. If the compound was dissolved in an excess of 0.1 N NaOH, the solution heated for 1 hr at 100° and back-titrated with acid, the values were in reasonable agreement with those calculated for saponification of the lactone (*Anal.* Calcd: 62.7. Found: 65.7, 65.0). Furthermore, the original compound gives on thin-layer chromatography a principal spot of R_F 0.75 (ethyl acetate–formic acid–water, 100:20:20, v/v) which shows an acid reaction with bromocresol green and a lactone color reaction with hydroxylamine–ferric chloride.

Amorphous preparations of the free acid were obtained by saponification of the lactone with aqueous sodium hydroxide, removal of Na^+ by passage through Dowex 50W-X8 (H^+ form), lyophilization of the effluent, and storage of the glassy product at 0°. Thin-layer chromatography showed only a very weak spot corresponding to the lactone and a strong, acidic spot at R_F 0.65.

An examination of the nuclear magnetic resonance spectra of the preparations of lactone and of free acid dissolved in D_2O indicated that, after standing for 30 min in solution at room temperature, the lactone is hydrolyzed extensively to the free acid. The resonance of proton A of the lactone appeared



as a doublet (J_{app} = 5 cps) at τ 4.90. The corresponding proton in the free acid gave rise to a similar doublet (J_{app} = 5 cps) at τ 5.75. The chemical shift difference corresponds to the usual alcohol → ester shift. The assignment of this resonance to the free acid was confirmed by examining the spectrum of an authentic sample. After 30 min, the lactone and free acid resonances showed approximately equal amounts, whereas at 45 min the ratio was 1:3, indicating that the lactone was still

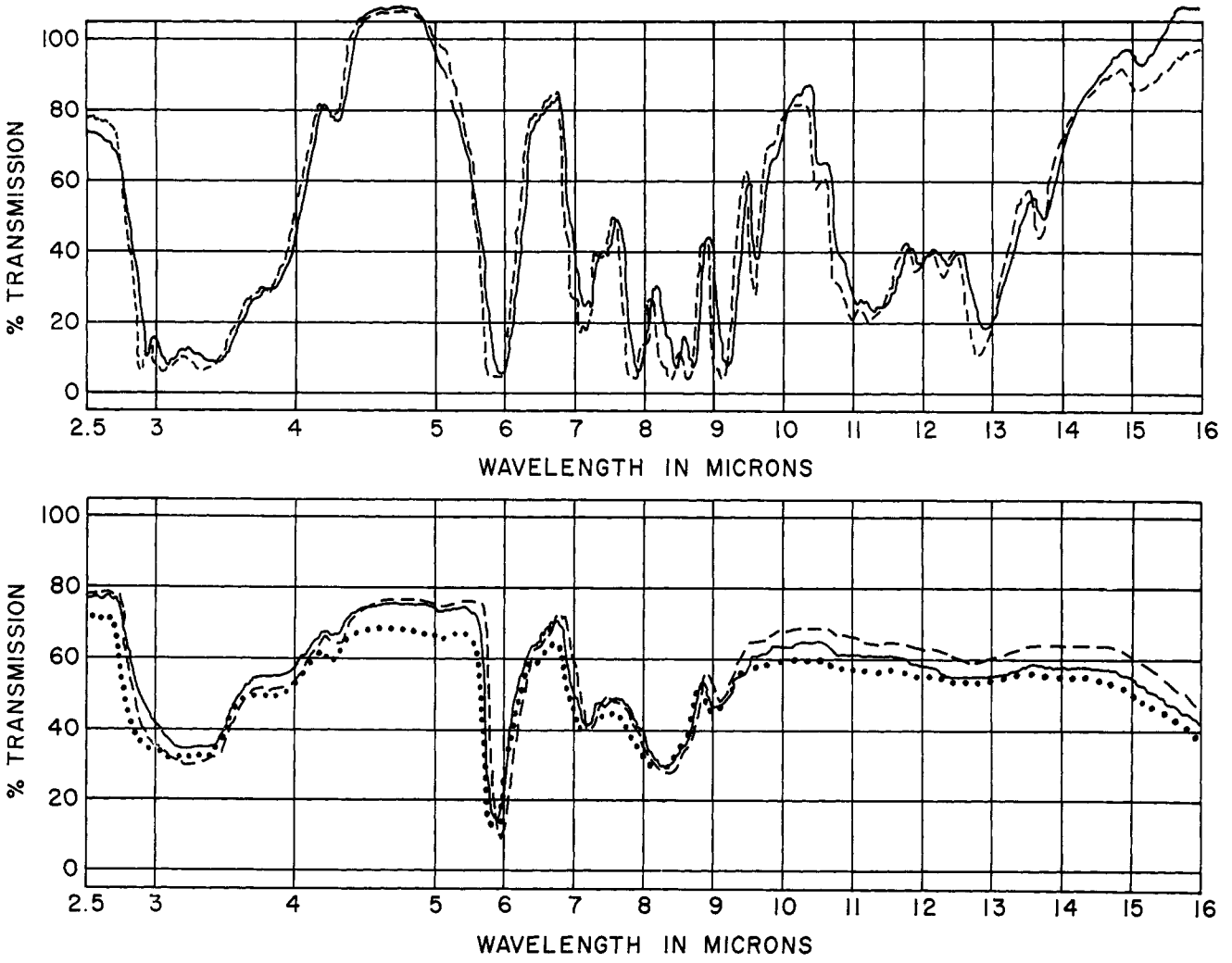


FIGURE 4: Infrared spectra of natural and synthetic *threo*-homoisocitric acid. Top chart: samples were run in KBr pellets on a Beckman IR-8 spectrophotometer. (- - - -) Crystalline, racemic *threo*-homoisocitric acid prepared by ozonolysis (offset 0.1 μ for comparison); (———) crystalline racemic *threo*-homoisocitric acid prepared according to the method of Yamashita (1958). Bottom chart: samples were run neat on NaCl plates. (· · · ·) Amorphous (−)-*threo*-homoisocitric acid prepared by ozonolysis; (———) amorphous natural homoisocitric acid isolated from mutant of *S. cerevisiae*; (- - - -) amorphous, racemic *threo*-homoisocitric acid prepared according to Yamashita (1958).

being converted into free acid and suggesting a preponderance of the free acid at equilibrium.

Racemic threo-Homoisocitric Acid. This compound was prepared by ozonolysis of methyl *trans*-2-acetoxy-3-cyclohexenecarboxylate according to the general procedure of Ackman *et al.* (1961), followed by oxidation with H_2O_2 and saponification of the product.

Methyl *trans*-2-acetoxy-3-cyclohexenecarboxylate (1.0 g) in 40 ml of solvent (glacial acetic acid–methyl formate, 3:1, v/v) was ozonized for 2 hr, using an ozonizer of the approximate design of Henne and Perilstein (1943). The reaction mixture was cooled in an ice bath during the ozonolysis. After complete ozonization, as indicated by the presence of large amounts of ozone in the effluent gases, 6 ml of 30% hydrogen peroxide was added, and the mixture was allowed to stand for 12–24 hr and then refluxed with stirring for 1 hr. The solvent was removed *in vacuo* and the residue was saponified by heating for 1 hr at 85° in 50 ml of methanolic KOH (5%) plus 10 ml of water. The solution was evaporated to a small volume and potassium ion was removed by passage through a column of Dowex 50W-X8 (H^+ form). The eluent and wash water were combined and evaporated to dryness, yielding 1.43 g of material which crystallized slowly on standing.

A portion of this product was fractionated on a silicic acid column according to the general method of Marvel and Rands (1950). The aliquot (0.3 g) in 2 ml of water was mixed with silicic acid to a dry powder and chloroform was added. The slurry of chloroform plus silicic acid was layered onto a silicic acid (30 g) column (25 × 2 cm), and the column was developed with a stepwise gradient of 5–65% 1-butanol in water-saturated chloroform without application of pressure. A representative elution pattern is shown in Figure 3. The fractions from peak IV (which corresponded to the position of synthetic homoisocitric acid prepared by the method of Yamashita (1958)) were pooled and evaporated *in vacuo* yielding 0.19 g of amorphous material. The properties of this material are given in Table II.

The remainder of the ozonolysis product was recrystallized

TABLE II: Properties of the Isomers of Homoisocitric Acid.

Properties	Racemic and Optical Isomers							
	(+,−)-*erythro*	(+)-*erythro*	(−)-*erythro*	(+,−)-*threo* (Yamashita, 1958)	(+,−)-*threo* (Ozonolysis)	(+)-*threo*	(−)-*threo*	Natural
$[\alpha]_{589}^{23}$ (deg) in acetone		+6.4 (*c* 3.27)	−4.4 (*c* 2.77)			+6 (*c* 1.95)	−8 (*c* 1.08) −9 (*c* 0.87) −190 (*c* 0.078)	−13.0 (*c* 1.22) −14.0 (*c* 0.70) −300 (*c* 0.120)
$[\alpha]_{589}^{23}$ (deg) in aqueous molybdate[c]		−265 (*c* 0.098)	+307 (*c* 1.56)			+115 (*c* 0.096)		
R_F thin-layer chromatography	0.65	0.65	0.65	0.66	0.65	0.66	0.65	0.66
Anal. Calcd for $C_7H_{10}O_7$: C, 40.78 H, 4.85		C, 42.96 H, 5.58	C, 47.75 H, 7.28	C, 40.72 H, 4.87	C, 40.85 H, 5.00 (crystalline) C, 44.28 H, 6.20 (amorphous)	C, 46.29 H, 6.36 (amorphous)	C, 40.51 H, 5.06 (amorphous) C, 47.59 H, 7.27 (amorphous)	C, 40.58[a] H, 5.03 (amorphous) C, 44.61 H, 6.14 (amorphous)
Substrate activity with homoaconitase (%)[b]	0	0	0	45	40	20	70–80	100

[a] Analyses were obtained on two different preparations of natural homoisocitric acid. The latter analysis giving high C and H values was determined on homoisocitric acid recovered from HI–P reaction mixture. This recovered material had a specific rotation of −14° and showed the same substrate activity with homoaconitase. [b] Based on 100% activity for natural homoisocitric acid; precision of assay approximately ±10%. [c] Fresh solutions of molybdate were allowed to stand overnight prior to use. This aging resulted in the precipitation of a considerable amount of white crystalline material from solution. The observed rotations of the isomers were very unstable in the freshly prepared solution while quite stable in the aged molybdate solution. Since a careful study of the effect of the different variables on the rotations was not undertaken, the values should be considered as qualitative.

from 20 ml of hot ethyl acetate by addition of ligroin. The yield of first crop was 0.28 g, mp 127–131°. Analytical data are given in Table II. Recrystallization of a second preparation of the compound from ether yielded a product mp 127–130°, the infrared spectrum of which is given in Figure 4.

Optical Isomers of Homoisocitric Acid. The general procedure was essentially identical with that for the preparation of racemic *threo*-homoisocitric acid. Since the yield of desired product was very substantially increased if the acetylated methyl esters were used instead of the free hydroxy acids, the resolved compounds were first esterified by treatment with excess diazomethane in ether solution, and then acetylated with acetyl chloride in pyridine using the general procedure of Bailey and Baylouny (1959). Analysis of the final products by gas–liquid partition chromatography indicated a nearly quantitative yield of the expected acetoxy methyl esters. Therefore, the products were ozonized routinely without purification. The yield of purified, amorphous homoisocitric acids obtained from 0.5 g of crude methyl 2-acetoxy-3-cyclohexenecarboxylates was 0.30–0.40 g (60–80%). Figure 2 indicates from which 2-hydroxy-3-cyclohexenecarboxylic acid each isomer of homoisocitric acid was derived. The properties of the homoisocitric acids are included in Table II.

Rotations of isomers were measured in acetone and in an aqueous solution containing 13% ammonium molybdate, 0.10 M sodium citrate, and 5% glacial acetic acid according to the method of Krebs and Eggleston (1943). While elemental analysis of the crystalline racemic products invariably agreed with the theoretical values, analytical results from chromatographically purified *amorphous* products (synthetic preparations as well as those isolated from cultures of the yeast mutant) were inconsistent. Some samples gave excellent analytical figures while others, prepared in an identical manner, were high in carbon and hydrogen. The results are consistent with the occlusion of variable amounts of solvent butanol in the glassy products, despite holding *in vacuo* over P_2O_5 at room temperature for many hours until an apparent constant weight of product was obtained.

Thin-layer chromatography on silicic acid of the purified homoisocitric acids in ethyl acetate–formic acid–water (100:20:20, v/v) usually indicated a single component (R_F 0.65) when sprayed with bromocresol green, corresponding to homoisocitric acid; however, a few preparations did show a small amount of a fast-running component.

Isolation of Natural Homoisocitric Acid. Homoisocitric acid was isolated from the culture medium of a lysine auxotroph

(Ly_{12}) of yeast (Lindegren's breeding stock of *Saccharomyces cerevisiae* strain 44174-2b) according to the method of Bhattacharjee and Strassman (1967). Homoisocitric acid was separated from citric, succinic, malic, and *cis*-homoaconitic acids, and homocitric acid lactone on columns of Dowex 1 (formate) according to the method of Maragoudakis and Strassman (1966). Homoisocitric acid was separated from homocitric acid by partition chromatography on columns of silicic acid according to the general method of Ramsey (1963).

Ly_{12} is a "leaky" mutant. Approximately 60 mg of homoisocitric acid was isolated from 10 l. of culture supernatant when the mutant was grown on minimal synthetic medium, and 30–40 mg was obtained from synthetic medium supplemented with L-lysine (30 mg/l.). The composition of the medium, a modified Burkholder's complete synthetic medium, is described by Maragoudakis and Strassman (1966).

Properties of the isolated homoisocitric acid are included in Table II.

Bioassay of Homoisocitric Acid Preparations with Homoaconitase. Homoaconitase was purified from baker's yeast according to the method of Strassman and Ceci (1966). The amount of homoisocitric acid converted into *cis*-homoaconitic acid was measured by the increase in optical density at 240 mμ. Since isomers of homoisocitric acid which were not substrates might be competitive inhibitors of homoaconitase, assays were run to equilibrium instead of obtaining initial rates. In this way small amounts of the natural isomer could be detected in the presence of large amounts of other isomers.

To a conical centrifuge tube was added 1.0 ml of a preparation of homoaconitase (4 mg of protein/ml) in 0.33 M potassium phosphate buffer (pH 8.6) and 0.2 ml of 0.01 M homoisocitric acid. The tubes were incubated at 25° for 2 hr, 0.3 ml of 70% perchloric acid was added, and centrifugation was at 15,000*g* for 10 min. The resultant supernatant was decanted and 0.3-ml aliquot was read in a microcell cuvet (0.35-ml capacity) at 240 mμ. Controls containing no substrate, and blanks in which perchlorate was added before substrate, were run with duplicate experimental tubes.

Table II provides assay data on a number of samples. For comparative purposes, all data are shown as *per cent* of activity of natural homoisocitrate. The substrate activity of synthetic (−)-*threo*-homoisocitric acid with homoaconitase was 70–80% of the activity of natural homoisocitrate. The preparations of (+)-*threo*-homoisocitric acid exhibited only 20% activity while both *erythro* forms showed no significant activity with the enzyme. The 70–80% activity of the partially resolved (−)-*threo*-homoisocitric acid and the 20% activity of the partially resolved (+)-*threo* isomer are in reasonable agreement with the optical purity achieved in the resolution of *trans*-2-hydroxy-3-cyclohexenecarboxylic acid (see Table I). The optical rotation at 589 mμ of natural homoisocitric acid had a negative value in acetone of −13 to −14° and a large negative value of −300° in aqueous molybdate solution. The synthetic *threo*-homoisocitric acid showing greater substrate activity also exhibited a negative rotation in acetone (−6 to −10°) and in aqueous molybdate (−150 to −190°). The magnitude of these rotations is again in general agreement with the optical purity achieved in the resolution.

As further evidence of the identity of the synthetic and natural materials, assays were run using a preparation of homoisocitric acid dehydrogenase (Strassman and Ceci, 1965). The results were consistent with those from homoaconitase, demonstrating that synthetic (−)-*threo*-homoisocitrate is a substrate for homoisocitric acid dehydrogenase.

Infrared Spectra. Infrared spectra (Figure 4) were recorded on a Beckman IR-8 spectrophotometer. The spectra in KBr pellets of crystalline homoisocitric acid prepared by the method of Yamashita (1958) and crystalline racemic *threo*-homoisocitric acid prepared as described in this paper were essentially identical. Infrared spectra of synthetic amorphous (−)-*threo*-homoisocitric acid and of amorphous natural homoisocitric acid, prepared by evaporation of an acetone solution of the compound on NaCl plates, were also identical but showed considerably less detail than the spectra of the crystalline compounds. This difference is attributed to the amorphous state of the synthetic and natural homoisocitric acids since racemic *threo*-homoisocitric acid gave an essentially identical spectrum when run by the same technique in the amorphous state.

Conversion of Homoisocitric Acids into 1,2,4-Butanetricarboxylic Acid (β-Carboxyadipic Acid). Reduction of homoisocitric acid to β-carboxyadipic acid was accomplished by refluxing 50–100-mg portions of the compound and 20 mg of red phosphorus in 1 ml of 47% hydrogen iodide at 110° for 7–24 hr. Iodide ion was removed by precipitation with silver acetate and excess silver ion by chromatography on Amberlite IR-120 (H^+ form). The product was purified on a column of silicic acid (20 g). The column was developed with a stepwise gradient of 5–65% 1-butanol in water-saturated chloroform. β-Carboxyadipic acid was eluted from the column with 35% 1-butanol while unreacted homoisocitric acid was eluted with 55–65% 1-butanol. Thus, both the reduction product and unreacted starting material were readily recovered from the reaction mixtures.

A striking difference was observed in the behavior of *erythro*-homoisocitric acids as compared with the *threo* isomers. The *erythro* compounds gave consistently high yields of reduction product of the predicted optical rotation. In contrast, the *threo*-homoisocitric acids gave much lower yields of product almost completely racemized. The reaction was 70–80% complete after 24 hr in the former case, while only a 20–30% yield of β-carboxyadipic acid was obtained with the *threo* isomers after 24 hr at 110°.

Table III gives representative data on the reduction products obtained from the isomers of synthetic homocitrate and from natural homoisocitrate. Reduction of (+)- and (−)-*erythro*-homoisocitric acid to (+)- and (−)-β-carboxyadipic acid, respectively, confirms the absolute configurations already assigned to the *erythro* isomers (Figure 2). On the other hand, it will be noted that there is a discrepancy in the direction of rotation of the reduction products of synthetic (−)-*threo*-homoisocitric acid as compared with natural homoisocitrate. The compound isolated from yeast culture yielded β-carboxyadipic acid with a small negative rotation, as predicted on the assumption of extensive racemization but a small degree of retention of configuration. The synthetic material, which is presumed to consist primarily of the natural isomer, yielded a product with a small positive rotation.

In subsequent experiments, additional observations were made which provide a reasonable explanation of this discrepancy. In one experiment, unreacted synthetic (−)-*threo*-homoisocitric acid was recovered from the HI–P reduction and was found to possess a higher specific rotation than the starting material (−7.5° *vs.* −6°). When this recovered material was

TABLE III: Properties of β-Carboxyadipic Acids Derived from HI–P Reduction of the Isomers of Homoisocitric Acid.

Starting Material	Yield (%)	Mp (°C)[b]	$[\alpha]_{589}^{23}$ (deg) in Acetone[b]	R_F (Thin-Layer Chromatography)	Anal.[c] C	Anal.[c] H
(+)-*erythro*-Homoisocitric acid	70	87–99	+22 (*c* 1.36)	0.86	44.42	5.41
(−)-*erythro*-Homoisocitric acid	70	94–101	−21.5 (*c* 0.87)	0.84	44.38	5.23
Racemic *threo*-homoisocitric acid[a]	70	121–122		0.86	44.47	5.46
(+)-*threo*-homoisocitric acid	20		−4.4 (*c* 0.446)	0.85	45.61	5.64
(−)-*threo*-Homoisocitric acid	25	85–116	+4 (*c* 0.61)	0.84	44.19	5.22
Natural homoisocitric acid	25	85–90	−1 (*c* 0.45)	0.85	46.02	5.99

[a] Racemic *threo*-homoisocitric acid was heated in a sealed tube at 160° for 7 hr with HI–P and worked up as described in this paper. All other samples were treated with HI–P as described in the text. [b] For comparison, the values reported by Freudenberg and Geiger (1952) are as follows: racemic β-carboxyadipic acid, mp 121–122°; (+)-β-carboxyadipic acid, mp 102°, $[\alpha]_{589}$ +26.9° (*c* 0.0893, acetone). [c] Calcd values for $C_7H_{10}O_6$: C, 44.21; H, 5.30.

subjected to a second treatment with HI–P, the recovered homoisocitric acid had an increased specific rotation of −10.5°. Furthermore, the β-carboxyadipic acid isolated from the first reduction had a specific rotation of +2.5°, while the crystalline product from the second treatment showed no measurable rotation. Entirely comparable results were obtained in experiments with synthetic (+)-*threo*-homoisocitric acid.

The most probable explanation of these results is that the synthetic preparations of optically active *threo*-homoisocitric acids are contaminated initially with a small amount of the optical isomers of *erythro*-homoisocitric acid of opposite rotation. (This in turn could be expected if the original *trans*-hydroxycyclohexenecarboxylic acid contained a small amount of the *cis* compound, in view of the much lower solubility of the brucine salts of the *cis* isomers.) Since the *erythro* forms have been shown to give a much higher yield of β-carboxyadipic acid and also a product of much higher rotation, the first HI–P reduction would be expected to remove most of the contaminating *erythro* isomer. Thus, the first crop of β-carboxyadipic acid would contain a significant excess of the optical isomer derived from the *erythro* contaminant and consequently would show an optical rotation greater in magnitude and opposite in direction to that predicted for the product from the pure *threo* isomer. On the other hand, the second treatment should yield β-carboxyadipic acid derived predominantly from *threo*-homoisocitric acid and therefore should show a much lower rotation. Likewise, the *threo*-homoisocitric acid recovered from successive HI–P treatments would be expected to show a higher specific rotation as the *erythro* contaminant of opposite rotation became depleted. The recovered material also showed significantly higher substrate activity with homoaconitase, as would be predicted.

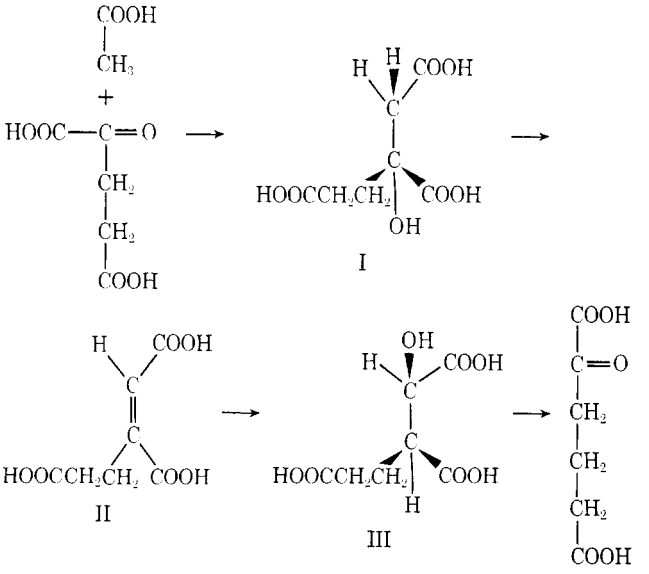


FIGURE 5: Stereochemistry of the conversion of homocitrate (I) into homoisocitrate (III).

On the basis of the above results, the configurational assignments of the four optical isomers of homoisocitric acid are confirmed by two independent methods (Figure 2). The absolute configuration of natural homoisocitric acid, shown in Figure 5, may be designated *threo*-D_s-homoisocitric acid (2-(*R*)-hydroxy-3(*S*)-carboxyadipic acid or 1-(*R*)-hydroxy-2(*S*)-1,2,4-butanetricarboxylic acid).

The stereochemistry of the enzyme-catalyzed rearrangement of homocitrate to homoisocitrate (Figure 5) is analogous to that of citrate to isocitrate. In each case the rearrangement appears to occur by *trans* elimination and *trans* addition of the elements of water, accompanied by a reversal of the position of attachment of the hydrogen and hydroxyl groups.

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