Substrate Activity of Structural Analogs of Isocitrate for Isocitrate Dehydrogenases from Bovine Heart†

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ABSTRACT: D-Garcinia acid (D-*threo*-1,2-dihydroxy-1,2,3-propanetricarboxylate), like D-*threo*-isocitrate, has an α-D$_S$-hydroxyl group and a β-L$_S$ configuration of the second carboxyl group. The maximal velocity of pyridine nucleotide reduction with D-garcinia acid is 8 and 21% of D-*threo*-isocitrate with the DPN-linked and TPN-linked isocitrate dehydrogenase from bovine heart, respectively. The other stereoisomers of hydroxycitrate [L-garcinia acid, D- and L-hibiscus acid (D- and L-*erythro*-1,2-dihydroxy-1,2,3-propanetricarboxylate)] are inactive. DL-*threo*-Homoisocitrate (DL-*threo*-1-hydroxy-1,2,4-butanetricarboxylate) supports DPN$^+$ reduction at 10–15% of the rate observed for isocitrate with the DPN-specific enzyme, but is not a substrate for TPN-linked isocitrate dehydrogenase. The values of apparent $S_{0.5}$ for total isocitrate and total garcinia acid are similar with both enzymes; the apparent $S_{0.5}$ of total homoisocitrate is two- to threefold higher than that of total isocitrate with the DPN-linked enzyme. Enzymatic oxidative decarboxylation of garcinia acid and homoisocitrate leads to formation of α-keto-β-hydroxyglutarate and α-ketoadipate, respectively. DL-Methylmalate (DL-1-hydroxy-2-methylsuccinate) is inactive as a substrate for either dehydrogenase as are the newly synthesized compounds: DL-*threo*-γ-isocitric amide (DL-*threo*-1-hydroxy-3-carbamyl-1,2-propanedicarboxylate), β-methyl-DL-isocitrate (DL-1-hydroxy-2-methyl-1,2,3-propanetricarboxylate), β-methyl-DL-garcinia acid (DL-*threo*-1-hydroxy-2-methoxy-1,2,3-propanetricarboxylate), DL-1-hydroxy-1,2,2-ethanetricarboxylate, and DL-1,4-dihydroxy-1,2-butanedicarboxylate.

Of the four stereoisomers of isocitrate, only one is active as substrate for isocitrate dehydrogenases from animal tissues. D-*threo*-Isocitrate[1] (Figure 1, I) is the substrate for the TPN-linked isocitrate dehydrogenase from porcine heart (Ochoa, 1951) and DPN-linked enzyme from bovine heart (Plaut and Sung, 1955). With both enzymes the α hydrogen of the substrate is transferred to the α position of the nicotinamide ring of the respective pyridine nucleotide coenzymes (Englard, 1960; Chen and Plaut, 1963a); the β-hydrogen of isocitrate is not lost during oxidation to α-ketoglutarate in either isocitrate dehydrogenase reaction (Lienhard and Rose, 1964; Chen and Plaut, 1963a).

The minimal requirements for substrate structure appear to be for an acid with an α-D$_S$-hydroxyl group and a β-L$_S$ configuration of the second carboxyl group[1] (Gawron et al., 1958). The nature of other substituents at the β carbon which might permit interaction of a compound with enzyme is largely unknown. However, there are indications that substitution of the β hydrogen of isocitrate by a hydroxyl group may lead to an active compound. Thus, Martius and Maué (1941) synthesized hydroxycitrate (1,2-dihydroxy-1,2,3-propanetricarboxylate) from aconitic acid and reported that only one of the four possible isomers led to reduction of Methylene Blue with cucumber seed extracts. The product(s) of the oxidation obtained with porcine heart homogenates was not characterized, but it differed from α-ketoglutarate. The natural hydroxycitric acid analogs L-garcinia acid and L-hibiscus acid (Figure 1, II) did not serve as substrates of TPN-linked isocitrate dehydrogenase (Lewis, 1969). However, natural garcinia acid is a potent inhibitor of citrate cleavage enzyme (Watson et al., 1969), inhibits hepatic fatty acid synthesis (Lowenstein, 1971), and is a positive effector of liver acetyl CoA carboxylase (Hackenschmidt et al., 1972). DL-Homoisocitrate prepared by the method of Yamashita (1958) has been shown by separate methods of synthesis to be DL-*threo*-homoisocitrate (Chilina et al., 1969). Although DL-*erythro*-homoisocitrate is inactive, one of the stereoisomers of DL-*threo*-homoisocitrate (Figure 1, IV) is active for homoisocitrate dehydrogenase from yeast (Rowley and Tucci, 1970), a step in lysine biosynthesis (Strassman and Weinhouse, 1953). However, DL-*threo*-homoisocitrate prepared by the method of Yamashita (1958) was reported not to be a substrate for TPN-linked isocitrate dehydrogenase from yeast (Strassman and Ceci, 1965).

A number of compounds have been tested here to determine further the specificity of isocitrate dehydrogenases from bovine heart. Of these, D-garcinia acid (Figure 1, III) was a substrate for both dehydrogenases, DL-*threo*-homoisocitrate was active for the DPN-linked enzyme and inactive with the TPN-specific dehydrogenase.

Experimental Procedures

Materials

DPN-linked isocitrate dehydrogenase was purified to homogeneity from bovine heart mitochondria (Giorgio et al., 1970). TPN-linked isocitrate dehydrogenase from the extramitochondrial fraction of bovine heart homogenate was purified by a modification of the method of Rose (1960). The final preparation had a specific activity of 8 μmol of

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[1] The configuration of isocitrate and structurally related derivatives is described by the nomenclature of Vickery (1962). The nomenclature for tricarboxylic acids and the corresponding lactones is in accord with useage in Chemical Abstracts.

TPNH formed min^{-1} mg of $protein^{-1}$ at 25° under the conditions of assay described by Siebert et al. (1957).

Nucleotides, Tris, and Hepes[2] were purchased from Sigma, inorganic chemicals of reagent grade from Mallinckrodt, and organic chemicals from Aldrich. The cation exchange resins AG 50W-X12 (200–400 mesh) and Bio-Rex 70 (200–400 mesh) were from Bio-Rad.

DL-*threo-Isocitric lactone* from Sigma was recrystallized from ethyl butyrate (Plaut et al., 1974).

DL-*Homoisocitric acid* (DL-*threo-1-hydroxy-1,2,4-butanetricarboxylic acid*) was synthesized by the method of Yamashita (1958), mp 127–129°.

DL-*Methylmalic acid* was prepared by catalytic reduction of diethyl oxalopropionate (Scherp, 1946) (mp 112–115°) and by deamination with HNO_2 of DL-*threo*-methylaspartate (Tatsumi et al., 1966) (mp 120°). Literature values for melting points for the DL-erythro and DL-threo isomers were 113–115 and 128–129°, respectively (Tatsumi et al., 1966).

DL-*β-Methylisocitric acid* (DL-*1-hydroxy-2-methyl-1,2,3-propanetricarboxylic acid*) was synthesized by two methods: one involving condensation of diethyl oxalopropionate and ethyl bromoacetate and, the other, an initial reaction of chloral with dipotassium methylsuccinate.[3]

Methods

Chromatography. Paper and thin-layer chromatography were done by the ascending procedure on the support media and under the conditions specified. The solvent systems used were: I, benzene–tetrahydrofuran–acetic acid (60:36:4); II, 90% phenol–H_2O–88% formic acid (83:17:1); III, isopropyl ether–88% formic acid–H_2O (7:2:1); IV, isopropyl ether–88% formic acid (3:2); V, 1-butanol–88% formic acid–H_2O (4:1:2).

Assays. The composition of assay media and the range of concentrations of isocitrate and the analogs used are specified in table legends. Incubations were done at 25° and the initial velocity of formation of DPNH or TPNH was followed at 340 nm.

Isocitric acid lactone and lactones of analogs were hydrolyzed under alkaline conditions as described previously (Chen and Plaut, 1963b).

Identification of Degradation Products from the Enzymatic Oxidation of Garcinia Acid. METHYLGLYOXAL 2,4-DINITROPHENYLOSAZONE. A reaction mixture containing 0.30 m*M* TPN^+, 3 m*M* DL-garcinia acid, 1.3 m*M* $MnSO_4$, and 167 m*M* NaHepes at pH 7.4 in 50 ml was incubated with enzyme at 25°. The reaction was terminated by the addition of a solution of 0.2 g of 2,4-dinitrophenylhydrazine in 12 ml of concentrated HCl when 0.198 μmol of TPNH/ml had formed. The mixture was left to stand at 25° for 18 hr and was then treated with three 200-ml portions of ethyl acetate. The ethyl acetate layers were combined and dried with 20 g of Na_2SO_4. The residue was removed by centrifugation and aliquots of the ethyl acetate extract were streaked on Mallinckrodt Chrom AR 1000 (1.4 ml per 20 × 28 cm sheet) and the sheets were developed for 1 hr with 0.6 *N* NaOH. Colored zones with values of R_f of 0.13, 0.34, 0.47, 0.69, and 0.79 were observed. The spots with R_f 0.79 (orange) and R_f 0.13 (purple) corresponded to the migration of authentic 2,4-dinitrophenylhydrazine and methylglyoxal 2,4-dinitrophenylosazone, respectively. The zone with R_f 0.13 was cut out of the wet sheets and stirred with 37 ml of ethyl acetate per sheet until all of the color had transferred to the solvent phase. The ethyl acetate layer was separated from the residue and evaporated to dryness under reduced pressure. The residue was dissolved in 0.6 *N* NaOH and examined by paper and thin-layer chromatography by the procedure of Beck (1955) for 2,4-dinitrophenylhydrazine derivatives of trioses. Only a single component could be detected on each of the chromatographic systems, which corresponded in migration to authentic methylglyoxal 2,4-dinitrophenylosazone prepared by the method of Baer and Fischer (1943).

The absorption spectrum in 0.6 *N* NaOH of the 2,4-dinitrophenylhydrazine derivative purified from the reaction corresponded to that of methylglyoxal 2,4-dinitrophenylosazone with a maximum at 550 nm, in agreement with the findings of Beck (1955). Spectrophotometric determination of the derivative from the reaction mixture in 0.6 *N* NaOH at 550 nm, using a mmolar absorbance of 68 for methylglyoxal 2,4-dinitrophenylosazone (Beck, 1955), gave a recovery of 0.130 μmol of methylglyoxal 2,4-dinitrophenylosazone/ml of mixture. This compared to formation of 0.198 μmol of TPNH/ml of incubation mixture.

1-HYDROXY-2-PROPANONE. The conditions of incubation of garcinia acid and TPN^+ were identical with those described above for the isolation of methylglyoxal 2,4-dinitrophenylosazone. The trichloroacetic acid filtrate of the reaction mixture was submitted to cold temperature vacuum distillation (Grant, 1946). The distillate was treated with *o*-aminobenzaldehyde according to the method of Forist and Speck (1950) for determination of 1-hydroxy-2-propanone. The blue fluorescent material formed in this reaction had the same migration in several systems of thin-layer chromatography as the expected condensation product 3-hydroxyquinaldine prepared by the method of Koenigs and Stockhausen (1902). The respective values of R_f for the product and authentic 3-hydroxyquinaldine were 0.71 and 0.70 on Eastman Chromagram cellulose 6065, 0.67, and 0.65 on Baker-flex DEAE-cellulose, and 0.93 and 0.93 on Eastman Chromagram silica 6060 with the solvent system 1-butanol–ethanol–0.5 *N* NH_4OH (7:1:2); development with 0.6 *N* NaOH on Mallinckrodt Chrom AR 1000 gave R_f values of 0.75 for both.

Preparation of Compounds. DL-*trans*-3-HYDROXY-5-OXO-2,3-TETRAHYDROFURANDICARBOXYLATE (DL-HIBISCUS ACID LACTONE).[4] Freshly prepared *cis*-aconitic anhydride (Malachowski and Maslowski, 1928) (4.68 g, 30 mmol) was dissolved in 45 ml of water at room temperature. To the solution was added 1.8 g (5.8 mmol) of solid $Ba(ClO_3)_2 \cdot H_2O$ and 30 ml of 1% osmium tetroxide in water (1.17 mmol). A second addition of the same quantities of $Ba(ClO_3)_2$ and osmium tetroxide was made after 30 min and the mixture was left at room temperature for 90 min, mixed with 3 g of Norit A, and filtered. The clear filtrate was passed through a 2 cm × 12 cm column of AG 50W-

[2] Abbreviation used is Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

[3] R. L. Beach and G. W. E. Plaut, unpublished work (1974). NMR spectra indicate that β-methylisocitrate formed from diethyl oxalopropionate and ethyl bromoacetate contains both of the possible DL isomer forms whereas the compound formed from chloral and methylsuccinate is a single DL isomer.

[4] The procedure of Boll et al. (1969), in which $AgClO_3$ is the oxidant, gave a product which had substrate activity for both isocitrate dehydrogenases; this was due to contamination with garcinia acid. The hibiscus acid prepared by the $Ba(ClO_3)_2$ procedure did not contain garcinia acid as tested by enzyme activity and paper chromatography.

X12 in the H^+ form. The acidic effluent was concentrated in a vacuum and the syrup formed was dried in a desiccator over NaOH pellets at 1 Torr overnight. The gummy residue was extracted continuously with about 100 ml of ether for 6 hr in a Soxhlet extractor. The white crystals separated from the cooled solvent were extracted again with about 50 ml of ether in a Soxhlet extractor for 48 hr. The product crystallized from the ether solution was collected and dried in vacuo at 60° (2.8 g, 49%), mp 183°. Anal. Calcd for $C_6H_6O_7$: C, 37.91; H, 3.18. Found: C, 37.83; H, 3.22. The synthetic DL-hibiscus acid lactone (R_f 0.46) and free acid (R_f 0.25) had the same mobilities by thin-layer chromatography on cellulose MN 300 G in solvent system IV as the corresponding samples from natural hibiscus acid.

DL-*cis*-3-HYDROXY-5-OXO-2,3-TETRAHYDROFURANDICARBOXYLATE (DL-GARCINIA ACID LACTONE) was synthesized from *trans*-aconitic acid by the method for hibiscus acid lactone described above.

Resolution of DL-*Garcinia Acid Lactone.* To 300 ml of water at 80° 10 g of DL-garcinia lactone (52.6 mmol) was added with stirring, followed by 17 g of recrystallized cinchonine (57.7 mmol) in portions. After 15 min, the reaction mixture was filtered hot and the filtrate was left to stand at room temperature for 16–18 hr. The precipitate formed was converted to (+)-garcinia lactone as described below. The supernatant solution was concentrated to 35 ml and treated at 95–100° with 3 g of cinchonine. The precipitate formed after standing for several days at room temperature was discarded and the (−)-garcinia lactone was recovered from the filtrate as described below.

The insoluble cinchonine salt of (+)-garcinia acid lactone was suspended in 50 ml of water and treated with 2 *N* KOH with vigorous stirring until alkaline. The suspension was filtered and the residue was washed with water until the washes were neutral. The filtrates were combined (approximately 180 ml) and passed through a 4.3 cm × 8 cm column of AG 50W-X12 (H^+ form). The acidic effluent was collected and evaporated to dryness in vacuo. The residue was dried in vacuo at 100° for 30–40 min, and extracted in a Soxhlet extractor with 70 ml of ether for 1–2 hr. The ether extract was treated with 10 ml of chloroform and the solution was evaporated until a precipitate appeared. The crystals of L-garcinia lactone formed on standing at room temperature were recrystallized from ether–chloroform in a yield of 1 g, mp 176–177°, $[\alpha]_D^{25.2}$ +106° (water).

D-(−)-Garcinia lactone was prepared from the water-soluble cinchonine salt by a method analogous to that used for the preparation of the L analog. The yield of recrystallized D-garcinia lactone was 1.04 g, mp 172–174°, $[\alpha]_D^{25.8}$ −98° (water).

L-Garcinia acid lactone ($[\alpha]_D^{20}$ +100°) and L-hibiscus acid lactone were isolated from *Garcinia cambogia* and *Hibiscus sabdariffa*, respectively (Lewis, 1969).[5]

DL-*cis-3-Methoxy-5-oxo-2,3-tetrahydrofurandicarboxylate* (*β-methyl*-DL-*garcinia lactone*). DIMETHYL DL-*cis*-3-METHOXY-5-OXO-2,3-TETRAHYDROFURANDICARBOXYLATE. To a stirred solution of 4 g (18.3 mmol) of dimethyl DL-*cis*-3-hydroxy-5-oxo-2,3-tetrahydrofurandicarboxylate (prepared by esterification of DL-garcinia lactone (Martius and Maué, 1941)) in 25 ml of CH_2Cl_2 containing 0.2 ml of freshly distilled boron trifluoride etherate at 0°, diazomethane in CH_2Cl_2 (Arndt, 1943) was added dropwise until the yellow color of the reagent persisted. The solvent was removed at 35° under a vacuum and the colorless hygroscopic syrup (4.2 g) was distilled at 145–150° and 0.65 Torr (2.9 g, 69%). Anal. Calcd for $C_9H_{12}O_7$: C, 46.56; H, 5.21. Found: C, 45.10; H, 5.73. Eq. Wt.: Calcd: 78.1. Found: 80.0.

DL-*cis*-3-METHOXY-5-OXO-2,3-TETRAHYDROFURANDICARBOXYLATE (β-METHYL-DL-GARCINIA LACTONE). One gram (4.3 mmol) of dimethyl DL-*cis*-3-methoxy-5-oxo-2,3-tetrahydrofurandicarboxylate was hydrolyzed in 20 ml of 0.97 *N* NaOH for 16–18 hr at room temperature. The solution was passed through a 1.5 cm × 8 cm column of AG 50W-X12 (H^+ form), and the acidic effluent was concentrated to a brown syrup in a flash evaporator and then dried at 100° under vacuum for 1 hr. The syrup (0.7 g) was taken up in 10 ml of ether, clarified with Norit A, and the solvent was evaporated. The residue was dissolved in 20 ml of acetone and 50 ml of benzene and cooled to 5°. Crystals formed within 2 weeks (250 mg, 27%), mp 95–96°: ν_{max}(Nujol) 1750 cm^{-1} (γ-lactone), 1700 cm^{-1} (COOH), 1125 cm^{-1} (O-CH_3); nuclear magnetic resonance (NMR) (acetone-d_6) ppm 3.87 (singlet, 1 H), 3.25 (singlet, 3 H), 2.90 (singlet, 2 H). Anal. Calcd for $C_7H_8O_8 \cdot 2H_2O$: C, 35.01; H, 5.04. Found: C, 35.10; H, 5.10. Chromatography of β-methylgarcinia lactone and garcinia lactone in solvent system V on Whatman 3MM paper gave R_f values of 0.86 and 0.77, respectively.

DL-*threo-1-Hydroxy-3-carbamyl-1,2-propanedicarboxylate* (DL-*γ-isocitric amide*). To 5 ml of concentrated NH_4OH saturated at 1–2° with NH_3 gas was added 500 mg of DL-*threo*-isocitric lactone. The reaction mixture was kept in an ice bath and a slow stream of NH_3 was passed through the solution for 2 hr. After 24 hr at 0°, the solution was concentrated to dryness under a vacuum and the residue was dissolved in 5 ml of water and passed through a 1 cm × 15 cm column of Bio-Rex 70 in the Li^+ form. The column was washed with 20 ml of water and the effluent was concentrated to dryness in vacuo at a bath temperature of 35°. The residue was dissolved in 5 ml of water followed by 10 ml of methanol. The compound crystallized overnight at 5–8°. The residue was dried in a vacuum at 65° and recovered in a yield of 170 mg: ν_{max}(Nujol) 3390 cm^{-1} (OH), 3150 cm^{-1} (NH_2), 1660 cm^{-1} (amide I), 1610 cm^{-1} (amide II), 1570, 1365 cm^{-1} (COO^-). Anal. Calcd for $C_6H_9NO_6Li_2$: N, 7.18. Found: N, 7.21. DL-γ-Isocitric amide had a migration on Whatman 3MM paper of R_f 0.36 and R_f 0.37 in solvent systems V and II, respectively; this compared to values of R_f for isocitric lactone and free isocitric acid of 0.62 and 0.50 in solvent V and 0.57 and 0.32 in solvent II, respectively.

DL-*1-Hydroxy-1,2,2-ethanetricarboxylic acid.* A solution of 5.2 g (40 mmol) of *trans*-epoxysuccinic acid (Liwschitz et al., 1962) in 75 ml of water was adjusted to pH 12 with NaOH, 2.16 g (44 mmol) of NaCN in 25 ml of water were added, and the mixture was left to stand for 16–18 hr at room temperature. The solution was refluxed for 1 hr, 1.60 g of NaOH was added, and it was heated for an additional 3 hr, then evaporated to one-half of its original volume to remove ammonia. The mixture was cooled to room temperature, adjusted to pH 7.9 with HCl, 6.4 g of $CaCl_2 \cdot 2H_2O$ in 50 ml of water was added, and the calcium salt precipitate was collected. The dried calcium salt (1.7 g) was dissolved in 3 ml of concentrated HCl and extracted with ether for 16–18 hr in a liquid–liquid extractor. The precipitate remaining after removal of ether was crystallized from

[5] We thank Dr. Y. S. Lewis for a generous gift of natural L-garcinia acid lactone which was used in the initial part of this work.

Table I: Comparison of Kinetic Constants of Isocitrate and Analogs with Isocitrate Dehydrogenases.[a]

Substrate	ADP (m*M*)	$S_{0.5}$ [b] (m*M*)	V_{max} (ΔA_{340} min^{-1})
DPN-linked enzyme			
DL-Isocitrate	0	18 (0.46)	0.83
DL-Isocitrate	0.3	10 (0.26)	0.90
DL-Garcinia acid	0	11	0.06
DL-Garcinia acid	0.3	5.7	0.07
DL-Hibiscus acid	0.3		0.00
L-Hibiscus acid (natural)	0.3		0.00
DL-Homocitrate	0	50	0.10
DL-Homocitrate	0.3	18	0.09
TPN-linked enzyme			
DL-Isocitrate	0	0.0057	0.29
DL-Garcinia acid	0	0.0102	0.06
L-Garcinia acid (natural)	0		0.00
DL-Hibiscus acid	0		0.00
L-Hibiscus acid	0		0.00
DL-Homoisocitrate	0		0.00

[a] DPN-linked enzyme. Incubated at 25° in a medium at pH 7.2 containing 167 m*M* NaHepes, 0.33 m*M* DPN$^+$, DL-isocitrate, or analogs in a range between 3.9 and 39 m*M*. With DL-isocitrate, total magnesium was varied between 1.1 and 0.18 m*M* to maintain free Mg^{2+} constant at 0.05 m*M*; the same concentrations of total magnesium were used with the substrate analogs. Under these conditions the ratios of total isocitrate (or analogs) to total magnesium declined from 39 to 26 with decreasing substrate concentrations. TPN-linked enzyme. Incubated at 25° in a medium at pH 7.4 containing 33.3 m*M* Tris-acetate, 1.33 m*M* $MnSO_4$, 0.33 m*M* EDTA, 0.03% gelatin, 0.1 m*M* TPN$^+$, and varied concentrations of isocitrate and substrate analogs. Comparisons of velocities with different substrates were made with identical concentrations of the DPN-linked dehydrogenase or TPN-linked enzyme. [b] The values for *total* DL-isocitrate, DL-garcinia acid, or DL-homoisocitrate were determined from Hill plots. The numbers in parentheses refer to $S_{0.5}$ of magnesium isocitrate$^-$ calculated from the stability constant of 0.521 m*M*$^{-1}$ (Grzybowski et al., 1970).

Table II: The Effect of the D and L Isomers of Garcinia Acid on DPN-Linked Isocitrate Dehydrogenase.

Compound	Expt 1[a] K_m app.[c] (m*M*)	Expt 2[b] K_m app.[c] (m*M*)	Expt 1[a] V_m (ΔA_{340} min^{-1})	Expt 2[b] V_m (ΔA_{340} min^{-1})
DL-Garcinia acid	1.48	1.24	0.027	0.142
D-Garcinia acid (resolved)	0.83	0.62	0.028	0.142
L-Garcinia acid (resolved)			0.000[d]	
L-Garcinia acid (natural)			0.000	0.000

[a] Incubated at 25° in a medium at pH 7.3 containing 167 m*M* Na-Hepes, 0.33 m*M* DPN$^+$, 0.10 m*M* ADP, and garcinia acids in a range between 1.0 and 3.3 m*M*. The ratio of molar concentrations of garcinia acids to $MnSO_4$ was maintained at 10 as the substrate concentration was varied. [b] Incubated at 25° in a medium at pH 7.2 containing 167 m*M* Hepes, 0.33 m*M* DPN$^+$, 0.3 m*M* ADP, and garcinia acids in a range between 0.16 and 1.6 m*M*; the concentration of total magnesium was fixed at 6.7 m*M*. [c] Expressed as concentration of total garcinia acid. [d] When tested at 11 m*M* this sample of resolved L-garcinia acid had 5% of the activity of the equivalent concentration of DL-garcinia acid.

ethyl acetate (270 mg). The residue was extracted with ether for 8 hr in a Soxhlet apparatus and dried, mp 139–141°. ν_{max}(Nujol) 3260, 920 cm^{-1} (OH), 1730, 1670 cm^{-1} (COOH). Anal. Calcd for $C_5H_6O_7$: C, 33.72; H, 3.40. Found: C, 34.01; H, 3.45. Eq. Wt.: Calcd: 59.4. Found: 61.2.

2-Oxo-3-(1-carboxy-1-hydroxymethyl)tetrahydrofuran (DL-*1,4-dihydroxy-1,2-butanedicarboxylic acid lactone*). 2-OXO-3-ETHOXALYLTETRAHYDROFURAN. A solution of sodium ethoxide, prepared from 11.5 g of metallic sodium (0.5 mol) and 250 ml of absolute ethanol in 375 ml of anhydrous ether, was treated at 5° with 73 g (0.5 mol) of distilled diethyl oxalate. After the mixture was stirred for 15 min, 43.05 g (0.5 mol) of γ-butyrolactone was added and the mixture was left at 5° for 5–12 days and the crystalline sodium salt was collected (98.3 g, 94%). Thirty grams of the sodium salt was dissolved in 70 ml of 3.5 *N* HCl at 5° and the aqueous phase was treated with four 100-ml portions of ether. The combined ether layers were washed with 25 ml of water, dried over Na_2SO_4 and evaporated to a syrup (26.5 g) which distilled at 125–128° and 0.7 Torr (18.3 g, 68%). Anal. Calcd for $C_8H_{10}O_5$: C, 51.62; H, 5.41. Found: C, 51.71; H, 5.47. NMR (acetone-d_6) ppm 1.30 (triplet, 3 H), 3.30 (multiplet, 2 H), 4.41 (multiplet, 5 H); enolic OH 10.82 disappears in 10% D_2O.

2-OXO-3-OXALYLTETRAHYDROFURAN. 2-Oxo-3-ethoxalyltetrahydrofuran (4.5 g, 24 mmol) was dissolved in 12 ml of concentrated HCl and incubated at room temperature for 16–18 hr. The crystals formed were collected (2.6 g) and recrystallized from 4 ml of acetone and 32 ml of benzene (1.6 g, 42%), mp 185–186° (after drying overnight, mp 174–176°). Anal. Calcd for $C_6H_6O_5$: C, 45.58; H, 3.82. Found: C, 45.83; H, 4.07. Eq. Wt.: Calcd: 158. Found: 156. NMR (acetone-d_6) ppm 2.73 (triplet, 2 H) and 4.38 (triplet, 2 H); enolic OH 9.22 disappears in 10% D_2O.

2-OXO-3-(1-CARBETHOXY-1-HYDROXYMETHYL)TETRAHYDROFURAN. 2-Oxo-3-ethoxalyltetrahydrofuran (18.6 g, 0.1 mol) in 200 ml of ethanol was hydrogenated in presence of 200 mg of PtO_2 under 27 psi of H_2 for 16–18 hr at room temperature in a Paar apparatus. The catalyst was removed by filtration and the solvent was removed under reduced pressure at 40°. The residue was crystallized from 50 ml of ethanol (9.5 g, 51%), mp 78–79°. Anal. Calcd for $C_8H_{12}O_5$: C, 51.06; H, 6.43. Found: C, 51.05; H, 6.49.

2-OXO-3-(1-CARBOXY-1-HYDROXYMETHYL)TETRAHYDROFURAN. 2-Oxo-3-(1-carbethoxy-1-hydroxymethyl)-tetrahydrofuran (6.4 g, 34 mmol) was refluxed in 100 ml of 3 *N* HCl for 1.5 hr. The solvent was removed under reduced pressure at 40°. The syrup was dissolved twice in 25-ml portions of water followed by evaporation to remove residual HCl and was then dried under a vacuum at 50° for 1 hr. The solid formed after cooling (5.1 g) was dissolved in 150 ml of boiling ethyl butyrate. Crystalline product was recovered after cooling to 5° for 16–24 hr (4.5 g, 83%): mp 146–148°; ν_{max}(Nujol) 3300, 1120, 1305 (2° OH), 1760 cm^{-1} (γ-lactone), 1705 (COOH); NMR (acetone-d_6) ppm 2.43 (multiplet, 2 H), 3.09–4.04 (multiplet, 1 H), 4.27 (multiplet, 2 H), 4.43 (doublet, 1 H). Found: C, 45.02; H, 5.28. Eq. Wt. (cold) Calcd: 160. Found: 161. Eq. Wt. (heated excess base) Calcd: 80.6. Found: 77.9.

The compound isolated probably exists as racemic mixtures of the two diastereoisomers. This would be consistent with the method of synthesis; namely, hydrogenation of the highly enolic ester (NMR absorption 10.82 ppm) 2-oxo-3-ethoxalyltetrahydrofuran over Adam's catalyst, and the high yield of product obtained. However, it is uncertain from the NMR spectrum whether one or two isomeric pairs are present.

Results

Hydroxycitric Acids (1,2-Dihydroxy-1,2,3-propanetri-

Table III: Measurement of Isocitrate Dehydrogenase Activity by TPNH and DPNH Formation and Dye Reduction.

Isocitrate Dehydrogenase	Substrate	(1) Activity[a] TPNH or DPNH Formation (nmoles per min per μg)	(2) 2,6-Dichlorophenol-indophenol Reduction (nmoles per min per μg)	(1)/(2) Ratio
TPN-linked	DL-Garcinia acid	1.68	1.69	0.99
TPN-linked	DL-Isocitrate	8.00	0.40	20
DPN-linked	DL-Garcinia acid	2.48	2.24	1.1
DPN-linked	DL-Isocitrate	25.0	0.84	30

[a] DPN-linked enzyme: 166 mM NaHepes at pH 7.2; 2 mM $MgSO_4$; 0.67 mM ADP; 0.33 mM DPN^+; 5.3 mM DL-garcinia acid or 5.3 mM DL-isocitrate; and enzyme at 25°. TPN-linked enzyme: 36.6 mM Tris-acetate at pH 7.2; 0.33 mM EDTA; 0.03% gelatin; 2 mM $MgSO_4$; 0.1 mM TPN^+; 1.33 mM DL-garcinia acid or 1.33 mM DL-isocitrate; and enzyme at 25°. Activities are expressed per μg of enzyme protein. When dye reduction was studied with either enzyme, 0.05 mM 2,6-dichlorophenolindophenol was added to the media. Reactions were initiated with enzyme. TPNH or DPNH formation was followed at 340 nm (ϵ 6.22 × $10^3 M^{-1}$ cm^{-1}) and dye reduction by decrease in absorbance at 600 nm (ϵ 16.8 × $10^3 M^{-1}$ cm^{-1}).

carboxylic Acids). No activity was found with the hibiscus acids where the hydroxyl groups at the α and β carbons are in the threo configuration (Table I). However, DL-garcinia acid, containing these hydroxyls in the erythro configuration (Boll et al., 1969), led to reduction of pyridine nucleotides with either dehydrogenase: the rates were 8 and 21% of those with DL-isocitrate for the DPN-linked and the TPN-linked enzymes, respectively. The D-garcinia acid was the active isomer for both dehydrogenases. With chemically resolved D-garcinia acid, apparent K_m values[6] with the DPN-linked enzyme were approximately half of those observed with DL-garcinia acid (Table II). The L isomer, obtained by chemical resolution or by isolation from *Garcinia cambogia,* was inactive either with the DPN-dependent enzyme (Table II), or the TPN-linked enzyme (Table I).

The present observation that only one of the isomers of hydroxycitrate is a substrate for isocitrate dehydrogenases is in accord with the earlier findings of Martius and Maué (1941) who found that (+)-garcinia acid, subsequently shown to be D-garcinia acid (Figure 1, III) (Boll et al., 1969), accelerated the bleaching of Methylene Blue catalyzed by cucumber seed extracts.

The activity of synthetic DL-garcinia acid was not due to contamination by isocitrate since paper chromatography showed that α-ketoglutarate was not a product of oxidation with either dehydrogenase. Attempts to characterize directly the expected product of oxidation (α-keto-β-hydroxyglutarate), either by chromatography of the free compound or after formation of the 2,4-dinitrophenylhydrazone, were not successful. However, methylglyoxal 2,4-dinitrophenylosazone was the major product of oxidation of garcinia acid isolated from incubation mixtures treated with 2,4-dinitrophenylhydrazine as described under Methods. This osazone was recovered in more than 65% yield compared to TPNH formed. Furthermore, 1-hydroxy-2-propanone was detected in cold distillates from incubation mixtures of the TPN-linked isocitrate dehydrogenase reaction with garcinia acid by the procedure of Forist and Speck (1950). The expected product of garcinia acid dehydrogenation, α-keto-β-hydroxyglutarate, can tautomerize to an enediol. The latter is likely to be susceptible to rearrangement to the easily decarboxylated substance α-hydroxy-β-ketoglutarate and, possibly, to spontaneous oxidation by molecular oxygen to α,β-dioxoglutarate. Either β-keto acid could be easily decarboxylated to trioses; 1-hydroxy-2-propanone could result from the loss of both carboxyl groups of α-hydroxy-β-ketoglutarate and methylglyoxal could be formed from α,β-dioxoglutarate. The reaction of either triose with excess 2,4-dinitrophenylhydrazine would yield the 2,4-dinitrophenylosazone of methylglyoxal. Since enediols such as ascorbic acid can be determined by titration with 2,6-dichlorophenolindophenol (Tillmans, 1927), it seemed possible to show enzymic formation from garcinia acid of the enediol form of α-hydroxy-β-ketoglutarate by following the reduction of dye. The rate of reduced pyridine nucleotide formation agreed closely with that of dye reduction, with DL-garcinia acid as substrate for either isocitrate dehydrogenase (Table III). Dye reduction is not due to interaction with reduced pyridine nucleotides, since with isocitrate as substrate loss of absorbance at 600 nm was negligible. The results in Table III are reported as initial velocities; parallel rates of reduction of pyridine nucleotides and the dye were maintained with prolonged incubation even when reaction progress was no longer linear with time. With magnesium ion as activator, similar results were obtained with both anaerobic and aerobic incubations, indicating a faster rate of oxidation of the enediol by the dye than by molecular oxygen. However, interaction with oxygen may be significant in the presence of manganous ion since with this activator (in experiments

[6] The values of K_m or $S_{0.5}$ for *total* garcinia acid in Table I and in experiments 1 and 2 of Table II permit comparisons of the relative values of constants within each experiment. However, the constants are not comparable between experiments where incubation conditions varied. Magnesium isocitrate⁻ has been reported to be the actual substrate of the DPN-linked enzyme and the values of $S_{0.5}$ calculated for magnesium DL-isocitrate shown in parentheses of Table I are in agreement with those reported previously (Plaut et al., 1974). By analogy it is likely that the divalent metal ion chelates of garcinia acid or homoisocitrate are the substrates of the enzyme, and presentation of constants based on chelate concentrations would be desirable. However, the precise stability constants of the divalent metal chelates of the substrate analogs are not known. Thus, the apparent large discrepancies in constants for total DL-garcinia acid shown in Table I and in experiment 1 of Table II are due to variations in the ratios of magnesium to garcinia acid as well as differences in pH and ADP concentration. In Table I a high ratio of isocitrate analog to magnesium concentration was maintained to hold most of the magnesium in the chelate form and to minimize the inhibitory effect of free Mg^{2+} (Plaut et al., 1974). In experiment 2 of Table II, total magnesium was held constant and was in excess (6.6 mM) of the variable concentrations of garcinia acids (0.16–1.6 mM) used; in experiment 1 (Table II) the concentration of substrate was varied at a constant ratio of [total garcinia acid]/[total $MnSO_4$] = 10.

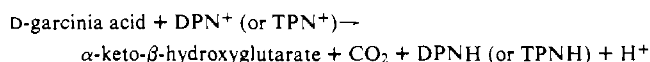
Table IV: Identification of Reaction Products Formed from Homoisocitrate and Isocitrate with DPN-Linked Isocitrate Dehydrogenase.[a]

Material	Chromatographic System[b]		
	I (R_f)	II (R_f)	III (R_f)
DL-Isocitrate + DPN$^+$ + enzyme	0.57	0.53	0.27
α-Ketoglutaric acid	0.59	0.53	0.28
DL-Homoisocitrate + DPN$^+$ + enzyme	0.70	0.61	0.45
α-Ketoadipic acid	0.68	0.61	0.45
Pyruvic acid	0.34		0.66
2,4-Dinitrophenylhydrazine	0.98	0.96	

[a] Incubations of substrates (2.4 mM) with the DPN-linked enzyme were done at 25° in a medium containing 118 mM NaHepes, 0.95 mM $MnSO_4$, 0.47 mM ADP, and 1.70 mM DPN$^+$ at pH 7.2. The formation of DPNH was followed at 340 nm and the reactions were terminated when from 4 to 20% of the substrate had been oxidized. [b] The solvent systems are described under Methods. I. Reaction mixtures (0.5 ml) were treated with 0.1 ml of 2,4-dinitrophenylhydrazine (0.3% in 2 N HCl) for 2 hr at 25°. The 2,4-dinitrophenylhydrazones were extracted from the solution with 5 ml of isopropyl ether. The ether layer was evaporated to dryness, the residue was then dissolved in 0.1 ml of 2-propanol and spotted on sheets of Eastman Chromagram 6060 (silica with fluorescent indicator) and developed for 40–60 min. Spots were detected with a Mineralight Model SL3660 lamp (Dittmann, 1968). II. The 2,4-dinitrophenylhydrazones were formed and extracted from the reaction mixtures as described under I. The extracts were spotted on sheets of Eastman Chromagram 6065 (cellulose) and the chromatograms were developed for 5 hr. III. The incubation mixtures (1 ml) were mixed with 20 ml of saturated $MgSO_4$ solution and 0.6 ml of concentrated H_2SO_4 and the free acids were extracted into ether for 16 hr in a liquid–liquid extractor. The solvent extracts were evaporated to 0.5–1 ml and spotted on sheets of Eastman Chromagram 6060. The chromatograms were developed for 2–3 hr. The sheets were dried, sprayed with 0.05% o-phenylenediamine-2HCl in 10% trichloroacetic acid and heated for 2 min at 100°. The fluorescent quinoxalinol derivatives of the keto acids were detected under uv light (Hockenhull and Floodgate, 1952).

not shown here) the observed dye reduction was appreciably slower than that of pyridine nucleotides when incubated in air; such a difference was not observed under anaerobic conditions.

Formation of α-keto-β-hydroxyglutarate and carbon dioxide from garcinia acid by isocitrate dehydrogenases is consistent with (i) the mode of action of these enzymes with isocitrate, (ii) the isolation of the degradation products 1-hydroxy-2-propanone and methylglyoxal 2,4-dinitrophenylosazone from reaction mixtures, and (iii) the parallel rates and stoichiometry of reduction of pyridine nucleotides and of 2,6-dichlorophenolindophenol (Table III). If the oxidation of the tautomeric form of α-keto-β-hydroxyglutarate by 2,6-dichlorophenolindophenol reflects formation of α,β-dioxoglutarate by two electron transfer, the reactions catalyzed by the isocitrate dehydrogenases can be represented by the equation



D-garcinia acid + DPN$^+$ (or TPN$^+$) → α-keto-β-hydroxyglutarate + CO_2 + DPNH (or TPNH) + H$^+$

DL-*Homoisocitrate.* In accord with the previous observation with TPN-linked isocitrate dehydrogenase from yeast (Strassman and Ceci, 1965) DL-homoisocitrate is not a substrate for this enzyme from bovine heart (Table I) with either Mg^{2+} or Mn^{2+} as the activator. It is doubtful whether the analog interacts significantly with the TPN-linked enzyme, since only slight inhibition of isocitrate oxidation by homoisocitrate was observed (apparent K_i/K_m ratio of 250–400). This suggests that the inhibition is not specific but may be due to removal by homoisocitrate of activating divalent cations by chelation.

Table V: Stoichiometry of DPNH and α-Ketoadipate Formation from DL-Homoisocitrate.[a]

Incubation Time (min)	DPNH (μmol/ml)	α-Keto acid (μmol/ml)
5	0.0175	0.0181
10	0.0241	0.0238
15	0.0290	0.0287
20	0.0325	0.0334

[a] The reaction mixture containing 118 mM NaHepes at pH 7.2, 0.95 mM $MnSO_4$, 0.47 mM ADP, 1.70 mM DPN$^+$, 2.40 mM DL-homoisocitrate, and 2.85 μg of enzyme/ml was incubated at 25° for the time periods indicated. DPNH was determined as described under Methods. α-Keto acid was determined by a microadaptation of the method of Friedemann and Haugen (1943) with α-ketoadipic acid as the standard.

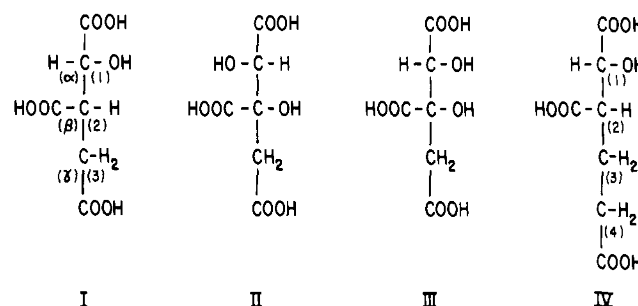


FIGURE 1: Structures of isocitric acids: I, D-*threo*-isocitric acid (D-*threo*-1-hydroxy-1,2,3-propanetricarboxylic acid); II, L-hibiscus acid (L-*erythro*-1,2-dihydroxy-1,2,3-propanetricarboxylic acid); III, D-garcinia acid (D-*threo*-1,2-dihydroxy-1,2,3-propanetricarboxylic acid); IV, D-*threo*-homoisocitric acid (D-*threo*-1-hydroxy-1,2,4-butanetricarboxylic acid).

However, DL-homoisocitrate does act as substrate for the DPN-linked enzyme. It supported DPN$^+$ reduction at a rate from 10 to 15% of that observed with isocitrate, with an apparent $S_{0.5}$ for total homoisocitrate two- to threefold higher than that for total isocitrate (Table I). Similar differences in relative velocities and $S_{0.5}$ were obtained when manganese (maintained constant at 1.3 mM while substrate varied) replaced magnesium ion as the activator.[7]

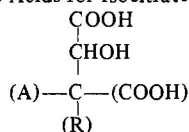
The product of homoisocitrate oxidation differs from α-ketoglutarate, and has chromatographic properties identical with α-ketoadipate (Table IV). This and the observation that stoichiometric amounts of DPNH and of the α-keto acid are formed from homoisocitrate (Table V) indicate that the reaction catalyzed by the DPN-linked isocitrate dehydrogenase is

homoisocitrate + DPN$^+$ → α-ketoadipate + CO_2 + DPNH + H$^+$

The Effect of ADP on the Oxidation of Analogs by DPN-Linked Isocitrate Dehydrogenase. ADP is a positive modifier for the enzyme from a number of animal tissues leading to a lowering of the apparent K_m of total isocitrate (Chen and Plaut, 1963b) or of magnesium isocitrate—the actual substrate of the enzyme—and a lowering of the Hill coefficient from n = 2 to n = 1 (Plaut et al., 1974). When

[7] T. Aogaichi, J. Fleming, and G. W. E. Plaut, unpublished observations (1970).

Table VI: Substrate Activities of α-Hydroxy-α, β-dicarboxylic Acids for Isocitrate Dehydrogenases (IDH).



Compound	β-Substituents		Substrate Activity[a]	
	A	R	DPN–IDH	TPN–IDH
D-*threo*-Isocitric acid[b]	H	CH_2COOH	+	+
D-Garcinia acid[b]	OH	CH_2COOH	+	+
L-Garcinia acid[b]	OH	CH_2COOH	–	–
DL-Hibiscus acid[b]	OH	CH_2COOH	–	–
DL-*threo*-Homoisocitric acid[b]	H	CH_2CH_2COOH	+	–
β-Methyl-DL-isocitric acid (1-hydroxy-2-methyl-1,2,3-propanetricarboxylic acid)[c]	CH_3	CH_2COOH	–	–
β-Methyl-DL-garcinia acid[c]	CH_3O	CH_2COOH	–	–
DL-*threo*-Methylmalic acid[c]	H	CH_3	–	–
DL-1-Hydroxy-1,2,2-ethanetricarboxylic acid[c]	H	COOH	–	–
DL-1,4-Dihydroxy-1,2-butanedicarboxylic acid[c]	H	CH_2CH_2OH	–	–
DL-*threo*-γ-Isocitric amide[c]	H	CH_2CONH_2	–	–

[a] +, active; –, inactive, i.e., less than 0.5% of the activity observed with optimal concentrations of isocitrate. [b] The assays were done as reported in Table I. [c] The conditions of assay for the DPN-linked enzyme were as described in footnote a of Table II; the concentrations of analogs varied between 1 and 8.9 m*M*. The activities of analogs with the TPN-linked dehydrogenase were measured in a medium containing 167 m*M* NaHepes at pH 7.4, 0.10 m*M* TPN^+, 1.33 m*M* $MnSO_4$, and analogs between 0.1 and 4 m*M*.

the ratio of concentrations of total isocitrate (or analogs) to total magnesium was kept relatively high as substrate concentration varied, ADP (0.3 m*M*) lowered $S_{0.5}$ for total isocitrate and the analogs (Table I). Furthermore, in the absence of ADP the Hill coefficient had a value of $n = 2$ with all of the substrates approaching a value of 1 with isocitrate and garcinia acid in the presence of ADP. However, under the incubation conditions of Table I, the Hill coefficient was not changed by ADP when DL-homoisocitrate was the substrate.

Discussion

The observation that of the hydroxycitric acids (DL-hibiscus acid and DL-garcinia acid) only D-garcinia acid serves as a substrate (Tables I and II) is in accord with the previous findings with D-*threo*-isocitrate that an α-D_S-hydroxyl group and a β-L_S configuration of the second carboxyl group (Figure 1, I) are minimal requirements for activity with either dehydrogenase. A need for a similar configuration of the hydroxyl group and carboxyl group at the α and β carbons, respectively, is also consistent with the activity of DL-*threo*-homoisocitrate (Figure 1, IV) with the DPN-linked enzyme (Table I).

While replacement of the hydrogen atom at the β carbon of D-*threo*-isocitrate by a hydroxyl substituent leads to an active substrate (i.e., D-garcinia acid), substitution by a methyl group (β-methyl-DL-isocitrate) or a methoxyl group (β-methyl-DL-garcinia acid) leads to loss of substrate activity (Table VI). The presence of the carboxyl group at the β carbon is essential at least for the activity of the DPN-linked dehydrogenase, since DL-α-hydroxyglutarate did not serve as a substrate for this enzyme (Plaut and Sung, 1955).

Only limited substitution for the carboxymethyl group attached to the β carbon of D-*threo*-isocitrate appears to be tolerated. Replacement of the carboxymethyl group by a methyl (DL-*threo*-methylmalate), carboxyl (DL-1-hydroxy-1,2,2-ethanetricarboxylic acid), hydroxyethyl (DL-1,4-dihydroxy-1,2-butanedicarboxylic acid), or carbamylmethyl group (DL-*threo*-γ-isocitric amide) resulted in compounds without substrate activity for either enzyme (Table VI). The compounds above are probably not bound to the substrate sites of the enzymes since they did not significantly inhibit activity with D-*threo*-isocitrate as substrate (Beach, Aogaichi, and Plaut, unpublished observations). However, reduction of oxaloacetate to D-malate by purified liver TPN-linked isocitrate dehydrogenase was reported (Illingworth and Tipton, 1970) indicating that in the direction of substrate reduction replacement of the carboxymethyl group by hydrogen is tolerated.

The inactivity of DL-*threo*-γ-isocitric amide and of DL-1,4-dihydroxy-1,2-butanedicarboxylic acid (Table VI) indicates a requirement for a carboxylate group as a part of the substituent at the β carbon of the substrate. However, the lack of activity of DL-1-hydroxy-1,2,2-ethanetricarboxylic acid suggests further that the β carbon and the carboxyl group must be separated by at least one carbon atom. DL-*threo*-Homoisocitrate, in which the β carbon and carboxylate are separated by two methylene groups, is a substrate for the DPN-linked enzyme, but it is neither a substrate nor a significant inhibitor for the TPN-specific isocitrate dehydrogenase (Table I).

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Rat Kidney L-α-Hydroxy Acid Oxidase: Isolation of Enzyme with One Flavine Coenzyme per Two Subunits†

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ABSTRACT: L-α-Hydroxy acid oxidase (listed as EC 1.4.3.2, L-amino acid: O_2 oxidoreductase) has been purified 100-fold from rat kidney to apparent homogeneity by gel electrophoresis. A subunit molecular weight of 47,500 was found by sodium dodecyl sulfate gel electrophoresis, but in contrast to previous reports, the enzyme has been found to have a molecular weight of ca. 200,000 by Sephadex gel filtration and by dodecyl sulfate gel electrophoresis of the enzyme cross-linked with dimethyl suberimidate. A somewhat higher value was found by sedimentation equilibrium, but a tetrameric structure for the active enzyme is definitely established. The enzyme was found to contain the FMN coenzyme at a concentration of one FMN/102,000 daltons or one flavine/two subunits, a highly unusual finding. This ratio was determined from spectroscopic analysis of the FMN in lyophilized samples of the enzyme and by titration of the coenzyme with the flavine specific enzyme inactivator 2-hydoxy-3-butynoate. The enzyme has the same specific activity as a crystalline sample of the enzyme reported to have twice as much flavine/milligram.

In the course of investigations into flavoenzyme reaction mechanisms, we have recently been concerned with the proposal that flavine-linked enzymes which oxidize α-hydroxy acids or α-amino acids generate transient α-carbanionic species during catalysis (Walsh et al., 1971). These studies have involved the detection of flavoenzyme-catalyzed elimination reactions with β-halo substrates (Walsh et al., 1971, 1973) and the susceptibility of certain bacterial flavoproteins to irreversible inactivation by the acetylenic suicide substrate 2-hydroxy-3-butynoate (Walsh et al., 1972a,b; Lederer, 1974). To extend these studies, we wished to isolate the previously described rat kidney L-hydroxy acid oxidase (Blanchard et al., 1945, 1946; Nakano and Danowski, 1966; Nakano et al., 1967), an unusual enzyme in possessing comparable oxidase activity toward both hydroxy acids and amino acids.

After the isolation of this enzyme and purification to apparent homogeneity by published procedures (Nakano and Danowski, 1966; Nakano et al., 1967), physical character-

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