This experiment, therefore, does not support the hypothesis that bromelain isolated in the presence of phenylmercuric acetate has undergone autodigestion. There remains the possibility that the results of the end-group analyses are related to the presence of the carbohydrate moiety. It is clear that only further work can clarify the nature of the heterogeneity that gives the analytical data obtained with purified preparations of bromelains.

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The Stereochemistry of Decarboxylation of Isocitrate by Isocitric Acid Dehydrogenase*

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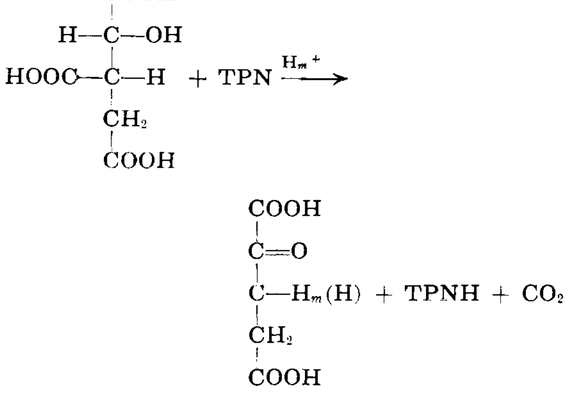
Stereospecifically β-tritiated α-ketoglutarate prepared by the oxidative decarboxylation of *threo*-D$_s$-isocitric acid with isocitric dehydrogenase in tritiated water undergoes complete exchange of its tritium with the medium hydrogen upon treatment with isocitric dehydrogenase under the conditions for the β-hydrogen exchange reaction. Therefore isocitric dehydrogenase acts upon the same β-hydrogen of α-ketoglutarate in both the oxidative decarboxylation and exchange reactions. The absolute configuration of stereospecifically β-tritiated α-ketoglutarate, which was prepared by the enzymic detritiation of randomly β-tritiated α-ketoglutarate, was determined from the known stereochemistries of the fumarase and aspartase reactions in the following way. Carbons 1–4 of this α-ketoglutarate were converted by a series of enzymic and chemical steps to β-tritiated L-aspartic and L-malic acids, which upon treatment with aspartase and fumarase, respectively, retained about 90% of their tritium. Comparison of the absolute configuration of the β-tritiated α-ketoglutarate with that of *threo*-D$_s$-isocitric acid revealed that in the oxidative decarboxylation of isocitric acid by isocitric dehydrogenase the replacement of the carboxyl group by a proton occurs with retention of configuration.

In the oxidative decarboxylation of *threo*-D$_s$-isocitric acid with TPN by isocitric dehydrogenase, a proton from the medium (H_m) takes the place of $-CO_2H$ in the β-position of α-ketoglutarate:



$$\begin{array}{c}\mathrm{COOH}\\ |\\ \mathrm{H{-}C{-}OH}\\ |\\ \mathrm{HOOC{-}C{-}H}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{TPN} \xrightarrow{H_m{}^+} \begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{C{=}O}\\ |\\ \mathrm{C{-}H_m(H)}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{TPNH} + \mathrm{CO_2} \quad (1)$$

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TABLE I
MAXIMAL TRITIUM EXCHANGE WITH CHEMICALLY AND ENZYMATICALLY PREPARED β-TRITIATED α-KETOGLUTARATES[a]

Experiment	Incubation Time (minutes)	Tritium in 0.4 ml of Effluent (cpm)	Per Cent of Total Tritium in the Medium
1	0[b]	5	0.3
	15	1180	64
	120	1730	94
	180	1730	94
2	0[b]	15	0.4
	120	1850	44
	180	1970	47
	240	2100	50

[a] In experiment 1, α-ketoglutarate labeled with tritium by carrying out the oxidative decarboxylation in tritiated water (0.63 μmole, 46,000 cpm) was incubated at 30° in 1 ml with TPNH (0.5 mM), MgCl$_2$ (0.6 mM), triethanolamine hydrochloride buffer (100 mM, *p*H 7.1), and isocitric dehydrogenase (0.6 unit). At various times the extent of tritium exchange was measured by separation of the medium from the α-ketoglutarate in the following manner. A 0.2-ml sample of the incubation mixture was diluted to 0.5 ml with water and applied to a 0.5-ml Dowex-1-acetate column; the column was washed three times with 0.5 ml of water, and the radioactivity of 0.4 ml of the resulting 2.0 ml effluent was counted with a liquid scintillation counter. In experiment 2, α-ketoglutarate randomly labeled with tritium in the β positions (0.63 μmole, 105,000 cpm) was treated with isocitric dehydrogenase under the same conditions as in experiment 1. No correction was made in either experiment for the nonenzymic detritiation, since a control experiment showed that it accounted for less than 2% of the total detritiation. [b] Before enzyme addition.

By establishing the absolute stereochemistry of such α-ketoglutarate-3-H$_m$ and comparing it with that now known for natural isocitrate (Kaneko *et al.*, 1960; Kaneko and Katsura, 1960), one may hope to limit the number of mechanisms that must be considered for the decarboxylation step of the reaction. From earlier work (Rose, 1960) it was established that only one of the β-hydrogens of α-ketoglutarate reacted in an exchange reaction with the medium. It will be shown that the same position is involved in the net decarboxylation reaction and that the replacement of —CO$_2$H by a proton occurs with retention of configuration.

MATERIALS AND METHODS

Tritiated Forms of α-Ketoglutarate.—Randomly β-tritiated α-ketoglutarate was prepared according to Rose (1960) by heating the disodium salt in tritiated water. α-Ketoglutarate tritiated in the β position that is not activated in the exchange reaction with isocitric dehydrogenase was prepared by incubation of the randomly labeled compound in nontritiated medium under exchange conditions similar to those shown in Table I. The third form of α-ketoglutarate-3-^{3}H was prepared by performing the net reaction (1) in tritiated medium as follows: Five 0.01-ml portions of 80 mM *threo*-D$_s$-isocitrate were added at 3-minute intervals to an incubation mixture at 25°, of total volume 0.45 ml, which contained TPNH (0.5 mM), MgCl$_2$ (6 mM), oxidized glutathione (20 mM), triethanolamine hydrochloride buffer (100 mM, *p*H 7.1), isocitric dehydrogenase (0.4 unit), glutathione reductase (1.0 unit), and enough tritiated water (1 curie/ml) to give a specific activity of 105,000 cpm/μatom of hydrogen. Three minutes after the final addition the reaction mixture was applied to a neutral Dowex-1-Cl$^-$ column (6 cm × 0.2 cm^2). After the column had been washed thoroughly with water to remove the tritiated water, the α-ketoglutarate was isolated by elution from the Dowex-1-Cl$^-$ column with 0.02 N HCl. The yield of α-ketoglutarate was 3 μmoles, and its specific activity was 73,000 cpm/μmole. The fact that the specific activity was less than that of the medium hydrogen is probably the result of discrimination against tritium in the enzymic reaction.

Enzymes.—Pig heart isocitric dehydrogenase specific for TPN was purchased from Boehringer und Soehne. It was observed to be free of the DPN-specific isocitric dehydrogenase and aconitase (less than 0.5% contamination). The enzyme had a specific activity of 1 unit/mg of protein when assayed by the increase in optical density at 340 mμ (TPNH formation) under the following conditions: TPN (0.5 mM), *threo*-D$_s$-isocitrate (1 mM), MgCl$_2$ (5 mM), and triethanolamine hydrochloride buffer (50 mM, *p*H 7.1). Throughout this paper a unit of enzyme is defined as an amount that catalyzes the reaction of 1 μmole of substrate per minute under the conditions specified, except in the case of isocitric dehydrogenase where the units always refer to the enzyme assayed under the above conditions.

Other enzymes used were: crystalline fumarase, prepared from pig heart according to the method of Massey (1952); aspartase, prepared from *B. cadaveris* and a gift of Dr. T. Emery; malic enzyme, prepared from chicken liver and a gift of Dr. M. Utter; and commercial preparations of glutathione reductase (type III of Sigma Chemical Co.) and glutamic dehydrogenase (Boehringer und Soehne).

Chemical and Enzyme Assays.—Aspartic acid was measured by the ninhydrin test (Rosen, 1957). When ammonia was present with the aspartic acid, a correction for the contribution of the ammonia to the ninhydrin test was made by measuring the ammonia by nesslerization. L-Malate was measured with malic enzyme by the increase in optical density at 340 mμ due to TPNH formation. Fumarate was determined by adding fumarase to the assay system for L-malate. α-Ketoglutarate was assayed with glutamic dehydrogenase by the decrease in optical density at 340 mμ due to DPNH oxidation, and *threo*-D$_s$-isocitrate was determined with isocitric dehydrogenase by the increase in optical density at 340 mμ due to TPNH formation. After activation according to the method of Morrison (1954), aconitase was measured by following the rate of formation of isocitrate from citrate spectrophotometrically at 340 mμ with excess TPN and isocitric dehydrogenase, and was found to be absent from the glutathione reductase. *threo*-D$_s$-Isocitric acid was a gift of Dr. H. Vickery.

RESULTS

Relative Stereospecificities of the Net and Exchange Reactions

Table I presents the results of an experiment designed to compare the stereospecificity of the exchange reaction with that of the carboxyl-replacement reaction. Exhaustive exchange was allowed to occur with either randomly β-tritiated α-ketoglutarate or α-ketoglutarate which resulted from the decarboxylative reaction of isocitrate in tritiated water. As will be noted, all the label of the latter compound was lost under conditions leading to only half-detritiation of the randomly labeled compound. Thus the stereospecificity at the carbon-3 position of isocitrate in decarboxylation is identical to

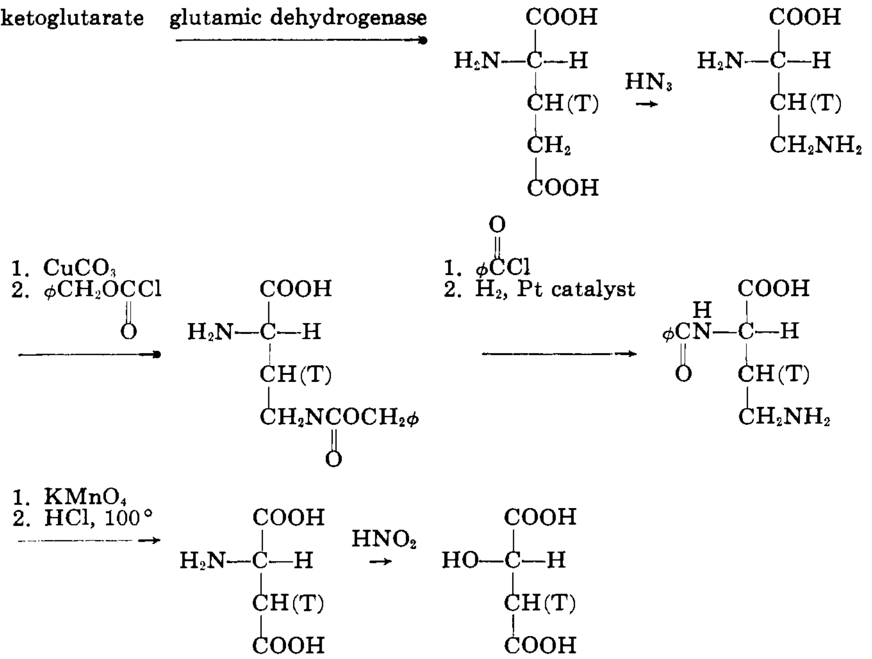

FIG. 1.—Conversion of stereospecifically β-tritiated α-ketoglutaric acid to L-aspartic and L-malic acids.

that for the exchange reaction at carbon-3 of α-ketoglutarate.

It should be noted that it is vital to the above conclusion that the tritium introduced into the α-ketoglutarate by carrying out the oxidative decarboxylation in tritiated water was actually introduced predominantly in the oxidative decarboxylation and not as the result of subsequent exchange of the α-ketoglutarate with the tritiated medium. In fact, it can be calculated from the relative rates of the oxidative decarboxylation and the exchange reaction that at most 35% of the tritium in this α-ketoglutarate could have been introduced by the exchange reaction. Moreover, since the exchange reaction requires TPNH and is strongly inhibited by *threo*-D$_s$-isocitrate (Rose, 1960), the conditions used for the conversion of isocitrate to α-ketoglutarate in tritiated water probably reduced the contribution of the exchange reaction to the labeling to a value much less than 35%.

Stereochemistry of the Enzymatic Labeling α-Ketoglutarate

To determine the position of α-ketoglutarate which is activated in the reaction with isocitric dehydrogenase, the tritiated compound must be related by reactions of unquestioned stereochemistry to a compound containing a hydrogen isotope in a known position. The approach was as follows: α-ketoglutarate stereospecifically tritiated in the β position that does not undergo the enzymic exchange reaction was converted to L-glutamic acid and thence to L-aspartic and L-malic acids by a series of reactions that did not change the absolute configuration of the β position (Fig. 1). The absolute configuration at the β position of the malic and aspartic acids was determined by relating them to chemically synthesized *threo*-β-deuterio-L-malic acid via the known stereospecificity of the fumarase and asparatase reactions (Englard, 1958; Krasna, 1958; Anet, 1960; Gawron *et al.*, 1961). Specifically, since it is known that *threo*-β-deuterio-L-aspartic acid and *threo*-β-deuterio-L-malic acid do not lose their deuterium upon conversion to fumarate as catalyzed by aspartase and furmarase, respectively, the absolute configuration of the β-carbon of the β-tritiated L-aspartic and L-malic acids derived from the α-ketoglutarate-3-^{3}H was established by whether or not these compounds retained their tritium in the aspartase and furmarase reactions, respectively. Figure 2 outlines these stereochemical relationships. It is evident that since the tritium label was initially in the nonexchangeable 3 position of α-ketoglutarate, the presence of the tritium in the fumarate would mean that in the oxidative decarboxylation the replacement of the carboxyl group of isocitrate by hydrogen from the medium occurs with retention of configuration, whereas the absence of tritium would mean inversion of configuration.

Conversion of α-Ketoglutarate to L-*Aspartic and* L-*Malic Acids.*—The α-ketoglutarate which was labeled with tritium in the 3 position that does not undergo the enzymic exchange reaction (see Materials and Methods) was converted to L-glutamate in the following way. To 4.5 μmoles of the α-ketoglutarate (specific activity 600,000 cpm/μmole) in 4.0 ml of triethanolamine hydrochloride buffer, *p*H 7.0 (150 μmoles), were added DPNH (4.7 μmoles), ammonium sulfate (100 μmoles), and glutamic dehydrogenase (3 units) in a volume of 3.0 ml. After 10 minutes of incubation, the reaction mixture was put on a Dowex-1-acetate column (7 cm × 0.75 cm^2), and the glutamic acid was eluted between 14 and 20 ml with 0.5 N acetic acid. The aqueous acetic acid was removed from the glutamic acid by flash evaporation, and the specific activity of the 3-tritiated L-glutamic acid was adjusted to 120 cpm/μmole by the addition of 20 mmoles of carrier L-glutamic acid.

The 3-tritiated L-glutamic acid (10 mmoles) was converted to L-2,4-diaminobutyric acid by a Schmidt degradation, according to Adamson (1939). Following the reaction with sodium azide in H_2SO_4, the diaminobutyric acid was isolated as the dihydrochloride by chromatography on Dowex-50-H$^+$ according to Pigretti and Stoppani (1961). Based on the radioactivity in the product, the yield of 2,4-diaminobutyric acid was 3.75 mmoles. The 2,4-diaminobutyric acid dihydrochloride was converted to a solution of its copper chelate by adaptation of the method of Synge (1948). To 7 ml of 0.53 M 2,4-diaminobutyric acid at 60° was added with shaking 0.55 g (2.5 mmoles) of $CuCO_3 \cdot Cu(OH)_2$. The reaction mixture was cooled, and the copper salts that precipitated were removed by filtration. Crystal-

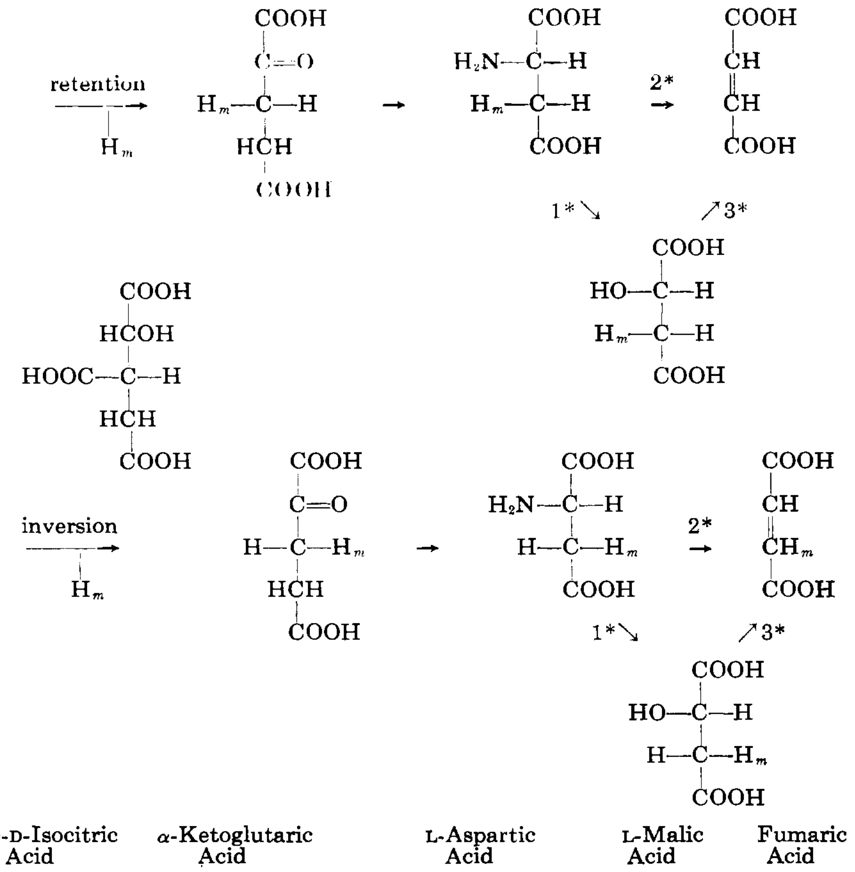

FIG. 2.—Stereochemical possibilities of the isocitric dehydrogenase reaction. 1*—Nitrous acid deamination. 2*—Aspartase. 3*—Fumarase.

line 4-carbobenzoxy-L-2,4-diaminobutyric acid was prepared from the solution of the copper chelate according to the carbobenzoxylation procedure of Barrass and Elmore (1957). The yield, based on radioactivity in the product and an assumed specific activity of 120 cpm/μmole, was 2.7 mmoles of the carbobenzoxy derivative. The melting range of the unrecrystallized derivative was 232–235° (dec.). The value reported is 235–236° (dec.) (Barrass and Elmore, 1957).

The 4-carbobenzoxy-L-2,4-diaminobutyric acid was converted to 2-benzoyl-L-2,4-diaminobutyric acid by adaptation of a method for the same conversion of ε-carbobenzoxylysine (Greenstein, 1937). A solution of 2.5 mmoles of the 4-carbobenzoxy derivative in 7.5 ml of 0.6 N NaOH was treated at 10° with 0.43 ml (3.75 mmoles) of benzoyl chloride and 6 ml of 0.6 N NaOH, both of which were added in portions and with stirring over 1 hour. After the reaction mixture had been stirred at room temperature for 30 minutes more, it was adjusted to about *p*H 3 with 4 N HCl. The 2-benzoyl-4-carbobenzoxy derivative separated as a gum, from which the remainder of the reaction mixture was decanted. The gum was extracted several times with ligroin in order to remove adhering benzoic acid, and was then dissolved in 30 ml of 50% methanol in water which contained 12 mmoles of HCl. The carbobenzoxy group was cleaved by subjecting this solution to hydrogenation over palladium catalyst for 1.5 hours. After the catalyst had been removed from the reaction mixture by filtration, the methanol was removed by evaporation at 30°. The concentrated solution was extracted with ethyl ether to remove benzoic acid. The acidic solution of the benzoyl derivative was neutralized with NaOH, with the hope that the benzoyl derivative would then crystallize. However, after concentrating the neutral solution to 10 ml by flash evaporation and cooling and scratching, no crystals appeared. The yield of the 2-benzoyl-L-2,4-diaminobutyric acid, based upon radioactivity in the above solution and an assumed specific activity of 120 cpm/μmole, was 2.3 mmoles.

The 2-benzoyl derivative was oxidized to 2-benzoyl-L-aspartic acid with permanganate by an adaptation of a method for the oxidation of *n*-propylamine to propionic acid (Mosbach *et al.*, 1951). To a solution (5 ml) which contained 1.1 mmoles of the 2-benzoyl derivative was added 15 ml of 0.10 M $KMnO_4$. The *p*H of the reaction mixture was immediately adjusted to 11 with 2 N NaOH and was maintained at this *p*H for 18 hours by periodic addition of NaOH. Precipitation of MnO_2 showed that oxidation was occurring. After 18 hours of reaction the unreacted permanganate was reduced to MnO_2 by the addition of 0.20 ml of 1 M sodium formate. After the insoluble MnO_2 had been removed by centrifugation, the supernatant was neutralized with HCl and concentrated to 5 ml by evaporation. Upon adjustment of the concentrate to less than *p*H 1 with 6 N HCl, a white crystalline precipitate, which was crude benzoyl-L-aspartic acid, appeared. The yield, based upon radioactivity in the product and assumed specific activity of 120 cpm/μmole, was 675 μmoles.

The benzoylaspartic acid was hydrolyzed and crude crystalline L-aspartic acid was isolated by following the procedure of Klosterman and Painter (1947). When this aspartic acid was analyzed by paper electrophoresis (1000 v for 1 hour in a *p*H 6 buffer consisting of 100 ml glacial acetic acid, 10 ml pyridine, and 890 ml water), it showed a strongly ninhydrin-positive spot for aspartic acid and a very weakly positive one for 2,4-diaminobutyric acid. A portion of the aspartic acid (150 μmoles) was further purified by chromatography on a Dowex-1-acetate column (9 cm × 1.5 cm^2). The aspartic acid was eluted between 80 and 100 ml with 0.5 N acetic acid; the acetic acid was removed from the aspartic acid by evaporation. The specific activity of the purified aspartic acid was 127 cpm/μmole, a value which, within experimental error, is the same as that of the starting L-glutamic acid. Thus there was no tritium loss during the conversion and therefore no racemization of the configuration of the β position.

A portion of the crude crystalline β-tritiated L-aspartic acid (300 μmoles) was converted to L-malic acid by the nitrous acid deamination procedure described by Englard (1958), and the malic acid was isolated from the reaction mixture by continuous extraction with ethyl ether (Englard, 1958). Further purification of the malic acid was achieved by chromatography on silica gel, according to the method of Varner (1955), followed by ion-exchange chromatography on Dowex-1-formate according to Busch *et al.* (1952). After removal of the formic acid by evaporation, titration revealed the presence of 152 μmoles of dicarboxylic acid in the malic acid fractions. Enzymic assay with malic enzyme indicated that 151 μmoles of L-malic acid was present. Thus all the acid in the peak was accounted for by L-malic acid, a fact which shows its purity. Moreover, since the nitrous acid deamination of aspartic acid proceeds with retention of configuration (Englard, 1958), the precursor aspartic acid must have been entirely the L isomer. The specific activity of the purified L-malic acid was 122 cpm/μmole, the same as the starting aspartic acid. Therefore there was no loss, and thus no randomization, of the tritium during the conversion.

Aspartase and Fumarase Treatments. – The β-tritiated L-aspartic acid (40 μmoles) which had been purified by chromatography on Dowex-1-acetate was incubated at *p*H 7 and 25° in a total volume of 1 ml with aspartase (2 units) and with fumarase (2.5 units), which was added to cause a greater net reaction of aspartate by removing the fumarate. After 45 minutes a sample of the incubation mixture in which the enzymes had been inactivated by heating was assayed for combined fumarate and L-malate; 20 μmoles had formed. After 55 minutes of incubation the water in the reaction mixture was recovered by microdistillation under reduced pressure at room temperature. The water was assayed at 405 cpm, which is only 16% of the tritium that possibly could have been found in the medium as the result of the reaction of the 20 μmoles. The residue from the distillation was dissolved in 2.0 ml of 0.05 N HCl and applied to a Dowex-50-H$^+$ column (5 cm × 0.3 cm^2). The fumaric and L-malic acids were washed from the column with water; the L-aspartic acid (and ammonium ion) was eluted with 1.5 N HCl. The specific activity of the combined fumaric and L-malic acids was 110 cpm/μmole, 87% of that of the starting aspartate, and the specific activity of the recovered aspartic acid was 114 cpm/μmole, 90% of that of the starting aspartic acid. Thus, as a first approximation, all the tritium in the L-aspartic acid was in the β position that was not removed in the aspartase reaction. Hence the aspartic acid was *threo*-β-tritiated L-aspartic acid, and the replacement of the carboxyl group in the oxidative decarboxylation of isocitrate must occur with *retention of configuration.* The 15% of the tritium which was in the other β position may be explained by a lack of complete stereochemical purity of the starting stereospecifically β-tritiated α-ketoglutarate. The exchange reaction used in the preparation of the stereospecifically labeled α-ketoglutarate may not have gone to completion and/or some randomization of the tritium in the stereospecifically labeled α-ketoglutarate may have occurred by a slow nonenzymatic ionization of the β-hydrogens, since they are α to a carbonyl group.

The purified β-tritiated L-malic acid was treated with fumarase in order to confirm the result obtained in the aspartase experiment. The L-malate (75 μmoles; specific activity, 122 cpm/μmole) was incubated at 25° in 5.5 ml with fumarase (15 units) and potassium phosphate buffer (10 mM), *p*H 7.3. The reaction was followed by the increase in optical density at 275 mμ due to fumarate formation. After 5 minutes equilibrium was reached. After 135 minutes of further incubation in order to insure isotopic equilibrium, the fumarate and L-malate were isolated and purified by chromatography on Dowex-1-formate according to the method of Busch *et al.* (1952), a method by which the two acids are well separated. The specific activity of the purified fumaric acid was 105 cpm/μmole, 86% of that of the starting L-malate; and the specific activity of the recovered L-malic acid was 129 cpm/μmole, 105% of that of the starting L-malate. The retention of the tritium in the malic and fumaric acids confirms the absolute configuration of the β-carbon determined by the aspartase treatment of the β-tritiated aspartic acid.

DISCUSSION

The present results lead to the conclusion that the oxidative decarboxylation of *threo*-D$_s$-isocitrate catalyzed by TPN-specific pig heart isocitric dehydrogenase occurs with retention of configuration in the replacement of the carboxyl group by a hydrogen atom.[1] The possibility that the replacement proceeds randomly is eliminated by the data in Table I which show that the stereochemistry of the oxidative decarboxylation and the exchange reaction are identical and confirm Rose's observation (1960) that the exchange reaction is stereospecific. The stereochemistry of the β-carbon of β-tritiated α-ketoglutarate specifically labeled by the exchange reaction was established by a series of chemical and enzymatic steps relating the β position of the α-ketoglutarate to that of chemically synthesized *threo*-β-deuterio-L-malic acid.

The stereochemistry of retention indicates a carbanion intermediate as the initial product of decarboxylation, since steric interference by the leaving carboxyl group makes it difficult to envision bond formation to a proton occurring simultaneously with cleavage of the carbon-carbon bond. Such a finding is consistent with the existence of an exchange reaction that is CO$_2$ independent (Rose, 1960) and supports a mechanism in which the immediate product of the oxidative decarboxylation is an enzyme-bound enol of α-ketoglutarate, a form which subsequently is stereospecifically protonated to yield the keto tautomer.

In its stereochemistry the isocitric dehydrogenase reaction is similar to the aldolase reaction, in which a proton approaches the intermediate formed between dihydroxyacetone phosphate and the enzyme upon cleavage of fructose-1,6-diphosphate from the same relative direction as that in which the glyceraldehyde-3-phosphate departed (Rose, 1958). In contrast, in the 6-phosphogluconate dehydrogenase reaction, which is an analogous β-oxidative decarboxylation, the replacement of the carboxyl group of D-6-phosphogluconate by a proton occurs with inversion of configuration (Lienhard and Rose, 1964). This difference between the two analogous reactions is consistent with the enol

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[1] Englard and Listowsky (1963) have recently reached the same conclusion by a different method.

mechanism, since the subsequent fate of an enzyme-bound enol intermediate need not be related stereochemically to the mode of enol formation by the earlier decarboxylation step. Other mechanisms, requiring active participation of a proton-donating group during decarboxylation, might be expected to result in a uniform stereochemistry for all β-oxidative decarboxylative reactions.

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The Mechanism of Action of 6-Phosphogluconate Dehydrogenase*

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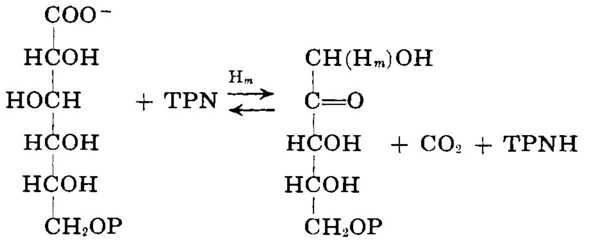
Stereospecifically 1-tritiated ribulose-5-phosphates have been prepared by the enzymic oxidative decarboxylation of D-6-phosphogluconate-2-^{3}H in unlabeled medium and of unlabeled D-6-phosphogluconate in tritiated water. The absolute configurations of carbon 1 of these ribulose-5-phosphates were established from the known stereochemistry of the glycolic acid oxidase reaction by converting carbons 1 and 2 of the ribulose-5-phosphates to the tritiated glycolic acids and determining that in the first case tritium was retained in the glyoxylic acid formed by the oxidation of the glycolic acid with the oxidase and that in the second case it was not. Comparison of the absolute configurations of carbon 1 of the 1-tritiated ribulose-5-phosphates with that of carbon 2 of D-6-phosphogluconate showed that the proton which replaces the carboxyl group in the enzymic oxidative decarboxylation does so with a net inversion of configuration. Moreover, it was found that 6-phosphogluconate dehydrogenase catalyzed the exchange with the medium hydrogen of the tritium in the ribulose-5-phosphate-1-^{3}H prepared by oxidative decarboxylation in tritiated water, but did not catalyze the exchange of the tritium in the ribulose-5-phosphate-1-^{3}H prepared from 6-phosphogluconate-2-^{3}H. The exchange required reduced nicotinamide adenine dinucleotide phosphate, but not CO_2. It was inhibited by D-6-phosphogluconate and nicotinamide adenine dinucleotide phosphate. On the basis of these observations, an enzyme-bound enol form of ribulose-5-phosphate is tentatively suggested as an intermediate in the oxidative decarboxylation of 6-phosphogluconate.

TPN-specific 6-phosphogluconate dehydrogenase catalyzes the reversible β-oxidative decarboxylation of D-6-phosphogluconate to D-ribulose-5-phosphate:



$$\begin{array}{llll}
\mathrm{COO^-} & & & \\
\mathrm{HCOH} & & \mathrm{CH(H_m)OH} & \\
\mathrm{HOCH} & + \mathrm{TPN} \overset{H_m}{\rightleftarrows} & \mathrm{C{=}O} & \\
\mathrm{HCOH} & & \mathrm{HCOH} & + \mathrm{CO_2} + \mathrm{TPNH} \\
\mathrm{HCOH} & & \mathrm{HCOH} & \\
\mathrm{CH_2OP} & & \mathrm{CH_2OP} &
\end{array}$$

On the basis of recent studies on the analogous isocitric dehydrogenase reaction (Z. Rose, 1960; Lienhard and Rose, 1964) and in accord with the known mechanisms for the decarboxylation of β-ketoacids (Bender and Breslow, 1962), one might expect that the immediate product of the oxidative decarboxylation would be the enzyme-bound enol form of D-ribulose-5-phosphate, and that the enol form would subsequently be protonated by a proton (H_m) in equilibrium with the medium. As a test of this reaction path, the characteristics of the exchange of the carbon-1 hydrogen of D-ribulose-5-phosphate with the hydrogen of the medium as catalyzed by TPN-specific 6-phosphogluconate dehydrogenase have been investigated. Moreover, the stereochemistry of the replacement of the —CO_2H of 6-phosphogluconic acid by H_m has been established by comparing the known absolute configuration of carbon-2 of D-6-

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