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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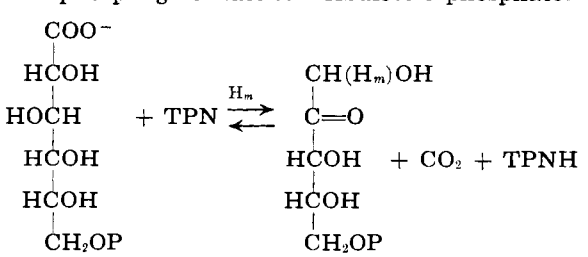
Stereospecifically 1-tritiated ribulose-5-phosphates have been prepared by the enzymic oxidative decarboxylation of D-6-phosphogluconate-2-^{3}H in unlabeled medium and of unlabeled D-6-phosphogluconate in tritiated water. The absolute configurations of carbon 1 of these ribulose-5-phosphates were established 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:



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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phosphogluconate with that of ribulose-5-phosphate tritiated enzymatically in the carbon-1 position.

Materials and Methods

6-Phosphogluconate Dehydrogenase Assay.—6-Phosphogluconate dehydrogenase was assayed spectrophotometrically by the increase in optical density at 340 mμ (TPNH formation) under the following conditions: TPN (0.5 mM), D-6-phosphogluconate (1.0 mM), and the conditions of temperature, buffer, and metal-ion concentration which are specified in the experiment. A unit of the dehydrogenase is defined as the amount which catalyzes the conversion of 1 μmole of substrate to product per minute under the above conditions; for other enzymes the definition is the same except that the conditions are as specified.

Enzymes.—Twice-recrystallized TPN-specific yeast 6-phosphogluconate dehydrogenase was prepared according to the method of Pontremoli *et al.* (1961) from *Candida utilis* kindly provided by the Lake States Yeast Corp., Rhinelander, Wisc. Its specific activity, when assayed at 25° with 15 mM $MgCl_2$ and 50 mM glycylglycine buffer, *p*H 7.6, was 23 units/mg of protein. The enzyme was stored at 0° as a suspension in 50% saturated ammonium sulfate solution–0.25 M glycylglycine buffer, *p*H 7.6. It was this enzyme preparation that was used in all the detritiation experiments; and when the preparation was so used, the suspension was centrifuged and the enzyme was dissolved in a diluent consisting of 0.005 M EDTA[1] and 1 mg/ml bovine serum albumin, *p*H 7.5. The crystalline enzyme was free of any detectable phosphopentose isomerase or D-xylulose-5-phosphate epimerase activity.

TPN-specific rat liver 6-phosphogluconate dehydrogenase was prepared according to the method of Glock and McLean (1953). Glycolic acid oxidase was purified from spinach leaves by the method of Frigerio and Harbury (1958) through the ammonium sulfate fractionation at alkaline *p*H. Lactic dehydrogenase, Type II from Sigma Chemical Co., was further purified by starch-gel electrophoresis by the procedure of Pfleiderer and Jeckel (1957). The purified enzyme contained no detectable phosphopentose isomerase or D-xylulose-5-phosphate epimerase. Yeast glutathione reductase, free of 6-phosphogluconate dehydogenase, was a gift of Dr. E. Racker. Catalase was purchased from C. F. Boehringer und Soehne.

Tritiated Forms of Ribulose-5-phosphate.—Ribulose-5-phosphate-1-^{3}H was prepared from 6-phosphogluconate with 6-phosphogluconate dehydrogenase in two ways: by carrying out the oxidative decarboxylation with 6-phosphogluconate-2-^{3}H and by carrying out the reaction in tritiated water.

For the first preparation, D-6-phosphogluconate-2-^{3}H was prepared by bromine oxidation of D-glucose-6-phosphate-2-^{3}H according to the method of Horecker (1955); the D-glucose-6-phosphate-2-^{3}H had been prepared by converting D-fructose-6-phosphate to D-glucose-6-phosphate in tritiated water with phosphoglucoisomerase (Rose and O'Connell, 1961). In its conversion to ribulose-5-phosphate the 6-phosphogluconate-2-^{3}H (20 μmoles; specific activity, 55,100 cpm/μmole) was incubated at 30° in 8.0 ml with rat liver 6-phosphogluconate dehydrogenase (0.75 unit), yeast glutathione reductase (2.4 units), TPN (10 μmoles), oxidized glutathione (17 μmoles), $MgCl_2$ (20 μmoles), EDTA (10 μmoles), and 0.05 M imidazole hydrochloride buffer, *p*H 7.0. When the optical density at 340 mμ of the reaction mixture began to increase, thus indicating the reaction of 17 μmoles of substrate, the mixture was deproteinized with trichloroacetic acid, extracted with ether to remove the trichloroacetic acid, and chromatographed on a Dowex-1-chloride column (10 cm × 0.75 cm^2). The ribulose-5-phosphate was eluted with 0.01 N HCl–0.02 N NaCl between 50 and 70 ml. It was adjusted to *p*H 5 with NH_4OH and stored in the frozen state.

Pontremoli and Mangiarotti (1962) have described a method for the preparation of the solid lithium salt of D-ribulose-5-phosphate from D-6-phosphogluconate with crystalline yeast 6-phosphogluconate dehydrogenase, a catalytic amount of TPN, and pyruvate plus lactic dehydrogenase as the TPN-regenerating system. For the conversion in tritiated water this method was followed with the exception that the volume was reduced from 8.0 ml to 1.5 ml in order to conserve tritiated water. The product lithium salt was stored under the conditions described by Pontremoli and Mangiarotti (1962). With a medium specific activity of 285,000 cpm/μatom of hydrogen, ribulose-6-phosphate (Li salt) of specific activity 140,000 cpm/μatom was obtained. The difference between the two values is probably the result of discrimination against tritium in the enzymic reaction.

In the course of using this ribulose-5-phosphate-1-^{3}H, which will be referred to as 1-T_m-ribulose-5-phosphate, to study the enzymic exchange reaction, it was discovered that the preparation contained a potent inhibitor of the exchange, which could be removed by chromatography on Dowex-1-chloride. Consequently, a day or two prior to its use the lithium salt was further purified in the following way. Several milligrams were dissolved in water and put on a Dowex-1-chloride column (5 cm × 0.2 cm^2). The ribulose-5-phosphate was eluted with 0.01 N HCl between about 20 and 30 ml. The effluent fractions were adjusted with stirring to *p*H 5 with 0.10 N NaOH and stored in the frozen state. In all cases, unless explicitly stated, it was 1-T_m-ribulose-5-phosphate, purified in this way, that was used in the various experiments. The specific activity of such purified 1-T_m-ribulose-5-phosphate was 127,000 cpm/μmole.

Detritiation Assay.—All or a portion of the detritiation incubation (volume generally 0.5 ml) was put on a small neutral Dowex-1-acetate column (about 0.5 ml of wet resin), and the column was subsequently washed three times with 0.5 ml of water. In this way the tritium in the medium was separated from that in the ribulose-5-phosphate, which was held by the column. A portion of the effluent was counted with a liquid scintillation counter. Since the entire chromatography procedure took about 4 minutes and since the incubation times were much longer, it was not necessary to inactivate the 6-phosphogluconate dehydrogenase before the assay.

When the 1-T_m-ribulose-5-phosphate was treated with a large amount of 6-phosphogluconate dehydrogenase under the conditions for the exchange reaction (see Table II), there was no further detritiation after 80% of the tritium had been released. This fact showed that 80% of the tritium was in the exchangeable position. All the rate data for detritiation have been expressed as μmoles of ribulose-5-phosphate detritiated during the incubation period. Such rates were calculated by multiplying the μmoles of 1-T_m-ribulose-5-phosphate present in the incubation by the fraction of the tritium in the exchangeable position which had exchanged. When more than 15% of the counts were released during an incubation, the observed detritiation rate was corrected, by use of the equation defining the kinetics of isotopic exchange reactions, to take into

[1] Abbreviation used in this work: EDTA, ethylenediaminetetraacetate.

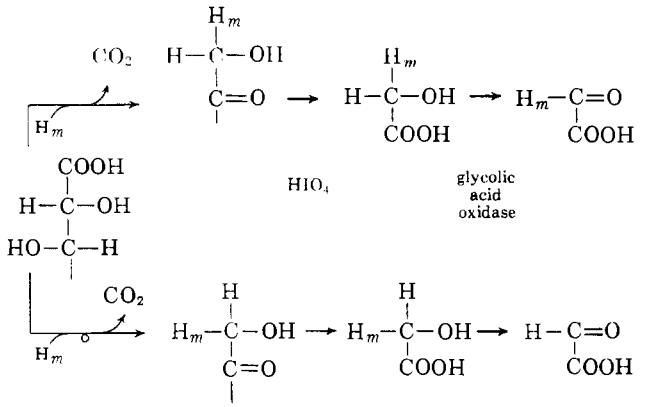

FIG. 1.—Stereochemical possibilities of the 6-phosphogluconate dehydrogenase reaction.

account the fact that the specific activity of the substrate had changed appreciably during the course of the reaction (Rose and Rieder, 1958). Such a correction was validated by the observation that the detritiation of the 1-T_m-ribulose-5-phosphate by 6-phosphogluconate dehydrogenase obeyed this equation up to at least 75% detritiation.

Assays for Compounds.—6-Phosphogluconate was measured enzymatically with 6-phosphogluconate dehydrogenase according to the method of Horecker (1955). Colorimetric tests were used for the measurement of ribulose-5-phosphate (Ashwell and Hickman, 1957), glycolic acid (Calkins, 1943), and glyoxylic acid (McFadden and Howes, 1960).

RESULTS

Absolute Configurations of 1-Tritiated Ribulose-5-phosphates

In order to determine the absolute configurations of the two forms of enzymatically prepared ribulose-5-phosphate-1-^{3}H, the method of Rose and O'Connell (1960) was employed. In this method, carbons 1 and 2 of ribulose-5-phosphate are converted by periodate oxidation to glycolic acid. The glycolic acid is subsequently oxidized to glyoxylic acid with the enzyme glycolic acid oxidase. Since glycolic acid oxidase is expected to select for removal the hydrogen of glycolic acid which is sterically equivalent to the α-hydrogen of the L form of lactic acid, for which the enzyme is specific (Rose, 1958),[2] the absolute configuration of the 1-tritiated ribulose-5-phosphate is determined by whether or not the tritium is retained in the glyoxylic acid. Figure 1 presents these conversions for the cases of retention and inversion of configuration in the 6-phosphogluconate dehydrogenase reaction.

In Table I the results of applying the method to the 1-tritiated ribulose-5-phosphates are presented. Note that when the tritium was introduced into the ribulose-5-phosphate from 6-phosphogluconate (with the rat liver dehydrogenase), 94% of the tritium in the glycolic acid was retained in the glyoxylic acid. Conversely, when the tritium was introduced from the medium (with the yeast dehydrogenase), 85% of the tritium in the glycolic acid was *not* retained in the glyoxylic acid. Thus, as a first approximation, the replacement of $-CO_2H$ by H_m in the decarboxylation as catalyzed by either enzyme occurs with *inversion of configuration* (see Fig. 1). The deviations from 100% (6% in one case and 15% in the other) can be explained by a slow nonenzymic ionization of the C-1 hydrogens of the ribulose-5-phosphates during their preparation. This idea is supported by the finding, to be presented in detail, that the net and exchange reactions are identically and almost completely stereospecific.

[2] This assumption is supported by substrate analogy for muscle L-lactic dehydrogenase (Rose, 1958). Further verification was obtained (Lienhard, 1964) using glycolic acid reductase in which the glycolic acid formed from glyoxylic acid-2-^{3}H and reduced DPN was found to give unlabeled glyoxylic acid upon oxidation by glycolic acid oxidase. Since glyoxylic acid reductase is known to reduce hydroxypyruvic acid to the D form of glyceric acid, this result is consistent with the use of substrate analogy for the designation of the absolute configuration of glycolic acid-2-^{3}H by reaction with glycolic acid oxidase.

TABLE I

ANALYSIS OF GLYCOLIC AND GLYOXYLIC ACIDS DERIVED FROM 1-TRITIATED RIBULOSE-5-PHOSPHATES[a]

Experiment	Compound	Specific Activity (cpm/μmole)
1	6-Phosphogluconate-2-^{3}H	55,100
	Glycolic acid	52,700
	Glyoxylic acid	49,600
2	1-T_m-Ribulose-5-phosphate	127,000
	Glycolic acid	129,000
	Glyoxylic acid	19,000

[a] In experiment 1, ribulose-5-phosphate-1-^{3}H (about 5 μmoles) which had been prepared from 6-phosphogluconate-2-^{3}H with the rat liver dehydrogenase was treated at 30° and *p*H 5 with periodate (100 μmoles) for 1 hour. Excess periodate was destroyed with ethylene glycol, and the glycolic acid was isolated by elution from a Dowex-1-acetate column with 2 N acetic acid. After the glycolic acid had been freed of acetic acid by flash evaporation, it was oxidized to glyoxylic acid under the following conditions: glycolic acid (2 mM), glycolic acid oxidase (0.2 unit), catalase (0.1 mg, to destroy H_2O_2 formed in the oxidation), flavin mononucleotide (0.2 mM), and 0.10 M Tris-acetate buffer, *p*H 8.3. After the reaction had proceeded to about 95% completion, the glyoxylic acid was isolated by chromatography under the conditions described above for the glycolic acid. In experiment 2, 1-T_m-ribulose-5-phosphate was analyzed by the procedure used in experiment 1.

Conditions for the Detritiation of 1-T_m-Ribulose-5-phosphate

Table II shows the results of a representative experiment to determine the requirements for the detritiation reaction. Note that of the substances necessary for the net reductive carboxylation only CO_2-HCO_3^- does not seem to be required for the detritiation, al-

TABLE II

REQUIREMENTS FOR THE DETRITIATION OF 1-T_m-RIBULOSE-5-PHOSPHATE[a]

Conditions	Detritiation (mμmoles/30 min)
(1) Control (0.5 mM TPNH, 10 mM $NaHCO_3$)	3.5
(2) No additions	3.5
(3) $NaHCO_3$ (10 mM)	3.6
(4) TPNH (0.5 mM)	103
(5) TPNH (0.5 mM); $NaHCO_3$ (10 mM)	172

[a] Each incubation contained in a total volume of 0.50 ml the following: 1-T_m-ribulose-5-phosphate (0.50 mM, 127,000 cpm/μmole), imidazole hydrochloride buffer (100 mM, *p*H 7.0), EDTA (2 mM), bovine serum albumin (1 mg/ml), 0.09 unit of enzyme in 0.10 ml of diluent or, in the control, 0.10 ml of diluent alone, and other additions as noted. In those cases where $NaHCO_3$ was present, the incubation was carried out in an atmosphere of 10% CO_2. Incubations were for 30 minutes at 30°.

though it is stimulatory. TPNH is absolutely necessary; other experiments have shown that TPN, nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide, adenosine-2′-phosphate, and inorganic phosphate cannot substitute for TPNH.

The CO_2-HCO_3^- stimulation of detritiation suggested that CO_2 (or HCO_3^-, since it is not known which form is the actual carboxylating species) might also be required for detritiation, but that this requirement was already satisfied by traces of CO_2 in the assay reagents. Consequently, an attempt was made to exclude CO_2 completely from a detritiation experiment by gassing with helium. First a test of the effectiveness of this method was made. In the main compartment of a Warburg flask were placed, in a volume of 0.80 ml, enzyme (0.3 unit), TPNH (0.6 mM), $Na_2{}^{14}CO_3$ (0.6 mM, 425,000 cpm), imidazole hydrochloride buffer (120 mM, *p*H 7.0), EDTA (2 mM), and bovine serum albumin (1 mg/ml). In the side arm were placed, in 0.20 ml, the buffer (120 mM) and $Na_2{}^{14}CO_3$ (2.5 mM). The flask was continuously gassed, with shaking and at 30°, with helium from which all CO_2 had been removed by passage through saturated barium hydroxide solution. After 1 hour of gassing less than 0.5% of the radioactivity remained. It can be calculated on this basis that if the incubation (buffered at *p*H 7.0) initially was in equilibrium with atmospheric CO_2 and contained no added $Na_2{}^{14}CO_3$, such gassing would reduce the CO_2-HCO_3^- concentration to less than 6×10^{-5} mM.

Two detritiation incubations, identical to that described above except that there was no $Na_2{}^{14}CO_3$ and the side arm contained 1-T_m-ribulose-5-phosphate (1.5 mM), were gassed for 1.5 hours, and then the substrate was tipped into the center compartment. In one case gassing was continued; in the other, it was ceased and $NaHCO_3$ (0.05 ml of 0.1 M) was added. After 45 minutes the extents of detritiation were determined. In the incubation without added $NaHCO_3$, 0.35 μmole was detritiated; in the other incubation 0.39 μmole was detritiated. If one assumes that in the instance with added $NaHCO_3$ the $NaHCO_3$ equilibrated within the volume of the Warburg flask (25 ml), it can be calculated that the CO_2-HCO_3^- concentration in solution was about 0.6 mM. Thus the detritiation rate at 6×10^{-5} mM CO_2-HCO_3^- was 90% of that at 0.6 mM. Consequently, if CO_2 is obligatory for detritiation it must have a K_m of about 10^{-8} M, a value which seems unlikely upon the basis of the measured K_m values for CO_2 in other enzymic reactions (Stickland, 1959a,b). Moreover, the stimulation of detritiation by CO_2 at higher concentrations (12 mM CO_2-HCO_3^-, Table II) indicates that CO_2 first interacts significantly with the enzyme at this higher concentration range. The tentative conclusion is that the detritiation reaction is independent of CO_2.

Characteristics of the Detritiation Reaction

Several properties of the detritiation reaction were examined in order to define the reaction further and especially to ascertain whether the reaction could be considered as representative of the initial step in the reductive carboxylation of ribulose-5-phosphate.

Stoichiometry of Detritiation.—Table III shows the results of the measurement of ribulose-5-phosphate at the beginning and end of a detritiation experiment. The fact that there was no detectable disappearance of the compound indicates that detritiation occurs as the result of an exchange reaction and not as part of a net conversion of ribulose-5-phosphate to some other substance.

Michaelis Constants for Detritiation.—With the use of the Lineweaver-Burk plot (1934), the K_m values were determined for TPNH and ribulose-5-phosphate in the detritiation reaction. With a constant TPNH concentration of 0.5 mM and with imidazole hydrochloride buffer (50 mM), *p*H 7.0, the value for ribulose-5-phosphate was 1.8×10^{-4} M. With a fixed ribulose-5-phosphate concentration of 0.72 mM and with imidazole hydrochloride buffer (100 mM), *p*H 7.0, the K_m for TPNH was 1.7×10^{-6} M.

Stereochemistry of the Detritiation Reaction.—If both the exchange reaction and oxidative decarboxylation occur in a completely stereospecific manner with respect to the carbon-1 hydrogens of ribulose-5-phosphate, and if the two reactions are identical in this stereochemistry, then 1-tritiated ribulose-5-phosphate generated from 2-tritiated 6-phosphogluconate with the dehydrogenase must not undergo detritiation. The

TABLE III

STOICHIOMETRY OF THE DETRITIATION OF 1-T_m-RIBULOSE-5-PHOSPHATE[a]

Time	Total Ribulose-5-phosphate (μmoles, in duplicate)	Total Detritiation (μmoles/hour)
0	1.20, 1.22	
1 hour	1.25, 1.25	2.0

[a] 1-T_m-Ribulose-5-phosphate (1 mM) was incubated in a total volume of 1.2 ml at 30° with enzyme (0.44 unit), TPNH (0.1 mM), imidazole hydrochloride buffer (100 mM, *p*H 7.0), EDTA (2 mM), and bovine serum albumin (1 mg/ml). Immediately after initiation of the reaction and after 1 hour the colorimetric test for ribulose-5-phosphate was run on duplicate 0.20-ml samples. Also, after 1 hour of incubation, an aliquot was assayed for detritiation.

TABLE IV

STEREOCHEMISTRY OF THE DETRITIATION OF 1-TRITIATED RIBULOSE-5-PHOSPHATE[a]

Conditions	Total Detritiation (mμmoles)
(1) Assay at once for detritiation	2
(2) Add 4.2 units of enzyme in 0.18 ml of diluent. Incubate 45 minutes at 30° and assay for detritiation	26
(3) Same as (2), except add 0.18 ml of diluent without enzyme	12
(4) Add, in 0.08 ml, 0.53 μmole of 1-T_m-ribulose-5-phosphate which was not purified by chromatography on Dowex-1-chloride. Also, add 4.2 units of enzyme in 0.18 ml of diluent. Incubate at 30° for 45 minutes and assay for detritiation	485
(5) Same as (4), except add 0.18 ml of diluent without enzyme	50

[a] 6-Phosphogluconate-2-^{3}H (2.5 μmoles, 125,000 cpm) was incubated in 2.0 ml with TPN (2.8 mM), enzyme (2.4 units), imidazole hydrochloride buffer (75 mM, *p*H 7.0), EDTA (1.5 mM), and bovine serum albumin (0.75 mg/ml). After 10 minutes the optical density at 340 mμ of a diluted aliquot showed that 95% of the 6-phosphogluconate had been converted to ribulose-5-phosphate. Then 0.35-ml portions (containing 0.41 unit of enzyme, 0.44 μmole of ribulose-5-phosphate with 22,000 cpm, 0.44 μmole of TPNH, and 0.54 μmole of TPN) were removed and treated as described above. In the calculation of the detritiation in samples 4 and 5, correction was made for the decrease of the specific activity of each stereochemically distinct carbon-1 hydrogen of ribulose-5-phosphate which resulted from the mixing of 1-T_m-ribulose-5-phosphate and ribulose-5-phosphate prepared from 6-phosphogluconate-2-^{3}H.

TABLE V

INHIBITION BY 6-PHOSPHOGLUCONATE AND TPN OF THE DETRITIATION OF 1-T_m-RIBULOSE-5-PHOSPHATE[a]

Experiment	Conditions	Detritiation (mμmoles/30 minutes)	Inhibition (%)
1	(1) Control (0.10 mM TPNH)	3.5	
	(2) TPNH (0.10 mM)	105	
	(3) TPNH (0.10 mM), TPN (1.9 mM)	49	55
2	(1) Control (0.20 mM TPNH)	4.3	
	(2) TPNH (0.20 mM)	81	
	(3) TPNH (0.20 mM), D-6-phosphogluconate (0.26 mM)	12.5	89

[a] Each incubation contained, in a total volume of 0.50 ml, 1-T_m-ribulose-5-phosphate (0.6 mM, 127,000 cpm/μmole), imidazole hydrochloride buffer (100 mM, *p*H 7.0), EDTA (2 mM), bovine serum albumin (1 mg/ml), 0.09 unit of enzyme in 0.09 ml of diluent or, in the control, 0.09 ml of diluent alone, and other components as noted. All incubations were for 30 minutes at 30°.

data in Table IV show that 4.2 units of enzyme catalyzed the detritiation of 14 mμmoles of ribulose-5-phosphate prepared from 2-tritiated 6-phosphogluconate, whereas the same amount of enzyme catalyzed the detritiation of 421 mμmoles (485 − 50 − 14) of 1-T_m-ribulose-5-phosphate. Thus, the rate of detritiation of the 1-tritiated ribulose-5-phosphate derived from 2-tritiated 6-phosphogluconate was only about 3% of that of the 1-T_m-ribulose-5-phosphate. Moreover, it was only twice that of the nonenzymic control. Thus, within a few per cent, the net and exchange reactions are completely and identically stereospecific.

Inhibition of Detritiation by D-6-Phosphogluconate and TPN.—Both D-6-phosphogluconate and TPN inhibited the exchange reaction (Table V). D-6-Phosphogluconate was an extremely effective inhibitor, causing an 89% decrease in detritiation when its concentration was about one-fourth that of the ribulose-5-phosphate. The fact that D-6-phosphogluconate and TPN inhibited in the absence of each other is in agreement with the tentative conclusion reached by Pontremoli *et al.* (1961) from kinetic studies on the net reaction that there is no compulsory order in the binding of 6-phosphogluconate and TPN.

DISCUSSION

The CO_2-independent exchange reaction that has been observed is most reasonably interpreted as occurring through the reversible formation of an enzyme-bound enol form of ribulose-5-phosphate. Moreover, the stereochemistry and the inhibitors of the exchange reaction are consistent with this enzyme-bound enol form being the initial intermediate in the reductive carboxylation of ribulose-5-phosphate and consequently, by the principle of microscopic reversibility (Hine, 1962), the immediate product of the oxidative decarboxylation of 6-phosphogluconate. This hypothesis is in keeping with the fact that an enol form as the initial product of decarboxylation is inherent in the known mechanisms for the facile nonenzymic decarboxylation of β-ketoacids (Bender and Breslow, 1962). However, it should be noted that the stereochemistry of inversion in the replacment of $-CO_2H$ by H_m makes it impossible to exclude the possibility that the net reaction occurs by a mechanism involving a direct electrophilic displacement of the carboxyl group by a proton, without the existence of a distinct enol intermediate.

It is interesting to compare these results with those from a similar study on the analogous isocitric dehydrogenase reaction. In this case, the fact that the enzyme catalyzed the detritiation of β-tritiated α-ketoglutarate was evidence for the enol intermediate (Z. Rose, 1960). However, in addition, the finding of a stereochemistry of retention in the replacement of $-CO_2H$ by H_m definitely excluded a simultaneous decarboxylation and protonation (Lienhard and Rose, 1964).

In considering the role of TPNH in the exchange reaction in the absence of CO_2, one must consider it to be other than that of a reductant, since on mechanistic grounds reduction would not be expected to precede carboxylation. It seems likely that TPNH forms a structural part of the "active site" that catalyzes enolization. Z. Rose (1960) has suggested a similar role for TPNH to account for its requirement in the detritiation of β-tritiated α-ketoglutarate by isocitric dehydrogenase. Also, in a study on the enolization of pyruvate by pyruvate kinase, adenosine triphosphate was required for enolization to occur (I. Rose, 1960). The fact that several substituted phosphates could substitute for adenosine triphosphate in the activation of enolization, but not in the phosphorylation of pyruvate, indicated that adenosine triphosphate was an activator of enolization as well as a phosphorylating agent.

The stereochemistry of inversion in the 6-phosphogluconate dehydrogenase reaction not only aids in the delineation of the mechanism of the reaction but also provides information about the metabolic fate of carbon-bound hydrogen. Rose and O'Connell (1960) have determined the absolute stereochemistry of the removal of hydrogen from carbon-1 of ribulose-5-phosphate in its conversion to ribose-5-phosphate as catalyzed by phosphopentose isomerase. A comparison of the two stereochemistries shows that the carbon-2 hydrogen of 6-phosphogluconate should be entirely retained as the carbon-1 hydrogen of ribose-5-phosphate in the conversion catalyzed by the dehydrogenase and isomerase. In fact, this prediction has been verified by the finding that the specific activity of the carbon-1 hydrogen of the ribose of RNA which was isolated from *E. coli* grown on 2-tritiated D-gluconate was about the same as that of the 2-tritiated gluconate (Lienhard, 1964).

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The Incorporation of Radioactive Inorganic Orthophosphate as Organic Phosphate by Collagen Fibrils *in vitro**

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The interaction of inorganic orthophosphate with reconstituted purified collagen fibrils obtained from a variety of tissues was studied *in vitro*. At initial solution concentrations of 1×10^{-2} M phosphate at 25°, collagen fibrils bound approximately 150–170 moles phosphate per mole collagen. Part of the bound phosphate was incorporated as organic phosphate. The acid and alkali stability and the electrophoretic behavior of the primary phosphate-32-labeled components make it unlikely that they are phosphorylated amino acids, but suggest that they are sugar phosphates. In addition to the readily dissociable and organic phosphate components, a fraction of the bound phosphate was very strongly complexed and difficult to separate from the collagen, but could be chemically extracted as inorganic orthophosphate. Therefore the interaction of collagen and inorganic orthophosphate is seen to encompass a wide spectrum of bond types.

An important consideration in understanding the mechanism underlying the nucleation of calcium and phosphate as apatite crystals by collagen fibrils *in vitro* (Neuman and Neuman, 1953; Sobel, 1955; Strates *et al.*, 1957; Glimcher *et al.*, 1957; Glimcher, 1959, 1960) are the interaction properties of collagen with calcium and phosphate ions.

Previous *in vitro* studies demonstrated that purified reconstituted collagen fibrils from fish swim bladder (ichthyocol) bound as much as 150 moles P/mole collagen, and that some of the bound phosphate had the characteristics of a covalent bond (Glimcher and Krane, 1962). The present study further characterizes the *in vitro* interaction of purified reconstituted collagen fibrils and inorganic orthophosphate.

EXPERIMENTAL

Preparation and Purification of Collagen.—Before the collagen was extracted, finely ground or hand-minced tissues were first washed for 24 hours in cold 1% NaCl, *p*H 7.4, extracted twice for 48 hours, and once for 24 hours at 2° with 3.9 M KCl, *p*H 8.3–8.5 (50–100 ml/g patted wet tissue), washed with 1% NaCl for 24 hours, and finally with cold distilled demineralized water until the fluid pressed from the tissue was chloride free. Acetic acid–soluble collagens from guinea pig skin and chicken leg tendon were obtained by extracting the washed tissues with approximately 15–20 volumes of 3% acetic acid at 2° for 48–72 hours. Rat tail tendon and mouse tail tendon were extracted in 20 volumes of 1% acetic acid, and carp swim bladder in 100 volumes of 0.05% acetic acid. The viscous collagen solutions were filtered either through Celite or through a 5-μ Millipore filter and prefilter using a specially designed pressure filtration device adapted to the standard large Millipore filter pressure unit.[1] The solutions were further clarified by centrifugation at 30,000 rpm for 2.5 hours in a Beckman-Spinco Model L ultracentrifuge.

The collagens, except for the carp swim-bladder collagen (ichthyocol), were purified as follows. Cold 25% NaCl, dissolved in the appropriate concentration of acetic acid, was added slowly with stirring at 2° to the clarified acetic acid solution of collagen until a final salt concentration of 5% was reached. The solution was stirred gently for approximately 1–2 hours, the stirring was discontinued, and the fluffy precipitate which settled out after 4–12 hours was collected by centrifugation at 6000–9000 rpm at 2°. The precipitate was washed three times by suspending and redispersing the collagen fibrils in cold 5% NaCl in the appropriate concentration of acetic acid, and was harvested at 6000–9000 rpm at 2°. The collagen fibrils were then redissolved in acetic acid, clarified by ultracentrifugation, and reprecipitated by the addition of NaCl. This procedure was repeated two more times. The collagen fibrils obtained from the last 5% NaCl precipitation were dissolved in 1% acetic acid and clarified by ultracentrifugation, cold concentrated NaCl in 1% acetic acid was added to a final concentration of 1% NaCl, and the *p*H was adjusted to 7.4 with NaOH. The temperature of the solution was then slowly raised to 25–30° and held for 0.5–1.0 hour (Gross and Kirk, 1958). The flocculent precipitate was separated from the supernatant, washed, redissolved in cold 1% acetic

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[1] Millipore Filter Corp., Bedford, Mass.