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Formation of L-Lyxonic and L-Xylonic Acids from L-Ascorbic Acid in Rat Kidney

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The enzymatic decarboxylation of L-ascorbic acid by rat kidney homogenates has been reported in an earlier study (1). It was shown that factors present in both the soluble and the particulate fractions were required for maximal activity. Evidence was presented suggesting that dehydro-L-ascorbic acid and 2,3-diketo-L-gulonic acid were intermediates in the decarboxylation of the vitamin. The reaction products were not identified at that time, although the formation of L-xylose and L-xylulose was excluded. More recently, evidence for the identity of the products as L-xylonic and L-lyxonic acids has been reported briefly (2).

An alternate ascorbic acid-metabolizing system present in the soluble fraction of guinea pig liver has been shown by Chan *et al.* (3). In this case, L-xylose was identified as a product of the decarboxylation of dehydroascorbic acid.

The present communication is concerned with a description of the partially purified enzyme system from rat kidney and with further documentation on the identification of L-lyxonic and L-xylonic acids as the major products arising from the decarboxylation of diketogulonic acid. The reactions studied are illustrated in Fig. 1.

EXPERIMENTAL PROCEDURE

Materials—L-Galactonolactone, prepared by the borohydride reduction of D-galacturonic acid (4), was converted to its calcium salt and subjected to a Ruff degradation (5). The resulting L-lyxose was oxidized by the method of Moore and Link (6) to L-lyxonic acid and isolated as the crystalline L-lyxonolactone, m.p. 105–107°, $[\alpha]_D^{24}$ − 78°. L-Ribonolactone was generously provided by Dr. H. Isbell of the National Bureau of Standards. D-Ribono- and L-arabonolactone were obtained from commercial sources. The lactones of the remaining four pentonic acids were prepared by bromine oxidation of the appropriate pentose in the presence of $CaCO_3$. Dehydroascorbic acid was prepared by the benzoquinone oxidation of L-ascorbic acid. Crystalline barium diketo-L-gulonate was synthesized as described by Penney and Zilva (7) and Curtin and King (8). Squash ascorbic acid oxidase was purified through the first ammonium sulfate step of Dawson and Magee (9) and was stable when stored as a suspension in saturated ammonium sulfate at 0° for at least a year. L-Ascorbic acid-6-C^{14} was purchased from the National Bureau of Standards.

Methods—Ascorbic acid, dehydroascorbic acid, and diketogulonic acid were measured by a modification of the method[1] of Roe and Keuther (10). The $FeCl_3$-hydroxamic acid assay of Lipmann and Tuttle (11) was used to determine the pentonic acid lactones. Protein was measured turbidimetrically (12) and by the phenol method (13).

The squash ascorbic acid oxidase activity was assayed by observing the disappearance of ultraviolet absorption at 260 mμ in the Beckman spectrophotometer. The incubation mixture consisted of 0.2 ml of Krebs-Ringer-phosphate buffer, pH 6.8, and 0.1 μmole of L-ascorbic acid in 1.0 ml of solution. The rate of decrease of optical density after addition of the enzyme was proportional to time and to enzyme concentration over a wide range of activity. A unit was defined as the amount of enzyme capable of producing a decrease of 1.0 optical density unit per minute under the above conditions. The preparation used in this work contained 200 units per mg of protein. In the presence of excess oxidase, the L-ascorbic acid was quantitatively oxidized.

Preparation of Kidney Enzyme—The kidneys from freshly killed Wistar strain rats were homogenized in 4 volumes of cold isotonic KCl and centrifuged for 45 minutes at 100,000 × *g* in a Spinco ultracentrifuge. The soluble enzyme preparation was brought to 50° and maintained at that temperature for 3 minutes. The precipitate which formed was removed by centrifugation and discarded. The remaining solution was treated with ammonium sulfate and the fractions precipitating between 60 and 75 and 75 and 90% of saturation were collected, dissolved separately in a small amount of water, and dialyzed overnight against 0.01 M K_2HPO_4. The major portion of the activity, usually recovered in the 60 to 75% ammonium sulfate fraction, represented a purification of about 5-fold over the original activity of the high speed supernatant fraction. Further attempts at purification by means of gel adsorption, solvent precipitation, and cellulose chromatography were unsuccessful.

The assay system employed for the decarboxylation reaction was initially developed with L-ascorbic acid as substrate. However, upon purification, the ability of the crude preparation to convert L-ascorbate to dehydroascorbate and diketogulonate was readily removed. Upon the addition of a slight excess of a partially purified squash ascorbic acid oxidase, a quantitative formation of these ketohexonic acids was observed. In the absence of the decarboxylating enzyme from rat kidney, there was no further metabolism of the dehydroascorbate and diketo-

[1] In an attempt to decrease the time required for the determination of ascorbic acid, the incubation conditions of the assay were altered in that the test samples were maintained at 57° for 45 minutes rather than at the lower temperature and for a longer incubation originally described (10). The modified procedure was carefully checked for proportionality, sensitivity, and specificity before being adopted.

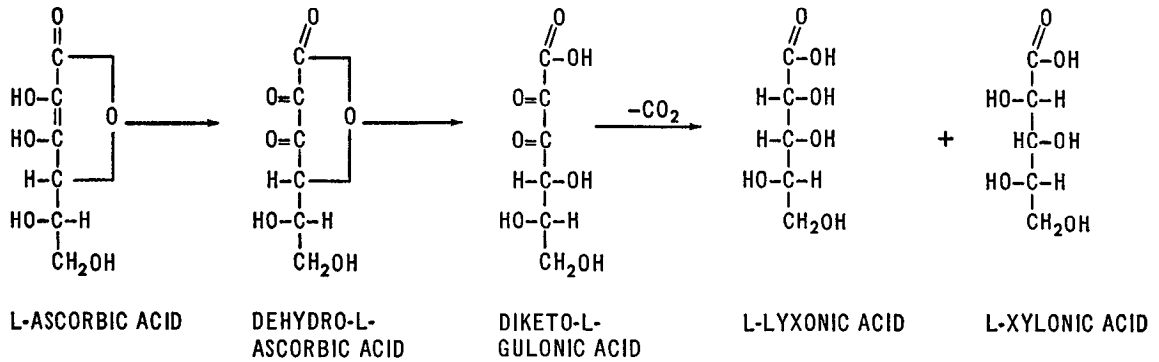

FIG. 1. Reaction sequence for formation of L-xylonic and L-lyxonic acids from diketo-L-gulonic acid

gulonate so formed and they could be recovered unchanged at the end of the incubation period. The routine assay contained 1 μmole of L-ascorbic acid, 19 μg of squash ascorbic acid oxidase, and varying amounts of the kidney enzyme brought to 1.0 ml with Krebs-Ringer-phosphate buffer at pH 6.8. The tubes were gassed with oxygen for 1 minute, stoppered, and incubated at 37° for 30 minutes. The reaction was stopped by the addition of trichloroacetic acid to make a final concentration of 4%. A unit is defined as that amount of enzyme required to catalyze the disappearance of 1 μmole of substrate under these conditions. The proportionality of the assay to enzyme concentration is shown in Fig. 2.

Isolation of Reaction Products—In an attempt to isolate and identify the reaction products resulting from the metabolism of L-ascorbic acid by the rat kidney enzyme preparation, a large scale incubation was performed. The reaction mixture contained 75 μmoles of L-ascorbic acid-6-C^{14} (1.73×10^4 c.p.m. per μmole), 10 ml of the dialyzed enzyme preparation, 20 units of squash ascorbic acid oxidase, and 15 ml of Krebs-Ringer-phosphate buffer, pH 6.8. The reaction flask was flushed with oxygen, stoppered, and incubated for 30 minutes at 37°. The reaction was terminated by the addition of trichloroacetic acid to give a final concentration 4%. The precipitate was removed by centrifugation and the supernatant solution was extracted several times with a 2-fold volume of ether to remove the excess trichloroacetic acid. At the end of the incubation period more than 90% of the substrate had disappeared.

The pH of the reaction mixture was brought to 6.8 with 1 N NaOH and the neutralized solution adsorbed on a column of Dowex-1-formate (1 × 24 cm). The column was washed with 100 ml of water and the elution initiated with 0.01 N formic acid. All chromatographic fractionations were carried out in the cold room at 0–3°. The flow rate was adjusted to 0.5 ml per minute and 10-ml fractions were collected. Aliquots of the various fractions were plated and their C^{14} content determined in a Geiger-Muller proportional counter.

The elution pattern of the radioactive products, illustrated in Fig. 3, revealed the presence of two incompletely separated peaks. The hatched area, corresponding to tubes 72 through 76, contained products common to both peaks and was rejected. The remaining tubes of each fraction were pooled and labeled Fraction A and Fraction B, respectively. Both fractions were concentrated to dryness in a vacuum at 40° to remove the formic acid and the residual material was dissolved in a small amount of water. The somewhat turbid solutions were treated with Darco and filtered to yield 1 to 2 ml of a clear filtrate.

Identification of Reaction Products—Colorimetric analysis of both fractions revealed the absence of pentoses (14), α-keto acids (15), and reducing sugars (16). Upon treatment with the $FeCl_3$-hydroxamic acid reagent (11) a strongly positive reaction was obtained in both fractions. When the samples were neutralized with 1 N NaOH before the addition of hydroxamic acid, there was a complete absence of color development. These results are

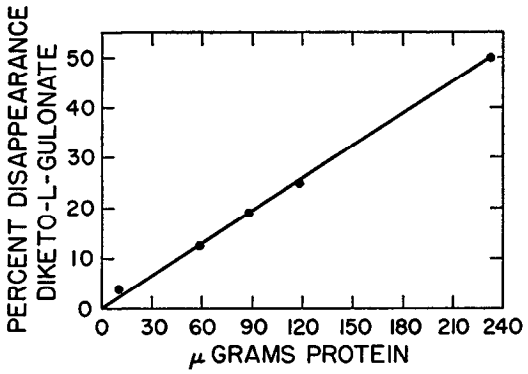

FIG. 2. Proportionality of disappearance of diketogulonic acid with increasing amounts of enzyme.

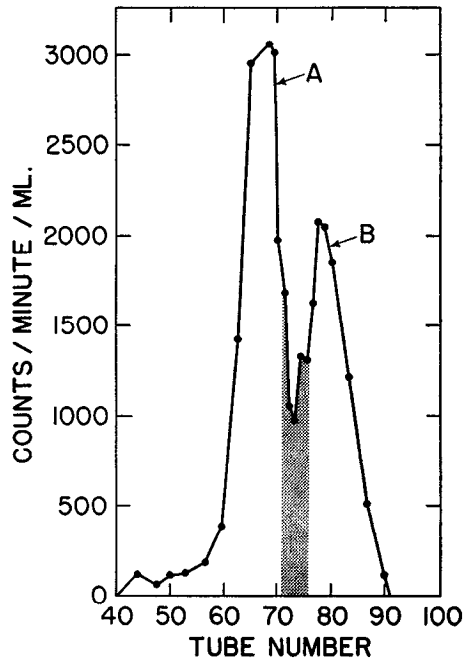

FIG. 3. Elution pattern of L-ascorbic acid metabolic products from a Dowex-1-formate column.

consistent with the behavior of the lactone of a carboxylic acid. Under neutral conditions, both samples rapidly consumed periodic acid with concomitant formation of formaldehyde as determined by chromotropic acid (17).

The metabolic samples were subjected to descending paper chromatography in four solvent systems as follows: (1) ethyl acetate-acetic acid-water (3:1:3); (2) water saturated butanol-formic acid (95:5); (3) *n*-propanol-formic acid-water (6:3:1); and (4) *n*-butanol-ethanol-water (5:1:4). The papers were developed with $AgNO_3$ (18) and with $FeCl_3$-hydroxylamine (19).

Fraction A was shown to contain a single rapidly moving component which migrated together with the lactones of ribonic, arabonic, and xylonic acids. Further chromatographic resolution of these lactones was unsuccessful. Fraction B similarly contained a single component which cochromatographed in all the solvent systems with lyxonolactone and could be clearly differentiated from Fraction A. In the solvent systems employed, both erythrono- and threonolactone migrated more rapidly than either of the metabolic products and could be eliminated from further consideration. The analogous hexonic acid lactones of gulonic and galactonic acids were similarly differentiated on the basis of their relatively slow rate of migration.

Upon the assumption that the specific radioactivity of the reaction products had remained unchanged from that of the original L-ascorbic acid-6-C^{14} used as the substrate, an estimation of the amount of product present in each fraction was calculated from the total radioactivity recovered. A comparison of the yield, calculated in this manner, with the yield calculated from the hydroxamic acid and the periodic-chromotropic acid data is summarized in Table I.

TABLE I

Analysis of metabolic reaction products

	Fraction A	Fraction B
Total C^{14} (c.p.m.)	3.15×10^5	1.78×10^5
% recovery of C^{14}	32.5	18.2
Yield in μmoles*	18.2	10.3
Yield in μmoles†	20.6	11.1
Yield in μmoles‡	16.5	8.0

* Calculated on the basis of radioactivity recovered.

† Calculated on the basis of the amount of formaldehyde liberated by periodic acid treatment as determined by chromotropic acid (17).

‡ Calculated on the basis of the amount of hydroxamic acid reacting material present (11).

TABLE II

Derivatives of Fraction A with carrier L-xylonolactone

	Brucine salt*	Xylitol pentaacetate†
1st crystallization (c.p.m./mg)	16.1	13.1
2nd crystallization (c.p.m./mg)	16.1	13.2
3rd crystallization (c.p.m./mg)	16.2	12.6
Melting point	177–178°	57–58°
$[\alpha]_D^{24}$	+24.3°	

* To an aliquot of Fraction A were added 75 mg of carrier L-xylonolactone. The brucine salt was prepared (21) and recrystallized successively from 90% ethanol.

† To an aliquot of Fraction A were added 60 mg of carrier L-xylonolactone and the sample reduced to xylitol with potassium borohydride (4). Xylitol pentaacetate was prepared (24) and recrystallized successively from petroleum ether-benzene (2:1).

TABLE III

Disappearance of dehydroascorbic acid and diketogulonic acid in presence of intact and heat inactivated enzyme

To the assay tubes containing either dehydroascorbate or diketogulonate, in the amounts listed in the table below, were added 10 units of the partially purified rat kidney enzyme and Krebs-Ringer-phosphate buffer, pH 6.8, to a final volume of 1.0 ml. In each case a boiled enzyme control, heated to 100° for 5 minutes, was included. The samples were incubated for 30 minutes at 37° and the reaction stopped by the addition of 0.04 ml of 100% trichloracetic acid. The residual substrate was assayed as described in the section on "Methods."

Enzyme	Zero time		30 minutes	
	Dehydroascorbic acid	Diketogulonic acid	Dehydroascorbic acid	Diketogulonic acid
	μmoles	μmoles	μmoles	μmoles
Active	10.0	0.0	1.65	4.00
Boiled	10.0	0.0	1.35	7.75
Active	0.0	6.0	0.0	1.85
Boiled	0.0	6.0	0.0	6.35

Identification of L-Xylonolactone—From a consideration of the structural configuration of the starting material and from the above described analytical values, the metabolic product isolated in Fraction A was presumed to be the lactone of L-xylonic acid. The optical rotation of this sample was strongly negative and approximated the reported value of −91.8° for L-xylonolactone (20). Identification of Fraction A as L-xylonolactone was obtained by carrier dilution experiments in which brucine L-xylonate and xylitol pentaacetate were prepared and crystallized to constant specific activity.[2] The physical constants of the derivatives are recorded in Table II.

Identification of L-Lyxonolactone—The identity of the second compound, Fraction B, which had been shown to cochromatograph with L-lyxonolactone was further established by carrier dilution experiments. To an aliquot of Fraction B were added 15 mg of carrier L-lyxonolactone and the phenylhydrazide derivative prepared (22). After several recrystallizations from 90% ethanol, the specific activity of the derivative remained constant at 307 c.p.m. per mg. The optical rotation of the product was $[\alpha]_D^{24}$ + 13.7° and the melting point was 163°. To check the specificity of this procedure for the L-isomer, the experiment was repeated with D-lyxonolactone as carrier. In this case, the radioactivity of the phenylhydrazide derivative fell to less than 5 c.p.m. per mg after the first crystallization.

Properties of Enzyme—The marked dependence of the decarboxylation reaction upon oxygen was completely eliminated when either dehydroascorbic or diketogulonic acid was substituted for L-ascorbic acid. With these substrates, the reaction proceeded equally well in air, oxygen, or helium.

In order to determine which of the two oxidation products of L-ascorbic acid was the immediate precursor of the decarboxyla-

[2] Attempts to prepare the phenylhydrazide derivative of L-xylonolactone according to the procedure of Neuberg (21) were unsuccessful although a crystalline product melting at 129°, in agreement with the reported value, was obtained. Further investigation revealed this compound to be the salt, phenylhydrazine acetate.

tion reaction, both compounds were incubated separately and the products determined. It can be seen from the data in Table III that dehydroascorbic acid was almost entirely converted to diketogulonic acid in the boiled enzyme and less than 10% disappearance of total ketohexonic acids was observed. When diketogulonic acid was incubated in the presence of the boiled enzyme, there was no disappearance of substrate nor was there any demonstrable formation of dehydroascorbic acid. In the presence of the untreated enzyme both substrates disappeared. Earlier studies (1) had previously established that the rate of decarboxylation was significantly greater when diketogulonate rather dehydroascorbate was used as substrate. These results are consistent with the concept that dehydroascorbic acid, formed by the oxidation of L-ascorbic acid, is converted to diketogulonic acid which undergoes decarboxylation. The data in Table III suggest that the conversion of dehydroascorbic acid to diketogulonic acid may occur nonenzymatically.

Specificity—The enzyme preparation described here does not appear to have a high degree of specificity. In addition to diketo-L-gulonic acid, the analogous diketohexonic acids prepared from D-ascorbic and D-araboascorbic acid were actively metabolized.[3] Paper chromatographic examination of the reaction products indicated, in each case, the formation of products indistinguishable from the expected pentonic acid lactones. For instance, D-xylonic acid and D-lyxonic acids were identified chromatographically as products of the decarboxylation of 2,3-diketo-D-ascorbic acid. The diketo analogue of D-glucoascorbic acid, on the other hand, proved to be completely inert in this system.

Enzyme Distribution—Survey experiments with various tissues have shown that the formation of pentonic acids from dehydroascorbic and diketogulonic acids is not restricted to the rat kidney and the reaction has been shown to take place in guinea pig, hog, and calf kidneys as well as in the livers of both rats and guinea pigs.

Inhibitors and Activators—A complete inhibition of enzymatic activity was observed upon the addition of 8-hydroxyquinoline, 10^{-5} M, diethyldithiocarbamate, 10^{-3} M, and *p*-chloromercuribenzoate, 4×10^{-3} M. Dialysis of the enzyme against 8-hydroxyquinoline, 10^{-3} M, followed by dialysis against 0.01 M potassium phosphate buffer at pH 8.5 to remove the inhibitor, resulted in complete loss in activity. The activity could not be restored by a Kochsaft or by the addition of Cu^{++}, Fe^{++}, Mg^{++}, or Mn^{++}, 10^{-3} M, to the dialyzed preparation, although some disappearance of diketogulonate was observed in the presence of Cu^{++}, 10^{-2} M, alone. The dialyzed enzyme was unaffected by the addition of glutathione, mercaptoethanol, or cysteine at a concentration of 10^{-3} M, although, here too, at higher concentrations, nonenzymatic decarboxylation of diketogulonate was observed. Ethylenediaminetetraacetate, 10^{-2} M, NaF, 10^{-2} M, KCN, NaN_3, and iodoacetate at 10^{-3} M were all without effect upon the activity of the enzyme.

DISCUSSION

Although the physiological function and metabolism of L-ascorbic acid has been studied intensively, little information of a specific nature is available concerning either the mode of action or the further metabolism of this vitamin. Small amounts of oxalate derived from L-ascorbic acid have been shown by experiments *in vivo* with rats (8), guinea pigs, (25), and human subjects (26), but no such oxalate formation has been shown by enzymes in mammalian tissues. More recently, Chan *et al.* (3) have reported the formation of L-xylose resulting from the action of a guinea pig liver supernatant fraction upon dehydroascorbic acid. Although the physiological significance of this observation is not yet clear, it is to be noted that the yield of this pentose constituted only a small fraction of the total amount of the overall reaction.

The results of the present study indicate that the major metabolic products of L-ascorbic acid metabolism in the rat kidney are L-xylonic and L-lyxonic acids. To the best of our knowledge, this is the first report of the formation of these pentonic acids in mammalian tissues. It is interesting to note, however, that an enzyme system in calf lens has been reported to carry out the DPN-linked oxidation of D-xylose with the formation of D-xylonic acid (27).

[3] The metabolism *in vivo* of D-ascorbic acid-1-C^{14} has been demonstrated in guinea pigs and rats (23).

SUMMARY

An enzyme system capable of decarboxylating dehydro-L-ascorbic acid and 2,3-diketo-L-gulonic acid has been partially purified from rat kidney. The products of the reaction have been identified as L-lyxonic and L-xylonic acids.

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