METABOLIC PRODUCTS OF L-ASCORBIC ACID*

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The carbon chain of L-ascorbic acid-C^{14} has been found to be converted almost entirely to carbon dioxide in the guinea pig (1–3), but a portion of the carbon in positions 1 and 2 gives rise to oxalic acid (1, 3). From tests with L-ascorbic acid-2,3,4,5,6-C^{14} in guinea pigs, the glucose isolated from liver glycogen was found to be uniformly labeled (2). This finding suggested a pathway for the catabolism of ascorbic acid via known patterns for other carbohydrates.

The results of the present study indicate that dehydroascorbic acid is a major intermediate in the breakdown process and that L-xylose and oxalate are formed as subsequent products. In the supernatant fraction from liver homogenate, decarboxylation of dehydroascorbic acid occurred, and L-xylose was isolated as a product. Further evidence of a pentose pathway of ascorbic acid metabolism involving L-xylose is furnished by the data on C^{14} distribution in glucose derived from liver glycogen after injection of ascorbic acid-3,4,5,6-C^{14} (4). The keto form of L-xylose can be transformed by way of xylitol to D-xylulose by the enzyme systems reported by Touster *et al.* (5) and Arcus and Edson (6).

EXPERIMENTAL

Normal male guinea pigs from Carworth Farms, Inc., and Hemlock Hollow Farms, 2 to 3 months old and weighing 300 to 400 gm., were used after a standardization period of 10 days or more in the laboratory.

Animals used for liver glycogen isolation were fed 500 mg. of glucose daily for 3 days in addition to the regular pellet diet and ascorbic acid supplement and were then fasted for 24 hours before injection with the isotopic compounds (7). 30 minutes before injection, 1.5 gm. of glucose

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were fed to stimulate glycogen synthesis. Glycogen was isolated according to the trichloroacetic acid procedure of Stetten and Stetten (8).

Glucose derived from the glycogen was degraded by two different methods, one according to Eisenberg (9) by way of gluconate to determine the radioactivity of carbons 1 and 6, and the other via glucosazone, which was oxidized with periodate (10) to determine the activity of carbons 1, 2, and 3 in the form of mesoxalaldehyde-1,2-bisphenylhydrazone.

Liver slices were prepared with a Stadie-Riggs microtome (11). Liver homogenates were prepared in a Potter-Elvehjem homogenizer, with the use of isotonic KCl and sucrose solutions as media. The mixture was kept at 0° during the 4 minute homogenization.

Liver mitochondria were isolated from 0.25 M sucrose homogenates according to the procedure of Lardy and Wellman (12). The supernatant fraction of liver homogenate was obtained by centrifugation at 22,000 $\times$ g for 2 hours.

All reactions were carried out in a Warburg constant volume respirometer at 37°. The flask contents are given for Tables I to IV. At the end of the reaction the mixture was centrifuged, and its supernatant fluid was passed through a Dowex 50-X12 column in the hydrogen form and a Duolite A-4 column in the carbonate form to give the neutral fraction. The Duolite column was eluted with 1 N formic acid. The neutral fraction and the formic eluate were each concentrated under a vacuum at 40–45° to the volume of 0.5 to 1.0 ml. and further separated by paper chromatography by using three different solvent systems: 1-butanol-acetic acid-water, 4:1:5, v/v (13); ethyl acetate-pyridine-water, 2:1:2, v/v (14); and 80 per cent phenol with 20 mg. of β-hydroxyquinoline per liter of solution (15).

The radioactive spots on the chromatogram were detected by direct scanning through a model D46A Q gas counter (Nuclear Instrument and Chemical Corporation) and by radioautography by using Blue Brand, Kodak medical x-ray films.

300 mg. of non-radioactive L-xylose were added to the mixture at the end of the reaction. After passage through the cation and anion exchange columns, the neutral fraction containing L-xylose-C^{14} was further purified by passage through a cellulose powder (Whatman standard grade) column with water-saturated 1-butanol as the developing solvent. The fractions containing xylose were detected with Tauber's reagent (16). When the combined fractions were concentrated to near dryness, L-xylose appeared as a white precipitate. It was recrystallized several times from ethanol-water until a constant specific activity was obtained. Samples from successive crystallizations were converted into $BaCO_3$ for activity determination. L-Xylosazone, prepared according to the method of Futterman and Roe (17), gave the same specific activity as the original xylose when converted to $BaCO_3$.

Radioactive samples of $BaCO_3$ and calcium oxalate were counted directly in a model D46A Q gas counter (Nuclear Instrument and Chemical Corporation). Other radioactive compounds were converted to $BaCO_3$ according to the method of Lindenbaum *et al.* (18) by using the reagents of Van Slyke *et al.* (19). All data were corrected for self-absorption, and the counting period was extended sufficiently long to bring the statistical deviation to within 2 per cent.

All isotopic compounds were recrystallized until the specific activity was constant, and their melting points agreed with the values in literature.

Ascorbic acid-1-C^{14} was synthesized by Salomon *et al.* (20). Ascorbic acid-2,3,4,5,6-C^{14} was synthesized by Rudoff *et al.* (2), by using a similar procedure, with uniformly labeled L-xylosone which was derived from photosynthetic D-glucose-U-C^{14}. L-Xylose-U-C^{14} was an intermediate in the synthesis of L-xylosone. Ascorbic acid-3,4,5,6-C^{14} was synthesized from L-xylose-U-C^{14}.[1]

Ascorbic acid oxidase from squash was kindly furnished by Dawson and Magee (21), and its activity was established by Nelson's method in Lovett-Janison and Nelson (22).

Cytochrome *c*, fructose 1,6-diphosphate, glucose 6-phosphate, and triphosphopyridine nucleotide were obtained from the Sigma Chemical Company. Diphosphopyridine nucleotide was obtained from the Pabst Laboratories.

RESULTS AND DISCUSSION

When ascorbic acid-1-C^{14} was administered to either the rat (23) or the guinea pig (1), the radioactive carbon was recovered in two end products, respiratory CO_2 and urinary oxalate. To find whether the latter is an intermediate in $C^{14}O_2$ production, Weinhouse and Friedmann (23) and Curtin and King (24) tested oxalic acid-C^{14} in the rat. Only 0.4 to 1.0 per cent of the substrate activity was found in the 24 hour respiratory CO_2. This very low conversion was confirmed in guinea pig liver slices in the present study. After incubation with 1 gm. of guinea pig liver slices at 37° for 1 hour, the substrate was quantitatively recovered, within ± 0.02 per cent.

Stotz *et al.* (25) have observed cytochrome *c* activity in the oxidation of ascorbic acid in a guinea pig liver preparation. In the present study addition of cytochrome *c* increased the production of $C^{14}O_2$ from ascorbic acid-1-C^{14} in liver slices, but there was no significant effect upon oxalic acid formation. The dependence of $C^{14}O_2$ production upon cytochrome *c* suggested that dehydroascorbic acid might be an intermediate, since liver mitochondria and cytochrome *c* constitute a known system for the oxidation of ascorbic acid to its dehydro form (26, 27).

[1] Becker, R. R., Dugal, L.-P., and King, C. G., unpublished.

Ascorbic acid oxidase from squash was also studied. The data recorded in Table I show that guinea pig liver homogenate plus either cytochrome *c* or ascorbic acid oxidase or both resulted in about the same yield of $C^{14}O_2$. In the control flasks with heated homogenate (Flask 1) or homogenate without such addition (Flask 2), only a small amount of activity was found in the CO_2. Hence, $C^{14}O_2$ did not derive directly from ascorbic acid-1-C^{14}, but from dehydroascorbic acid produced either by the oxidase or the homogenate-cytochrome *c* system.

The above conclusion was further tested in an experiment with the mitochondrial and supernatant fractions of liver homogenate. As shown in

TABLE I

Yield of $C^{14}O_2$ from Ascorbic Acid-1-C^{14} in Guinea Pig Liver Homogenate

Flask No.	Addition	Activity in CO_2
		per cent
1	Heated homogenate + cytochrome *c*	1.3
2	Homogenate only	0.4
3	+ cytochrome *c*	10.1
4	+ ascorbic acid oxidase	11.8
5	+ cytochrome *c* and ascorbic acid oxidase	12.0

Each flask contained 0.5 ml. of 0.1 M phosphate buffer, pH 7.3, 0.5 ml. of 0.5 M KCl, 0.5 ml. of "10 per cent" liver homogenate in 0.15 M KCl, and 3.7 μmoles of ascorbic acid-1-C^{14}. 0.5 ml. of 4×10^{-4} M cytochrome *c* was added to Flasks 1, 3, and 5. 0.2 ml. of ascorbic acid oxidase, with 5.4 Nelson units, was added to Flasks 4 and 5. Water was added to make a total volume of 3.0 ml. per flask. In Flask 1, the homogenate was heated at 80° for 5 minutes. Incubation was carried out at 37° in an air atmosphere for 1 hour.

Table II, the flask containing only catalytic amounts of cytochrome *c* and the supernatant fraction resulted in a low yield of $C^{14}O_2$. The terminal oxidation system is known to be located mainly in the mitochondria (28). Only in Flasks 1, 2, and 4, where oxidizing systems for ascorbic acid were present, was there a large yield of $C^{14}O_2$. The relatively large yield of $C^{14}O_2$ in Flask 4 shows that the factor responsible for the decarboxylation was present in the supernatant fraction. This is in agreement with the findings of De Salegui *et al.* (29), who studied the disappearance of dehydroascorbic acid in rat liver homogenate fractions.

Tests with chemically prepared dehydroascorbic acid-1-C^{14} as substrate are recorded in Fig. 1. The control tests with heated homogenate and without any tissue preparation resulted in a yield of about 5 per cent of the activity in CO_2. Increasing yields of $C^{14}O_2$ were proportional to the amounts of the supernatant fluid, and the formation of oxalic acid was

TABLE II

Yield of $C^{14}O_2$ from Ascorbic Acid-1-C^{14} in Guinea Pig Liver Fractions

Flask No.	Tissue fraction	Addition	Activity in CO_2
			per cent
1	Mitochondria	AAO*	9.5
2	"	Cytochrome *c*	7.9
3	"	0	0.5
4	Supernatant	AAO	16.9
5	"	Cytochrome	2.5
6	"	0	0.3

Each flask contained 0.5 ml. of 0.5 M KCl, 0.5 ml. of 0.1 M phosphate buffer, pH 7.3, 0.5 ml. of either "10 per cent" mitochondria suspension or "10 per cent" supernatant fluid of the homogenate in 0.25 M sucrose solution, and 3.3 μmoles of ascorbic acid-1-C^{14}. 0.3 ml. of AAO, with 8.1 Nelson units, was added to Flasks 1 and 4. 0.5 ml. of 4×10^{-4} M cytochrome *c* was added to Flasks 2 and 5. Water was added to make a total volume of 3.0 ml. The incubation was carried out at 37° in an air atmosphere for 1 hour.

* Ascorbic acid oxidase.

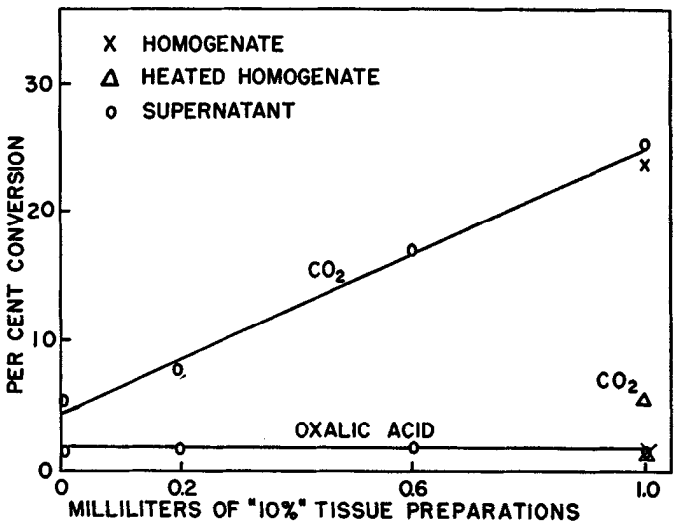

FIG. 1. Yield of oxalic acid-C^{14} and $C^{14}O_2$ from L-dehydroascorbic acid-1-C^{14} in guinea pig liver preparations. The reaction mixture contained 0.8 ml. of 0.1 M phosphate buffer (pH 7.0), 1.0 ml. of 0.15 M KCl, and tissue preparation in 0.15 M KCl, 1.0 ml. of water, and 3.8 μmoles of L-dehydroascorbic acid-1-C^{14}. Incubation was for 1 hour at 37° in an air atmosphere.

independent of the presence of the tissue preparations. In tissue slices, and in homogenates also, the formation of oxalic acid appeared to be non-enzymatic. When the whole homogenate was used, the yield of $C^{14}O_2$

was the same as that obtained from the corresponding amount of the supernatant fraction.

For the purpose of studying the remaining carbon chain of the vitamin after loss of the carboxyl carbon, ascorbic acid-2,3,4,5,6-C^{14} was used. In contrast to the findings *in vivo* of Rudoff *et al.* (2), the $C^{14}O_2$ production in liver slices was insignificant in comparison with the yield from ascorbic acid-1-C^{14} (Table III). The results indicated that carbon 1 of ascorbic acid was eliminated by decarboxylation and suggested that major cleavage between carbons 3 and 4 was unlikely. Therefore the investigation was directed toward testing the hypothesis that a pentose metabolic pathway might account for the observed uniform labeling of glucose derived from ascorbic acid-2,3,4,5,6-C^{14} (2).

TABLE III

Yield of $C^{14}O_2$ from Ascorbic Acid-1-C^{14} and Ascorbic Acid-2,3,4,5,6-C^{14}

Experiment No.	Period	Ascorbic acid-1-C^{14}	Ascorbic acid-2,3,4,5,6-C^{14}
	hrs.	*per cent*	*per cent*
1	2	49.6	0.7
2	2	52.7	0.7
3	1	38.7	0.2
4	1	25.5	0.1

Each flask contained 8.2 ml. of the Krebs-Ringer phosphate solution, pH 7.0, 0.8 ml. of 4×10^{-4} M cytochrome *c*, 1 gm. of guinea pig liver slices, and 9 to 12 μmoles of ascorbic acid-C^{14} in 0.5 ml. of water. Incubation was carried out at 37° in an air atmosphere.

Isolation of intermediates from the reaction mixture was attempted by separation of the supernatant fraction into neutral and acidic portions with ion exchange columns. Each fraction was further separated by paper chromatography.

In addition to being converted enzymatically into various compounds, ascorbic acid decomposes non-enzymatically into small fragments during both the reaction period and the separation process. Therefore it was not surprising to find a large number of radioactive components in the acidic fraction, but there were only three or four prominent radioactive spots in the chromatograms of the neutral fraction. The R_F values of these components were compared with those of known compounds in three different solvent systems. Three of the compounds were identified as xylose, xylulose, and glucose, respectively. Xylulose and glucose showed much less radioactivity than xylose. The carrier technique was used to isolate radioactive L-xylose from the reaction mixture. Purification of the sugar was accomplished by cellulose powder column fractionation, followed by re-

crystallization until constant specific activity was obtained. In a 2 hour incubation with guinea pig liver slices, the conversion of ascorbic acid-2,3,4,5,6-C^{14} to L-xylose was 0.09 per cent. However, this value is twice that found in an *in vivo* experiment in which L-xylose was isolated from a 24 hour urine sample after injection of 4.1 μmoles of ascorbic acid-2,3,4,-5,6-C^{14}. In control experiments, xylose, xylulose, and glucose were not formed from the non-enzymatic breakdown of dehydroascorbic acid.

TABLE IV

Yield of L-Xylose-C^{14} from Dehydroascorbic Acid-2,3,4,5,6-C^{14} in Supernatant Fraction of Guinea Pig Liver Homogenate

Experiment No.	Gas phase	Addition	Per cent activity isolated as L-xylose
1, a	Air		0.10
1, b	"	L-Xylose "trap," 10 mg.	0.11
2, a	"		0.11
2, b	"	DPN, 10 μmoles, + fructose 1,6-diphosphate, 15 μmoles	0.62
3, a; 4, a	N_2	DPN, 4 μmoles, + fructose 1,6-diphosphate, 5 μmoles	2.0, 1.5
3, b; 4, b	"	TPN, 4 μmoles, + glucose 6-phosphate, 10 μmoles	2.9, 3.0

Experiments 1 and 2, each flask contained 4.0 ml. of "10 per cent" supernatant fraction of liver homogenate in 0.15 M KCl, 3.0 ml. of 0.1 M phosphate buffer at pH 7.0, 10 to 12 μmoles of dehydroascorbic acid-2,3,4,5,6-C^{14}, supplements, and water to make the final volume 10 ml. In Experiments 3 and 4, each flask contained 1.0 ml. of "20 per cent" supernatant fraction of liver homogenate in 0.15 M KCl, 0.8 ml. of 0.1 M phosphate buffer at pH 7.0, 5 to 6 μmoles of dehydroascorbic acid-2,3,4,5,6-C^{14}, 0.2 ml. of 0.1 M nicotinamide, 0.1 ml. of 0.1 M $MgCl_2$, supplements, and water to make the final volume 2.8 ml.

Touster *et al.* (5) have described an enzyme system in the insoluble fraction of ruptured mitochondria, capable of transforming L-xylulose to xylitol, which in turn can be converted to D-xylulose and metabolized via the pentose cycle (30). It is possible that L-xylose is in equilibrium with its keto form which leads to the terminal breakdown pathway. Hence elimination of the L-xylulose-reducing enzyme in mitochondria might effect an accumulation of L-xylose in the catabolism of ascorbic acid. In Experiment 1, Table IV, dehydroascorbic acid-2,3,4,5,6-C^{14} was incubated in the supernatant fraction of liver homogenate. There was no apparent increase in the conversion either with or without an L-xylose "trap." However, upon addition of diphosphopyridine nucleotide (DPN) there was a 6-fold increase in the conversion, as shown in Experiment 2. When the test was conducted in an atmosphere of nitrogen, the yield of L-xylose was

raised to 2 per cent by addition of DPN and to 3 per cent with triphosphopyridine nucleotide (TPN) (Experiments 3 and 4, Table IV).

Ascorbic acid-3,4,5,6-C^{14} was then prepared and used in further tests of the pathway toward glucose. Decarboxylation of this ascorbic acid should yield L-xylose-2,3,4,5-C^{14}, which can be converted to D-xylulose-1,2,3,4-C^{14} and then via the pentose cycle to form glucose-1,2,3,-4,5-C^{14}. The degree to which xylose and xylulose are equilibrated *in vivo* has not been established. Due to partial randomization of the 3-carbon fragments in the animal, a portion of the theoretical yield of C^{14} in carbon atom 1 was found in carbon 6, but the major part of the conversion was in agreement with theory, as shown in Table V.

TABLE V

Distribution of C^{14} in Glucose Derived from Ascorbic Acid-3,4,5,6-C^{14}

Carbon atom	C.p.m. per μatom C $\times 10^{-2}$	Total glucose-C^{14}	
		Found	Calculated*
		per cent	*per cent*
1-6	5.2	100	100
1, 2, 3	5.3	53	60
1	4.8	15	20
2, 3 (Difference)	6.0	38	40
4, 5 "	6.4	40	40
6	2.3	7	0

* Based upon metabolism entirely through the hexose monophosphate shunt.

Uniformly labeled L-xylose was injected into a guinea pig, conditioned for glycogenesis, to test the degree of over-all conversion and excretion from this initial substrate. Within 6 hours, 3.17 per cent of the activity was recovered in respiratory CO_2, 1.85 per cent in liver glycogen, and 63 per cent in the urine, of which 92.5 per cent was isolated as L-xylose. Rudoff *et al.* (2) have used ascorbic acid-2,3,4,5,6-C^{14} in a similar experiment and found 3.28 per cent of the labeled carbon in respiratory CO_2, 6.4 per cent in the urine, and 0.52 per cent in liver glycogen at the end of 6 hours. Even though more than half of the injected L-xylose was excreted, the yield of radioactive carbon found in liver glycogen was about 3.5 times greater than was found after administration of ascorbic acid-2,3,4,5,6-C^{14}. These results, supplementing the isolation of L-xylose from *in vivo* and *in vitro* experiments with labeled ascorbic acid, support the hypothesis that L-xylose and L-xylulose are normal intermediates in the reactions by which ascorbic acid is converted to glucose. The formation of L-xylulose from L-gulonolactone has been reported recently by Burns and Kanfer (31) and Bublitz, Grollman, and Lehninger (32).

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SUMMARY

1. Studies of L-ascorbic acid-1-C^{14} and related compounds in preparations of guinea pig liver indicated that L-dehydroascorbic acid is a major intermediate in the metabolic pathway toward carbon dioxide, oxalate, L-xylose, glucose, and glycogen.

2. Production of $C^{14}O_2$ from L-dehydroascorbic acid-1-C^{14} was largely dependent upon the supernatant fraction from liver homogenate, but the yield of oxalic acid-C^{14} was independent of the presence of liver tissue.

3. L-Xylose-C^{14} was isolated from incubation mixtures of guinea pig liver homogenate fractions with either L-ascorbic acid-2,3,4,5,6-C^{14} or L-dehydroascorbic acid-2,3,4,5,6-C^{14}, but conversion to the dehydro form of the vitamin was a necessary intermediate step. The yield of L-xylose-C^{14} was increased 20- to 30-fold by addition of diphosphopyridine nucleotide or triphosphopyridine nucleotide to the homogenate supernatant fraction.

4. Glycogen formation from L-xylose-U-C^{14} in the guinea pig was 3.5 times greater than from L-ascorbic acid-2,3,4,5,6-C^{14} under comparable conditions.

5. All of the available data are in agreement with the hypothesis that L-xylose is formed as an intermediate during the conversion of ascorbic acid to glucose. This conclusion is further supported by the observed distribution of C^{14} in the carbon chain of glucose prepared from guinea pig liver glycogen derived *in vivo* from injected L-ascorbic acid-3,4,5,6-C^{14}.

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