Synthesis of L-ascorbic-4-^{3}H acid*

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SUMMARY

A method is presented for preparation of L-xyloascorbic-4-^{3}H acid 9.5μC/mg by the exchange of 8 M dipotassium ascorbate in tritium-labeled water by heating 20 hours at 100°C. The data confirms the assumptions of Brenner [8], that alkaline isomerization of ascorbic acid proceeds through a carbanion on carbon-4. A study of the radiation stability of crystalline ascorbic acid indicates that it is not unusually radiation sensitive.

INTRODUCTION

The nutritional importance of ascorbic acid as the antiscorbutic factor has been recognized for many years [1, 2, 3]. Much is known about the biosynthesis of ascorbic acid in plants and animals. There are now a number of biochemical reactions which show a specific need for ascorbic acid. The importance of ascorbic acid has been indicated in the hydroxylation of proline [4] and 3,4-dihydroxyphenylethylamine [5]. More recently it has been shown that ascorbic acid functions as a specific substrate in the enzymic oxidation of NADH in certain tissues [6]. In spite of all that is known about ascorbic acid, the details of its mode of action remain uncertain. The availability of labeled ascorbic acid is important to such future studies. Ascorbic-1-^{14}C acid is commercially available, but no tritium-labeled product has been described. This paper presents a method to label ascorbic acid in the 4-position with tritium. The method is easy, and gives a fair yield.

Of the four hydrogens of ascorbic acid that could be replaced with tritium the one on C-4 is the most promising. Weigl [7] suggests that C-4 is chemically the most likely to have a labile hydrogen. More recently, Brenner, *et al.* [8] showed that L-xyloascorbic acid heated in the presence of excess base, racemizes at the C-4 carbon to produce a mixture of epimers, L-xyloascorbic acid and

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L-araboascorbic acid (L-isoascorbic acid). We have repeated Brenner's experiments on the racemization of ascorbic acid and have shown that when the epimerization reaction is carried out in the presence of tritium-labeled aqueous methanol solution, radioactivity is incorporated into the ascorbic acid during the reaction. This isomerization was then further investigated and shown to take place when a solution of dipotassium ascorbate in water was heated to approximately 100 °C for twenty hours. This method was then utilized to synthesize high specific activity L-xyloascorbic-4-^{3}H acid.

Identity of the various products formed during labeling and isomerization was controlled by thin layer chromatography, using a developing solvent of butyronitrile acetonitrile water [8]. This procedure very effectively separates L-xyloascorbic acid and L-araboascorbic acid and permits one to distinguish clearly between L-xyloascorbic acid and dehydroascorbic acid, the oxidation product of ascorbic acid.

EXPERIMENTAL

Synthesis method I

To a 200 ml solution of 50% (by volume) aqueous methanol was added 11.2 g KOH (0.2 moles), 0.02 ml water containing 1.26 m curies tritium and 17.6 g L-xyloascorbic acid (0.1 mole). This solution was refluxed for 17 hours. After cooling, the mixture was acidified with Dowex 50 in the acid form and filtered. The filtrate was eluted with 600 ml distilled water and lyophilized to dryness. The yellow-brown solid obtained was taken up in water and lyophilized again. The product was then recrystallized from acetonitrile and the resulting crystals were dissolved in water and lyophilized three additional times to remove exchangeable tritium. At this point, a 100 mg sample was withdrawn. The material was recrystallized from acetonitrile twice more with 100 mg samples withdrawn after each recrystallization. During the last recrystallization the solution was treated with decolorizing carbon. Aliquots of the three samples were counted in a liquid scintillation counter. The activity of the three samples were:

Sample	Activity
1	2.10×10^{-4} μC/mg.
2	2.10×10^{-4} μC/mg.
3	2.21×10^{-4} μC/mg.

The final yield was 2.13 grams of L-xyloascorbic-4-^{3}H acid. The material chromatographed as a single iodine-sensitive spot by the method of Brenner, *et al.* [8] and showed no evidence for araboascorbic acid.

Synthesis method II

A solution of 15 g of L-xyloascorbic acid and 9.57 g of KOH in a convenient amount of water was lyophilized to dryness. The product was a light tan glassy hygroscopic solid. Dipotassium ascorbate, 14.3 g, was placed in a heavy-walled pyrex test tube (30 mm OD by 20 cm long) and dissolved in 7 ml of water containing 3 curies HTO. The contents of the tube were frozen in dry ice, and the tube was evacuated and sealed. The tube was heated at 92-100 °C for twenty hours, cooled, opened, and placed in a lyophilizing flask. The tritium water was removed from the dark brown solution under vacuum and back-distilled into a second tube containing another 14.3 g of dipotassium ascorbate. This tube was then sealed under vacuum, heated for twenty hours at approximately 100 °C, and dried by lyophilization as before. The color of the material in the second run was a light tan. Approximately 25 ml of water were back distilled into the lyophilizing flask containing the two open tubes; the material was dissolved and the water removed under vacuum.

The lyophilized residue was dissolved in water and treated with enough Dowex 50 in the acid form to bring the pH of the solution to 2. The Dowex suspension was filtered, washed, and the 500 ml of resulting solution were shell frozen and lyophilized. The light brown solid residue was extracted eight times at reflux temperature with quantities of acetonitrile ranging from 100 to 400 ml. Crystals from the first extraction (100 ml) contained 2.2 g mixed L-arabo- and L-xyloascorbic acid as shown by thin film chromatography. The 2.9 g of material crystallized from the other extractions were chromatographically pure L-xylo-ascorbic-4-^{3}H acid. Liquid scintillation counting gave the specific activity of this material as 9.5 μC/mg. This corresponds to a 15% material yield and a 1% radiochemical yield. The product material was stored in evacuated sealed glass tubes. All of the high specific activity water was recovered.

Studies of alkaline stability of AsH_2

Various concentrations of ascorbic acid and KOH in water were heated in evacuated sealed tubes at 100 °C for 17 hours. The concentrations of the solutions ranged from 0.6 M ascorbate and 1 M KOH to 15.8 M ascorbate and 33.5 M KOH. Dipotassium ascorbate is much more soluble than ascorbic acid and increased KOH concentrations are necessary to dissolve a large amount of ascorbic acid. After heating, the colors of the solutions ranged from pale orange for the more dilute solutions, to dark brown for the more concentrated solutions. Decomposition appeared to increase with increasing concentrations of ascorbic acid and KOH. After treatment with Dowex 50 in the acid form, the material from each tube was chromatographed as described previously. In every case clean spots were observed for L-xylo- and L-araboascorbic acid using an iodine spray.

Thin film chromatography

The thin film chromatography experiments were run using silica gel G (E. Merck, A.G., Darmstadt, Germany) and the developing solvent system of Brenner. The developing solvent is acetonitrile butyronitrile water (66: 33: 2). In this system, we observed the following approximate Rf values: L-xylo-ascorbic acid, 0.26; L-araboascorbic acid, 0.40; dehydro-L-xyloascorbic acid, 0.48; and L-2,3-diketogulonic acid, 0.08. Generalized spots were developed with a concentrated sulphuric acid spray, and the ascorbic acid spots were detected with a spray solution of iodine in chloroform. This gives a positive spot. Sprays of iodine in methanol give a negative spot—that is, a white spot on a brown background—which is what would be expected if the ascorbic acid reacted with free iodine to give oxidized ascorbic acid and iodide ion.

Radiation stability of solid ascorbic acid

40 to 50 mg samples of ascorbic acid (U.S. Pharmacopeia Reference Standard) were weighed into small glass tubes and sealed under vacuum. These tubes were irradiated in a ^{137}Ce gamma irradiation source for varying lengths of time in a field of 5×10^5 rads per hour. The samples were analyzed for total ascorbate by the method of Shaffert and Kingsley [9]. In this procedure the ascorbic acid is treated with dinitrophenylhydrazine, and the derivative formed is dissolved in 85% sulphuric acid and read colorimetrically at 515 mμ. This analytical procedure gives « total ascorbate » and does not distinguish between radiolitically-formed ketones and those of the ascorbic acid.

Radioactive assays

All samples were analyzed for radioactivity using liquid scintillation counting. The solvent system was 7 g PPO, 0.3 g dimethyl POPOP, and 100 g naphthalene dissolved in 1 liter dioxane [10]. After counting, all samples were spiked with tritium labeled toluene standard and recounted to determine the counting efficiency of the particular sample.

RESULTS AND DISCUSSION

Confirmation of C-4 proton exchange

Brenner, *et al.* [8] suggest that the mechanism of the alkaline isomerization reaction of ascorbic acid involves the formation of the C-4 cation (Figure 1). While this is a likely reaction, alternate possibilities, such as reactions involving opening of the lactone ring, cannot be excluded. Our observation of the exchange labeling with tritium under the conditions that Brenner used indicates that the isomerization of xyloascorbic acid to a mixture of xylo- and araboascorbic acid

probably proceeds through the carbanion, as proposed. Such data, however, do not uniquely prove that the tritium incorporated is attached to the number four carbon atom *.

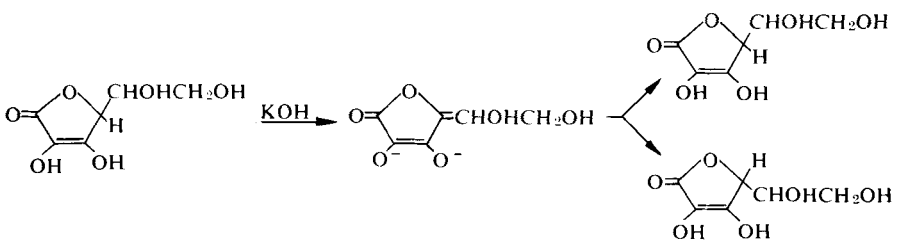

Fig. 1

In a study of the infrared spectra of deuterated ascorbic acid, Weigl [7] interprets variations in the infrared spectrum of normal and deuterated ascorbic acid as indicative of C-4 hydrogen exchange. The condition for the exchange was the mere dissolving of ascorbic acid in deuterated water at room temperature for 16 hours. It would appear from our studies that this conclusion must be incorrect. We have been able to work with ascorbic acid labeled with tritium in the number four position in room temperature solutions for protracted periods of time without loss of labels. This would indicate that ready exchange of this hydrogen does not occur as postulated. In addition, the studies of Brenner, confirmed by us, show that ascorbic acid in water solution does not isomerize to a mixture of arabo- and xyloascorbic acid, as would be necessary if Weigl's postulated exchange occurred.

The specific activity of the L-xyloascorbic-4-^{3}H acid obtained in synthesis Method I, corresponds to a 44% exchange of the carbon-4 hydrogen with the hydrogens of the aqueous methanolic solution. This corresponds to a somewhat lower rate of isomerization of the xyloascorbic acid to araboascorbic acid than was observed by Brenner for a comparable reflux time. However, this slower rate can be explained on the basis of a lower reflux temperature for our solution because of the elevation of Boulder. The barometric pressure here is approximately 620 mm Hg.

* We have also made studies on ascorbic-4-d_1 acid, prepared by Method II, described above, and preliminary studies on the nuclear magnetic resonance spectra of this molecule show that the deuterium has been incorporated on the number four carbon atom. Details of these experiments will be reported at a later date. The combination of our deuterium and tritium experiments together with the isomerization studies of Brenner show conclusively that in strong, hot, alkaline solutions there is exchange isomerization of the number four hydrogen in ascorbic acid.

Preparation of high specific activity L-xyloascorbic-4-^{3}H acid

The procedure of Brenner is not satisfactory for the preparation of high specific activity tritium-labeled ascorbate. The total number of exchangeable hydrogens in this solution is so large that it would require a tremendous amount of high specific activity tritium to give a reasonable amount of tritium-labeled ascorbic acid. We therefore investigated the possibility of carrying out this exchange isomerization reaction in alkaline KOH solutions containing limited amounts of water and no methanol. In strongly alkaline solutions, ascorbic acid forms the di-anion and the dipotassium salt can be isolated as a hygroscopic glass when solutions of dipotassium ascorbate are dried under vacuum. Our experiments show that concentrated alkaline solutions of ascorbic acid can be heated in the absence of air for as many as 20 hours without complete decomposition of the resulting xylo-araboascorbic acid mixture. The most concentrated solution that we studied was 15.8 M in dipotassium ascorbate and 1 M in KOH. A solution at this concentration showed excess browning and was of no value in exchange labeling of tritium. In one experiment, a tube containing a curie of tritium in 2 ml water and enough dipotassium ascorbate to make the solution 15.8 M formed a charred mass on heating and yielded only a trace of labeled ascorbate. There seems to be increased decomposition in the reaction due to self-irradiation from the tritium.

Contrary to Brenner's report, excess alkali is not necessary to effect the exchange isomerization. Loss by decomposition is also dependent on the amount of oxygen left in the sealed tubes. This loss was lowered by evacuating the tubes containing the dry material and distilling water into them under vacuum. It is possible to lower the decomposition by flushing the solution with nitrogen prior to evacuation and sealing. This decomposition would appear to be a chain reaction initiated by oxygen since the quantitative amounts of oxygen in these experiments were small compared to the dipotassium ascorbate.

The procedure for the exchange labeling of ascorbic acid as presented has not been optimized. It should be possible by a careful study of exchange conditions and the purification procedure to increase both the gravimetric and radiochemical yields. Since the principle object of this work was to obtain tritiated ascorbic acid, the volume of acetonitrile used in the first extraction was small (100 ml). All of the more soluble arabo-isomer dissolved in this volume, allowing pure xyloascorbic acid crystals to be harvested from the later extractions. The first two crops of crystals from the first extraction were mixtures of epimers. On prolonged standing, pure L-araboascorbic acid crystals will form.

Radiation stability

Ascorbic acid in the crystalline form is moderately unstable and in aqueous solution is quite unstable. The question arises, then, is ascorbic acid also unusually sensitive to decomposition by ionizing radiation? In order to inves-

tigate this question, a number of samples were subjected to gamma irradiation and analyzed by the method of Shaffert and Kingsley [9]. The results of these experiments are presented in table 1. These data do not show any great sensitivity of ascorbic acid to ionizing radiation. After 10^8 rads the irradiated samples were neither appreciably discolored nor changed in physical appearance.

These results indicate that the ascorbic-4-^{3}H-acid should not be unduly sensitive to self-irradiation. In addition, the tritium labelled material shows no change in appearance or chromatographic purity after six months storage in vacuo.

TABLE 1. Radiation sensitivity of crystalline ascorbic acid.*

Sample weight (mg)	Radiation dose Rads $\times 10^{-6}$	Percent decomposition
35.8	control	0
43.5	control	0
45.7	9.1	1.7
39.7	9.1	0.4
50.2	12.4	0.4
36.6	12.4	−0.7
43.7	56	1.1
40.6	56	2.8
45.9	118	0.5
42.0	118	1.0

* Reference Standard, U.S. Pharmacopeia. L-xyloascorbic acid.

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