Synthesis of Ascorbic-6-^{14}C Acid*

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SUMMARY

L-ascorbic-6-^{14}C acid with a specific activity of 3.25 μCi/mg was prepared from D-glucose-1-^{14}C in an overall yield of 10 %. Synthetic steps were : glucose to sorbitol, sorbose, diisopropylidene sorbose, potassium diisopropylidene 2-ketogulonate, 2-ketogulonic acid and ascorbic acid. In a similar manner L-ascorbic-5-^{14}C acid was prepared from D-glucose-2-^{14}C. Conversion of 2-keto-L-gulonic acid to ascorbic acid was done in 0.5 M trifluoroacetic acid solution at 115 °C with 2 % acetone as a decomposition inhibitor. The reaction probably proceeds via an enediol intermediate.

INTRODUCTION

Ascorbic-1-^{14}C acid [1, 2] (AsH_2) has been available commercially for some years. Labels in other positions [3] would be of considerable biochemical and nutritional interest but have not been offered commercially, probably because procedures described in the literature have not worked satisfactorily. The preparation of uniformly labeled ascorbate by the procedure of Reichstein [4] is reported by Bothner-By *et al.*[5] using alkaline lactone formation. However, when this laboratory group attempted to use this method to prepare ascorbic-6-^{14}C acid, the final steps did not work properly in the high specific activity run, and no product was obtained. Dayton [6] used a similar method with acid lactone formation to prepare ascorbate-6-^{14}C, but yields were poor.

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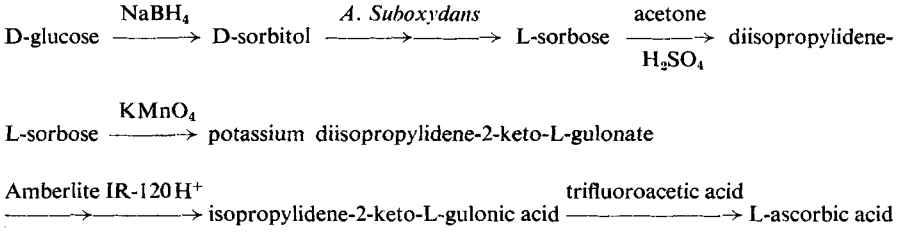
Other workers have not been able to make these methods work, especially in high specific activity runs. In recent years two commercial firms have undertaken preparations of ascorbic-6-^{14}C acid for us but have been unable to obtain a product in a reasonable time. In general, preparation of labeled precursors to 2-keto-L-gulonate go fairly well, but the lactonization reaction to ascorbic acid itself has been the source of most trouble.

In this paper we present a detailed procedure for the preparation of ascorbic acid from glucose on a one gram scale. Synthetic steps were :



A simple modern procedure for reducing the glucose to sorbitol is used. Extensive studies on the lactonization of 2-keto-L-gulonate to ascorbic acid are also reported. The best conditions for ring closure employ the use of trifluoroacetic acid (TFA) with acetone as a decomposition inhibitor. A probable mechanism for this reaction is proposed, which accounts for the difficulties involved in the ring closure step. The experimental procedure has been used to prepare ascorbic-6-^{14}C acid and ascorbic-5-^{14}C acid.

Experimental

D-sorbitol-1-^{14}C.

A solution of 150 mg (4 mmoles) sodium borohydride in 1 ml of 10 N ammonium hydroxide was added dropwise with constant stirring to a solution of D-glucose-1-^{14}C (230 mg/10 mCi, 1.3 mmoles) in 9 ml water in a 50 ml round-bottom flask. The temperature of the reaction solution was kept at 0 °C throughout the addition and for 30 minutes thereafter. The solution was then warmed to room temperature and stirred for 4 1/2 hours to insure completion of the reaction. The ammonium hydroxide was neutralized by adding slowly 1 ml of glacial acetic acid in 4 ml of water to the solution which had been re-cooled to 0-4 °C. The stirrer was removed and washed with water which was added to the reaction mixture. The solution was concentrated to dryness under vacuum on a Rotavac at 40 °C. Ten ml of methanol was added

and thoroughly mixed into the white viscous concentrate. The methanol was removed under vacuum; this treatment was repeated 6-7 times or until a white powdery product was obtained. The identity of this material as D-sorbitol was verified by chromatography on Whatman No. 1 paper with ethyl acetate : pyridine : water (8 : 2 : 1) for 12-18 hours, $R_{sorbitol}$: $R_{glucose}$, 0.87. In preliminary runs D-sorbitol was recrystallized from 90-95 % ethanol. M.p., 99.0 °C.

L-SORBOSE-6-^{14}C.

Acetobacter Suboxydans 621 (American Type Culture Collection) were cultured in 100 ml sterile broth containing 10 % D-sorbitol and 1 % Diffco yeast extract for 5 days. The bacteria were harvested by centrifugation at 500 × g. Approximately 20-25 mg of the pellet were transferred to a solution of D-sorbitol-1-^{14}C (218 mg/9.5 mCi) and D-sorbitol (770 mg) in 10 ml sterilized water, pH 5.5 to 6.5. The solution was incubated 30-32 °C until it contained less than 15 % D-sorbitol-1-^{14}C. The mixture was analyzed by paper chromatography in ethyl acetate : pyridine : water as described above. Sorbitol, glucose and sorbose standards were located by silver nitrate in acetone spray followed by ethanolic sodium hydroxide spray [7]. The migration of sorbitol and sorbose were measured with respect to D-glucose (R_g 1.0); D-sorbitol, R_g, 0.87; L-sorbose, R_g, 1.20. The percent conversion was determined by the distribution of the carbon 14. If no conversion was apparent within three days, the D-sorbitol-1-^{14}C solution was spiked with a sterilized solution of 5 mg Diffco yeast extract in 0.5 ml of water. If spiking with yeast extract didn't work within 10 days, the solution was centrifuged at 27,000 × *g*, and the supernatant was transferred to a sterile flask and reinoculated with fresh bacteria. In the high specific activity run this reinoculation procedure was repeated six times, often at intervals longer than ten days. Although 80-90 % conversions were achieved with unlabeled D-sorbitol solutions within 2-4 days, only 53 % conversion to L-sorbose-6-^{14}C over a period of several months was obtained.

The sorbose-6-^{14}C was purified by paper chromatography on nineteen sheets of Whatman paper No. 3, preferably acid washed. Approximately 0.5 ml of the solution (115 mg/ml) was spread along 48 cm of baseline. The papers were chromatographed as described above. The sorbose-6-^{14}C region was located by exposure to X-ray film for 2-3 hours. This region was cut from the paper, and the L-sorbose-6-^{14}C was eluted with water. The solutions were combined, and the water was removed by lyophilization. The L-sorbose-6-^{14}C residue was extracted with 70 ml of boiling 85 % ethanol. The extract was concentrated under vacuum, and the L-sorbose-6-^{14}C was dried overnight *in vacuo*. A 49 % yield or 4.7 mCi were recovered. Most of the unrecovered radioactivity was converted to a water soluble material with a low R_g.

2,3;4,6,-DI-O-ISOPROPYLIDENE-L-SORBOFURANOSE-6-^{14}C [5, 6].

A solution of 2 ml conc. sulfuric acid in 50 ml of acetone was saturated with N_2 at 0° C and added to 1 g (5.5 mmoles) solid L-sorbose (L-sorbose-6-^{14}C, 470 mg/4.7 mCi and commercial L-sorbose, 530 mg) under N_2 atmosphere in a 125 ml flask. The mixture was stirred continuously. After 30 minutes it was warmed to room temperature for 5-10 minutes, taking care to minimize evaporation of acetone with the N_2 sweep. The mixture was returned to the ice bath. The warming and cooling was repeated four times or until the L-sorbose had dissolved. The pale yellow solution was refrigerated overnight.

The diisopropylidene sorbose solution was added slowly with stirring to a cold solution of 8 g potassium carbonate in 60 ml of water. The original flask was washed with 200 ml of reagent grade acetone. The salts which precipitated as the acetone is added and as the mixture cools to 0 °C were removed by vacuum filtration and thoroughly washed with 90 ml acetone. The acetone was removed under vacuum, and the remaining water, 30-40 ml, was extracted with 7 × 100 ml of ether. The ether was concentrated to dryness leaving a yellow syrupy residue. The syrup was analyzed by T.L.C. as described below and gave one spot with sulfuric acid development, R_f 0.76, corresponding to 2,3;4,6-di-O-isopropylidene-L-sorbofuranose. 4.2 mCi were present in this fraction.

POTASSIUM 2,3;4,6-DI-O-ISOPROPYLIDENE-2-KETO-L-GULONATE-6-^{14}C.

A solution of 2.2 g (13.9 mmoles) potassium permanganate in 60 ml of water was added dropwise to the solution of diisopropylidene sorbose-6-^{14}C in 14 ml of 4 % potassium hydroxide. The addition was carried out slowly over a period of an hour always keeping the temperature of the reaction between —1° to 2° C with an ice-salt bath. The reaction mixture was allowed to stand at room temperature overnight. The excess potassium permanganate was destroyed by adding 50 ml of ethanol. The mixture was heated at 40 °C for 10 minutes to coagulate the manganese dioxide which was then removed by vacuum filtration. The filtrate was adjusted to pH 8.5 by bubbling carbon dioxide through it and concentrated to dryness. The white residue which remained was extracted with 8 × 25 ml of boiling absolute ethanol. The ethanol was removed, and the white material was extracted with 3 × 20 ml ether to remove any remaining diisopropylidene sorbose. The amount of potassium 2,3;4,6-di-O-isopropylidene-2-keto-L-gulonate-6-^{14}C was 1.59 g/2.7 mCi, R_f of 0.90 when analyzed by T.L.C. as described below. From this material 0.16 g/0.27 mCi was removed and stored under N_2 in a sealed tube for other studies. The yield of potassium diisopropylidene-2-keto-L-gulonate-6-^{14}C from L-sorbose-6-^{14}C was 64 % which was considerably lower than unlabeled runs which usually gave yields of 85 %.

2,3-O-ISOPROPYLIDENE-2-KETO-L-GULONIC-6-^{14}C.

Amberlite 1R-120 H^+, 20 to 25 g, pretreated with 2 N trichloroacetic acid, was added to a solution of potassium 2,3;4,6-di-O-isopropylidene-2-keto-gulonate-6-^{14}C (1.43 g, 4.6 mmoles) in 25 ml of water saturated and maintained in a nitrogen atmosphere. The mixture was stirred under nitrogen for one hour at room temperature. The Amberlite was removed and thoroughly washed with water. The washings were combined with the original filtrate. Analysis of this solution by T.L.C. showed one spot at R_f 0.60, which was presumed to be 2,3-O-isopropylidene-2-keto-L-gulonic-6-^{14}C acid. The aqueous solution was concentrated to a syrup under vacuum.

L-ASCORBIC-6-^{14}C ACID.

The syrup of 2,3-O-isopropylidene-2-keto-L-gulonic-6-^{14}C acid was dissolved in 20 ml 0.5 N trifluoroacetic acid saturated with nitrogen and aliquoted evenly into five pyrex 22 $\times$ 175 mm test tubes which had been previously constricted for sealing. The original flask was washed serially with 2 $\times$ 20 ml and 1 $\times$ 15 ml of 0.5 N trifluoroacetic acid, and the washings were aliquoted into the tubes. Each tube, which now contained 0.3 g (1.7 mmole) of material in 15 ml of 0.5 N trifluoroacetic acid, was flushed with nitrogen, and 0.3 ml acetone was added. Nitrogen was bubbled through the solutions for ten minutes, and the tubes were sealed. They were heated in an oil bath at 115° $\pm$ 1 °C for 100 to 110 minutes or until the solutions began to darken. The tubes were removed, immediately cooled in an ice bath and stored at -20 °C overnight. L-ascorbic acid (30 mg) was added to each tube as they were opened. The solutions were centrifuged at 27,000 $\times$ g for 10 minutes, and the supernatants were combined and extracted with 3 $\times$ 80 ml ether. The ether extracts were washed with 20 ml water, which was added to the aqueous solutions. The aqueous solution containing 1.7 mCi carbon-14 was concentrated to dryness.

The residue was dissolved in 135 ml boiling acetonitrile, and the solution was concentrated by nitrogen sweep to about 70 ml. Crude ascorbic acid crystals formed and were separated by decantation. The ascorbic acid was recrystallized under N_2 from 100 ml of hot acetonitrile to which 150 mg of commercial L-ascorbic acid was added. L-ascorbic-6-^{14}C acid (0.24 g, 0.77 mCi, sp. act., 3.25 μCi/mg), was collected, m.p. 187.0 °C. Radioassay on T.L.C. indicated at least 98.6 % of the carbon-14 was present as L-ascorbic-6-^{14}C acid, R_f 0.26. A second crop of material (96 mg, 0.079 mCi, spec. act. 0.82 μCi/mg m.p. 186.0-187.0 °C) was obtained after spiking the original liquor with 90 mg ascorbic acid. Radioassay indicated that only 86 % of the carbon-14 was present as ascorbic acid. A third crop of L-ascorbic-6-^{14}C acid (76 mg, 0.092 mCi, spec. act. 1.22 μCi/mg m.p. 187.5 °C) was obtained after spiking the first recrystallization liquor with 80 mg L-ascorbic acid. Radioassay indicated 96 % of the carbon-14 label was present as ascorbic acid. The yield

from D-glucose-6-^{14}C corrected for the 0.27 mCi of potassium diisopropylidene-2-keto-L-gulonate-6-^{14}C removed was 10 %; from L-sorbose, 22 % and from the isopropylidene-2-keto-L-gulonate acid, 48 %.

L-ASCORBIC-5-^{14}C ACID.

The above procedure was used in a preliminary run to prepare L-ascorbic-5-^{14}C acid from D-glucose-2-^{14}C (100 μCi). Most of the 100 μCi of carbon-14 material was lost in the preparation of L-sorbose-5-^{14}C, but 0.3 μCi/0.3 g of L-sorbose-5-^{14}C was recovered, which was converted to potassium diisopropylidene-2-keto-gulonate-5-^{14}C. This material was used in confirming the best method to convert labeled material to ascorbate. The method described above for L-ascorbic-6-^{14}C was tested with 0.9 g (0.05 μCi) of potassium diisopropylidene-2-keto-L-gulonate-5-^{14}C. L-ascorbic-5-^{14}C acid (0.113g, 0.02 μCi) was obtained in 42 % yield, m.p. 185.0° C.

ANALYTICAL TECHNIQUES

Thin layer chromatography was used to analyze all products except D-glucose, D-sorbitol and L-sorbose. Plates (20 × 20 cm) were covered with a 0.25 mm layer silica gel G (15 g in 60 ml of 5 % methanol, 1.8 g of metaphosphoric acid solution). The solvent system used to develop the plates was acetonitrile : butyronitrile : water, 66 : 33 : 2. The ascorbic acid was detected by spraying with 5 % iodine in chloroform. Ascorbic acid appears as a yellow spot. Other products were detected by spraying with sulfuric acid and heating the plates at 120 °C for 30 minutes.

If the distribution of the carbon-14 was to be determined, standards were run simultaneously on the same plate, and the ascorbate was located by spraying with the 5 % iodine in chloroform. The plate was appropriately sectioned, and the silica gel from each section was scraped off and placed in a scintillation vial. The silica was covered with 0.5 to 1.0 ml water and shaken, and the water was solubilized in Bio-Solv-3-toluene cocktail * (120 ml of Bio-Solv-3, 1 liter of toluene, 7 g of PPO and 0.42 g dimethyl POPOP). The samples were counted by a liquid scintillation counter, spiked with ^{14}C-toluene standard and recounted. From these data dpm and millicuries were calculated.

RESULTS AND DISCUSSION

The experimental procedure for synthesis of ascorbic-6-^{14}C from glucose-1-^{14}C has been presented in considerable detail because many of the steps require careful technique. Monitoring progress of the synthesis by T.L.C.

* Beckman Instrument Co., Fullerton, Calif., U.S.A.

and paper chromatography is essential during trial runs and especially during the high specific activity preparation in order to assure adequate purity and yields of intermediate products to give a final crystalline product.

D-sorbitol-1-^{14}C was prepared by direct reduction of glucose-1-^{14}C with sodium borohydride, a convenient reagent for small scale synthesis [8]. Complete removal of the borate after reduction by volatilization of methyl borate is essential if sorbitol is to be obtained in a crystalline form and to prevent possible poisoning of the *A. Suboxydans*. The bacterial oxidation of unlabeled sorbitol, either commercial material or prepared from glucose with borohydride, gave yields of 70-85 % in a few days, The labeled sorbitol was converted in yields of only 40-50 % and required long incubation times, weeks to months. In the labeled runs aeriation and shaking made no improvement in yield although they are used in commercial processes [9]. Purification of labeled sorbitol by anion exchange resin or Sephadex G-10 chromatography did not increase rate or yield of conversion. Sterile techniques, millipore filtration and high speed centrifugation were used to control microbial contamination in this very slow reaction.

The method described by Dayton [6] was followed in preparing both the diisopropylidene sorbose and the potassium diisopropylidene-2-keto-gulonate. Satisfactory yields were obtained without crystallizing products before proceeding to the next step. Yields of diisopropylidene sorbose was 89-95 % based on sorbose. Yields of potassium diisopropylidene-2-ketogulonate was 85-95 % based on sorbose if the reaction temperature did not rise more than 2 °C during the addition of the potassium permanganate and if this fluctuation of temperature occurred in the region of -2 °C to 2 °C. In the preparation of potassium diisopropylidene-2-ketogulonate-6-^{14}C, a yield of only 64 % based on diisopropylidene sorbose was obtained.

Removal of potassium ion from the diisopropylidene-2-ketogulonate solution was made by treatment with Amberlite 1R-120 H^+ resin to facilitate the crystallization of free ascorbic acid in the final step. This treatment also removes one of the isopropylidene groups. Assignment of the product as 2,3-isopropylidene-2-ketogulonic acid was based on T.L.C. behavior and subsequent hydrolysis of this material to 2-ketogulonic acid, m.p. 169-170 °C, R_f 0.02, and the fact that isopropylidene residues on potential reducing groups are more firmly attached [10]. The 2,3-isopropylidene group is rapidly lost when the compound is heated in 0.5 N trifluoroacetic acid above 80-90 °C, as described in the lactonization step. Free 2-keto-L-gulonic acid is easily prepared. A solution of isopropylidene-2-ketogulonic acid in 0.5 to 2 N trifluoroacetic acid is converted to 2-ketogulonic acid overnight at room temperature or by heating a few minutes at 80-90 °C. Yields are good, and the product is easily recrystallized from ethanol.

LACTONIZATION OF 2-KETO-L-GULONIC ACID.

Proper conditions for conversion of 2-keto-L-gulonic acid to ascorbic acid required extensive study. Earlier work by this laboratory group (R. Bevill and E. Baker, unpublished data) on a synthesis via the methyl ester derivative and alkaline lactonization was unsuccessful. No product was obtained even though the apparent yield of the 2-keto-L-gulonic acid was satisfactory. Dayton [6] did not use alkaline lactonization although he had the help of Bothner-By [5] who had used alkaline lactonization in an earlier preparation of ascorbic acid. For these reasons and because of the difficulty in working with sodium metholate solutions on a small scale we chose to reinvestigate acid lactonization.

Hay *et al.*[11] have reported on patented procedures for the conversion of 2-keto-L-gulonic acid to ascorbic acid in acid solutions. In addition acid lactonization was used by Dayton and Ault [6, 12]. Data on the comparative ability of four strong acids to catalyze this reaction are presented in Table I. The reactions were carried out in sealed tubes in solutions saturated in nitrogen and containing comparable amounts of the respective acids. The reaction mixtures were assayed by T.L.C., and comparative yields were approximated by color development. All four acids are capable of giving some conversion of the 2-keto-L-gulonic acid to ascorbic acid, but the least decomposition and the best yield of ascorbic acid occurred using TFA. In addition TFA has the great advantage in small scale synthesis because it can be readily removed under vacuum.

Results of further experiments to determine the optimum conditions for the lactonization reaction with TFA are given in Table II. Comparable conversions of 2-ketogulonic acid to ascorbic acid are obtained with 2 N TFA,

TABLE I. Acid Conversion of 2-Ketogulonic Acid to Ascorbic Acid.

	3.3 M H_3PO_4 or TCA		2 M TFA		2.8 M HCl	
	45 °C 24 hr	100 °C 1 hr	45 °C 24 hr	100 °C 1 hr	45 °C 24 hr	100 °C 1 hr
Ratio of L-Ascorbic Acid to 2-Ketogulonic Acid	0[a]/100	20/80	5/95	40/60	1/99	90[b]/10

[a] Ascorbate spot was visible with iodine in chloroform, but not with sulfuric acid development.

[b] Extensive charring and degradation of material occurred.

TABLE II. Conversion of 2-Ketogulonate to Ascorbic Acid with Trifluoroacetic Acid [a].

Condition	Ratio of ascorbic acid to 2-ketogulonic acid	
	1.0 N TFA	0.5 N TFA
2 hours at 100 °C	>95/5[b]	80/20
1 1/2 hours at 115 °C	>95/5[b]	90/10
2 1/4 hours at 115 °C	—	95/5[b]
1/2 hour at 120 °C + 1 hour at 110° C[c]	>95/5[b]	85/15

[a] Based on comparative intensity of the ascorbic acid spot to the 2-ketogulonic acid spot. These data do not consider material lost by decomposition.

[b] In these reactions a considerable amount of charred material was present.

15 minutes at 120 °C and with 0.5 N TFA, 1-2 hours and 115 °C. However, there is less charring with the lower acid concentration, and the reaction is more readily controlled. There is always the danger that a reaction that gives some decomposition in a test run will result in extensive decomposition in a high specific activity run. Therefore, the milder lactonization conditions were chosen.

The decomposition of ascorbic acid in these reaction mixtures appeared to be autocatalytic. Acetone was found to retard the degradation of ascorbic

TABLE III. Carbon-14 Assay of Products from TFA Catalyzed Lactonization of 2-Keto-L-gulonic Acid [a].

Material	Ascorbic Acid mCi	2-Ketogulonate mCi	Decomposition products, mCi
Ether extract	—	—	0.68[b]
Crop 1 : AsH_2-6-^{14}C	0.77	0.01	
Crop 2 : AsH_2-6-^{14}C	0.07	0.01	
Crop 3 : AsH_2-6-^{14}C	0.086	0.004	
Combined liquors	0.28	0.22	0.29
Totals	1.20	0.24	0.97

[a] Recovery of carbon-14 based on 2.43 mCi starting activity : crystalline AsH_2, 38 %; AsH_2 present in liquor, 11 %; total AsH_2, 49 %; 2-ketogulonate, 10 %; decomposition products, 40 %; total, 99 %.

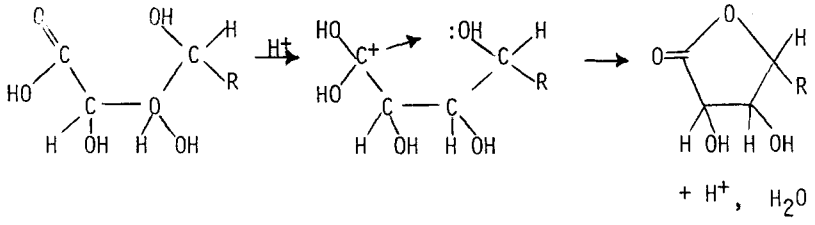
[b] Contained no ascorbic acid or starting compounds.

acid in hot acid solutions and was used in all the experiments described above. It is probable that the acetone acts as a free radical scavenger, and thereby retards the onset of rapid decomposition. In any case once the hot solution begins to darken appreciably the reaction should be terminated. At that time the rate of decomposition of ascorbic acid is faster than the rate of of synthesis by lactone formation.

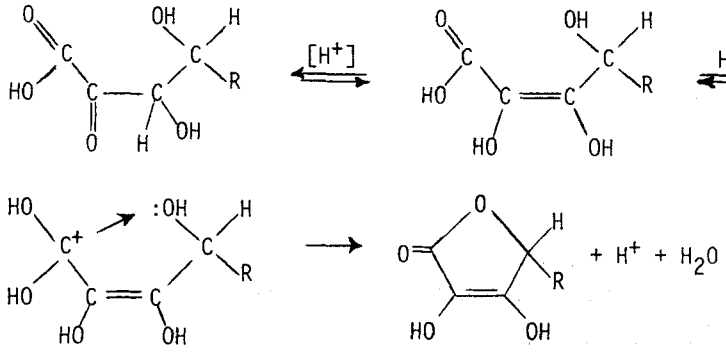
Table III summarizes the material balance for the high specific activity conversion of 2-ketogulonic acid to ascorbic acid.

Mechanism

The experiments discussed above indicate that formation of the γ-lactone of 2-ketogulonic acid requires drastic conditions. In contrast gulonic acid and many other sugar acids form gamma lactones under mild conditions. Such lactonizations usually proceed through a carbonium ion intermediate formed by preliminary protonization of the carboxylic acid group :



The severe conditions required for the synthesis of ascorbic acid indicate that this is not a likely mechanism. In general it is not easy to form a carbonium ion intermediate if there is an adjacent keto group. On the other hand, carbonium ions of carboxylic acids form readily if adjacent to an enediol group. If lactonization of 2-ketogulonic acid proceeds through a cis enediol intermediate, the mechanism of cyclization would be :



In this mechanism it should be emphasized that three prior equilibrium steps precede lactonization : hydroxyketone $\rightleftarrows$ enediol; *trans*-enediol $\rightleftarrows$ *cis*-enediol; and *cis*-enediol $\rightleftarrows$ protonated *cis*-enediol. The formation of the enediol is catalyzed by strong acids, and high temperatures give an appreciable concentration of the critical intermediate. Thus optimum conditions for lactonization of 2-ketogulonic acid in TFA require only modest acid concentrations and as high a temperature as is consistent with the thermal stability of the reactants and product. There is no reason to postulate an actual 2-ketogulonolactone intermediate in this synthesis.

The enediol intermediate in alkaline lactonization of methyl 2-ketogulonate has been suggested by Reichstein *et al.*[4]. Thus these two processes for ring closure are roughly parallel, but one is acid catalyzed and the other alkali catalyzed.

An alternate mechanism for ring closure is that the 2-ketogulonate might be acid isomerized to 3-ketogulonate, which could undergo lactonization and isomerization to ascorbic acid. Under the conditions of the reaction the 3-keto acid should be rapidly decarboxylated, and we observed no evidence for CO_2 formation in this synthesis. It seems more likely that the rather stable carbonium ion of the enediol is converted to the lactone with high efficiency.

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