Journal of Research of the National Bureau of Standards Vol. 59, No. 4, October 1957 Research Paper 2800

Synthesis of C^{14}-Labeled L-Sorbose and L-Ascorbic Acid[1]

H. L. Frush and H. S. Isbell

A method is presented for preparing C^{14}-labeled L-ascorbic acid (vitamin C) from labeled barium D-gluconate or D-glucose. The process is adapted from the commercial synthesis of vitamin C through sorbitol, L-sorbose and potassium di-*O*-isopropylidene-2-*keto*-L-gulonate. In the cyanohydrin synthesis of labeled D-glucose, barium D-gluconate is used as an intermediate. For preparing labeled L-ascorbic acid, the gluconate is converted to the lactone. This is efficiently reduced, by means of sodium borohydride, directly to sorbitol, obviating the intermediate preparation of D-glucose.

If labeled D-glucose is more readily available than the corresponding gluconic acid (as for instance D-glucose-6-C^{14} or randomly labeled D-glucose), the sugar is reduced to sorbitol by the sodium borohydride method; the yield is nearly quantitative.

In a preparation of L-ascorbic acid-6-C^{14} from barium D-gluconate-1-C^{14} the yield was 41.5 percent, based on the gluconate, or 67.8 percent, based on the intermediate L-sorbose. A mixture of Methyl Cellosolve, ethylene dichloride, and hydrogen chloride was found to be effective in converting potassium di-*O*-isopropylidene-2-*keto*-L-gulonate to L-ascorbic acid.

1. Introduction

Carbon-14-labeled L-ascorbic acid (vitamin C) is a useful tool for studying many problems in biology and medicine. Before the present work, 1-labeled L-ascorbic acid had been prepared from L-xylosone through the addition of C^{14}-labeled cyanide [1],[2] and randomly labeled L-ascorbic acid had been prepared from randomly labeled D-glucose [2] by the process of Reichstein and Grüssner [3]. In the present commercial process for synthesizing L-ascorbic acid, the following substances are successively prepared from D-glucose: Sorbitol; L-sorbose; di-*O*-isopropylidene-L-sorbose; potassium di-*O*-isopropylidene-2-*keto*-L-gulonate; and finally L-ascorbic acid. The process differs from that of Reichstein and Grüssner chiefly in the manner of converting L-sorbose to diacetone L-sorbose, and potassium di-*O*-isopropylidene-2-*keto*-L-gulonate to L-ascorbic acid [4, 5]. Because D-glucose or D-gluconic acid labeled in position 1, 2, or 6 is now readily available, it is possible to prepare L-ascorbic acid labeled respectively in position 6, 5, or 1. This paper reports improved procedures, adapted from the commercial process, for preparing C^{14}-labeled L-ascorbic acid.

2. Discussion of Experimental Methods

The Bureau has developed efficient methods for converting barium carbonate-C^{14} to sodium cyanide-C^{14} [6]; for successively preparing, from sodium cyanide-C^{14}, C^{14}-labeled cyanohydrins, aldonic acids, and lactones [7]; for reducing lactones directly to glycitols [8]; and for preparing D-glucose-6-C^{14} [9].

The method reported here for preparing labeled L-ascorbic acid starts with labeled D-glucose, or barium D-gluconate (which is converted to D-glucono-δ-lactone). After reduction of the sugar,[3] or lactone, with sodium borohydride, the resulting crude sorbitol is not crystallized, but oxidized to L-sorbose with *Acetobacter suboxydans* [11, 12]. Preliminary study of this oxidation with nonradioactive sorbitol showed a maximum yield of L-sorbose of 95 percent in 40 hr. The yield obtained with crude sorbitol-6-C^{14}, however, was lower (69%), and the fermentation proceeded more slowly than that of nonradioactive sorbitol used as a control.

The next intermediate, 2,3-4,6-di-*O*-isopropylidene-L-sorbose-6-C^{14}, was prepared from L-sorbose-6-C^{14}, acetone, and sulfuric acid under conditions previously reported by Slobodin [4]. The di-*O*-isopropylidene-L-sorbose, when oxidized with potassium permanganate in alkaline solution [3], gave crude potassium di-*O*-isopropylidene-2-*keto*-L-gulonate in high yield. This intermediate, treated with a mixture of hydrochloric acid, methanol, and chloroform, under conditions similar to those described by Elger [5], yielded about 50 percent of L-ascorbic acid-6-C^{14}. However, the reaction mixture turned dark and formed a small amount of humuslike material. To improve the last step, other solvents and conditions were studied. It was found that mixtures of hydrochloric acid and Methyl Cellosolve (ethylene glycol monomethyl ether) considerably improve the yield of L-ascorbic acid, which crystallizes readily following the addition of ethylene dichloride.

[1] Part of a project on the development of methods for the synthesis of radioactive carbohydrates sponsored by the Division of Research, Atomic Energy Commission.

[2] Figures in brackets indicate the literature references at the end of this paper.

[3] Sodium borohydride reduces sugars almost quantitatively to glycitols in alkaline solution [10].

The accompanying diagram gives a summary of the steps in the synthesis. The over-all yield of L-ascorbic acid-6-C^{14} from barium D-gluconate-1-C^{14} was 41.5 percent, and from L-sorbose-6-C^{14}, 67.8 percent.

Barium D-gluconate-1-C^{14}
↓ (89.2%)
Sorbitol-6-C^{14} (in solution)
(D-Glucitol-1-C^{14})
↓ (68.7%)
L-Sorbose-6-C^{14}
↓ (84.9%)
Di-*O*-isopropylidene-L-sorbose-6-C^{14}
↓ (94.5%)
Potassium di-*O*-isopropylidene-2-*keto*-L-gulonate-6-C^{14}
↓ (84.5%)
L-Ascorbic acid-6-C^{14}
(over-all yield 41.5%)

3. Experimental Procedures

3.1. Sorbitol-6-C^{14} (or D-Glucitol-1-C^{14})

Sorbitol-6-C^{14} was obtained by sodium borohydride reduction of D-glucono-δ-lactone-1-C^{14} [8]. The process began with barium D-gluconate-1-C^{14} and was conducted in the following manner: A 1.38-g quantity of barium D-gluconate-1-C^{14} having an activity of 1,756 microcuries (μc) [4] was dissolved in water and passed through a column containing 20 ml of cation exchange resin.[5] The combined effluent and washings were concentrated under vacuum in a 100-ml flask to a thin sirup, which was lactonized by alternate addition of Methyl Cellosolve and evaporation in a current of air. After 8 or 10 evaporations over the course of three weeks, lactonization was complete and the product appeared to be entirely crystalline. The flask was then cooled in an ice bath, and 50 ml of 0.05-*M* aqueous boric acid and 10 ml of Amberlite IR 120 resin were added. With efficient mechanical stirring, 50 ml of freshly prepared 0.3-*M* sodium borohydride was added during 5 min. Stirring was continued for 30 min, and a second 50-ml portion of the borohydride solution was added as before. After an additional 30 min, 10 ml of 1-*N* sodium hydroxide was added, and the mixture was stored overnight at about 5° C. It was then passed through a column containing 100 ml of cation exchange resin. The effluent and washings were concentrated in a rotating vacuum still at 30° C to a volume of about 10 ml.

In order to remove boric acid as volatile methyl borate, 50 ml of methanol was added and the solution was evaporated under reduced pressure. This procedure was repeated, and after five additions of methanol, each followed by evaporation to a sirup, a test for boric acid with acidified turmeric paper was negative. The material was dissolved in water, and aqueous sodium hydroxide was added until a permanent pink color was obtained with phenolphthalein. The solution was then passed through a column containing 30 ml of mixed cation (see footnote 5) and anion [6] exchange resin. The effluent and wash liquor were concentrated in a vacuum to a volume of 20 ml. An assay of the solution by a direct count of a portion in formamide [13] showed 1,566 μc of activity. Hence, 89.2 percent of the barium D-gluconate-1-C^{14} had been reduced, after conversion to the lactone. The product, sorbitol-6-C^{14}, was not crystallized, but used directly for the preparation of L-sorbose-6-C^{14}.

3.2. L-Sorbose-6-C^{14}

To study the oxidation of sorbitol to L-sorbose, a solution was prepared from 3.64 g of nonradioactive sorbitol, 1.0 g of yeast extract, 0.6 g of potassium dihydrogen phosphate and 200 ml of water. Aliquots (25 ml) were placed in 125-ml Erlenmeyer flasks and sterilized for 15 min at a steam pressure of 15 lb. Each flask was then inoculated with 5 drops of a previously prepared inoculum of *Acetobacter suboxydans*, and incubated at 30° C. (The inoculum was prepared by growth, at 30° C for 45 hr, of a fresh culture of *Acetobacter suboxydans* on a medium containing 2 g of sorbitol, 0.2 g of yeast extract, and 40 ml of water.)

At intervals, one of the flasks was removed from the incubator, and the volume of solution was adjusted to 25 ml at 20° C; 0.2 g of Filtercel (diatomaceous earth) was added, and the mixture was filtered through a dry paper into a 2-dm saccharimeter tube. The optical rotation was measured, and the yield of sorbose was calculated from the specific rotation of L-sorbose (−43.4°). Calculated yields of 23.3, 95.7, 94.4, 92.1, 88.1, and 83.0 percent were obtained after incubation times of 20, 40, 45, 49, 66, and 94 hr, respectively. Thus, under the conditions used, L-sorbose reached a maximum in about 40 hr.

For the preparation of L-sorbose-6-C^{14}, the solution described in section 3.1, containing approximately 4.2 mM of sorbitol-6-C^{14}, and having an activity of 1,566 μc was divided into two equal parts. Each of these was transferred, with sufficient water to give a volume of 25 ml, to a 125-ml Erlenmeyer flask containing 125 mg of yeast extract and 75 mg of potassium dihydrogen phosphate. The mixture was sterilized at 15 lb of pressure for 15 min and then incubated at 30° C with five drops of a previously prepared inoculum of *Acetobacter suboxydans*. After 72 hr, the fermentation mixtures had approximately the same amount of turbidity as that observed for control experiments with nonradioactive sorbitol at the point of maximum sorbose content (40 hr). At this point the contents of the two flasks were combined and treated with 5 ml of 20-percent aqueous zinc sulfate, followed by sufficient aqueous barium hydroxide to give a blue color

[4] The work described in sections 3.1–3.2, 3.3, and 3.4–3.5, was carried out with materials having specific activities, respectively, of 370, 259, and 60 μc/mM.
[5] Amberlite IR 120–H, Rohm and Haas Co., Philadelphia, Pa.
[6] Duolite A4, Chemical Process Co., Redwood City, Calif.

with bromothymol blue. After the addition of 0.5 *g* of Filtercel, the solution was filtered, and the precipitate was washed thoroughly, and then discarded. The filtrate was passed slowly through a column containing 35 ml of a mixture of cation and anion exchange resin; a conductivity test of the combined solution and washings showed nearly complete absence of ionic impurities.[7] The product was concentrated under reduced pressure to a thick sirup, diluted with about 1 ml of methanol, and treated with 2-propanol to the point of incipient turbidity. It was then seeded with L-sorbose and allowed to stand for several days, after which a crop of crystalline L-sorbose-6-C^{14} was separated; the recrystallized product had an activity of 934 μc. An additional 142 μc of L-sorbose-6-C^{14} was separated from the mother liquor by use of a total of 500 mg of carrier L-sorbose in three portions. The total radioactivity, 1,076 μc, was 68.7 percent of that of the crude sorbitol-6-C^{14}.

3.3. Di-*O*-Isopropylidene-L-sorbose-6-C^{14}

One ml of concentrated sulfuric acid was added dropwise to 25 ml of dry acetone at 0° C. Twenty milliliters of this solution was placed in a 200-ml round-bottomed flask, together with 540 mg (3mM) of finely powdered L-sorbose-6-C^{14} having an activity of 777 μc (see footnote 4). The mixture was stoppered, and shaken mechanically until all of the L-sorbose had dissolved (1 to 2 hr). The pale yellow solution was placed first in a refrigerator at 0° C for 18 hr, and then in a freezer at −20° C for 24 hr. The cold material was diluted with a solution of 3 g of potassium carbonate in 25 ml of ice water, followed by 100 ml of acetone. Precipitated potassium sulfate was removed by filtration, washed with acetone, and discarded. The acetone solution was concentrated on a rotary vacuum still nearly to dryness, and the residue was carefully extracted in a hot water bath with successive 50-ml portions of heptane. The extracts were removed by a vacuum siphon arrangement to a 200-ml round-bottomed flask. Following the fourth extraction, the residue was dissolved in acetone, and the solvent was evaporated as completely as possible under vacuum. After one additional extraction of the residue, the combined heptane extracts were concentrated under vacuum to a sirup, which crystallized when seeded with di-*O*-isopropylidene-L-sorbose. The material, when recrystallized from hot heptane, had an activity of 611 μc. By use of 500 mg of nonradioactive di-*O*-isopropylidene-L-sorbose, material having an additional 49μc of activity was recovered from the mother liquor, to give a total radiochemical yield of 84.9 percent.

3.4. Potassium Di-*O*-isopropylidene-2-*keto*-L-gulonate-6-C^{14}

A solution containing 500 mg of di-*O*-isopropylidene-L-sorbose-6-C^{14} (having an activity of 128 μc, see footnote 4) and 0.225 g of potassium hydroxide in 5 ml of water was cooled to 0° C, and a solution of 0.43 g of potassium permanganate in 10 ml of water was added dropwise, with shaking, over the course of ½ hr. Then the mixture was mechanically shaken for 4 hr. At the end of this time it was heated to 50° C to coagulate the manganese dioxide, and 2 ml of ethanol was added to remove unreacted permanganate. The solution was filtered, and the residue washed with water and discarded; the filtrate was saturated with carbon dioxide and freeze-dried. To recover any remaining di-*O*-isopropylidene-L-sorbose, the dry residue was extracted 4 times with 10-ml portions of ether. However, the ether extract contained less than 1 μc, and hence was discarded. The main product, containing 121 μc of carbon-14, was used without purification for the production of ascorbic acid.

3.5. Conversion of Potassium Di-*O*-isopropylidene-2-*keto*-L-gulonate-6-C^{14} to L-Ascorbic Acid-6-C^{14} by Hydrogen Chloride in Methanol

The solution of crude potassium di-*O*-isopropylidene-2-*keto*-L-gulonate-6-C^{14} (2.0 mM having an activity of 121 μc) was passed through a column containing 10 ml of cation exchange resin. The effluent was concentrated under vacuum to a sirup, which was transferred with a little ethanol to a 10-ml glass ampoule. The sirup was then dehydrated by the repeated addition and evaporation of absolute ethanol, and finally was dissolved in 0.2 ml of methanol containing 20 percent of hydrogen chloride. After the addition of 2 ml of chloroform, the ampoule was sealed and stored at room temperature. In the course of 3 weeks, a crop of L-ascorbic acid-6-C^{14} crystallized from solution; the crystals were separated and washed with acetic acid. After recrystallization from glacial acetic acid with the addition of chloroform to the point of incipient turbidity, a crop of L-ascorbic acid-6-C^{14} having an activity of 71 μc was obtained. The dark-colored mother liquors were diluted with an equal volume of toluene and concentrated in a rotary vacuum still to a thick sirup. By use of 400 mg of L-ascorbic acid as carrier, and cocrystallization from acetic acid, material having an additional 20 μc of radioactivity was obtained, to give a radiochemical yield of 75.2 percent.

3.6. Conversion of Potassium Di-*O*-isopropylidene-2-*keto*-L-gulonate to L-Ascorbic Acid by Hydrogen Chloride in Methyl Cellosolve

To study the conversion step, 1-mM samples of potassium di-*O*-isopropylidene-2-*keto*-L-gulonate in Methyl Cellosolve-hydrogen chloride mixtures were stored at 40° C under nitrogen in sealed glass tubes, and the yield of L-ascorbic acid was determined by titration with iodine in acid solution. The results are reported in table 1. Relatively little color developed, and the yields of L-ascorbic acid were somewhat higher than those obtained in comparable experiments with the conventional methanol-hydrogen chloride mixtures.

[7] Test made with Barnstead Purity Meter, Barnstead Still and Sterilizer Co., Inc., Boston, Mass.

In view of the results given in table 1, the following procedure was selected for converting the *keto*-acid to L-ascorbic acid: A 2-mM sample of potassium di-*O*-isopropylidene-2-*keto*-L-gulonate in aqueous solution was passed through a column containing 10 ml of ice-cold cation exchange resin. The effluent was concentrated under vacuum to 10 ml and was then freeze-dried. The residue was dissolved in a mixture of 1 ml of concentrated hydrochloric acid and 7.5 ml of Methyl Cellosolve. The solution was diluted with 15 ml of Methyl Cellosolve and stored under nitrogen at 40° for 1 week. Then it was concentrated under vacuum to about 1 ml, diluted with 5 ml of Methyl Cellosolve, reconcentrated to 1 ml, and diluted with 5 ml of ethylene dichloride. In the course of 24 hr, a crop of crystalline L-ascorbic acid formed. The mother liquor was separated, and the product was washed with acetone. It was recrystallized by dissolving it in Methyl Cellosolve, concentrating the solution under vacuum to a low volume, and adding 5 ml of ethylene dichloride. The recrystallized product weighed 265 mg; titration with iodine showed the presence of 1.49 mM of L-ascorbic acid. The mother liquor contained 0.20 mM by analysis, making the total yield in the last step 1.69 mM, or 84.5 percent.

TABLE 1. *Yields of ascorbic acid from potassium di-O-isopropylidene-2-keto-L-gulonate in Methyl Cellosolve-hydrogen chloride mixtures*

Rearrangement mixture [a]		Temperature	Reaction time	Yield of ascorbic acid [b]
Methyl Cellosolve	Concentrated HCl			
ml	*ml*	° *C*	*hr*	%
4	0.4	40	140	54
			304	80
			520	82.8
4	1.2	40	67	71
			140	84
			304	82
8	0.4	40	140	48
			304	76
			520	79.2

[a] One millimole of potassium di-*O*-isopropylidene-2-*keto*-L-gulonate plus the materials listed below.
[b] Calculated from titration of the product with iodine in acid solution.

The authors are grateful to Galen Wampler for assistance in the experimental work.

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WASHINGTON, April 18, 1957.

U. S. GOVERNMENT PRINTING OFFICE: 1957