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A GENERAL PROCEDURE FOR THE PREPARATION OF LABELED L-ASCORBIC ACID FROM LABELED D-GLUCOSE*

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ABSTRACT

A simple, three-step conversion of 1,2-*O*-isopropylidene-α-D-glucofuranose into L-ascorbic acid, originally described by Bakke and Theander, was used to prepare L-[4-^{14}C]ascorbic acid from milligram amounts of D-[3-^{14}C]glucopyranose in 28% radioisotopic yield. In addition, L-[6-^{14}C]- and L-[U-^{14}C]-ascorbic acid were prepared from D-[1-^{14}C]- and D-[U-^{14}C]-glucopyranose, respectively. The procedure is useful for the synthesis of L-ascorbic acid bearing isotopic hydrogen, carbon, or oxygen atoms at specific positions, subject only to the availability of starting material.

INTRODUCTION

To the general methods for the synthesis[1] of L-ascorbic acid has been added a novel procedure involving the one-step oxidation of 1,2-*O*-isopropylidene-α-D-glucofuranose[2] or 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone[3], hydrolysis of the protecting group, and reduction with borohydride. We have used this new technique to prepare labeled L-ascorbic acids under conditions where labeled starting-material was limited to milligram amounts, and now report the synthesis of L-[4-^{14}C]-, -[6-^{14}C]-, and -[U-^{14}C]-ascorbic acid.

EXPERIMENTAL

General. — D-[3-^{14}C]-, -[6-^{14}C]-, and -[U-^{14}C]-Glucose (**1**) were purchased from New England Nuclear Corporation. 1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose[4] (**2**), 1,2-*O*-isopropylidene-α-D-glucofuranose[5] (**3**), 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone[6] (**3d**), and 1,2-*O*-isopropylidene-α-D-*xylo*-5-hexulofuranurono-6,3-lactone monohydrate[7] (**4**) were prepared for use as standard com-

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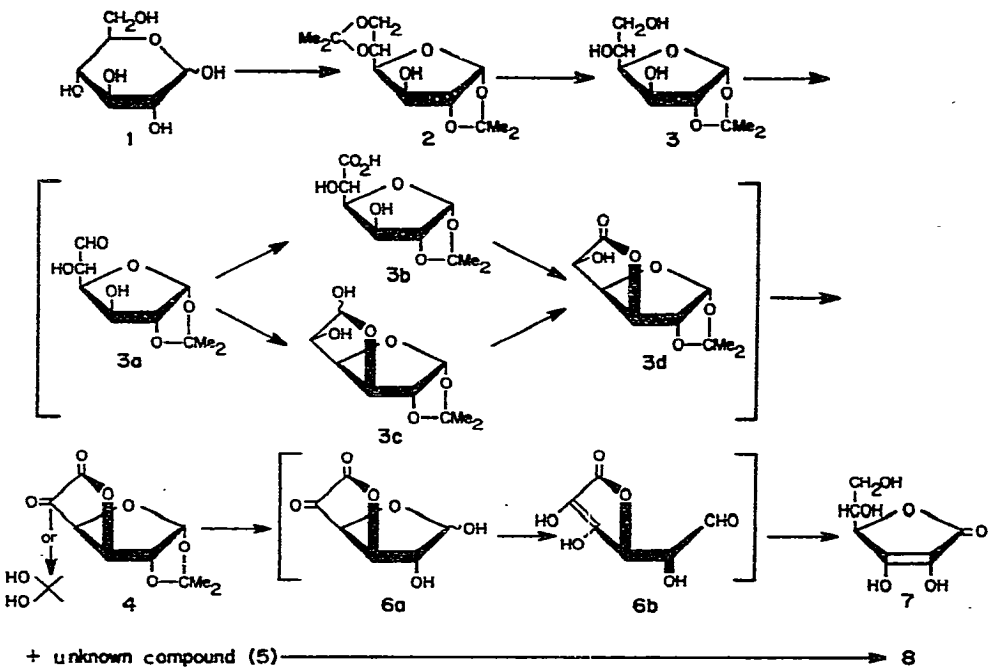

pounds by methods described in the references indicated. D-Glucofuranurono-6,3-lactone and L-ascorbic acid (7) were purchased from Aldrich Chemical Co. The latter was recrystallized from glacial acetic acid prior to use.

Thin-layer chromatography (t.l.c.) of 7 was performed on 0.25-mm, cellulose plates (Quantum Industries) in 18:4:3:1 ethyl acetate–water–acetic acid–formic acid and, for isopropylidene derivatives, on 0.25-mm plates of silica gel (Quantum Industries) in 16:1 chloroform–methanol. Compound 7 was detected by spraying with 0.3% Bromocresol Green. Other compounds were detected by spraying with 10% sulfuric acid in ethanol, and charring at 120°. Mobilities were determined relative to the solvent front (R_F) or to 2 (R_{II}). Radiochemical yields were estimated by scanning the plates with a Packard radiochromatogram scanner, and then integrating the resulting peaks.

Evaporations were performed with dry nitrogen gas at 25°, unless otherwise noted. All solvents were degassed by boiling, or purged with nitrogen, prior to storage in air-tight containers.

The procedure employed is exemplified by a description of the synthesis of L-[4-^{14}C]ascorbic acid (7).

1,2-O-Isopropylidene-α-D-[3-^{14}C]glucofuranose (3). — D-[3-^{14}C]Glucopyranose[8] (1) (50 μCi, 19 μmol) and Dowex 50 X8-400 (H^+) ion-exchange resin (100 mg, recycled once between the Na^+ and H^+ forms, and dried from acetone) were stirred with anhydrous acetone (2 mL) for 6 h at 25° in a glass-stoppered flask. The solution was then transferred by pipet to a 10-mL, conical flask, and the resin was washed

repeatedly with small portions of anhydrous acetone which were combined with the main solution. The solvent was evaporated, leaving white crystals of **2** (97% radioisotopic yield, R_F 0.6). Without purification, **2** was dissolved in 77% acetic acid (4 mL) and the solvent evaporated, to give primarily **3** (74%, R_{II} 0.2) plus **2** (20%) and **1** (6%, R_{II} 0.0).

*1,2-O-Isopropylidene-α-*D-xylo-*[3-^{14}C]hexulofuranurono-6,3-lactone* (**4**). — To the crude preparation of **3** was added an aqueous suspension of freshly hydrogenated, Adams catalyst (35 mg in 1.2 mL, adjusted to pH 3 with acetic acid). The flask was sealed with a rubber septum pierced by gas inlet- and outlet-needles, and agitated for 3 h at 45° under a stream of oxygen. Labeled carbon dioxide was collected from the outlet gas-stream by two successive gas-dispersion bottles, each containing M KOH (100 mL). In preliminary studies, virtually all of the labeled carbon dioxide was found in the first trap.

On completion of the oxidation, the catalyst was damp-filtered, and cautiously washed with methanol (10 mL). The filtrate and the methanol wash were combined, evaporated in a 2-ml glass ampoule containing unlabeled **4** (37 μmol) as the carrier. T.l.c. on silica gel gave two radioactive spots, **4** (50% radioisotopic yield from **1**, R_{II} 0.9) and **5**, an unknown compound that remained at the origin (46% radioisotopic yield from **1**).

L-*[4-^{14}C]Ascorbic acid* (**7**). — Unlabeled **7** (137 μmol) was added to the dried mixture of **4** and **5**, and the solids were dissolved in 0.1M sulfuric acid (2 mL). The addition of carrier **7** greatly lessened the radiochemical losses in subsequent steps. The ampoule was purged with nitrogen, sealed, and kept for 50 min at 90° to hydrolyze off the isopropylidene group. While still warm, the ampoule was opened, and the acid was neutralized by the addition of barium carbonate (0.76 mmol). The precipitate was removed by centrifugation, and washed twice with water (1 mL). The washings were combined with the original, supernatant liquor, and to this solution, at 4°, was added potassium borohydride (0.29 mmol). After 45 min, Dowex 50 X8-400 (H^+) ion-exchange resin (104 mg) was cautiously added to decompose the excess of borohydride, and then removed by centrifugation. The supernatant liquor was applied to a column (1 × 12 cm) of Dowex 1 X8-400 ($HCOO^-$) ion-exchange resin, and rinsed through the column with water to remove neutral, labeled material (8.5% of the starting radioactivity). Acidic components were eluted (220 ml/h) by a gradient of dilute formic acid prepared by adding, at the rate of column flow, 0.1M formic acid (500 mL) to water (250 mL)[9]. Radioactive components appeared in two major peaks that were eluted between 222 and 300 mL (Peak I) and between 318 and 414 mL (Peak II). The identity of Peak I (**8**), possibly the hydrolysis and reduction product of **5**, is still under investigation. Titration of Peak II with 2,6-dichlorophenolindophenol[10] provided a quantitative estimate of **7** (0.14 mmol). The overall, radiochemical yield of **7** was 28%, and its specific activity was 92 mCi/mmol. From Peak II, fractions containing **7** were combined, concentrated to a small volume at 40° under diminished pressure, and stored as a lyophilized solid in sealed ampoules

at −20°. T.l.c. of the product revealed a single component (R_F 0.4) corresponding to authentic **7**.

Degradation of labeled L-*ascorbic acids.* — Oxidative cleavage of **7** by sodium hypoiodite to oxalic acid (C-1 + C-2 of labeled **7**) and L-threonic acid was followed by periodate oxidation[11] of the latter to carbon dioxide (C-3), formic acid (C-4 + C-5), and formaldehyde (C-6). For **7** prepared from D-[3-^{14}C]glucose, the degradation was modified, in that a portion of the L-threonic acid fragment was further oxidized by nitric acid[12] to yield L-(+)-threaric acid, which was recovered by ion exchange[13]. Subsequent degradation by periodate[14] yielded carbon dioxide (C-3 + C-6 of labeled **7**) and formic acid (C-4 + C-5 of labeled **7**). Formic acid was separately oxidized to carbon dioxide by mercurous chloride[15].

RESULTS AND DISCUSSION

Small amounts (2 to 30 mg) of **1** gave excellent yields (>97%) of **2** when anhydrous acetone and a dry, strong cationic resin (H^+) were used. Replacement of the resin by sulfuric acid caused considerable formation of byproduct, and low yields (<30%) of **2**. Although **2** has been suggested[2] as a starting material for subsequent oxidation to **4**, the weakly acidic conditions of oxidation delayed hydrolysis of the 5,6-*O*-isopropylidene group, lowering the rate of oxidation of C-5 and C-6 and resulting in incomplete conversion into **4** and **5**. Therefore, **2** was treated with 77% acetic acid[5] to remove the 5,6-*O*-isopropylidene group prior to oxidation. This treatment converted ~70% of **2** into **3**, but it also hydrolyzed some **3** to **1** (7%). Residual **1** was oxidized to carbon dioxide in the subsequent step.

Oxidation of **3** by Pt/O_2 gave **4** in 50% radioisotopic yield from **1**. Another 46%

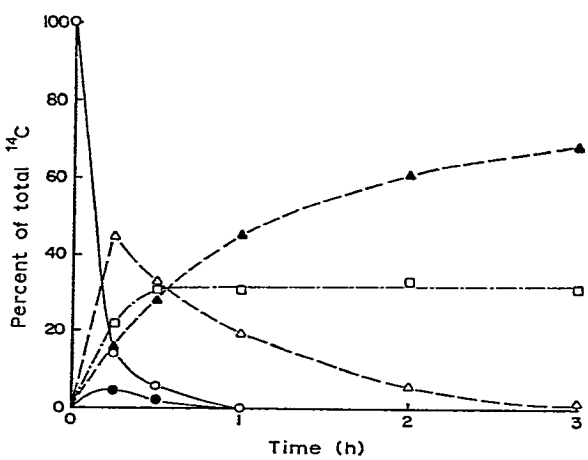

Fig. 1. A time-course study of the oxidation of 1,2-*O*-isopropylidene-α-D-[1-^{14}C]glucofuranose by Pt/O_2. {Conditions are described in the text. ⊙—⊙, 1,2-*O*-isopropylidene-α-D-[1-^{14}C]glucofuranose (**3**); ●—●, **2a**; △--△, 1,2-*O*-isopropylidene-α-D-[1-^{14}C]glucofuranurono-6,3-lactone (**3d**); ▲--▲, 1,2-*O*-isopropylidene-α-D-*xylo*-[1-^{14}C]hexulofuranurono-6,3-lactone monohydrate (**4**): □-·-□, **5**}.

appeared as **5** (the identity of which is still uncertain). A preliminary, time-course study (by t.l.c.) of the appearance of intermediates and products of the oxidation of 1,2-*O*-isopropylidene-α-D-[1-^{14}C]glucofuranose is shown in Fig. 1. A transient intermediate (R_{II} 0.35), seen only in the initial stage of the oxidation, may be the putative aldehyde[2] **3a**. Appearance of **3d** (R_{II} 0.6) closely followed the consumption of **3** during the first few minutes of oxidation, and then declined as further oxidation converted it into **4**. Formation of **5** (R_{II} 0.0) appeared to parallel that of **3d**, but, unlike **3d**, this highly polar compound did not undergo further oxidation to **4**.

Assuming that oxidation of **3** to **3a** is the initial step of the oxidation, two alternatives may be proposed for the formation of **3d**: (*1*) oxidation of C-6 to the carboxyl level (**3b**) followed by lactonization to **3d**, or (*2*) intramolecular cyclization of the aldehyde (**3a**) to a hemiacetal (**3c**), followed by oxidation of C-6. In either case, the final step is oxidation at C-5 to afford **4**. To ensure complete oxidation, a ratio of catalyst to substrate (**3**) of at least 10 : 1 was necessary; lessening of this ratio resulted in incomplete oxidation of **3**, accumulation of intermediate compounds (especially **3d**), and poor yields of **4** and **5**.

Acid hydrolysis of **4** yielded the purported compound *aldehydo*-L-*threo*-[4-^{14}C]hex-4-enurono-6,3-lactone[2,3]. Presumably, the immediate product of hydrolysis, **6a**, underwent spontaneous enolization to **6b**, which was readily assayed by titration with 2,6-dichlorophenolindophenol under conditions similar to the assay[10] of **7**. As **6b** was readily oxidized, precautions were taken to prevent its decomposition; these included the addition of unlabeled **4** and **7** prior to hydrolysis of labeled **4**, degassing of solvents, and use of a nitrogen atmosphere in a sealed ampoule for

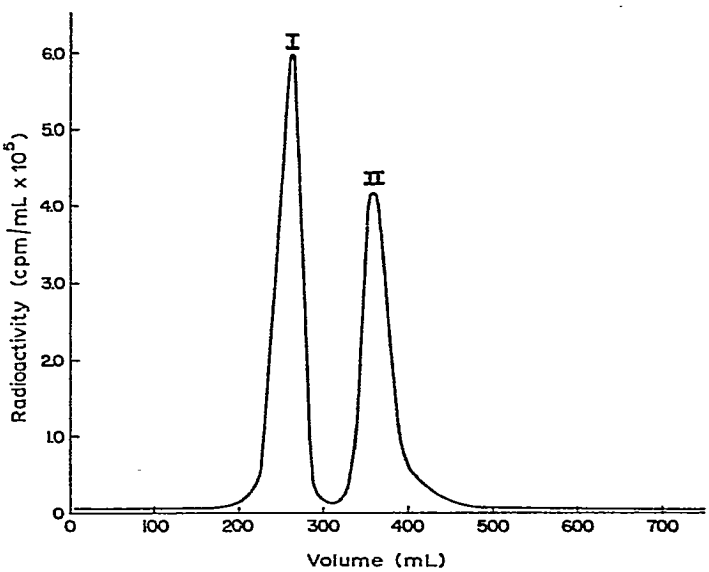

Fig. 2. Ion-exchange (Dowex 1 X8-400, formate) separation of L-[4-^{14}C]ascorbic acid (**7**, Peak II) from unknown compound **8** (Peak I). (Chromatographic conditions are described in the text.)

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN LABELED L-ASCORBIC ACID

Carbon number in L-*ascorbic acid*	*Percent of total activity From* D-[*3*-^{14}*C*]*glucose*	*From* D-[*1*-^{14}*C*]*glucose*	*From* D-[*U*-^{14}*C*]*glucose*
1 + 2	1	<1	34
3	n.d.[a]	<1	17
4 + 5	96	<1	32
6	<1	98	17
3 + 6	3	n.d.	n.d.

[a]n.d. = not determined.

conversion into **6b**. Under these conditions, the yield of **6b** from **4** was increased by 30% over the value reported[2].

Reduction of **6b** to **7** was virtually quantitative. The desired product (**7**) was accompanied by an acidic compound **8** (possibly derived from **5**). These two products were readily separated by ion-exchange, column chromatography (see Fig. 2), with **8** appearing as Peak I, and **7** as Peak II. When unlabeled **7** (10 mg) was carried through all of the manipulations involved in the hydrolysis of **4** and the reduction of **6b**, the recovery was >95%.

Degradation of preparations of labeled **7** gave the anticipated distributions of label (see Table I).

The procedure used here is generally applicable to a variety of syntheses of labeled **7**, limited only by the availability of starting material. D-Glucose specifically labeled with ^{2}H or ^{3}H on C-1, C-2, or C-3 would be converted into the corresponding **7** labeled on C-6, C-5, or C-4, respectively. Use of sodium [^{2}H]- or [^{3}H]-borohydride would also provide the corresponding C-6-labeled **7**. Appropriate choice of labeled reactants offers the opportunity to prepare many other labeled compounds of biochemical interest, especially those of D-glucuronic acid and its derivatives[1,6]. Synthesis of **7** by the present method augments a host of methods already described in the literature[2,3,17]. The virtue of the Bakke and Theander synthesis[2] is its simplicity, high radioisotopic yield, and versatility.

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