A New Derivative of Vitamin C and its Application to the Synthesis of Labelled Ascorbic Acid

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1. The preparation of a mono-*O*-cyclohexylidene derivative of L-ascorbic acid is described. 2. The new compound is shielded by the cyclohexanone group at C-5 and C-6 of the ascorbic acid molecule, while the double bond between C-2 and C-3 is kept intact. 3. The double bond of the new derivative is more resistant to oxidation than its parent compound. 4. Ascorbic acid is easily regenerated by mild acid hydrolysis. 5. The new derivative facilitates the synthesis of ^{14}C -labelled vitamin C.

The known instability of ascorbic acid causes marked difficulty in handling this material and has prompted us to search for a stable derivative from which the vitamin can be regenerated. Vargha (1932) reported the preparation of 5,6-mono-*O*-isopropylidene-L-ascorbic acid in 70% yield, by treatment of ascorbic acid with acetone in the presence of cupric sulphate as catalyst. The isopropylidene derivative hydrolysed in water to ascorbic acid.

This paper describes the preparation of a new derivative of L-ascorbic acid obtained by condensation of vitamin C with cyclohexanone in the presence of catalytic amounts of strong acids. The cyclohexanone derivative is more stable than its parent compound. Nevertheless, ascorbic acid can be easily regenerated by hydrolysis with dilute acids. The new derivative was identified as 5,6-mono-*O*-cyclohexylidene-L-ascorbic acid, an analogue of 5,6-mono-*O*-isopropylidene-L-ascorbic acid.

EXPERIMENTAL

Materials. Cyclohexanone (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.), was redistilled before use, b.p. 155°. Vitamin C (E. Merck A.-G., Darmstadt, Germany) had m.p. 188°. The Na^{14}CN was prepared by zinc reduction of $\text{Na}_2^{14}\text{CN}_2$ (S. Von Schuching, unpublished work). L-[1- ^{14}C]Ascorbic acid, specific activity 0.1 $\mu\text{C}/\text{mg.}$, was obtained from L-[1- ^{14}C]ascorbic acid, specific activity 7 $\mu\text{C}/\text{mg.}$ (synthesized as described below), by dilution with carrier ascorbic acid.

Melting points. These were taken on a Fischer-Jones melting-point apparatus and are uncorrected.

Paper chromatography and paper electrophoresis. Paper chromatography was carried out by using the ascending technique in butan-1-ol-acetic acid-water (4:1:5, by vol.) for 18 hr. on Whatman 3MM paper (Partridge & Westall, 1948; Mapson & Partridge, 1949). Paper electrophoresis was carried out as described by Bourne, Hutson & Weigel (1960) at pH 5 and 10 v/cm. on Whatman no. 1 paper in 0.9M-molybdate buffer for 2 hr. Sorbitol was used as a

Scheme 1.

standard for the comparison of rates of migration (M_s). Since no indicator could be found for the universal detection of ascorbic acid and its derivatives on the paper strips, L-[1- 14 C]ascorbic acid, specific activity 0.1 μ C/mg., was used as a starting material. The migration of the compound under study was revealed by the position of radioactivity on the paper chromatogram by scanning the strip with a windowless paper-chromatogram scanner.

Assay of radioactivity. Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer. The material to be analysed was dissolved in ethanol, and 0.1 ml. of the solute was added to 15 ml. of liquid-scintillation fluid, containing 4 g. of 2,5-diphenyloxazole and 0.5 g. of 2,2'-p-phenylenebis-(5-phenyloxazole)/l. of toluene (Packard Instrument Co., La Grange, Ill., U.S.A.). A high-voltage setting of 2-600 was used. The efficiency for 14 C counting under these conditions was 58%, as determined with an internal standard.

Preparation and properties of 5,6-mono-O-cyclohexylidene-L-ascorbic acid (Scheme 1). L-Ascorbic acid (1.0 g., 5.7 m-moles) was covered with 10 ml. of redistilled cyclohexanone, and 0.14 ml. of conc. H_2SO_4 was added. The solution became clear after shaking for 10 min., and was allowed to stand for an additional 12 hr. The solution was then diluted with 50 ml. of methanol and neutralized with solid $NaHCO_3$. After removal of the precipitated Na_2SO_4 by filtration, the solution was concentrated to a small volume *in vacuo* at 65° and *n*-heptane (500 ml.) was added. The resulting precipitate was crystallized from acetone (10 ml.) and *n*-heptane (500 ml.) and recrystallized from acetone (10 ml.) and methylcyclohexane (500 ml.). The yield was 1.1 g. (77%).

The cyclohexylidene derivative of ascorbic acid is freely soluble in light petroleum and water, and insoluble in *n*-heptane and methylcyclohexane.

The new compound had m.p. 185° (decomp.) and $[\alpha]_D^{25} + 37.0^\circ$ (c 2 in methanol) (Found: C, 56.1; H, 6.4. $C_{12}H_{16}O_6$ requires C, 56.2; H, 6.2%).

A sample (0.25 g., 1.0 m-mole) of this material was dissolved in 100 ml. of water and a portion titrated with 0.1 N-NaOH. It required exactly 1 equiv. of the alkali (with phenolphthalein as indicator). The formation of the monosodium salt corresponds to the neutralization of the enol group of ascorbic acid at C-3 (Rosenberg, 1942, p. 295).

The monosodium salt gave an intense blue colour with aq. $FeCl_3$, indicating another free enol group at C-2 (Reichstein & Oppenauer, 1934).

Titration of a sample with indophenol dye (Rosenberg, 1942, p. 316) required 1 equiv. of the reagent, which proved that the double bond is retained. No change in titre was observed after passing O_2 from a tank through a 5% solution of the monocyclohexylidene derivative in water, which indicates an increased stability of the derivative.

The following studies were carried out with derivatives prepared from L-[1- 14 C]ascorbic acid, specific activity 0.1 μ C/mg.

Paper chromatography of the new compound revealed a spot with R_F 1.0 (R_F for ascorbic acid is 0.37). During paper electrophoresis, the substance migrated towards the anode, the M_s value being 0.79 (M_s for ascorbic acid is 1.53).

L-[1- 14 C]Ascorbic acid. A sample (1 g., 3.9 m-moles) of 5,6-mono-O-cyclohexylidene-L-[1- 14 C]ascorbic acid, specific activity 0.1 μ C/mg., was covered with 50 ml. of 10% formic acid. The progress of hydrolysis was followed by paper chromatography, and was complete after 24 hr. at room

temperature. The formic acid solution was evaporated to dryness and the residue washed with *n*-heptane to remove cyclohexanone. The residue was next dissolved in ethanol (15 ml.), and peroxide-free ether (150 ml.) was added. Some flocculent material was filtered off and the ether-ethanol solution was concentrated to 1.0 ml. and stored at 4°. The resulting crystals weighed 490 mg. (77% yield), m.p. 186°. The peak of radioactivity coincided with the position of unlabelled ascorbic acid on a paper chromatogram.

5,6-Mono-O-cyclohexylidene-3-methyl-L-ascorbic acid (Scheme 1). An ether suspension of 5,6-mono-O-cyclohexylidene-L-[1- 14 C]ascorbic acid (2.0 g., 7.8 m-moles), specific activity 0.1 μ C/mg., was cooled to 0° and an excess of diazomethane in cold ether was added (Fieser, 1955). The mixture was allowed to come to room temperature and a slow evolution of N_2 took place. The ether and excess of diazomethane were removed by spontaneous evaporation, leaving an oily residue that resisted all attempts at crystallization. The oily residue was dried thoroughly; it had no boiling point but decomposed at 150-160°. The ester did not reduce 2,6-dichlorophenol-indophenol; it did not migrate during paper electrophoresis, but moved on a paper chromatogram (R_F 1.0). The presence of a methoxy group was shown by boiling 1 g. of the material with an excess of hydriodic acid and collecting the resulting methyl iodide in ethanolic $AgNO_3$ (Richter-Anschütz, 1928). Without prior neutralization a blue colour was obtained with $FeCl_3$, indicating a free enol group at C-2 of the ascorbic acid molecule and a methyl group at C-3. Analysis corresponded to the addition of 1 methyl group/mol. (Found: C, 57.1; H, 6.8. $C_{13}H_{18}O_6$ requires C, 57.7; H, 6.8%).

Synthesis of L-[1- 14 C]ascorbic acid with 5,6-mono-O-cyclohexylidene-L-ascorbic acid as an intermediate. L-Xylosone (1.2 g., 8 m-moles) was condensed with $Na^{14}CN$ (0.294 g., 6 m-moles, 10 mc of ^{14}C) according to the procedure of Salomon, Burns & King (1952), but no carrier ascorbic acid was added before hydrolysis of the imino-L-ascorbic acid. The solution after hydrolysis was evaporated *in vacuo* at 40°. The syrup was thoroughly dried at room temperature at high vacuum. Then 10 ml. of redistilled cyclohexanone and 0.14 ml. of conc. H_2SO_4 were added to the residue and the mixture was shaken for 10 min. and kept at room temperature for another 12 hr. Ethanol (50 ml.) was added and also enough $NaHCO_3$ to neutralize the solution. The resulting salts were filtered off, the solution was concentrated *in vacuo* at 65° to 10 ml. and *n*-heptane (500 ml.) was added. After 24 hr. at -20°, 5,6-mono-O-cyclohexylidene-L-[1- 14 C]ascorbic acid settled partly at the bottom of the flask as a sticky mass, while another part floated in the heptane layer. The *n*-heptane layer was filtered and the precipitates were combined. Further purification was carried out by reprecipitation from acetone and *n*-heptane and also from acetone and methylcyclohexane. Separation from inorganic ions, which interfere with the crystallization of ascorbic acid, is thus achieved. The air-dried material was hydrolysed to free ascorbic acid with 10% formic acid as described above. L-[1- 14 C]Ascorbic acid in water was further purified by passage over a Duolite-A-4 resin column (Chemical Process Co., Redwood City, Calif., U.S.A.) in the acetate form (column 2 cm. $\times$ 20 cm., resin bed 10 cm.).

The eluate was evaporated, taken up in 30 ml. of ethanol and 300 ml. of peroxide-free ether was added. A flocculent

precipitate was filtered off and the ethanol-ether solution concentrated to a small volume and kept at 4°; 510 mg. of L-[1-¹⁴C]ascorbic acid, specific activity 7 μ C/mg. (36% radiochemical yield), was obtained, m.p. 186°. A mixed m.p. with an authentic sample of L-ascorbic acid showed no depression. Radioactive purity was established by scanning a paper chromatogram with a windowless scanner. A single peak coincided with the position of unlabelled ascorbic acid on the paper strip.

An alternative procedure for hydrolysing the derivative to ascorbic acid consists in stirring monocyclohexylidene-L-ascorbic acid with a tenfold excess of Dowex-50 (H⁺ form) for 1 hr. in 50 ml. of water. After the resin had been filtered off and the solution evaporated, L-ascorbic acid was obtained in crystalline form. This eliminates the use of an anion-exchange column in the acetate form which was previously employed in preparing ¹⁴C-labelled ascorbic acid.

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