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Synthesis of the 2-Methyl Ether of L-Ascorbic Acid: Stability, Vitamin Activity, and Carbon-13 Nuclear Magnetic Resonance Spectrum Compared to Those of the 1- and 3-Methyl Ethers

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Reaction of L-ascorbic acid (4) with dimethyl sulfate (1.6 equiv) at pH 10.5 and 60 °C gave 76% crystalline 2-*O*-methyl-L-ascorbic acid (2) along with 15% of a byproduct (9). The byproduct formed a lactone during isolation and was tentatively identified as methyl 2-*O*-methyl-γ-L-*lyxo*-hex-3-ulofuranosidonolactone (7). Compound 7 was quantitatively converted to 2 in boiling methanolic hydrogen chloride. 5,6-*O*-Isopropylidene-L-ascorbic acid (5) was 2-methylated by dimethyl sulfate at pH 10.5 and 60 °C, and removal of the 5,6-acetal blocking group gave 2 in 85% yield from 4. The 2-methyl ether (2) showed only marginal vitamin C activity in the tobacco hornworm and guinea pig. The structures of the 1-, 2-, and 3-methyl ethers (1, 2, and 3) were confirmed by ^{13}C NMR and UV spectroscopy. Under aerobic conditions at pH 7, the order of stabilities of the methyl ethers was 2 ≫ 3 > 1.

The occurrence of 2-*O*-methyl-L-ascorbic acid (2) (Figure 1) in the urine of guinea pigs and rats (Blaschke and Hertting, 1971; Gazave et al., 1975) suggested that this compound might be an important metabolite that would be more stable than L-ascorbic acid (4). In this paper we describe the synthesis of crystalline 2 starting from either L-ascrobic acid (4) or 5,6-*O*-isopropylidene-L-ascorbic acid (5, Figure 2). The stability of 2 in water under aerobic conditions at pH 2, 7, and 10 was compared with that of 4, 3-*O*-methyl-L-ascorbic acid (3), and 1-*O*-methyl-L-ascorbic acid (1) at pH 7 and 10. The structures of 1, 2, and 3 were verified by UV and ^{13}C NMR spectroscopy. We also tested the growth promoting activity of 2 in vertebrate and invertebrate animal species.

EXPERIMENTAL SECTION

General. Melting points were determined with a Thomas-Hoover Unimelt apparatus. Solutions were evaporated under reduced pressure below 50 °C. TLC was performed on plates coated with silica gel G (Brinkman Instruments, Inc., Westbury, NY). Components were located by using an ultraviolet lamp, by spraying with 1% ferric chloride in 95% ethanol (Vestling and Rebstock, 1946) or by spraying with 50% aqueous sulfuric acid and charring on a hot plate. A Beckman DB-G (Beckman Instruments Inc., Palo Alto, CA) or a Cary 14 recording spectrophotometer (Varian Associates, Palo Alto, CA) was used to record UV spectra. ^{13}C NMR spectra were recorded on a Varian Model XL-100-15 spectrometer interfaced to a Nicolet 1180 digital computer and to a Nicolet 1093B pulse Fourier transform system with quadrature phase detection. In aqueous solution dioxane was used as an internal reference and the shifts were calculated relative to tetramethylsilane (Me_4Si): $\delta_{Me_4Si} = \delta_{dioxane} + 67.40$ ppm. In organic solutions Me_4Si was added as an internal reference standard.

High-performance liquid chromatography (HPLC) was carried out at 25 °C using a Waters Model 6000A pump (Waters Associates, Inc., Milford, MA) fitted with a loop injector and a Beckman Model 100-10 UV detector. Retention times and integrations were recorded using a computing integrator (Waters Data Module, Waters Associates Inc.). The stainless steel column (250 × 4.0 mm) contained a reverse-phase packing (BioSil ODS-10, Bio-Rad Laboratories, Richmond, CA). Samples (20 μL) were injected and components were eluted isocratically with 0.05 M phosphate buffer (pH 3) at a flow rate of 1.0 mL min^{-1}. Peaks were detected at 255 nm. The retention times of compounds 4, 2, and 3 were approximately 4.3, 6.1, and 9.8 min, respectively.

1-*O*- and 3-*O*-Methyl-L-ascorbic Acid (1 and 3). Methylation of L-ascorbic acid by diazomethane (a hazardous chemical: handle with caution) was done essentially as described by Reichstein et al. (1934) and Haworth et al. (1934). To a rapidly stirred solution of 4 (2.5 g) in dry methanol (<0.1% water, 30 mL) maintained at 0–5 °C was added dropwise, until a yellow color persisted, a solution of diazomethane (2.7–2.9 g, 0.07 mol) in ethyl ether (125 mL). The diazomethane had been generated previously (DeBoer and Backer, 1967) from *N*-methyl-*N*-nitroso-*p*-

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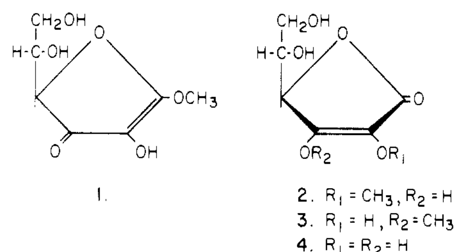

Figure 1. L-Ascorbic acid (4) and its 1-, 2-, and 3-methyl ethers (1, 2, and 3).

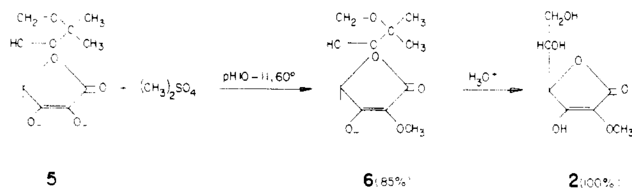

Figure 2. Synthesis of 2-*O*-methyl-L-ascorbic acid (2) from 5,6-*O*-isopropylidene-L-ascorbic acid (5).

toluenesulfonamide (Diazald, 21.5 g, Aldrich Chemical Co., Milwaukee, WI). After addition of diazomethane, thin-layer chromatography using chloroform–methanol (4:1 v/v) showed the reaction mixture contained one major component (R_f 0.5) and three minor ones (R_f 0.8, 0.7, and 0.3). All components gave a blue color with ferric chloride spray, but the blue color at R_f 0.3 faded within minutes. The solvent was removed under vacuum to ~5 mL, at which time dry acetone (20 mL) was added and the mixture evaporated again to 5 mL. The residue was held at 0 °C several days, and the crystalline mass was triturated with dry acetone (20 mL). The mixture was filtered to give a white residue and an acetone solution. The insoluble residue, which had an R_f of 0.3, was collected and rinsed 3 times with acetone to give 1-*O*-methyl-L-ascorbic acid: 0.2 g; mp 152–153 °C; UV data, pH 3–7, λ_{max} 282 nm (ϵ 12.7 × 10^3), and pH 11, λ_{max} 325 nm (ϵ 11.5 × 10^3). Titration with 0.04 M acid and alkali gave pK_1 = 5.7, while the shift of λ_{max} with pH gave pK_2 = 9.1 (Albert and Serjeant, 1962). At pH 2 and 25 °C UV showed a 20 μM solution of 1 hydrolyzed to 4 (λ_{max} 245 nm) in 30 min; at pH 10.6 compound 1 disappeared with a half-life of 14 h as determined by UV, but ascorbic acid was not detected. Haworth et al. (1934) reported 1-*O*-methyl-L-ascorbic acid with mp 162 °C and λ_{max} 280 with ϵ 11.0 × 10^3 in water and λ_{max} 320 nm in alkali.

The acetone solution, upon addition of hexane and cooling, gave crystalline 3-*O*-methyl-L-ascorbic acid (3): yield, 1.2 g (50%); mp 119–122 °C; UV data, pH 2, λ_{max} 243 nm, (ϵ 8.5 × 10^3), pH 6, λ_{max} 243 nm (ϵ 8.5 × 10^3), and pH 12, λ_{max} 275 nm (ϵ 6.74 × 10^3). Haworth et al. (1934) reported a mp of 121 °C, UV in water (40 mg/L), λ_{max} 245 nm with ϵ 10 000, and UV in dilute alkali, λ_{max} 275 nm. The ionization constant of 3 was determined by UV to be 7.9, in agreement with Radford et al. (1979), who reported pK_a = 7.8.

2-*O*-Methyl-L-ascorbic Acid (2) from 5,6-*O*-Isopropylidene-L-ascorbic Acid (5). 5,6-*O*-Isoproylidene-L-ascorbic acid (5) was prepared in nearly quantitative yield by the method of Jackson and Jones (1969). Whiile nitrogen was bubbled through water (100 mL) at 60 °C, 5,6-*O*-isopropylidene-L-ascorbic acid (10.8 g, 0.05 mol) and aqueous sodium hydroxide (10 M) were added in rapid succession to pH 10.5. To this solution was added, by using a syringe pump, dimethyl sulfate (5.3 mL, 0.055 mol) over a period of 1 h while maintaining a temperature of 60 °C and pH 10.5. The mixture was kept at pH 10.5 and 60 °C for an additional hour, cooled to 25 °C, adjusted to pH 3 by using cold sulfuric acid (1 M), and extracted with ethyl acetate (3 × 300 mL). The organic layers were combined and dried over anhydrous sodium sulfate. Evaporation of the solvent gave syrupy, 5,6-*O*-isopropylidene-2-*O*-methyl-L-ascorbic acid (6), which crystallized from a mixture of ethyl acetate and petroleum ether. The yield was 8.5 g (75%) of material with mp 110–115 °C and λ_{max} 259 nm [ϵ (14.3–14.7) × 10^3] at pH 6 and 10. Elemental analysis: calculated for $C_{10}H_{14}O_6$: C, 52.17; H, 6.13. Found: C, 52.41; H, 5.97.

A mixture of 5,6-*O*-isopropylidene-2-*O*-methyl-L-ascorbic acid (6, 1.0 g) in water (15 mL) was stirred at 25 °C with a strongly acidic cation-exchange resin (3 g, wet resin) in the hydrogen-ion form. After 3.5 h, TLC (chloroform methanol, 4:1) showed the starting material (R_f 0.7) disappeared and a new material (R_f 0.4) formed. In a separate experiment using HPLC we found no loss of the 2-methyl ether (2) for 24 h in aqueous acid at pH 2 and 25 °C. The ion-exchange resin was removed by filtration, and the filtrate was evaporated to a syrup that crystallized after several additions and evaporations of ethyl acetate. The material was recrystallized from ethyl acetate to give platelets of 2 with mp 129–131 °C. The specific rotation was independent of temperature between 25 and 60°C, but was dependent on pH: pH 2.5, $[\alpha]^{25}_D$ +30°; pH 8.0, $[\alpha]^{25}_D$ +90°; pH 14, $[\alpha]^{25}_D$ +110°. Elemental anal. Calcd for $C_7H_{10}O_6$; C, 44.20; H, 5.30. Found: C, 44.38; H, 5.27. UV data: pH 2, λ_{max} 239 nm (ϵ 9.06 × 10^3); pH 7, λ_{max} 259 nm (ϵ 15.32 × 10^3). The pK_a of 2 was determined by UV to be 3.2. Haworth et al. (1937) reported syrupy 2 with $[\alpha]_D$ +10° (*c* 1.5, water) and λ_{max} 245 nm (ϵ 5000) in aqueous solution and λ_{max} 260 nm (ϵ 6500) in dilute alkali.

2-*O*-Methyl-L-ascorbic Acid (2) from L-Ascorbic Acid (4). L-Ascorbic acid (4, 10 g, 57 mmol) was methylated with 1.1, 1.6, or 2.2 equiv of dimethyl sulfate as described for the 5,6-*O*-isopropylidene derivative (5). The pH of the reaction mixture remained constant within 30 min after the addition of dimethyl sulfate. The amount of alkali consumed during methylation was equal to the sum of the moles of 4 and dimethyl sulfate. The yield of 2 was determined by HPLC to be 62, 76, and 51%, respectively, using 1.1, 1.6, and 2.2 equiv of dimethyl sulfate. The 3-methyl ether was not detected, but 25%, 8% and a trace of starting material (4) remained in the reactions done with 1.1, 1.6, and 2.2 equiv of methylating agent, respectively. The reaction mixtures were cooled, adjusted to pH 2 with 1 M aqueous sulfuric acid, extracted with 1-butanol or ethyl acetate (3 × 60 mL), and dried over sodium sulfate. TLC (chloroform methanol, 4:1) of the organic extract showed, besides the 2-methyl ether (R_f 0.3), a byproduct (R_f 0.9) that was not detected by UV or ferric chloride and that increased with increasing equivalents of dimethyl sulfate.

The 2-methyl ether (2) was isolated from the extract of the reaction mixture in which 1.6 equiv of dimethyl sulfate was used. Ethyl acetate was evaporated until the volume reached ~30 mL, whereupon cooling gave 5.3 g (49%) of crystals with mp 128–130 °C.

Compound (2) was also isolated in pure form by using ion-exchange chromatography. The reaction mixture from 5 g of 4 reacted with 1.6 g of dimethyl sulfate was diluted with water, the solution passed through a column of Amberlite-IR-120 (hydrogen form), and the effluent added to a column (28 × 610 nm) of a weakly basic anion-exchange resin (Type AG-3, 200–400 mesh, Bio-Rad Laboratories, Inc.) previously converted to the free base form by using 1 M sodium hydroxide. The column was developed with

0.08 M ammonium acetate at 1 mL min^{-1}, and 15-mL fractions were collected. The fractions were examined for the presence of the 2-methyl ether (**2**) by UV and for the presence of methyl sulfuric acid by wet ashing followed by detection of sulfate ion. Methyl sulfuric acid began to elute at fraction 81. Fractions 11–76 were combined and passed through a column of Amberlite IR-120, and the effluent was concentrated to a syrup that crystallized from ethyl acetate: yield, 3.5 g (65%); mp 129–131 °C.

Methyl 2-*O*-Methyl-α-L-*lyxo*-hex-3-ulofuranosidono-γ-lactone (7). L-Ascorbic acid (10 g) was methylated as previously described by using 1.6 equiv of dimethyl sulfate. After being cooled and acidified to pH 2, the reaction mixture was extracted with ethyl acetate (3 × 100 mL), the organic layer washed with water (3 × 50 mL) and then dried over sodium sulfate. The solvent was removed and the residue chromotographed on a column (25 × 610 mm) of silica gel (250 g) by using a 3:2 mixture of chloroform and ethyl acetate. The first component off the column was a syrup (2.1 g) that gave very weak UV absorption between 245 and 280 nm and a rotation of $[\alpha]_D$ −22° (chloroform, *c* 5). The byproduct (**7**) did not react with diazomethane in methanol. Its ^{13}C NMR spectrum in chloroform gave two quaternary carbons (δ 171.7 and 108.8), three methine carbons (δ 87.5, 79.6, and 73.8), one methylene carbon (δ 75.3), and two methoxyl carbons (δ 59.0 and 51.6).

The byproduct (**7**) was also prepared starting from crystalline 2,3-di-*O*-methyl-L-ascorbic acid (**8**) mp 63–64 °C. The 2,3-dimethyl ether (1 g) was treated with a saturated barium hydroxide solution at 25 °C to pH 12. After 10 min the mixture was acidified to pH 2, readjusted to pH 5, and filtered to remove barium sulfate, and the filtrate was evaporated to a syrup. TLC in chloroform–methanol (4:1) showed **8** (R_f 0.8) was completely converted to **7** (R_f 0.9). Haworth et al. (1934) originally prepared **7** from **8** using this procedure. Those investigators named compound **7** "isodimethyl ascorbic acid".

The byproduct and isodimethylascorbic acid were each treated with barium hydroxide to pH 12, and carbon dioxide was passed through the solutions to pH 7. After barium carbonate was removed by filtration, the ^{13}C NMR spectra were measured. Both spectra were identical; they showed two quaternary carbons (δ 177.5 and 109.1), three methine carbons (δ 82.9, 82.0, and 74.2), one methylene carbon (δ 76.), and two methoxyl carbons (δ 59.0 and 50.2) in accord with **9**. The byproduct and isodimethylascorbic acid both gave the same mono-*p*-nitrobenzoate ester with mp and mixed mp 178–180 °C. Anal. Calcd for $C_{15}H_{15}O_9N$: C, 50.99, H, 4.25; N, 3.97. Found: C, 50.21; H, 4.45; N. 3.79.

The syrup byproduct (**7**, 0.2 g) was refluxed in 1.0% methanolic hydrogen chloride. After 24 h TLC and UV analysis at 239 nm (ε 9.1 × 10^3) indicated quantitative conversion of **7** to 2-*O*-methyl-L-ascorbic acid (**2**). Evaporation of the solvent and addition of ethyl acetate gave 0.2 g of crystalline **2**.

Methyl 2-*O*-Methyl-α-L-*lyxo*-hex-3-uloside (11). Compound (**7**) (0.2 g) was dissolved in tetrahydrofuran (20 mL) and the solution was added dropwise to a stirred mixture of lithium aluminum hydride (0.5 g) in tetrahydrofuran (100 mL). The mixture was warmed to 60 °C for 1 h and cooled, and ethyl acetate (15 mL) was added dropwise. After slow addition of water (2 mL) the mixture was stirred 1 h and filtered. The filtrate was dried over sodium sulfate and concentrated to a syrup that was purified by column chromatography (20 mm × 250 mm) on silica gel using chloroform–methanol (14:1 v/v). TLC with

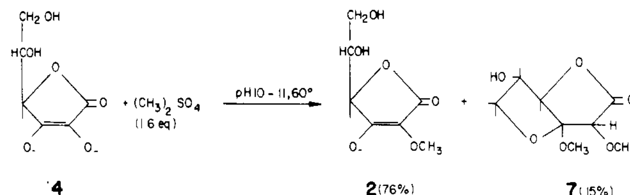

Figure 3. Synthesis of 2-*O*-methyl-L-ascorbic acid (**2**) and byproduct (**7**) from L-ascorbic acid (**4**).

chloroform–methanol (4:1 v/v) showed the purified syrup (0.2 g) had R_f 0.7 compared to 0.9 for the starting material (**7**). The ^{13}C NMR spectrum was consistent with methyl 2-*O*-methyl-δ-L-*lyxo*-hex-3-uloside (**11**); it showed a single anomeric carbon (C-3, 108.9 ppm), a methylene substituted with an *O*-alkyl group (C-6 at 73.5 ppm), a methylene substituted with a free OH (C-1, 61.4 ppm), a methine carbon with many long-range proton couplings (C-5, 77.2), two methine carbons (C-2 or C-4, 82.1 or 82.3 ppm), an anomeric methoxyl with no long-range proton couplings ($3\text{-}OCH_3$, 49.7 ppm) and a methoxyl with long-range proton couplings ($2\text{-}OCH_3$, 60.4 ppm). The above assignments were consistent with the spectrum of α-L-*xylo*-hex-3-ulofuranose (Angyal et al., 1976) with the following chemical shifts: C-1, 62.5 ppm; C-2, 72.4 ppm; C-3, 107.4 ppm; C-4, 80.9 ppm; C-5, 76.7 ppm; C-6, 74.2 ppm.

Stability of 1, 2, 3, and 4 under Aerobic Conditions. Solutions (1 mM) of **2**, **3**, and **4** were prepared in 0.1 M sodium phosphate buffers at pH 2, 7, and 10, while solutions of **1** were prepared at pH 7 and 10. Oxygen was bubbled though the solutions at 25 mL min^{-1}, and the decline in UV absorbance at the λ_{max} of each compound was followed to determine loss of ascorbate or its methyl ethers.

Bioassay of 2 for Vitamin C Activity. The 2- and 3-methyl ethers (**2** and **3**) were assayed for vitamin C activity by using the tobacco hornworm, *Manduca sexta* L., according to the procedure of Kramer et al. (1978). The 2-methyl ether (**2**) was also assayed in the guinea pig as described by Collins and Elvehjem (1958). English short-hair guinea pigs (Small Stock Industries, Inc., Pea Ridge, AR) were housed three animals to a cage and fed Purina guinea pig ration during a 2-week acclimation period. The young pigs (190–250 g) were then divided into three groups of six animals. One group was fed a diet void of vitamin C (Reid–Briggs guinea pig diet, ICN Nutritional Biochemicals, Cleveland, OH; Reid and Briggs, 1953), the second group received the same diet but supplemented with 5.0 mg of L-ascorbic acid (kg of body weight)$^{-1}$ day^{-1}, and the third group received 5.4 mg of **2** per (kg of body weight)$^{-1}$ day^{-1}. The vitamin C was administered orally with a calibrated syringe and freshly prepared solutions containing 50 mg of **4** and 54 mg of **2** per 10 mL of water. The feed consumption and weights of the animals were recorded daily.

RESULTS AND DISCUSSION

For synthesis of 2-*O*-methyl-L-ascorbic acid, we recommend starting from 5,6-*O*-isopropylidene-L-ascorbic acid (**5**) instead of L-ascorbic acid because no side product was detected by TLC. In alkali at pH 10–11, the 2-oxyanion of **5** attacks dimethyl sulfate rapidly to give an 85% yield of crystalline 5,6-*O*-isopropylidene-2-*O*-methyl-L-ascorbic acid (**6**, Figure 2). Mild acid hydrolysis of the 5,6-acetonated derivative gave quantitatively the 2-methyl ether (**2**), which crystallized from ethyl acetate.

Methylation of L-ascorbic acid (**4**) with dimethyl sulfate (1.6 equiv) at pH 10–11 gave predominantly **2** (76%, Figure 3). However, the methylation reaction was complicated

Table I. Carbon-13 NMR Chemical Shifts in D_2O in ppm Downfield from Me_4Si[a]

compound	pH	C-1	C-2	C-3	C-4	C-5	C-6
1[b]	3.6	178.1	115.2	190.1	84.9	70.3	62.6
1[b]	10.1	179.1	123.3	190.3	83.3	70.4	62.8
2[c]	2.0	173.7	122.5	162.1	77.0	69.7	62.8
2[c]	7.0	179.4	119.3	178.1	79.3	70.4	63.3
3[d]	2.0	174.1	119.2	155.9	76.9	69.8	62.8
3[d,e]	7.0	174.8	120.4	155.0	76.8	70.0	63.0
3[d]	9.3	179.1	130.3	147.7	76.3	70.6	63.4
4	2.1	175.7	120.4	158.4	78.9	71.6	64.9
4	7.1	179.7	115.7	177.9	81.0	72.3	65.3
7[f]		171.7	87.5	108.8	79.6	73.8	75.3
9[g]	7.0	177.5	82.9	109.1	82.0	74.2	76.9
11[h]	7.0	61.4	82.1-82.3	108.9	82.1-82.3	77.2	73.5

[a] Dioxane as the internal reference. [b] Signal of $-OCH_3$ 58.7 and 57.6 ppm at pH 3.6 and 10.1, respectively. Compound 1 unstable at pH 10 after 0.5 h and at pH 3.6 after ~4 h. The spectrum at pH 10.1 is the monoanion form (1a). [c] Signal for $-OCH_3$ 61.2 and 61.6 ppm at pH 2 and 7, respectively. [d] Tetramethylsilane used as the external reference. Signal for $-OCH_3$ at 60.1 and 60.3 at pH 2 and 7, respectively. [e] Values agree with those of Radford et al. (1979). [f] Spectrum recorded in chloroform. Methoxyl peaks at 59.0 and 51.6 ppm. [g] Barium salt. Methoxyl peaks at 59.0 and 50.2 ppm. [h] Glycosidic methoxyl at 49.7 ppm and 2-methoxyl at 60.4 ppm.

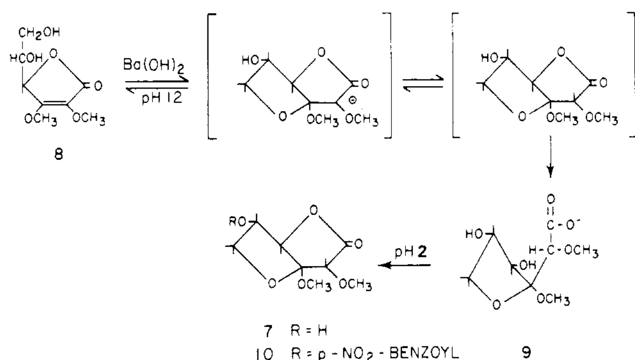

Figure 4. Mechanism proposed for formation of methyl 2-*O*-methyl-α-L-*lyxo*-hex-3-ulofuranosidono-γ-lactone (7) during methylation of L-ascorbic acid (4) with dimethyl sulfate.

by a side reaction that produced the methyl α-furanoside of 2-*O*-methyl-L-*lyxo*-or L-*xylo*-hex-3-ulosonic acid (**9**, Figure 4). Compound **9** formed the lactone (**7**) when the methylation reaction mixture was acidified to pH 2. Extraction with 1-butanol or ethyl acetate removed both **2** and methyl 2-*O*-methyl-α-L-*lyxo*-hex-3-ulofuranosidonic acid lactone (**7**) from the aqueous reaction mixture. We did not investigate whether the epimeric methyl 2-*O*-methyl-α-L-*xylo*-hex-3-ulofuranosidonic acid remained in the aqueous phase. The xylo isomer would probably not lactonize easily in the L-xylo configuration because the 2-OCH_3 is endo to the bicyclic ring system.

The yield of **2** from **4** went through an optimum depending on the quantity of dimethyl sulfate used. HPLC analysis showed that 1.1, 1.6, and 2.2 equiv of dimethyl sulfate gave 62, 76 and 51% of **2** and 25%, 8%, and a trace of starting material, respectively. At the same time, the amount of **7** isolated by ethyl acetate extraction of the acidified reaction mixtures increased from 2 to 15 to 30%.

One can postulate that the 2-methyl ether (**2**) is formed rapidly from the dianion of **4** and dimethyl sulfate. Subsequent 3-methylation of **2** gives the intermediate 2,3-dimethyl ether (**8**, Figure 4), which rearranges to the furanoside (**9**) in alkali. Apparently the driving force for formation of **9** from **8** is the ionization of the carboxylic acid (Figure 4). In contrast, the 1-, 2- and 3-monomethyl ethers (**1**, **2**, and **3**) are relatively stable in alkali at pH 10–12 because each has an ionizable 2- or 3-OH, which apparently stabilizes the lactone ring.

Haworth et al. (1937) first prepared "isodimethylascorbic acid" (**7**) by treatment of 2,3-di-*O*-methyl-L-ascorbic acid (**8**) with aqueous barium hydroxide. After removal of barium ion with sulfuric acid, those workers isolated **7** by removal of water followed by vacuum distillation. We confirmed those results, except we purified **7** by chromatography on silica gel.

We have assigned isodimethyl-L-ascorbic acid to structure **7**, which is methyl 2-*O*-methyl-α-L-*lyxo*-hex-3-ulofuranosidono-γ-lactone. The structure was assigned based on the formation of **7** from **8**, its ^{13}C NMR spectrum, and its chemical behavior toward diazomethane, *p*-nitrobenzoyl chloride, and methanolic hydrogen chloride.

In spite of the creation of two new asymmetric carbons when **9** (or **7**) was formed from **8**, the ^{13}C spectra of the ethyl acetate extract of the acidified reaction mixture showed the predominance (>90%) of one stereoisomer (**9**). In the spectrum of **7** in chloroform, the signal of C-1 appeared at 171.7 ppm, but no vinyl carbons were found at 120–150 ppm (Table I). We found **7** would not react with diazomethane, but was smoothly reduced with lithium aluminum hydride to give the glycoside (**11**) (Figure 5); thus, the C-1 signal is that of a lactone carbonyl. The signal at 75.3 ppm was identified by decoupling experiments as C-6. The C-6 signal in L-ascorbic acid is at 63.0 ppm, but it shifts to 76.8 ppm when the 6-OH adds to C-3 to give the bicyclic hydrated form of dehydro-L-ascorbic acid (Hvoslev and Pedersen, 1979). The signals at 108.8 ppm (C-3) and 59.0 ppm in the spectrum of **7** are typical of anomeric and methoxy carbons in glycosides (Perlin et al., 1970). The shifts of C-6, C-3, and the methoxyl carbon confirm the presence of a furanoside ring in **7**.

The α-furanoside ring that was assigned to **7** is similar to the α-furanose rings in D-*threo*-pentulose and L-*xylo*-3-hexulose, two ketoses whose ^{13}C shifts were examined by Angyal et al. (1976). The shifts of the two model compounds were useful in tentatively assigning the signals of C-2, C-4, and C-5 in compound **7**. The signal at 87.5 ppm in **7**, which was a methine carbon, was assigned to C-2 based on the facts that substitution of OMe for OH shifts the signal of an α-carbon 10 ppm downfield (Stothers, 1972), and the lowest field signal of the methine carbons in D-*threo*-pentulofuranose and L-*xylo*-3-hexulofuranose was at 81.4 ppm. Of the two remaining methine carbons in **7**, the signal at 79.6 ppm was assigned to C-4 while the signal at 73.8 ppm was assigned to C-5. In the model furanoses the ring carbon adjacent to the anomeric center resonated 4-5 ppm to lower field than the ring carbon that was in the β-position to the anomeric carbon. Even in the β-furanoses, the difference was 2–3 ppm to lower field for the adjacent ring carbons than for the β-ring carbons.

Compound **7** was assigned the α-L-*lyxo* configuration

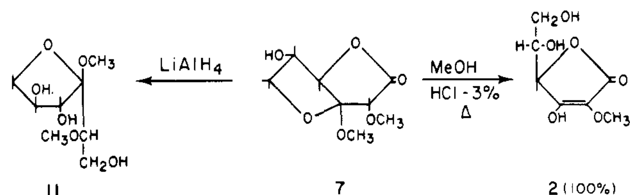

Figure 5. Reduction of the lactone (7) to methyl 2-*O*-methyl-α-L-*lyxo*-hex-3-ulofuranoside (11) and methanolysis of 7 to give 2-*O*-methyl-L-ascorbic acid (2).

based on arguments similar to those used by Goshima et al. (1973) to characterize methyl α-L-*lyxo*- and -*xylo*-hex-3-ulofuranosidono-γ-lactone. The anomeric configuration in 7 was designated α because of the preferred cis fusion of the two rings in the bicyclo[3.3.0]octanes. The configuration of C-2 in 7 was assumed to be controlled by the more stable exo orientation of the 2-OCH_3. When Goshima et al. (1973) boiled 4 in methanol containing boron trifluoride for 10 h, they isolated methyl α-L-*lyxo*-hex-3-ulofuranosidono-δ-lactone (10%) and its C-2 epimer (3%). Those authors found the predominant glycoside with the α-L-*lyxo* configuration to have a specific rotation of +2.7° in methanol, while we found the 2-methyl ether (7) had $[\alpha]^{25}_D$ - 22° in chloroform. The epimeric methyl α-L-*xylo*-hex-3-ulofuranosidono-δ-lactone gave a rotation of +75° in methanol.

The ^{13}C spectrum of 9 supported the hydrolysis of the lactone ring when 7 was treated with aqueous barium hydroxide. Ionization shifted the signal of C-1 downfield 5.8 ppm in agreement with saponification of methyl esters (Stothers, 1972). Ionization also caused the C-2 signal to shift upfield by 4.6 ppm instead of the normal downfield shift (Table I). Apparently the rigid bicyclic system in 7 caused the anomalous upfield shift in the 2-carbon when the lactone ring was opened and C-1 ionized.

We hypothesize that the mechanism given in Figure 4 explains the stereoselectivity when the furanoside (9) is produced during methylation of 4 with dimethyl sulfate. The intermediate 2,3-dimethyl-L-ascorbic acid (8) cannot ionize to stabilize the enone system, so that 6-hydroxyl attacks at C-3 (Michael addition reaction) to give a cis fusion of the rings with formation of an α-L-furanoside. Proton transfer at the intermediate C-2 carbanion is rapid compared to alkaline hydrolysis of the lactone ring, which leads to the more thermodynamically favorable L-*lyxo* configuration of the 3-hexulose.

The results on methylation suggest that any reaction of 4 in alkali that gives a 2,3-di-O-substituted intermediate is susceptible to Michael addition, giving the 6,3-furanose ring. The 6,3-furanose ring also forms under acidic conditions (Goshima et al., 1973).

Prior investigators prepared 2 by acid-catalyzed methanolysis of isodimethylascorbic acid (7) as originally reported by Haworth et al. (1937). We also found that 7 was converted quantitatively to 2 by methanolysis (Figure 5). Recently Bowers-Komro et al. (1982) confirmed in vitro formation of 2 when equal molar amounts of 4 and *S*-adenosylmethionine were incubated with catechol *O*-methyltransferase in the presence of Mg^{2+}. Bowers-Komro et al. (1982) synthesized 2 unequivocally by reaction of diazomethane with 3-*O*-benzoyl-5,6-*O*-isopropylidene-L-ascorbic acid.

The structures of the 1-, 2-, and 3-methyl ethers of L-ascorbic acid were readily verified by ^{13}C NMR (Figure 6) and UV spectra at different pHs (Paukstelis et al., 1982). The 1-methyl ether (1) has pK_1 = 5.7 and pK_2 = 9.1 (Figure 7), the 2-methyl ether pK = 3.2, the 3-methyl ether

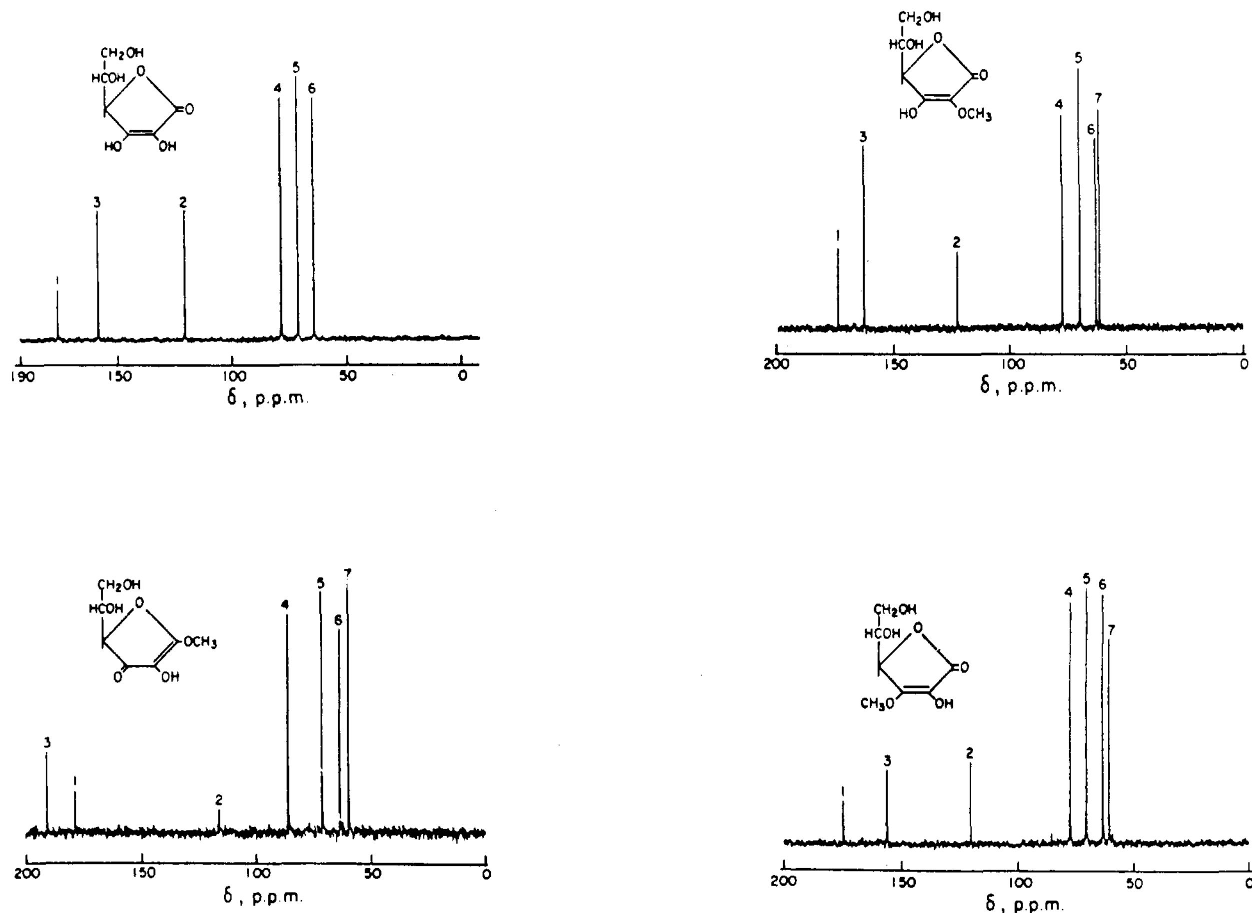

Figure 6. Carbon-13 NMR spectra of the unionized forms of L-ascorbic acid (4) and its 1-, 2-, and 3-methyl ethers (1, 2, and 3).

Table II. UV Spectral Properties and Ionization Constants of L-Ascorbic Acid and Its 1-, 2-, and 3-Methyl Ethers

derivative of L-ascorbic acid	acid (pH 2.0) λ_{max}, nm	ϵ, mM	neutral (pH 7.0) λ_{max}, nm	ϵ, mM	base (pH 10.0) λ_{max}, nm	ϵ, mM	pK_1	pK_2
free acid	243	10.0	265	16.5			4.17	11.79
1-*O*-methyl	282	12.7	282	12.7	325	11.5	5.7	9.1[c]
2-*O*-methyl	239	9.1	259	15.3	259[a]	15.3	3.2	
3-*O*-methyl	243	8.5	244	8.5	275[b]	6.7	7.9	

[a] pH 11.0. [b] pH 12. [c] Formation of C-4 carbanion.

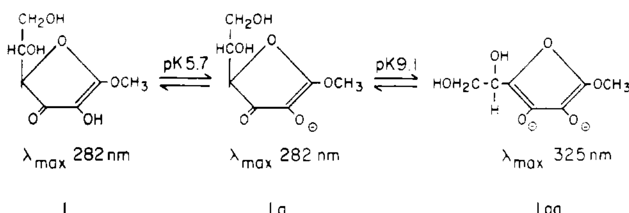

Figure 7. Ionization of 1-*O*-methyl-L-ascorbic acid (1).

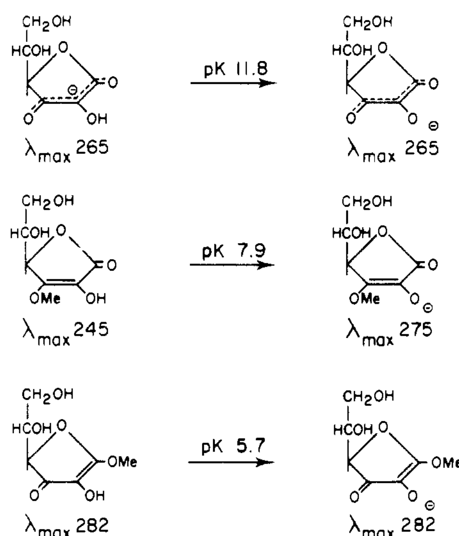

Figure 8. Ionization of L-ascorbic acid (4) and its 1- and 3-methyl ethers (1 and 3).

p*K* = 7.9, and the parent compound (4) (Crawford and Crawford, 1980) pK_1 = 4.17 and pK_2 = 11.79. The 1- and 3-methoxy groups change the ionization constant of the 2-OH in 4 by 6 and 4 orders of magnitude, respectively (Figure 8).

The ^{13}C spectrum of a 1 M solution of 1 showed the 1-methyl ether autohydrolyzed at pH 3.6 over a period of hours at 29 °C, in agreement with the earlier report of Haworth et al. (1937). The spectrum of 1 at pH 3.6 was measured immediately after dissolution and 4 h of pulsing. The spectrum showed 1 was completely hydrolyzed in 24 h to a mixture of only L-ascorbic acid (4) and methanol (δ 48). In confirmation of those results, the λ_{max} of the solution changed from an initial value of 282 nm to a final value of 243 nm.

The spectrum of 1 at pH 3.6 is shown in Figure 6; the chemical shifts are given in Table I. The signal furtherest downfield at 190.1 ppm was assigned to the carbonyl at C-3, while that at 178.1 ppm was assigned to the enolic C-1. The assignment of the carbonyl to C-3 was supported by the ~4-ppm deshielding of C-4 (84.9 ppm) in 1 compared to its signal (78.9 ppm) in 4. The remaining vinyl signal at 115.2 ppm in 1 was assigned to C-2. The signals at 70.3 and 62.6 ppm, respectively, which were similar to those of C-5 and C-6 in 4, showed that the 5-OH and 6-OH in 1 were not methylated nor was the 6-OH involved in a 6,3-furanose ring.

Table III. Stability (Percent Remaining of 1-*O*-Methyl-L-ascorbic Acid (1), 2-*O*-Methyl-L-ascorbic Acid (2), 3-*O*-Methyl-L-ascorbic Acid (3), and L-Ascorbic Acid (4) in Phosphate Buffer (0.1 M) at 25 °C under Aerobic Conditions

compound[a]	pH	time, h 2	4	6	24	$t_{1/2}$, h
1[b]	2	90	82	74	12	13
2	2	97	97	97	97	≫24
3	2	97	97	97	97	≫24
4	2	89	81	73	8	13
1	7	90	80	70	50	24
2	7	100	100	100	100	≫24
3	7	100	100	100	80	>24
4	7	53	23	13	0	2
1	10	90	80	65	30	14
2	10	100	98	96	96	≫24
3	10	96	92	88	80	>24
4	10	0	0	0	0	<<1

[a] Initial molarity was 1 mM. [b] The 1-methyl ether (1) was hydrolyzed to 4 in 30 min at pH 2.

When a solution of 1 in D_2O was adjusted to pH 10.1 by using sodium in D_2O, the signal of C-2 shifted immediately downfield ~8 ppm, while the other carbons resonated at practically the same frequencies as observed at pH 3.6 (Table I). However, after 2 h the signal for C-4 disappeared because H-4 underwent deuterium exchange. At the same time, the signals of C-5, C-6, and OCH_3 remained at 70.4, 62.8, and 57.6 ppm, respectively, while the signals of C-1, C-2, and C-3 were lost in the spectral noise, apparently due to an increase in their relaxation times. The acidity constant for ionization at C-4 was determined by UV to be 9.1. The relatively low p*K* of C_4-H is thought to be due to formation of the tautomeric dianion (**1aa**, Figure 7) containing the stable furan ring. The ionization of **1a** to **1aa** is accompanied by a bathochromic shift of 43 nm (Figure 7 and Table II).

Ionization of the 3-OH on 4 and its 2-methyl ether (2) caused the signal of C-3 to shift downfield ~20 ppm, while the signal of C-2 shifted upfield 3–5 ppm (Figure 6; Table I). At the same time the absorption maximum of 4 and 2 shifted from 239–243 nm at pH 2 to 265 nm at pH 7 (Table II). In contrast, ionization of the 2-OH in 3 caused C-3 to shift upfield 7.3 ppm, while the signal of C-2 shifted downfield 10.1 ppm. The UV absorption maximum of 3 changed from 245 nm at pH 2 to 275 nm at pH 10. The 2- and 3-methyl ethers did not form the bicyclic ring system in alkali as did the 2,3-dimethyl ether. The ^{13}C shifts of the C-2 and C-3 carbons remained in the vinyl region in 2 and 3, and the shift of C-6 also did not change substantially at alkaline pH (Table I).

The stabilities of 1, 2, 3, and 4 to oxygen at 25 °C were compared in aqueous phosphate buffer (0.1 M) at pH 2, 7, and 10 (Table III). At pH 2 and 7, the 2-methyl ether was stable whereas the 3-methyl ether was slightly labile.

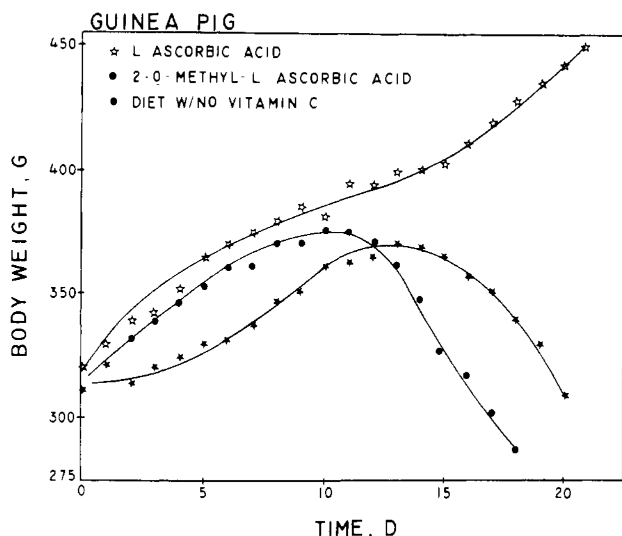

Figure 9. Growth curves for guinea pigs fed (☆) 5 mg (kg of body weight)$^{-1}$ day^{-1} L-ascorbic acid, (★) 5.4 mg (kg of body weight)$^{-1}$ day^{-1} of the 2-methyl ether (2), and (●) no vitamin C control.

L-Ascorbic acid (4) was oxidized almost completely under those conditions in 24 h. The 1-methyl ether was somewhat more stable than 4 at pH 7.0. At pH 10, the order of stabilities was 2 ≫ 3 > 1 ≫ 4 (Table III).

The 1-methyl ether rapidly hydrolyzed at pH 2 and room temperature, whereas the 2-methyl ether was stable. Vestling and Rebstock (1946) reported the 3-methyl ether was not hydrolyzed at 25 °C and pH 1.6 over a period of several hours.

Insect and guinea pig bioassays were done on the 2-methyl ether (2) to determine vitamin C activity. The 3-methyl ether (3) was also tested in the insect assay. Kramer et al. (1978) have shown that the tobacco hornworm (*M. sexta* L.) requires a minimum of 0.5 mM L-ascorbic acid in its diet for normal development. Moreover, the vitamin C activity of various compounds in the hornworm paralleled their activities in the guinea pig.

The 2- and 3-methyl ethers (2 and 3) were marginally active (<5% activity) in promoting growth in the hornworm. At a diet concentration of 1 mM of 2 and 3, 22 ± 12 and 20 ± 19%, respectively, of the experimental animals attained normal larval weight. But the growth curves of the test animals were delayed as much as 2 weeks compared to the controls receiving 4. Most of the test animals failed to complete larval development and exhibited ecdysial failure and premature sclerotization problems. The results on 3 confirm the findings of Vestling and Redstock (1946), whose investigation on guinea pigs showed 3 to have approximately 10% of the vitamin activity of 4.

The 2-methyl ether (2) was also fed to guinea pigs. The data in Figure 9 show the three groups of animals gained weight equally until days 10–13 of the experimental period. From days 10–18 the animals given no L-ascorbic acid lost weight rapidly and showed clinical signs of scurvy, whereas the control group given ascorbic acid at 5 mg (kg of body weight)$^{-1}$ day^{-1} continued to grow and remained healthy. Collins and Elvehjem (1958) used growth rates to determine that the minimum level of L-ascorbic acid required by guinea pigs is 5 mg/kg of body weight. The animals receiving the 2-methyl ether (2) at 5.4 mg (kg of body weight)$^{-1}$ day^{-1} also developed scurvy beginning on day 13. The animals receiving the 2-methyl ether did not appear as ill and as inactive as those not receiving the vitamin. The delay in onset of weight loss for the animals receiving 2 also indicated the 2-methyl ether effects a marginal level of vitamin C activity.

1-*O*-Methyl-L-ascorbic acid has been shown to have strong antiscorbutic activity in the guinea pig, probably due to its ease of hydrolysis in the stomach (Gould et al., 1949). We did not test the activity of 1.

2-*O*-Methyl-L-ascorbic acid (2) has been reported in rat urine (Blaschke and Hertting, 1971) and in guinea pig urine (Gazave et al., 1975). We found 0.1 g/100 mL 2 but no 3 in human urine. The human urine was acidified to pH 2 and extracted with ethyl acetate, and the extracts were analyzed by HPLC.

In summary, we have described two procedures for preparing 2-*O*-methyl-L-ascorbic acid and compared its properties to those of L-ascorbic acid and its 1- and 3-*O*-methyl derivatives. The 2-methyl ether, which is a metabolite present in human, rat, and guinea pig urine, is vastly more stable in solution than L-ascorbic acid and somewhat more than the 1- and 3-*O*-methyl derivatives. Unfortunately, the 2- and 3-methyl ethers exhibit no substantial vitamin activity. Apparently 2-*O*-methyl-L-ascorbic acid is a minor metabolic product in animals utilized for excretory purposes.

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Measurements of Oxygen-18/Oxygen-16 Stable Isotope Ratio in Citrus Juice: A Comparison of Preparation Methods

Eli Cohen* and Israel Saguy

The $^{18}O/^{16}O$ ratios of the water of a number of citrus juices were measured by utilizing two preparation methods (i.e., distillation and charcoal treatment). The charcoal preparation method not only was proven to be a rapid method that circumvented the need for distillation but also was more accurate and reliable in accounting for the real isotopic ratio of $^{18}O/^{16}O$ in citrus juices. Data obtained by utilizing the charcoal preparation method were ca. 0.5‰ higher than those obtained by distillation. This discrepancy was related to incomplete water distillation. The superior accuracy obtained with the charcoal method indicates that it should be possible to utilize the isotope ratio for the detection of citrus juice adulteration.

In recent years the study of stable isotopes in food products revealed the possibiity of applying new and advanced methods to detect adulteration. For instance, the isotope ratio of $^{13}C/^{12}C$ was utilized to detect adulteration in honey (Doner and White, 1977; Doner et al., 1979; White and Doner, 1978a,b; Ziegler et al., 1977), apple juice (Doner and Phillips, 1981; Doner et al., 1980), whisky (Simpkins and Rigby, 1982), fruit juice concentrates (Parker, 1982), wine (Dunbar, 1982a), grape juice (Dunbar, 1982b), and citrus juice (Doner and Bills, 1981; Nissenbaum et al., 1974).

Bricout (1973) differentiated between a natural fruit juice and a dilute concentrate, by measuring the isotopic composition of their water. On the basis of measuring the stable isotopes deuterium and ^{18}O, he concluded that isotopic analysis allowed a very confident distinction between natural fruit juice and reconstituted juice. According to Nissenbaum et al. (1974), the technique is based on the fact that the rainwater or irrigation water, when transported from the roots to the fruit, is fractionated, probably by evapotranspiration, in such a way that the light isotopes (hydrogen and ^{16}O) are lost preferentially to the heavy isotopes (deuterium and ^{18}O).

Variations in the heavy isotope ^{18}O content and the ratio $^{18}O/^{16}O$ in a number of marine water and freshwater samples in different places in the world were reviewed by Epstein and Mayeda (1953). In Israel the ^{18}O values for rain- and groundwater were reported to be −5‰ and −4.5 to −5.5‰, respectively, and for deuterium −25‰ in a rainwater and −15 to −25‰ in groundwater (Gat and Dansgaard, 1972).

Nissenbaum et al. (1974) reported that the deuterium content of citrus juice was too widespread to be applicable in detecting addition of water to citrus juice. Hence, deuterium measurement was precluded from being utilized as a routine detection method for adulteration.

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A rapid technique for sample preparation that circumvents the need for distillation required normally in isotopes determination was proposed by Nissenbaum and Feld (1980). They also indicated that a major drawback in applying isotopic techniques on a routine basis is the need to distill the sample prior to analysis. Furthermore, the low-temperature vacuum distillation used (Bricout, 1973) is a tedious and time-consuming procedure.

This research was undertaken with the aim of reviewing the determination of $^{18}O/^{16}O$ and to furnish a rapid, easy-to-use, accurate, and reliable method to be utilized in the routine preparation and determination of stable isotopes in citrus juice.

MATERIALS AND METHODS

Samples of natural citrus juice (orange and grapefruit) and reconstituted citrus juice were blended (in order to obtain a broad range of isotopic composition) and prepared for the analysis of $^{18}O/^{16}O$ by adapting two methods: vacuum distillation (Bricout, 1971) and charcoal treatment (Nissenbaum and Feld, 1980).

Due to several drawbacks detected in the aforementioned methods, the following revised preparation procedures were employed. (a) Distillation method: (1) A sample of about 5 g was frozen in one leg of a Ritenberg "trousers" (Figure 1) at ca. −60 °C by using an acetone/dry ice bath. (2) The Ritenberg trousers was attached and the system was evacuated by using an oil vacuum pump. When vacuum was established (10^{-2} mm Hg), the Ritenberg trousers was cut off from the vacuum system by using a high-vacuum stopcock. (3) The sample was then heated slowly from −60 to +50 °C, at a rate of 20 °C/h. The water vapor was condensed and collected in the other leg of the Ritenberg trousers by using an acetone/dry ice trap. (4) The distillation was ended when all water had evaporated and a constant weight was obtained (after ~24 h). (b) Charcoal treatment: (1) To exclude suspended particles, samples (ca. 10 g) were centrifuged for 10 min at 15 000 rpm, in closed cuvettes. (2) The supernatant was mixed with activated charcoal powder (washed with acid; BDH Chemicals, Ltd., England; activated at 105 °C in a vacuum oven overnight) for 60 min in a closed flask. (3) The slurry was centrifuged at 15 000 rpm for 10 min in closed cuvettes

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