MAGNETIC RESONANCE IN CHEMISTRY, VOL. 34, 116–122 (1996)

^{17}O NMR of L-Ascorbic Acid. New Spectroscopy of an Old Molecule

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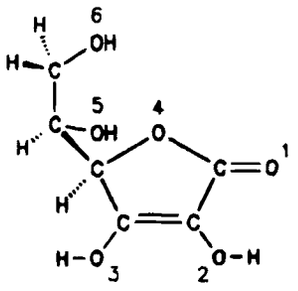
Natural abundance ^{17}O NMR spectra of L-ascorbic acid were recorded in aqueous solution as a function of pH. The water solvent peak was suppressed through the use of ^{17}O-depleted water and inversion–recovery. The ^{17}O NMR resonances were assigned from chemical shift comparison and pH titration behaviour. The resonance of the β-enol oxygen could also be located because of its isotopic exchange with water. Charge densities and bond orders were calculated for α-hydroxytetronic acid at the 6–31G SCF level. Their variations with pH related well with the ^{17}O titration shifts in L-ascorbic acid.**

KEY WORDS L-ascorbic acid; α-hydroxytetronic acid; ^{17}O NMR; pH titration; *ab initio* MO calculations

INTRODUCTION

L-Ascorbic acid (**1**) or vitamin C has been implicated in a large variety of biological phenomena,[1,2] and for this reason several ^{1}H and ^{13}C NMR spectroscopic studies have been undertaken[3–8] for a detailed structural description of this molecule in its different ionization states. To our knowledge, however, no ^{17}O NMR spectra of L-ascorbic acid have so far been reported, although valuable information could be expected from this technique[9] because of the large number of oxygen atoms with multiple functionalities. The difficulties in recording a natural abundance ^{17}O NMR spectrum of L-ascorbic acid in aqueous solution are due not only to the low natural abundance of ^{17}O (0.037%) and the large quadrupole coupling constants which lead to broad resonances,[9–11] but also to the numerous resonances, expected to resonate over an extended chemical shift range. In order to obtain a useful spectrum,[10,11] avoidance of rolling baseline distortions[12] and pulse power decline[12] and also the suppression of the intense solvent water signal[13] were required. In this paper, we report the first ^{17}O NMR spectra of L-ascorbic acid as a function of pH. An attempt is made to assign the ^{17}O resonances of L-ascorbic acid and to rationalize the ^{17}O chemical shifts on going from the free acid to the deprotonated species. Also, a comparison of the ^{17}O titration shifts with those from ^{13}C NMR spectroscopy[7] is made in order to obtain a unique picture of the electronic charge distribution in the different ionization states of L-ascorbic acid.



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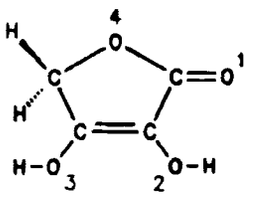

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For a better understanding of the electronic parameters which play a role in the variations of the ^{17}O and ^{13}C chemical shifts in L-ascorbic acid, reference was taken to the *ab initio* calculations performed recently by Bäcker *et al.*[14] for α-hydroxytetronic acid (**2**) at the STO 6–31G** SCF level with full geometry optimization. Using Mulliken's population analysis, atomic charges and bond orders have been evaluated for the different ionization states of α-hydroxytetronic acid.[15] Here these data are discussed with respect to the chemical shifts of L-ascorbic acid. Reference is also made to the crystallographic bond lengths obtained from x-ray analysis.[16,17]

EXPERIMENTAL

Materials

L-Ascorbic acid was purchased from Merck (Darmstadt, Germany). For the natural abundance ^{17}O NMR measurements, *ca.* 1 M solutions of L-ascorbic acid were prepared in ordinary or ^{17}O-depleted water and NaCl was

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CCC 0749–1581/96/020116–07

Received 10 May 1995
Accepted (revised) 4 October 1995

added to bring the solution to an ionic strength of 1 M. All samples contained 0.001 M EDTA to eliminate the influence of paramagnetic metal ion impurities and/or free radical formation on the linewidths. ^{17}O-depleted water was obtained from Alfa Products (Karlsruhe, Germany) (^{17}O content *ca.* 1×10^{-3}%). ^{17}O-enriched water was obtained from Ventron (Karlsruhe, Germany). For the pH titrations, the pH of the initially acidic solution was adjusted upwards with NaOH directly in the NMR tube while purging with nitrogen.

Enrichment in ^{17}O

A 5 g (28 mmol) amount of L-ascorbic acid was dissolved in 10 ml (555 mmol) of enriched water (1 at% ^{17}O). The solution was stirred for 12 h at 60 °C and evaporated to dryness under reduced pressure. The solid was monitored by ^{1}H NMR spectrometry and its enrichment in ^{17}O (*ca.* 0.8 at%) was determined by mass spectrometry and by integration of the ^{17}O NMR resonances, either by direct comparison with the natural abundance intensities or from the intensity ratio of the labelled compound to *tert*-butanol as reference.

NMR measurements

The ^{17}O NMR spectra were obtained at 48.4 MHz using a Bruker AM-360 spectrometer. Sample tubes of 10 mm o.d. were employed. 1,4-Dioxane was used as an external reference.[18] The sample temperature was set at 60 ± 1 °C. Typical spectra settings for ^{17}O were as follows:[12,19] 62 kHz spectral width, 8 ms acquisition time at 1K data points, 90° pulse angle corresponding to a *ca.* 16 μs pulse width and 10 ms recycle delay. The natural abundance samples typically required 50 000 acquisitions for an adequate signal-to-noise ratio and resolution (application of Gaussian–exponential functions). Several spectra were recorded with a RIDE sequence[12,20] for suppression of acoustic ringing combined with inversion–recovery for water elimination.[13]

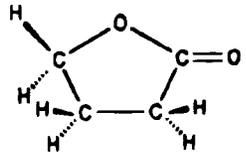
RESULTS AND DISCUSSION

Figure 1 shows natural abundance ^{17}O NMR spectra of 1 M L-ascorbic acid in ^{17}O-depleted water at 60 °C at two different pH values. The use of ^{17}O-depleted water gave a *ca.* 30-fold suppression of the water signal intensity. At acidic pH [Fig. 1(A)], four out of the six ^{17}O resonances which were expected from the functionally different oxygen atoms of L-ascorbic acid, could be detected. They appeared within a chemical shift range of 300 ppm with linewidths of *ca.* 500 Hz. It was clear that the missing resonances of L-ascorbic acid were hidden beneath the residual water resonance. They became easily visible, however, when, in addition to ^{17}O-depleted water, an inversion–recovery method was applied (inset in Fig. 1). It was indeed most advantageous to combine inversion–recovery with a pulse sequence for elimination of the baseline distortions due to acoustic ringing (e.g. RIDE).[20]

It has been proven by ^{13}C NMR spectrometry[7,8] that L-ascorbic acid in aqueous solution, as in the crystal,[16] exists exclusively as the tautomer **1** without any equilibrium between other tautomeric forms.[7] Theoretical studies of the solvent effect on the stability of the tautomeric forms of α-hydroxytetronic acid showed the same tendency.[21] The assignments of the ^{17}O resonances were first attempted by comparison of their chemical shifts with those known for simple mono- and bifunctional oxygen derivatives.[9] Subsequently, they were confirmed by the observation of the ^{17}O chemical shifts on pH titration and by the study of the isotopic oxygen exchange with water.

Assignment of the ^{17}O resonances

The lactone carbonyl resonance of the neutral free L-ascorbic acid occurred at 283 ppm (Table 1), at lower frequency than that reported[22] for γ-butyrolactone (**3**) (340 ppm) and also that of the corresponding α,β-unsaturated lactone **4** (327 ppm). This tendency of the carbonyl chemical shift in L-ascorbic acid was consistent with an increased single-bond character of this group,[9] corresponding to increased electron delocalization by conjugation. However, since the spectra of the model compounds were acquired in acetonitrile,[22] a low-frequency shift for the carbonyl resonance of L-ascorbic acid was also expected from hydrogen bonding to the solvent water.[9] The resonance for the singly bonded oxygen of the lactone moiety of free L-ascorbic acid occurred at 153 ppm (Table 1), again at lower fre-



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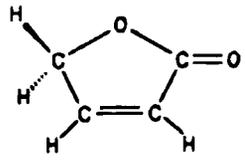

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Table 1. ^{17}O chemical shifts[a] of the titratable oxygens of L-ascorbic acid in the different ionization states

Resonance	Chemical shift (ppm)[b] δ_1[c]	δ_2[d]	δ_3[e]
O-1	283	245	235
O-2	25	5	68
O-3	90	216	175
O-4	153	138	127

[a] Measured in 1 M aqueous solutions at 60 °C. The chemical shifts were obtained from non-linear least-squares fits of one-proton titration curves to the experimental data.
[b] Chemical shifts were measured relative to 1,4-dioxane used as external reference, +0.2 ppm relative to water. The errors of the chemical shifts were estimated to be ±2 ppm.
[c] δ_1 is the chemical shift at acidic pH (neutral free acid).
[d] δ_2 is the chemical shift at neutral pH (anion).
[e] δ_3 is the extrapolated shift for the dianion.

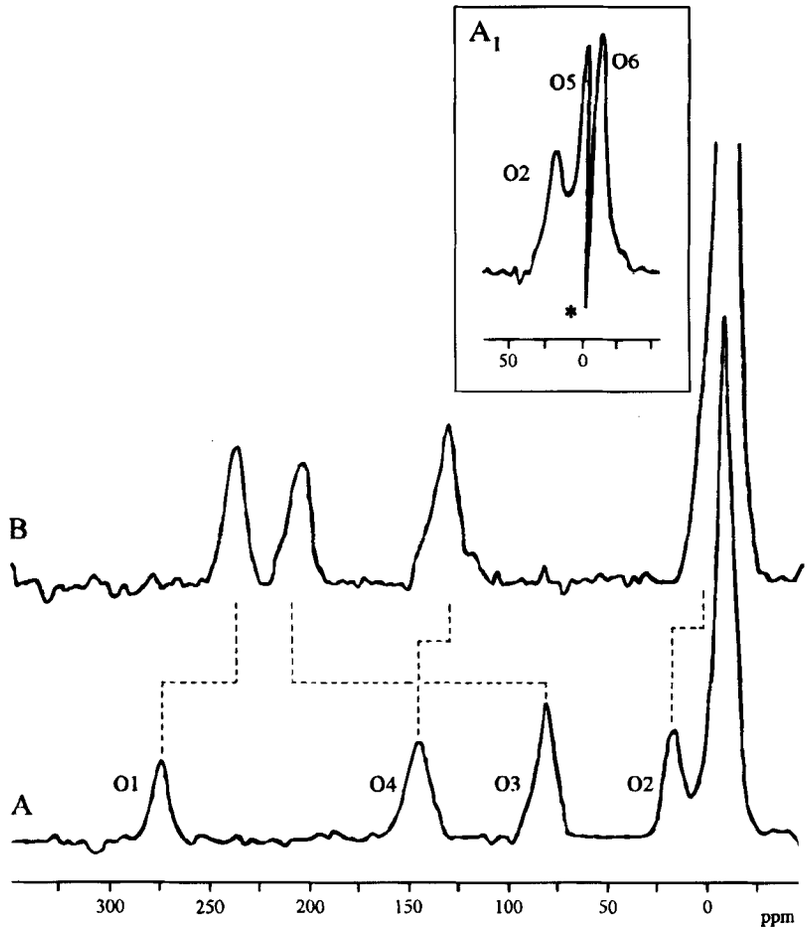


Figure 1. Natural abundance ^{17}O NMR spectra of a 1 M solution of L-ascorbic acid in ^{17}O-depleted water at (A) pH 2 and (B) pH 7; at 60 °C. The inset (A_1) shows the low-frequency region of the spectrum at pH 2 recorded with inversion–recovery for suppression of the water signal (*).

quency than those reported for **3** (179 ppm) and **4** (173 ppm).[22] The resonances at 90 and 25 ppm (Table 1) were attributed to the oxygens of the enediol group. To our knowledge, no ^{17}O chemical shift data of enediols are available for comparison. ^{17}O chemical shifts of enols have been reported for derivatives where the enol forms were stabilized by strong intramolecular hydrogen bonds (95–125 ppm)[23–25] and for phenols with aromatic stabilization (69–73 ppm).[9,26] Frey *et al.*[27] recently reported ^{17}O data for several simple aryl-substituted enols which are stabilized by steric effects[28] rather than intramolecular hydrogen bonding. The ^{17}O chemical shifts were found to depend on the steric bulk of the α-substituent and the solvent (70–99 ppm in chloroform). However, the large difference between the chemical shifts of the enediol oxygens O-2 and O-3 was similar to that observed earlier[7] for the chemical shifts of the carbons C-2 (118.8 ppm) and C-3 (157.0 ppm). The high-frequency shift of C-3 was realized as typical for cyclic α,β-unsaturated carbonyl compounds because of the admixture of charged resonance structures.[24] We tentatively assigned the resonance at 90 ppm to the β-enol oxygen and that at 25 ppm to the α-enol oxygen.

The resonances at *ca.* −6 and 5 ppm, only detectable after water signal suppression (Fig. 1, inset), were assigned to the hydroxyl oxygens O-5 and O-6 of the L-ascorbic acid side-chain. The ^{17}O chemical shift of −6 ppm is typical for primary alcohols, e.g. in ethylene glycol (−5.3 ppm),[26] or in carbohydrates.[10,29] The resonance at 5 ppm can then be attributed to O-5 of the secondary alcohol group, in agreement with the chemical shift range found in carbohydrates.[10]

pH dependence of the ^{17}O chemical shifts

The variation of the ^{17}O chemical shifts of L-ascorbic acid with pH is presented in Fig. 2. Fast exchange was observed between the species of different ionization states (Scheme 1) giving rise to a single average spectrum. Table 2 gives the ^{17}O titration shifts arising from the deprotonation reactions of this diacid with pK_a values of 4.2 and 11.6.[30] For comparison, the ^{13}C titration shifts obtained by Berger[7] are also given. Inspection of Fig. 2 shows that the successive deprotonation of the enediol group causes characteristic shifts of all ^{17}O resonances except of those at low frequency attributed to the alcohol groups of the side-chain. The latter observation confirms that the side-chain of L-ascorbic acid participates only slightly in the redistribution of the electronic charges on deprotonation. Figure 2 shows that the first deprotonation step between pH 2 and 6 strongly affected the resonance at 90 ppm, undergoing a

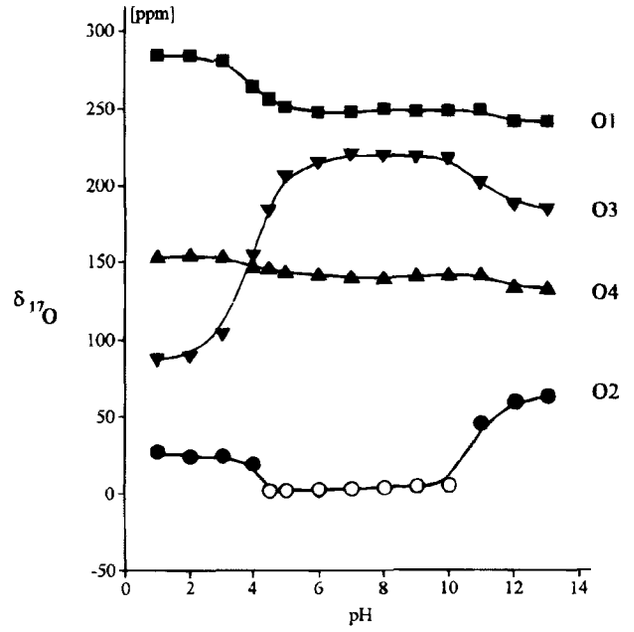


Figure 2. pH titration shifts of the ^{17}O resonances of L-ascorbic acid in a 1 M solution in water at 60 °C. The solid lines correspond to non-linear least-squares fits to one-proton titration curves to the experimental data. Open circles indicate the position of the composite line from water oxygen and O-2, O-5 and O-6 of L-ascorbic acid.

Table 2. ^{17}O and ^{13}C titration shifts of L-ascorbic acid

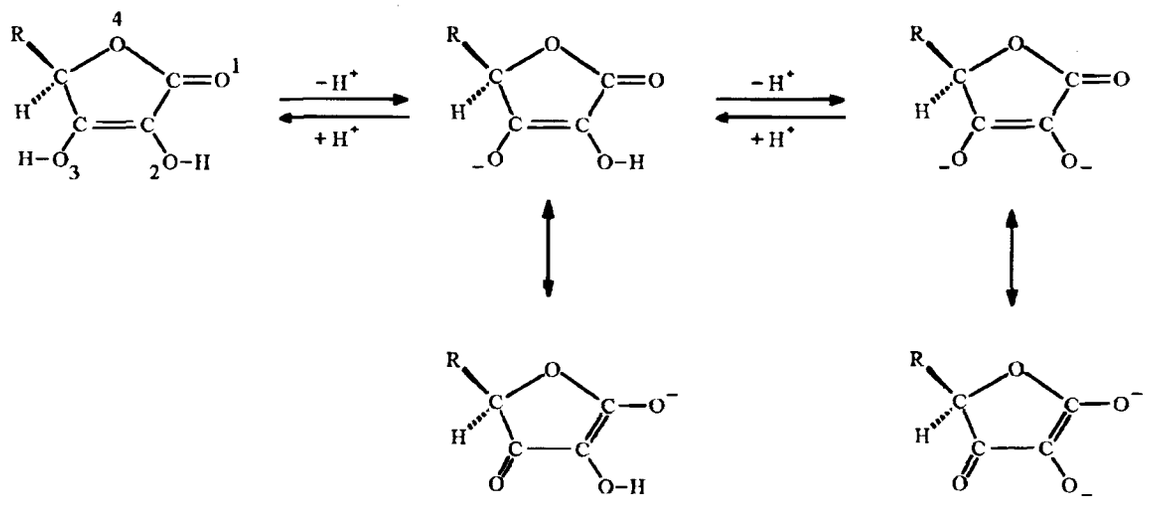
Resonance	Δ_{12}[a] ^{17}O	Δ_{12}[a] ^{13}C	Δ_{23}[b] ^{17}O	Δ_{23}[b] ^{13}C
O-1/C-1	−38	+4.0	−10	+3.3
O-2/C-2	−21	−4.7	+64	+8.3
O-3/C-3	+126	+19.8	−41	−6.0
O-4/C-4	−15	+2.1	−10	−0.5

[a] Δ_{12} are the chemical shift changes on going from the free acid to the anion. Positive values indicate deshielding.
[b] Δ_{23} are the chemical shift changes on going from the anion to the dianion.

chemical shift change of +126 ppm. Since this is the pH range over which O-3 ionizes[16,17] (Scheme 1), the assignment of this resonance to the β-enol group was confirmed. The carbonyl resonance at 283 ppm, in contrast, was shifted by −38 ppm, resulting in very close resonance positions for O-3 and O-1 in the monoanion. This observation clearly pointed to a resonance-stabilized structure of the monoanion in which the negative charge is delocalized to approximately equal extents between O-3 and O-1 (Scheme 1). The titration shift of O-3 was much larger than that observed for ionization of simple carboxylic acids.[31,32] However, the shifts of the resonances of the other ring oxygens O-2 and O-4 also exceeded those observed for adjacent oxygens in carboxylic acids.[32]

The results from ^{17}O NMR spectrometry were comparable to those from ^{13}C NMR spectrometry.[7,8] The strong deshielding of O-3 (+126 ppm) on going from the free acid to the monoanion of L-ascorbic acid paralleled that of C-3 (+19 ppm), and the close approach of the resonance positions of O-3 and O-1 resembled that of C-3 and C-1. It is well known that both the carbon[33] and oxygen[32] resonances of carboxylic acids undergo high frequency shifts on deprotonation, the mechanism of which is not understood, however.[32,33] Both the titration shifts of carboxyl carbons and oxygens are linearly correlated with the pK_a values of the titrating groups.[32,33] From the p$K_a \approx 4.2$ of L-ascorbic acid, titration shifts of $\Delta\delta(^{17}O) = +21$ ppm and $\Delta\delta(^{13}C) = +4.5$ ppm would be predicted. Although these shifts are much smaller than those observed experimentally, the assignment of the O-3 resonance is beyond question.

No substantial shift changes were observed for the ^{17}O resonances between pH 6 and 9, as has been noticed for the ^{13}C resonances.[7] Above pH 10, O-2 began to ionize (Scheme 1), inducing a movement of the corresponding resonance to high frequency while the O-3 resonance was shifted to low frequency, reversing the trend seen between pH 2 and 6. In this second transition from the mono- to the dianion of L-ascorbic acid, the results from ^{17}O NMR corresponded well with those from ^{13}C NMR, especially since also a reversal of



Scheme 1

the chemical shift directions of C-2 and C-3 was observed. Since O-1 and O-4 continued to show increased shielding (Fig. 1), the tendency towards increased electron delocalization seems to be continued when O-2 is ionized.

Oxygen exchange

When recording the ^{17}O NMR spectrum of L-ascorbic acid at acidic pH in ^{17}O-depleted water, the resonance at 90 ppm was observed to disappear with time. This observation allowed confirmation of its assignment to the acidic β-enol group. Conversely, it was possible to prepare 3-^{17}O-enriched L-ascorbic acid (see Experimental), an oxygen exchange reaction which can be assumed to be similar to that in phenolic compounds.[34] Figure 3 shows the spectrum of [3-^{17}O]-L-ascorbic acid in normal water. It can be seen that the α-enol group was also ^{17}O enriched, although to a much smaller extent. The kinetics of exchange of O-3 were measured by following the decrease in the area of the resonance from [3-^{17}O]-L-ascorbic acid in ^{17}O-depleted water over time. The inset in Fig. 3 shows the experimental plot of the ^{17}O exchange data. The rate constant for oxygen exchange determined under these conditions (60 °C, pH 2) was $(5.2 \pm 0.3) \times 10^{-6}$ s^{-1}. This value is at least one order of magnitude smaller than the rate of carbonyl oxygen exchange in aromatic aldehydes and ketones,[35] but much larger than in saturated alcohols.

Understanding the ^{17}O titration shifts

It has been established[9] that the paramagnetic term σ_p is the major contributor to the total screening constant in ^{17}O and ^{13}C chemical shifts. This term may be written as

$$\sigma_p^A = -\text{constant} \times (\Delta E)^{-1} \langle r^{-3} \rangle \sum_B Q_{AB} \qquad (1)$$

where A is the nucleus under investigation and B represents all other nuclei in the molecule, $\langle r^{-3} \rangle$ is the average value of r^{-3} for the 2p orbital under consideration, ΔE is the effective electronic excitation energy and Q_{AA} and Q_{AB} are the charge and bond order terms, respectively.

On deprotonation of L-ascorbic acid, a modification of the terms which contribute to σ_p is expected. Although the terms in Eqn (1) are not independent,[36] it is useful to discuss them separately in order to evaluate their relative importance for the ^{17}O and ^{13}C chemical shifts of L-ascorbic acid.

In order to understand better the electronic parameters which play an important role in the variations of the ^{17}O and ^{13}C chemical shifts in L-ascorbic acid, we made reference to α-hydroxytetronic acid (**2**), for which Bäcker *et al.*[14] recently performed *ab initio* calculations at the STO 6–31G** SCF level. The latter structure differs from that of L-ascorbic acid only in so far as the side-chain is missing. Although this has important consequences for the biological activity of these molecules, no direct interaction of the alcohol groups of the side-chain with the lactone ring has been reported. Bäcker *et al.*[14] showed that a correct quantum mechanical description of **2** requires polarization functions on the hydrogen atoms (at least 6–31G**) in order to yield tautomer **1** as the most stable form. A Mulliken orbital population analysis gave the oxygen net charges (Table 3) and the π-bond orders (Table 4). The same calculations were subsequently extended[15] for the mono- and dianion of α-hydroxytetronic acid. They are contained in Tables 3 and 4, together with the most important C—C and C—O bond lengths obtained from x-ray analysis[16,17] of L-ascorbic acid. In general, the exploitation of atomic charge variations leads to reliable results,[37] in contrast to the absolute atomic charges, which depend on the method of calculations.[14,38] It is

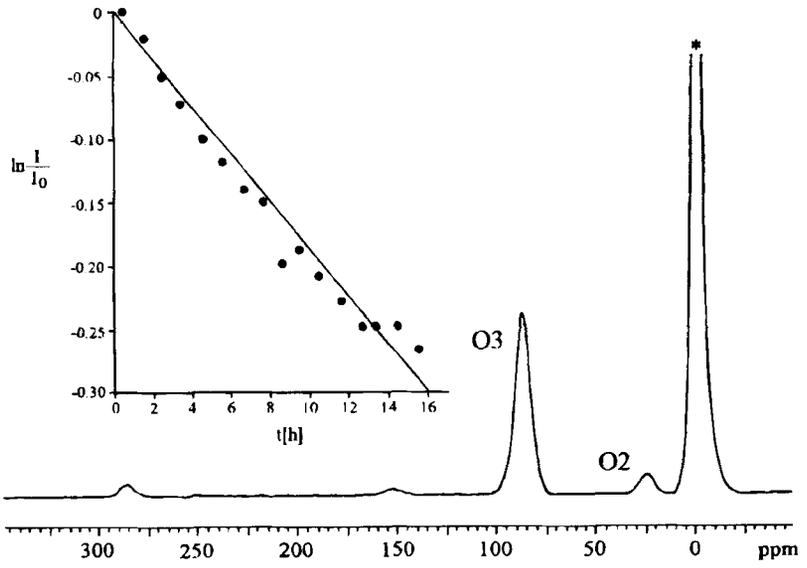


Figure 3. ^{17}O NMR spectrum of ^{17}O-enriched L-ascorbic acid in normal water at pH 2. The enrichment was *ca.* 0.8 at% in O-3 and *ca.* 0.05 at% in O-2. The inset shows a semi-logarithmic plot of the decay of the O-3 resonance intensity in ^{17}O-depleted water.

Table 3. Total net charges of the oxygen atoms of α-hydroxytetronic acid in the three ionization states[a]

Atom	Neutral	Monoanion	Dianion
O-1	−0.580	−0.717	−0.753
O-2	−0.676	−0.710	−0.967
O-3	−0.637	−0.740	−0.887
O-4	−0.634	−0.676	−0.706

[a] Obtained through a Mulliken population analysis on STO 6–31G** wavefunctions.[14,15]

assumed that qualitative trends of charge variations on going from the neutral species to anions and dianions are reproduced correctly, even if no diffuse functions[39] are included in the description of the latter.

An attempt can now be made to rationalize the ^{17}O chemical shifts in the different ionization states of L-ascorbic acid in terms of the calculated variations in the electron populations at the oxygen atoms. NMR shift charge relationships have been verified for ether and carbonyl oxygen atoms on a number of occasions.[40] Usually, correlations were examined for the local π-charges, assuming that the charge on the oxygen depends mainly on the delocalization of the oxygen lone pairs through conjugation. In all cases the increase in π-electron charge at the oxygen atom was indicative of a low-frequency ^{17}O chemical shift. Indeed, the chemical shifts of the lactone carbonyl and ether groups to low frequency can be explained by the increase in the charge density at these oxygens following both deprotonation reactions. The latter is clearly evidenced from the data in Table 3, and is also qualitatively reflected in the resonance structures in Scheme 1. The charge density on the carbonyl carbon, on the other hand, was calculated to decrease (result from Ref. 15), with a concomitant ^{13}C shift to high frequency (Table 2). Hence the bond order term can be excluded as a determining factor for the ^{13}C carbonyl chemical shifts since a decrease in the π-bond order on deprotonation would mean shielding of *both* the ^{17}O and ^{13}C nuclei, which is opposite to the direction observed for the ^{13}C chemical shifts (Table 2).

Table 4. π-Bond orders[a] of the carbon–oxygen bonds in the three ionization states of α-hydroxytetronic acid and bond lengths[b] in L-ascorbic acid and its anion

	π-Bond order			Bond length (Å)	
Bond	Neutral	Anion	Dianion	Neutral	Anion
C-1—O-1	0.613	0.579	0.557	1.216	1.233
C-1—C-2	0.400	0.445	0.393	1.452	1.416
C-1—O-4	0.260	0.223	0.143	1.355	1.358
C-2—O-2	0.250	0.195	0.440	1.361	1.384
C-2—C-3	0.567	0.429	0.432	1.338	1.373
C-3—O-3	0.263	0.547	0.449	1.326	1.287
C-3—C-4	0.404	0.400	0.317	1.493	1.516
C-4—O-4	0.195	0.157	0.125	1.444	1.448

[a] Obtained through a Mulliken population analysis on STO 6–31G** wave functions.[14,15]

[b] Distances from x-ray of L-ascorbic acid[16] and sodium ascorbate.[17]

The situation was more complex when the enol oxygens were considered. Although the charge density of the β-enol oxygen increases strongly during the first deprotonation step, and comparably that of the α-enol oxygen during the second, the observed chemical shifts for both O-3 and O-2 were towards high frequency. Therefore, no explanation of the shifts on grounds of charge density changes seems possible. In contrast, the increase in the charge density of O-3 and O-2 on deprotonation of the other hydroxyl group was in agreement with the experimentally observed shielding.

Looking at the results obtained for the π-bond orders (Table 4), the high-frequency shift of the β-enol at pK_{a_1} and that of the α-enol at pK_{a_2} can be explained by the dramatic changes in this parameter. The order of the C-3—O-3 bond increased from 0.263 to 0.547 and that of the C-2—O-2 bond from 0.195 to 0.440, indicating in both cases a strong increase in the double-bond character. A similar deshielding effect was observed for the deprotonation of carboxylic acids.[32] The case of the β-enol is also reflected in the crystal,[16,17] where strong shortening of the C-3—O-3 bond was observed on going from the neutral to the anionic species. Confirmation of the dominant contribution of the π-bond order term was also obtained from the ^{13}C chemical shifts which titrated with the same sign in the pH ranges considered ($\Delta_{12} = +126$ and $+19.8$ ppm for O-3 and C-3, respectively, and $\Delta_{23} = +64$ and $+8.3$ ppm for O-2 and C-2, respectively, Table 2). Even the relative magnitude of the ^{17}O and ^{13}C titration shifts was well conserved, and the shift values reflected the amount of change in the π-bond order. In conclusion, ^{17}O and ^{13}C chemical shifts of the enol groups in L-ascorbic acid seem to be strongly dominated, in their proper deprotonation step, by the large change in π-bond order.

ΔE is the most problematic factor in Eqn (1). This arbitrary[36] term is generally replaced by an average excitation energy and to a first approximation is equal to the lowest excitation energy. For a carbonyl group this transition is of n–π* character. Unfortunately, the absorptions from the n–π* transitions of L-ascorbic acid are strongly overlapped by those from the π–π* transitions.[41] However, as with the consideration of the π-bond order changes, the ΔE term seems not to be the dominant term because of the different chemical shift directions for C-1 and O-1 of L-ascorbic acid.

CONCLUSION

It is evident that ^{17}O NMR chemical shifts in L-ascorbic acid are very sensitive to changes in the nature of oxygen bonding. The ^{17}O chemical shift directions, in combination with those of ^{13}C, allow differentiation between changes of electric charge densities, π-bond order and, possibly, excitation energies. ^{17}O isotopic enrichment of the β-enol oxygen may be exploited in future studies of the reactivity of this site. e.g. in metal ion complexation.

Acknowledgements

We are grateful to the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for financial support.

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