

The Structure of Dehydroascorbic Acid in Solution

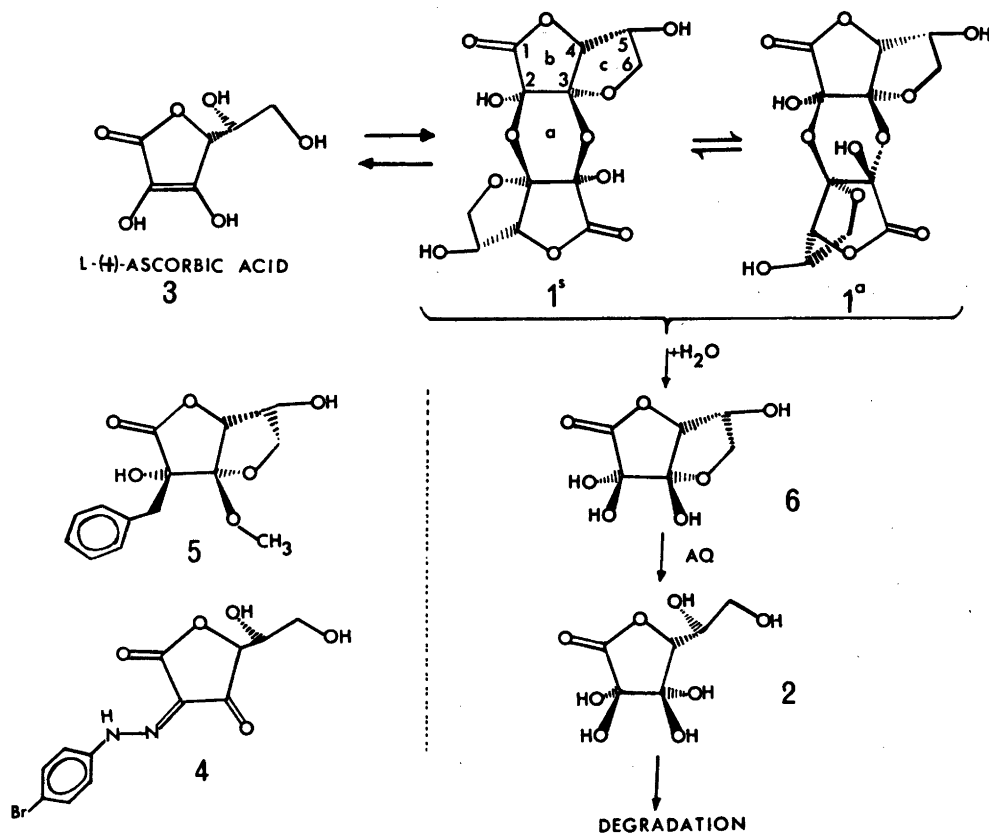
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Crystalline dehydroascorbic acid is composed of dimeric molecules with twofold symmetry. Our ^1H and ^{13}C NMR spectra show that this dimer is unstable in solution and transforms to other species. In dimethylformamide and dimethyl sulfoxide at room temperature an equilibrium is set up between the symmetric dimer and an asymmetric anomer. The standard molar free energy difference is determined to be $3.0 \pm 0.5 \text{ kJ mol}^{-1}$ in favour of

the asymmetric anomer. Either dimer reacts with water to form a bicyclic hydrated monomer. In aqueous solution at room temperature the furanose ring of this monomer opens to a free sidechain as in ascorbic acid.

From the reaction mixture formed by oxidation of L-ascorbic acid [3 (Scheme 1)] a crystalline compound can be isolated.^{1,2} This com-



Scheme 1.

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pound is a dimeric form of dehydroascorbic acid (DHA), with a system of five fused rings (I^s).³

The molecule has twofold symmetry with the central dioxane ring (a) in a distorted boat conformation. The γ -lactone ring (b) is nearly planar, and the furanose ring (c) is in the envelope conformation with C(6) out of the plane.

Our paper deals with ^{13}C and ^1H NMR studies of DHA in different solvents. It should therefore be noted that the symmetrical dimer only contains six distinct carbon atoms. These are numbered as shown in (I^s). The molecule, furthermore, carries four distinct hydrogen atoms which are expected to form an ABCD spin system [H(6)H(6') H(5)H(4)].

In two recent communications, Berger⁴ and Matusch⁵ have reported on ^{13}C NMR studies of DHA. Berger has oxidized L-ascorbic acid in aqueous solution by iodine, and observed a six-peak spectrum which was interpreted in terms of the hydrated monomer (2) Matusch has performed the same experiment, but interpreted the six peaks in terms of the symmetrical dimer (I^s). Furthermore, he observed that the spectrum changed with time: six new lines grew in intensity while the original six peaks diminished. His explanation for this was that the dimer splits up to form the hydrated monomer (2).

We have for some time worked on structural aspects of compounds related to ascorbic acid, including studies similar to those of Berger and Matusch, but our observations and conclusions differ from theirs, as we will show below.

EXPERIMENTAL

Crystalline DHA was prepared by a method similar to that given by Müller-Mulot,³ using 1,4-benzoquinone as oxidant, but *N,N*-dimethylformamide (DMF) as solvent. Although the product was washed carefully, the NMR spectra showed that some formic acid was present. DMF when used as solvent for DHA was dried by means of micro-sieve (4 Å).

The ^{13}C NMR spectra were recorded on a JEOL FX-60 NMR spectrometer operating at 15 MHz. The ^1H NMR spectra were recorded on a VARIAN HR-100 NMR spectrometer operating at 98 MHz. The simulated ^1H spectra were calculated using the simulation program included in the software for JEOL FX-60. The least-squares iteration was performed on a NICOLET 1180 computer using the program ITRCAL (from Nicolet Inst. Corp.).

RESULTS

Dehydroascorbic acid dimers. A surprisingly complex ^{13}C NMR spectrum was obtained at room temperature of DHA dissolved in DMF (Fig. 1b) or dimethyl sulfoxide (DMSO) (Fig. 4a). Whereas Matusch reported a simple six-peak spectrum of DHA in DMSO, we observed a total of 18 major resolved peaks when both spectra were considered. The spectrum of the reaction mixture obtained when L-ascorbic acid was oxidized in DMF was almost identical to that of DHA in the same solvent (Fig. 1a).

A symmetric dimer should give a six-peak ^{13}C NMR spectrum. The presence of 3×6 lines indicates that the original symmetric dimer present in the solid state must have changed when dissolved in DMF.

The spectrum of DHA in DMF or DMSO at room temperature did not change with time. It requires only a few minutes to obtain a ^{13}C spectrum, but the spectrum of a freshly made sample showed that the change had already taken place before the spectrum was recorded.

However, when we dissolved DHA in DMF at -50°C a simple six-peak spectrum was recorded (Fig. 2), but the chemical shifts are different from those reported by Matusch. On heating the solution slowly and recording the spectra at 10 K intervals, we noticed that above -10°C all the room temperature lines could be detected. At room temperature the original and the latter samples gave identical spectra. The process was not reversible, as no changes in the spectrum were detected upon renewed cooling.

It is reasonable to assume that at low temperature the sample contains dissolved molecules of the symmetric dimer. The resulting six peaks can also be seen at room temperature, but with reduced intensity as shown in Fig. 1a. The spectrum recorded at room temperature shows that the solution contains one or more molecular species besides the symmetric dimer.

The new species must account for 12 peaks. There are two peaks of equal intensity in the carbonyl region in DMSO solution at δ 168.1 and 168.7. These must be assigned to the C(1) type carbon atoms. Similarly, from off-resonance and non-decoupled spectra, the remaining peaks could be assigned to two carbon atoms of type C(2), two of type C(3) and so on. The as-

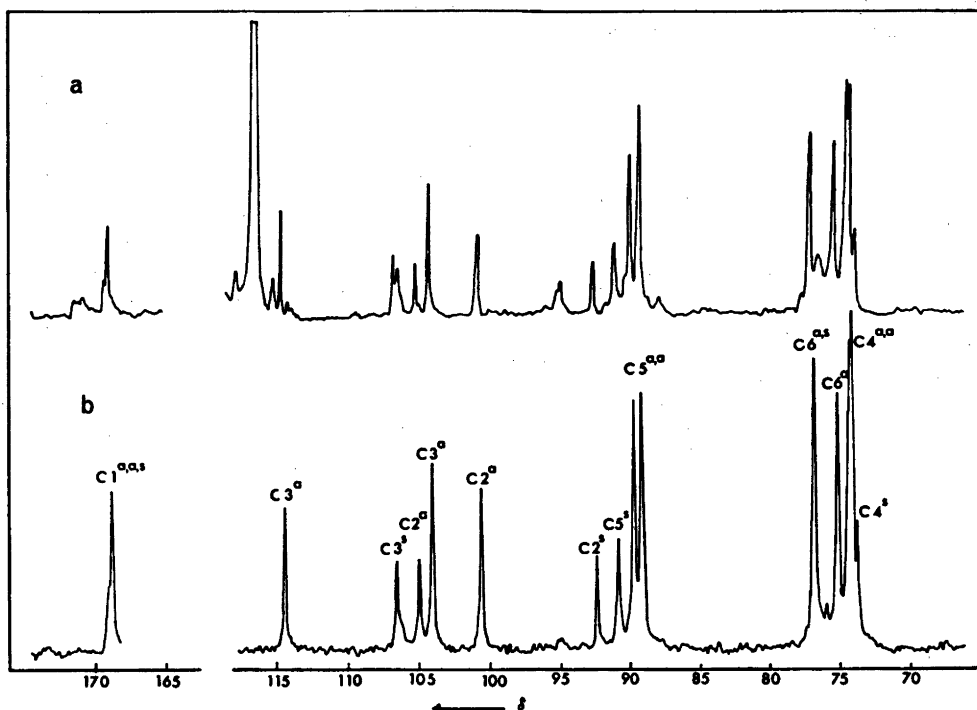


Fig. 1. 15 MHz proton decoupled ^{13}C NMR spectra of DHA at 28 °C. (a) DHA as present in the reaction mixture after the oxidation of L-ascorbic acid in DMF. 68° pulse angle, 2 s between pulses. (b) Crystalline DHA dissolved in DMF. 45° pulse angle, 3 s between pulses. The assignment of the peaks refer to 1. (See text; the superscripts s and a refer to the symmetric and asymmetric dimer, respectively.)

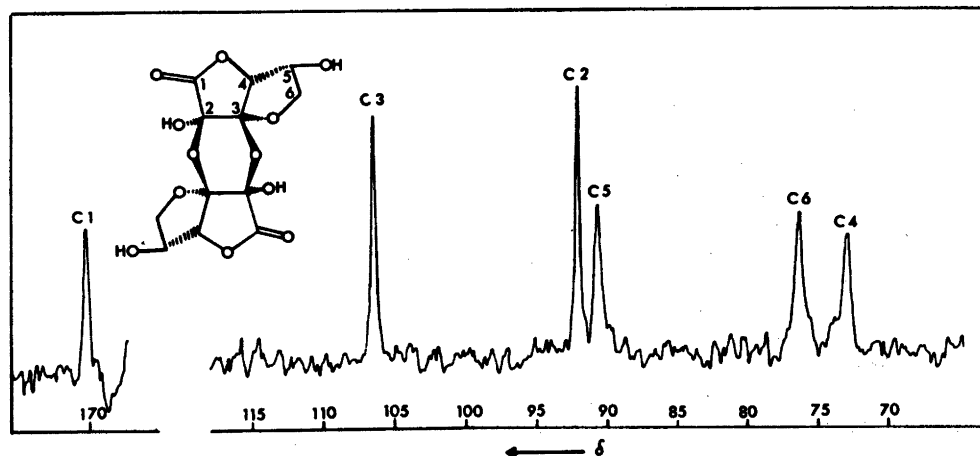


Fig. 2. 15 MHz proton decoupled ^{13}C NMR spectrum of DHA dissolved in DMF at -50 °C.

Table 1. ^{13}C NMR chemical shifts (δ) for compounds related to L-ascorbic acid (in ppm referred to internal TMS at 28 °C).

Cpd	Solvent	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)
I^s	DMF	169.1	92.3	106.5	73.7	90.8	76.8
I^s	DMSO	169.1	91.6	105.6	73.0	90.3	76.3
I^a	DMF	168.9	100.5	104.0	74.1	89.1	75.1
		168.9	104.9	114.4	74.3	89.7	76.8
I^a	DMSO	168.1	99.7	103.4	73.2	88.3	74.5
		168.7	104.2	113.9	73.4	89.1	76.3
2	D ₂ O	171.9	95.1	97.1	74.9	68.6	63.0
4 ^a	DMF	165.9	122.4	193.8	82.6	71.7	62.4
5 ^b	DMSO	172.8	79.7	109.3	73.8	86.9	74.7
6	DMF ^c	173.5	92.2	106.6	74.1	88.6	76.0
6	DMSO ^c	173.8	92.1	106.4	74.0	88.6	76.2
6	D ₂ O	174.2	92.0	106.3	73.5	88.2	76.8

^a *p*-BrC₆H₄: C(1) 141.3, *o*-C 119.5, *m*-C 133.1, *p*-C 119.8. ^b PhCH₂, 40.5, Me 50.4; Ph: C(1) 134.6, *o*-C 130.7, *m*-C 127.8, *p*-C 126.9. ^c Containing 10 % D₂O.

signments are given in Table 1. The spectra therefore indicate that a fraction of the symmetric dimer must either have changed to two different monomers, or to another, but asymmetric dimer.

It is expected that if a dimer of DHA breaks up into two monomers in non-aqueous solution, these should be identical and C(2) should have carbonyl character with a chemical shift of about δ 180. No peaks were detected in this region of the spectrum. The two peaks assigned to C(2) are at about δ 105 and 100 (which are somewhat larger than for C(2) in the symmetric dimer (δ 92)). We therefore find it unreasonable that the observed peaks can be assigned to monomers.

An asymmetric dimer I^a can be formed from a symmetric molecule by opening of the dioxane ring at C(2) and closing it again using the other hydroxyl group at the same carbon atom. Each molecule may split up on either C(2)–O or C(2')–O', but produce the same asymmetric dimer due to the original twofold symmetry. Models show that the dioxane ring then changes from a distorted boat to a distorted chair conformation (Fig. 3). Such a change would induce changes in the chemical shifts of the magnitude observed and presented in Table 1. The changes are largest for C(2) and C(3)

and fairly small for carbon atoms outside the dioxane ring.

The symmetric and asymmetric dimers differ only in configuration at one hemiacetal center C(2). The two dimers are therefore anomers. The equilibration taking place when the symmetric dimer is dissolved is therefore accompanied by mutarotation.

This interpretation of the ^{13}C spectra on the basis of an equilibrium of two anomers was corroborated by measurements of integrated intensities of the different resolved peaks in a spectrum recorded under conditions selected to minimize differences in T_1 and NOE.

From the measured intensities the mol fraction of the asymmetric dimer was found to be 0.77 ± 0.04 . The asymmetric dimer is hence the stabler anomer in these solutions at room temperature. The relative amounts of the two anomers correspond to a difference in standard free energy (G°) of 3.0 ± 0.5 kJ mol⁻¹ in favour of the asymmetric dimer.

We now proceeded to add 10 % water to DMF/DMSO solutions of DHA. In our spectra, six new peaks emerged with increasing in-

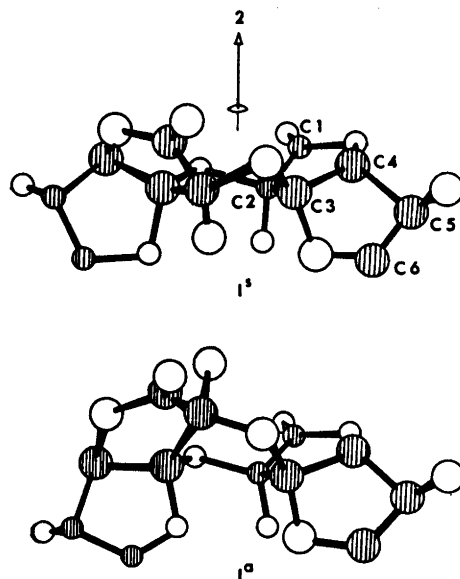


Fig. 3. Perspective drawings of symmetric and of asymmetric DHA dimers (I^s) and (I^a). For comparison, the moiety to the right is drawn with unchanged orientation.

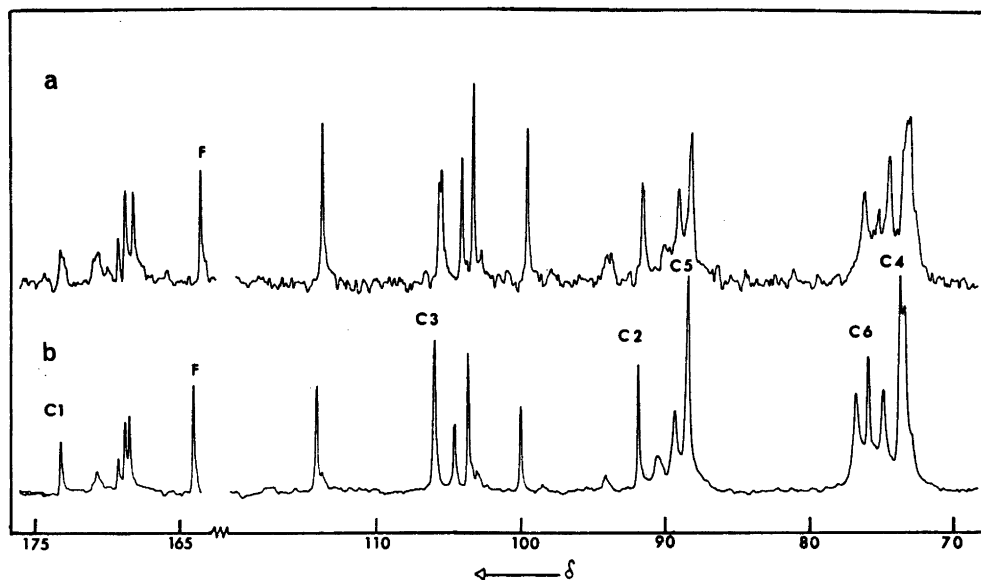


Fig. 4. 15 MHz proton decoupled ^{13}C NMR spectra of dehydroascorbic acid. (a) DHA dissolved in DMSO. 45° pulse angle, 2 s between pulses. (b) Spectrum 1 h after adding 10% D_2O to (a). The numbered peaks increase in intensity with time. C (1) – C (6) refer to (δ). Same recording conditions as in (a). F indicates formic acid impurity.

tensity, whereas the original peaks decreased uniformly with time (Fig. 4b). After a few days only a six-peak spectrum resulted, with chemical shifts close to the values reported by Matusch.⁵ These changes show that the dimer reacts with water to form a hydrated monomer. We ascribe the spectrum reported by Matusch to this monomer. The constitution of this compound will be discussed below.

Dehydroascorbic acid monomers. Crystalline dehydroascorbic acid is only slightly soluble in cold water, but dissolves at 60°C by reacting with water to produce a hydrated monomer.⁶ This is confirmed by the simple six-peak ^{13}C NMR spectrum obtained from a freshly prepared solution of DHA in water as shown in Fig. 5a. The chemical shifts given in Table 1 show that this monomer is the same compound as was formed in DMF or DMSO by addition of 10% water. The spectrum changed, however, in a few days, showing instability of the compound. The original six peaks were replaced by another set of six peaks after some time.

In order to determine the constitution of these two compounds we have compared their ^1H

and ^{13}C NMR spectra with spectra from monomeric ascorbates which have molecular structures known from X-ray analyses: L-ascorbic acid (3),⁷ the *p*-bromophenylhydrazone

Table 2. ^1H NMR chemical shifts (δ) and coupling constants (J , Hz) for L-ascorbic acid (3) and related compounds at 98 MHz.

	3	4 ^a	5 ^b	6
Solvent	D_2O	<i>c</i>	DMSO	D_2O
$\delta(6)$	3.788	3.537	4.204	4.269
$\delta(6')$	3.788	3.537	3.721	4.167
$\delta(5)$	4.100	4.000	4.169	4.679
$\delta(4)$	4.985	4.920	3.929	4.714
$J(6,6')$	—	—	−9.6	−10.4
$J(6,5)$	6.6	7.0	6.4	5.7
$J(6',5)$	6.6	7.0	4.5	2.6
$J(5,4)$	2.0	0.9	0.0	0.9

^a *p*- BrC_6H_4 δ 7.65. ^b PhCH_2 : 2.946 and 3.054, J −13.6 Hz; Me δ 3.436, Ph δ 7.276. ^c DMSO- CDCl_3 , 2:1 (v/v).

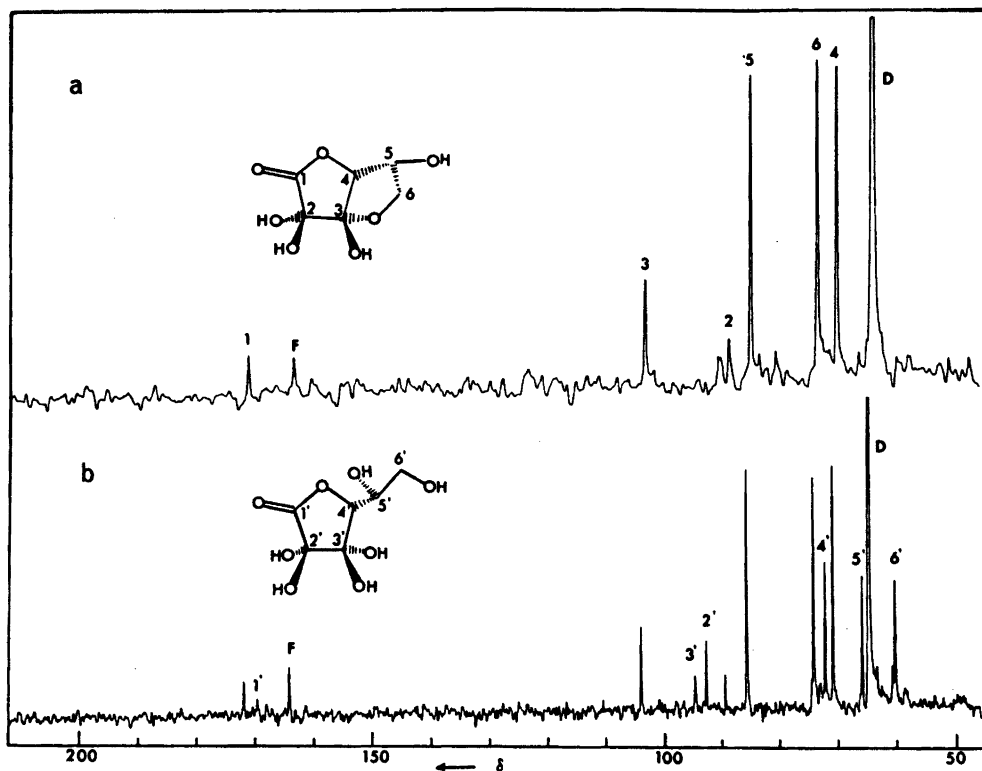


Fig. 5. 15 MHz proton decoupled ^{13}C NMR spectra of DHA in D_2O at 28°C . (a) Freshly prepared solution. 45° pulse angle, 3 s between pulses. (b) 90 h later. 45° pulse angle, 2 s between pulses. Dioxan (D) as reference. F indicates formic acid impurity.

(4)⁸ of DHA and the methyl glycoside (5)⁹ of 2-*C*-benzyl-3-keto-*L*-lyxo-hexulosonic acid lactone. The ^1H NMR spectrum of (4) shown in Fig. 6 is closely similar to that of (3). Both can be analyzed on the basis of a A_2BX spin system (Table 2). The ^1H spectrum of (5) (Fig. 7), however, is markedly different and can be analyzed on the basis of an ABMX spin system. This difference is due to the fact that in (3) and (4) the protons are situated on a free side-chain, whereas in (5) on a furanose ring. The most characteristic difference is that the two protons on C(6) appear magnetically equivalent in a free furanose side-chain, but they are clearly non-equivalent in the furanose ring.

The ^1H spectrum obtained from a freshly prepared aqueous solution of DHA could indeed be analyzed on the basis of an ABMX

spin system (Fig. 8). The two protons on C(6) are clearly non-equivalent, and we conclude that this compound is the hydrated monomer (6). ^{13}C NMR spectra support this conclusion, as the shifts for C(3), C(4), C(5) and C(6) are very close to the values for the dimers.

For the reference substances (3) and (4), however, the C(3), C(5) and C(6) peaks are shifted to higher fields, obviously due to the opening of the furanose ring. This feature proved useful when we compared the new six-peak spectrum found in aqueous solution with spectra from (3) and (4). The same shifts were observed, and we therefore conclude that the new compound is the hydrated monomer (2) with a free side-chain.

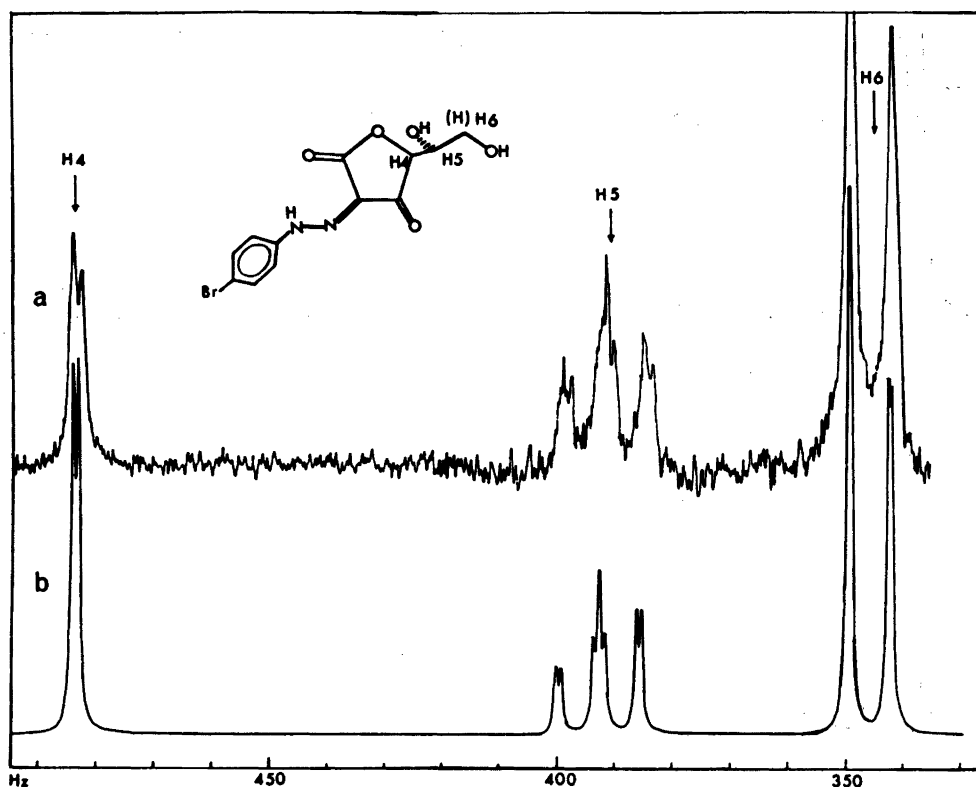


Fig. 6. Part of the 98 MHz ^1H NMR spectrum of *p*-bromophenylhydrazone (4) of DHA dissolved in DMSO at 28°C. (a) Observed spectrum. (b) Calculated spectrum.

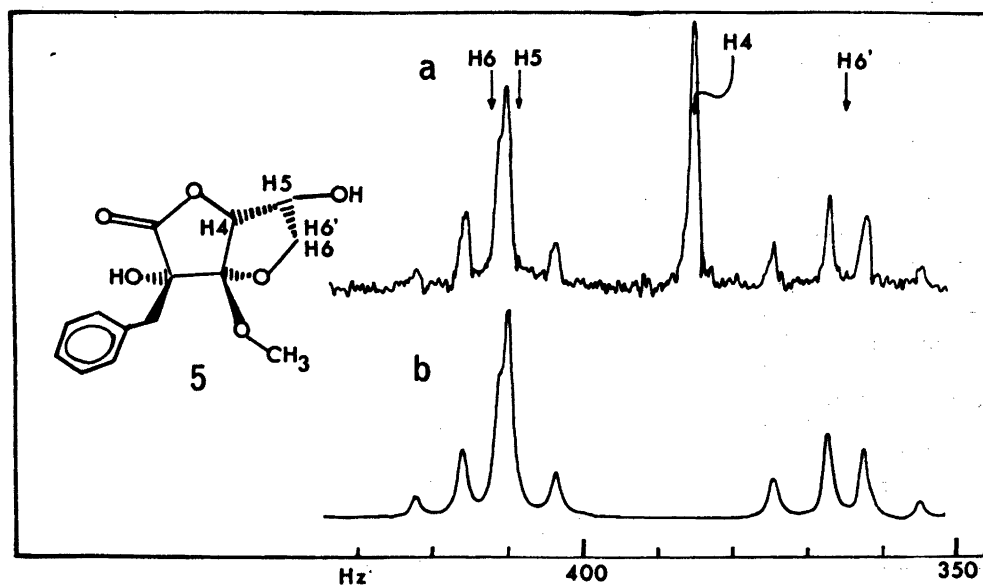


Fig. 7. Part of the 98 MHz ^1H NMR spectra of the methyl glycoside (5) of 2-*C*-benzyl-3 keto-*L*-lyxo-hexulosonic acid lactone dissolved in DMSO at 28°C. (a) Observed. (b) Calculated.

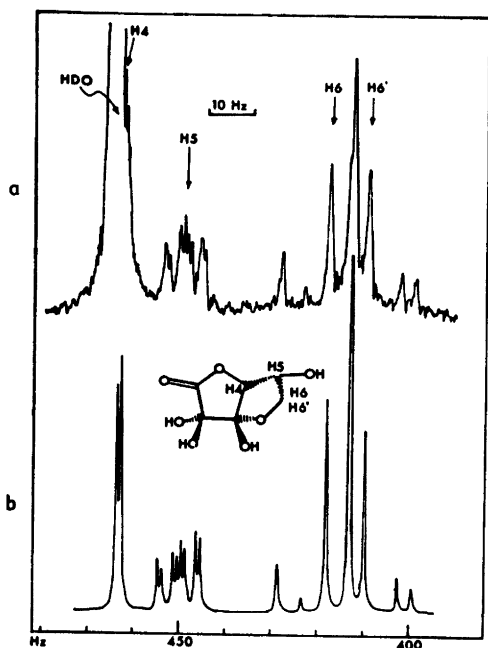


Fig. 8. 98 MHz ^1H NMR spectrum of DHA in freshly prepared aqueous solution at 28 °C. (a) Observed spectrum. (b) Calculated spectrum.

DISCUSSION

It is well known that aqueous solutions of vitamin C are unstable and are easily oxidized to dehydroascorbic acid. The latter eventually degrades to other species, and the useful biological function is lost. The evasive nature of DHA in solution has so far complicated the analysis of its molecular structure, and different opinions have been expressed in the literature. Our observations and conclusions are summarized in Scheme 1, which also includes the reference structures used to support the interpretation of the NMR spectra.

We have used 1,4-benzoquinone to oxidize L-ascorbic acid in DMF because it gives high yield and a relatively pure product. It is noteworthy that the reaction mixture contained DHA in dimeric form even before the components prescribed by Müller-Mulot² were added. Earlier it was thought that dimerization took place after the addition of certain acids or other ingredients, but these do not seem to accomplish the dimerization, but rather facilitate the crystallization by lowering the

solubility of the symmetric dimer. The existence of an equilibrium of two dimeric anomers in DMF or DMSO requires reinterpretation of the mutarotation investigated by Müller-Mulot,¹⁰ who presumed a monomerization process. We also observed high sensitivity of the dimers to the presence of water, and as little as 0.4 % H_2O in a 0.1 M solution of DHA is sufficient to change all dimers to hydrated monomers. This may explain the adverse molecular weight determinations by Albers *et al.*¹¹ and by Dietz.¹²

The oxidation pathway is probably complex. It is an open question whether the dimer is formed as a primary product or formed subsequently in a secondary reaction. We could not detect any peaks in our ^{13}C spectra of a monomer with three $\text{C}=\text{O}$ groups in the ring. However, a spectrum of such a molecule has been reported by Matusch (using iodine as oxidant).

The proposed structure of the asymmetric dimer represents the simplest possible alternative, and satisfies the spectral data and structural reasonability. The two dimers found in solution are not unique, however, because at least six models could be constructed from two hydrated monomers (6). At least four of these seem to be structurally plausible. There could be two explanations to why only two are detected: either these two have lowest energy, or one or both are formed directly in the oxidation process. Models indicate that the two dimers found are lower in energy than the other isomers, but more work is necessary to clarify this point. If water is present during the oxidation of L-ascorbic acid, or when crystalline DHA goes into solution, the bicyclic monomer (6) is eventually formed. This is in agreement with Pfeilsticker,⁶ who also reports to have isolated the solid monomer from water by rapid cooling and lyophilization.¹³ In another set of experiments, we have observed that alcohols and certain other substances react with DHA in the same way as water. These results will be published shortly.

We wish to conclude our discussion by stating that if competition occurs between the hydroxyl groups of water and of other ascorbates during the oxidation of ascorbic acid, the former are preferred. A hydrated monomer should hence have higher stability in aqueous solutions, such as, *e.g.*, in body fluids, than any version of a dimer. In other physiological systems the

presence of dimers seems more likely, and they are presumably useful as storage version for the vitamin because of their higher lipid solubility and stability towards oxidative degradation.¹⁴

Note added in proof. We have recently had access to a JEOL FX 60 QS NMR spectrometer making it possible to obtain the ¹H decoupled ¹³C NMR spectrum of crystalline DHA. The spectrum contains six well-resolved peaks (Fig. 9) from (*I*^s) present in the crystals. The chemical shifts are C(1) 175.2, C(2) 95.4, C(3) 107.0, C(4) 73.4, C(5) 93.8, and C(6) 77.8. These values confirm the interpretation given above (Table 1). Compared to the chemical shifts for (*I*^s) in dissolved form C(3), C(4) and C(6) are unshifted, whereas C(5) and C(2) are shifted 3.0 ppm and C(1) 6.1 ppm to low field. These shifts are probably due to the breaking of the hydrogen bonds when the crystals dissolve as only carbon atoms directly bonded to oxygen atoms are affected.

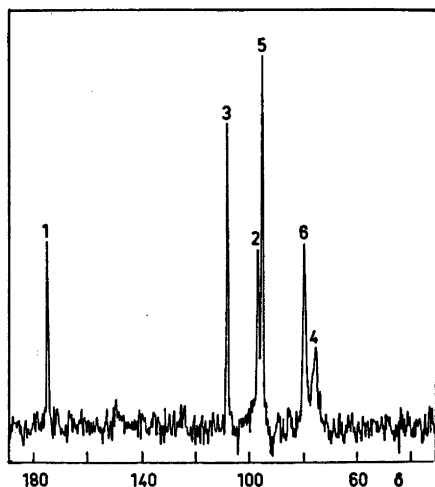


Fig. 9 15 MHz proton decoupled ¹³C NMR spectrum of crystalline DHA at room temperature. (Proton enhanced with magic angle spinning, 400 pulses).

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