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Assay of Ascorbic Acid in Plants Tissues, I: ^{1}H and ^{13}C NMR Study of the Complex between Cu(I) and 2,2′-Biquinoline

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The Shieh's method, originally devised for the analysis of ascorbic acid (AsA) in pharmaceutical preparations and based on the reaction of this acid with Cu(I) and 2,2′-biquinoline, was adapted to determine AsA in biological samples and particularly in plant tissues. The proposed assay is compared with some current methods for AsA determination. Moreover the system Cu(I)-biquinoline is studied by ^{1}H and ^{13}C NMR spectroscopy, and a coordination model for the complex is proposed.

INTRODUCTION

The determination of ascorbic acid (AsA) in biological samples is usually accomplished by one of the following methods: (i) redox reactions based on Fe(II) oxidation followed by chelation with substances such as ferredozine[1] or dipyridyl[2]; (ii) reduction of AsA to dehydroascorbic acid (DHA) followed by the formation of a characteristic osazone with dinitrophenylhydrazine[3]; (iii) optical-enzymatic assays in the presence of ascorbic acid oxidase[4]; (iv) liquid chromatographic methods[5].

All the above methods are not suitable for the assay of AsA in plant tissues because of interferences produced by phenolic substances such as chlorogenic and caffeic acids, well represented in plants. A recent method, originally devised for the determination of AsA in pharmaceutical preparations[6], was therefore adapted to the above purposes.

2, 2′-Biquinoline (biq), also known as cuproine, is a much-used reagent for the spectrophotometric determination of Cu(I)[7–9]. Despite its wide use no detailed work on the spectroscopic properties of this complex in solution has been published. The aim of this work was to evaluate the suitability of the biquinoline method[6] for AsA determination in biological samples, including plant material, where most methods suffer interferences caused by phenolic compounds. In order

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to gain better knowledge of the Cu(I)-biq complex an ^{1}H and ^{13}C NMR study in $CDCl_3$ solution was also performed.

EXPERIMENTAL

2, 2'-Biquinoline, copper (I) chloride and all NMR solvents were A.C.S. Aldrich reagents.

Preparation of complex

The complex $[(biq)_2Cu]$ Cl has been prepared according to literature methods[10]; elemental analysis has been performed on a Perkin Elmer 240B CHN-analyzer; calculated for $C_{36}H_{24}N_4Cl$ Cu: C, 70.63, N, 9.15, H, 3.92. Found: C, 68.61, N, 7.79, H, 4.35%. Although the observed Δ% in analytical figures of the cuprous complex (e.g. Δ% C = 2.02, Δ% N = 1.36) are beyond what is generally considered acceptable, this difference is due to the instability of the Cu(I) complex in the solid state.

NMR Studies

The spectra were recorded by a CTF-20 Varian spectrometer at 37°C, in 8 and 5 mm tubes for ^{13}C and ^{1}H measurements respectively. The chemical shifts are reported as δ (ppm) downfield from TMS. The ^{1}H spectra were recorded with a sweep width of 1200Hz; a 24 ms pulse and 8192 data points were employed. The ^{13}C spectra were recorded with a sweep width of 4000Hz; a 10 ms pulse was employed with a 2s delay between pulses and 8192 data points. ^{1}H and ^{13}C spectra have been recorded of pure biq (0.01M in $CDCl_3$) and of different Cu(I)-biq mixtures prepared by adding weighed amounts of solid CuCl to the biquinoline solution. The CuCl was completely dissolved in $CDCl_3$ by complexation; when the [Cu]/[biq] ratio was greater than 1 : 2 the salt was in excess and did not dissolve.

Preparation of samples

Samples of fresh plant material were homogenized in a Waring blender with 9 parts of cold 4% metaphosphoric acid, HPO_3; dried seeds were first milled in a hand mortar and then treated in the same way. Both types of homogenates were then centrifuged at 25,000 × g and the clear supernant was used for AsA determinations.

The deep purple color of the Cu(I)-biquinoline complex is due to an intense absorption band at 545 nm with a molar absorptivity, ε, of 6300 $M^{-1}cm^{-1}$ at 25°C: these spectral features are very temperature dependent. We worked at 37°C where we find that Beer's law is satisfied over a concentration range of 0.05 ÷ 1 mM.

RESULTS AND DISCUSSION

The Shieh's method, based on the reaction showed in Fig. 1, suffers phenolic

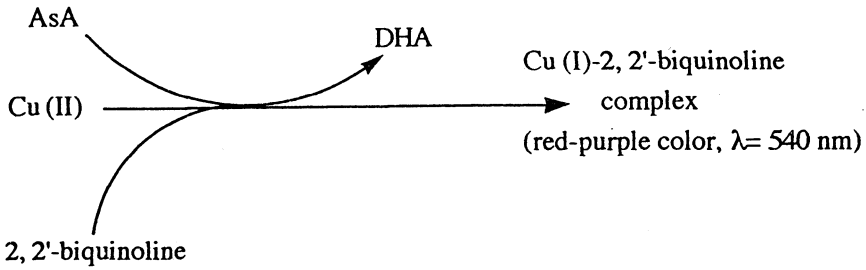

Fig. 1 Mechanism of formation of the Cu(I)-2,2'-biquinoline complex

interferences when used on plant tissues. For removal of these substances a new procedure was developed and tested, whose experimental steps are:

(i) directly homogenize the samples in 4% metaphosphoric acid;

(ii) centrifuge to obtain a clear solution;

(iii) add the copper-biquinoline reagent (CBQ) to the clear supernatant and leave at 37°C for 30 min.;

(iv) read the developed red-purple color at 540 nm; the color is stable for about 1 h.

To ensure the validity of the method, AsA concentration in some samples of plant material was measured, both with our method and with some of the established methods currently recommended even in a very recent review[12] on AsA determination, *i.e.* the dinitrophenylhydrazine (DNP) and the optical-enzymatic method (OPE). The results are shown in Table 1.

TABLE 1
ASCORBIC ACID CONTENT IN PLANT TISSUES AS ASSAYED BY THE PRESENT METHOD (CBQ), DNP AND OPTICAL-ENZYMATIC METHOD (OPE)

Sample	CBQ	DNP	OPE
	mg AsA/100 g fresh or dried material		
Spinach (leaves)	34.6 ± 0.78	36.2 ± 0.60	33.8 ± 0.47
Orange (juice)	43.2 ± 0.82	39.6 ± 0.63	40.4 ± 0.68
Tomato (juice)	11.5 ± 0.23	10.7 ± 0.24	9.3 ± 0.20
Broad bean (fresh seeds)	31.1 ± 0.54	30.0 ± 0.49	32.6 ± 0.75
Broad bean (dry seeds)	8.0 ± 0.10	8.9 ± 0.13	7.4 ± 0.09
Peas (Fresh seeds)	19.6 ± 0.45	20.7 ± 0.40	22.2 ± 0.47
Chick-pea (dry seeds)	0.9 ± 0.02	1.4 ± 0.03	1.1 ± 0.02
Kidney bean (dry seeds)	2.2 ± 0.06	1.9 ± 0.04	2.0 ± 0.05

The above values represent the mean ± SD of five replicate measurements for each sample.

A comparative analysis of these results shows that the modified biquinoline method can be considered equivalent to other methods: in fact a t test at the α

level = 0.01 shows that thee is no significant difference between the results obtained with the three methods. It is nevertheless the simplest to perform and furthermore it is inexpensive. On the basis of its reliability and its simplicity, this method was extended to different biological systems and it is now currently used for the determination of AsA in blood and platelets of thalassemic subjects. In fact AsA plays an important role in iron adsorption at intestinal level and its frequent monitoring through a simple method is therefore required.

The complex Cu(I)-biquinoline (Fig. 2A), which is responsible of the color development in this AsA assay, has been better characterized in this study, where results of ^{1}H and ^{13}C NMR measurements are here below reported together with a tentative discussion on a coordination model.

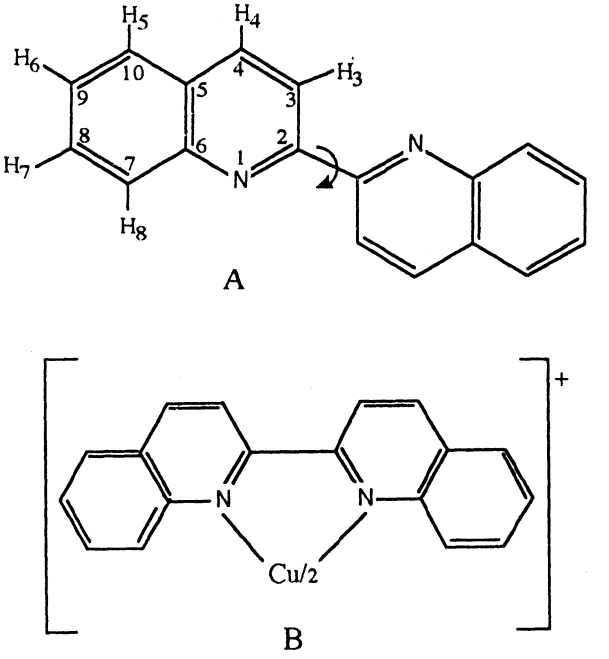

Fig. 2 Structural formulae of 2,2'-biquinoline (A) and of the Cu(I)-2,2'-biquinoline complex (B)

The rotation in 2,2'-biquinoline (Fig. 2B) of the two quinoline moieties around the 2-2' bond gives a mirror plane in addition to the C2 rotation axis; a spin system which may be regarded as $(A\ B\ C\ D\ E\ F)_2$ in the ^{1}H spectrum is therefore expected. In Fig. 3A the ^{1}H spectrum of pure biquinoline is reported; the ^{1}H assignments were made on he basis of Drake and Jones[11]. In Fig. 4A the ^{13}C spectrum is shown; the ^{13}C assignments on the basis of analogous compounds. The NMR parameters are reported in Table 2.

TABLE 2

^1H and ^{13}C chemical shifts δ(ppm) downfield from TMS and coupling constants J(Hz) for 2,2'-biquinoline (80 MHz spectra).

^1H NMR

	δ_3	δ_4	δ_5	δ_6	δ_7	δ_8	
pure biquinoline	8.7	8.2	7.7	7.4	7.6	8.1	
biquinoline-Cu(I)	8.7	8.3	7.8	7.4	7.5	8.0	
	J_{34}	J_{56}	J_{57}	J_{58}	J_{67}	J_{68}	J_{78}
pure biquinoline	8.4	7.3	1.2	0.5	6.4	1.1	7.8

^{13}C NMR

	δ_2	δ_3	δ_4	δ_5	δ_6	δ_7	δ_8	δ_9	δ_{10}
pure biquinoline	150.0	113.1	130.4	122.2	141.9	121.3	123.7	120.6	123.2
biquinoline-Cu(I)	149.7	113.1	130.4	122.2	141.5	121.3	123.4	120.6	123.2

The reported results are optimized with the program LAOCOON III (11).

The addition of increasing amounts of CuCl induced progressive variations in both ^{13}C and ^1H spectra, until the [Cu(I)/[biq] ratio 1 : 2 was reached; the spectra obtained in these latter conditions are reported in Figs 3B and 4B, while the parameters for the complexed biquinoline are also reported in Table 2. An analysis of these results allows some considerations on the coordination mechanism:

(a) an increase in line width with Cu(I) concentration is clearly visible, due to the complexation: a further increase in line width, that can be accounted for by both an increase of the viscosity of the medium and in addition by some slight variation of relaxation times on complex formation has been compensated for by subtracting the half height line width of the $CHCl_3$ from all other lines of the spectrum. On the contrary any effect due to paramagnetic Cu(II) species can be excluded because more drastic widenings should be produced;

(b) protons 4 and 5 are shifted downfield, protons 3 and 6 are not affected while 7 and 8 ones show a highfield shift;

(c) only the resonances at carbon atoms 2, 6 and 8 are affected, showing a highfield shift.

Furthermore we point out that for each kind of nuclei there is only a single signal: this latter finding excludes any lack of symmetry both between the two different biquinolines in the complex and between the two different quinoline moieties in each biquinoline. In order to explain the differences in the direction of the chemical shifts of the various protons a tetrahedral structure can be tentatively hypothesized: in a square planar structure, in fact, only an inductive effect should be exerted by the Cu(I) bindng which results in a downfield shift; in a tetrahedral structure on the contrary the ring current effect of the two

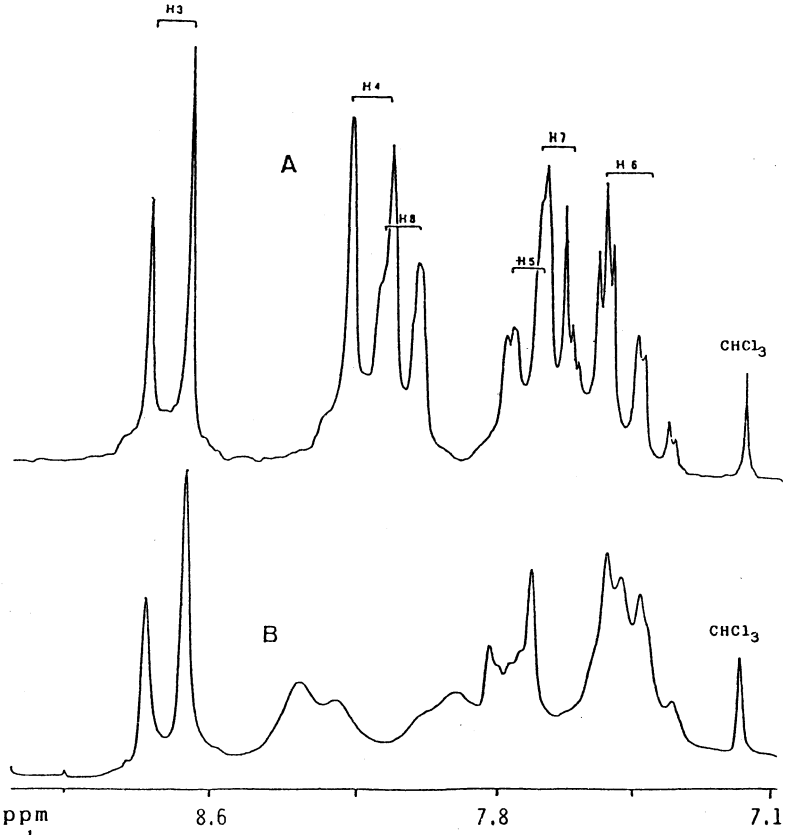

Fig. 3 ^{1}H NMR spectra of pure biquinoline (A) and of the Cu(I)-biquinoline complex(B) in $CDCl_3$ solution.

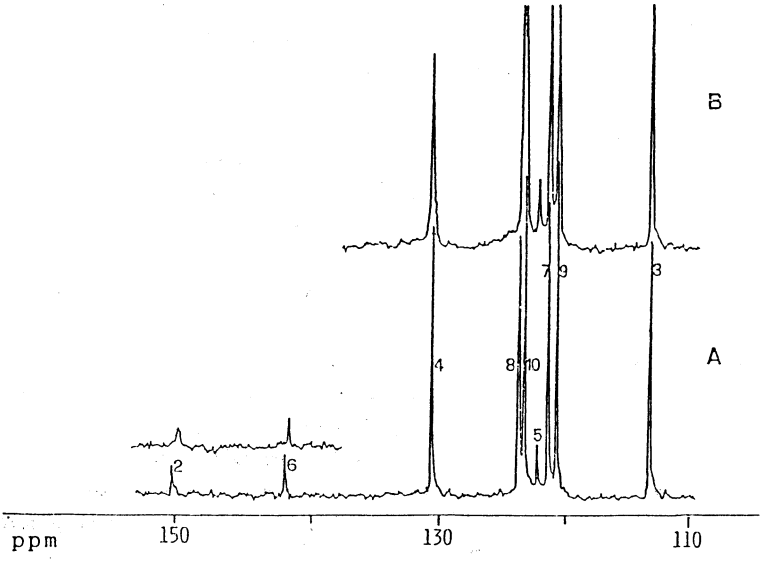

Fig. 4 ^{13}C NMR spectra of pure biquinoline (A) and of the Cu(I)-biquinoline complex(B) in $CDCl_3$ solution.

biquinolines, which are perpendicular to each other, acts in a different way on the various protons. H-4 and H-5, which lie out of the field of the ring current feel only inductive effects, while H-7 and H-8 are above all affected by the ring current effect; in atoms H-3 and H-6 these two opposite effets seem to neutralize each other and no chemicals shift variation is evident on complexation.

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