REACTION OF BIOFLAVONOIDS WITH COPPER(II) ACETATE IN AQUEOUS SOLUTION

N. B. Mel′nikova, I. D. Ioffe, and L. A. Tsareva

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The reaction of dihydroquercetin, quercetin, and rutin with Cu(II) acetate in aqueous solution is studied. Quercetin and dihydroquercetin form complexes with Cu. The water-insoluble complexes are characterized as chelates of the 3,4-positions of the main γ-pyrone ring. Electronic spectroscopy showed the presence of stable polynuclear rutin complexes of composition rutin:Cu = 1:x, where x > 2.

Key words: rutin, dihydroquercetin, quercetin, Cu complexes, flavonoid.

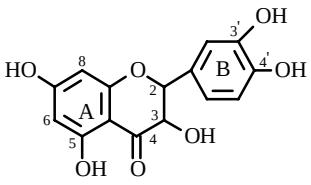
Flavonoids are used in medicine, pharmacology, and dietology as unique phytopreparations with a wide spectrum of activity [1-4]. Flavonoids are low-molecular-weight bioregulators in humans. They are interesting primarily because of their specific reactivity in redox processes of enzymes and proteins [5-7].

Copper-containing enzymes occupy a special position among enzymes that catalyze redox processes involving flavonoids [5-9]. Copper is a transition metal and has two common valence states. This enables Cu-containing proteins, depending on the nature and geometry of the ligands, to span a wide range of redox potentials and to bind reversibly oxygen and carbon monoxide [5, 8].

An example of the important role of Cu complexes with flavonols is Cu-containing quercetin-2,3-dioxygenase, which catalyzes oxygenolysis of 3-hydroxyflavones to the corresponding products of oxidative cleavage of the heterocyclic ring [9].

Despite the great interest in the mechanism of reaction of bioflavonoids and Cu ions, data on the composition, structure, and complex-formation features are incomplete and contradictory.

We studied the reaction in aqueous solution of Cu(II) acetate and bioflavonoids with the 3′,4′-*o*-phenol structure, i.e., dihydroquercetin (DHQ) (**1**), quercetin (Q) (**2**), and rutin (**3**).



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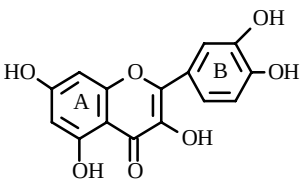

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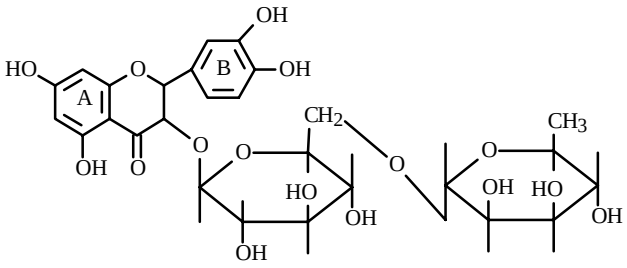

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The reaction of bioflavonoids and Cu(II) acetate was studied in the range 293-343 K at component concentrations varying from the solubility limit of the bioflavonoids in aqueous solution at the given temperature to $C \approx 1 \cdot 10^{-5}$ M and with the bioflavonoid:Cu(II) acetate mole ratio varying over a wide range (1:x, where x = 0.5-32).

The blue color disappears when Cu(II) acetate solution is added to a saturated aqueous solution of flavonoids. The solutions become deep yellow.

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TABLE 1. Analytical Data for Reaction Mixture of Dihydroquercetin (DHQ, $3 \cdot 10^{-3}$ M) and Cu (II) Acetate (T = 309 K, τ = 2 h)

DHQ:Cu^{2+} ratio	Precipitate color	Remaining DHQ, % (±5%)			Cu content***				
					Solution				in alcohol-insoluble part of ppt., % (±5%)
		Solution*	Precipitate**	Total	[Cu^{+}], % (±3%)	[Cu^{2+}], % (±3%)	Cu^{+}/Cu^{2+}	Cu, mg ion/g	
1:0.5	Green	13	30	43	20	1	20:1	2.6 ±5	20
1:1	Yellowish-green	5	15	20	20	8	2.5:1	2.2 ±3	20
1:2	Brown	3	12	15	15	10	1.5:1	2.7 ±1	15

*UV-spectral analysis at λ_{max} = 290 nm;
**cyanidine chloride test;
***spectrophotocolorimetric method using bicinchonic acid. Cu content in total precipitate 10% (±5%).

TABLE 2. Comparative Reaction Data for Cu(II) Acetate and Bioflavonoids (T = 309 K, τ = 2 h, aqueous solution, [Cu^{2+}]:[bioflavonoid] = 1:1)

Biofalvonoid	Appearance		Solution			
	Solution	Precipitate	Fraction of remaining flavonoid (±5%), %	Cu^{+}:Cu^{2+}	pH decrease, ΔpH	Flavonoid fraction (±5%), %
DHQ	Yellow, white flakes	Yellowish-green	5	2.5:1	0.20	15
Q	Yellowish-brown	Brown	5	100:1	0.15	10-30
Rutin	Bright yellow homogeneous	-	10-30	100:1	-	-

Cu content in total precipitate 10% (±5%), in alcohol-insoluble part 20%.

A mixture of saturated rutin solution and Cu(II) acetate remains homogeneous at any rutin:Cu^{2+} mole ratio. Adding Cu(II) acetate to saturated aqueous solutions of Q and DHQ causes rapid formation of precipitates. The color changes from green to brown. The rate of precipitate formation depends on the mole ratio of the components (Tables 1 and 2).

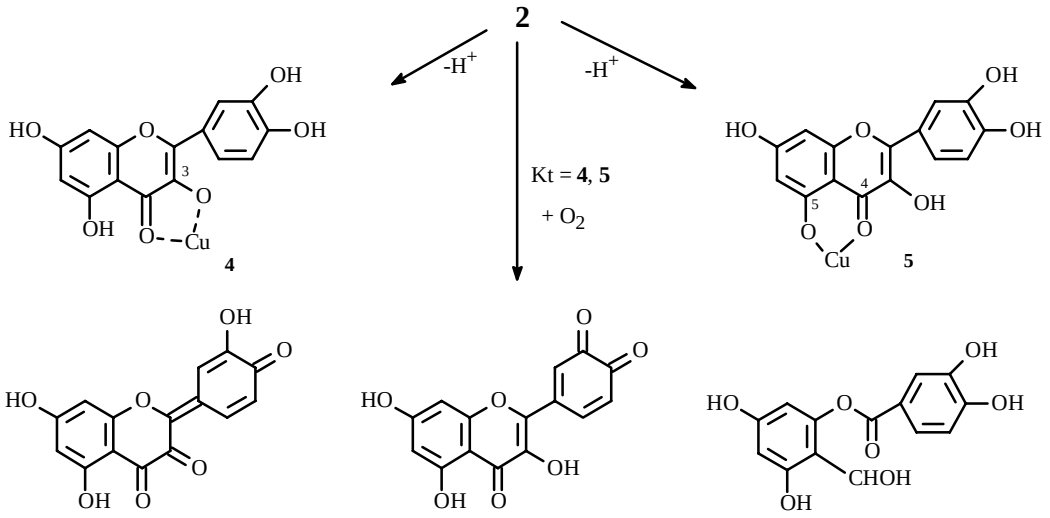
Table 1 gives an example of the reaction of **1** and $Cu(OAc)_2$ at 309 K. The effect of the flavonoid:Cu mole ratio on the nature of the reaction is shown. Increasing the fraction of Cu not only decreases the concentration of unreacted DHQ but also decreases the Cu content in the alcohol-insoluble part of the precipitate.

IR spectra of the reaction products obtained over several time intervals (from 1-2 min to several days) are identical. The main reaction of **1** with $Cu(OAc)_2$ occurs in 1-2 min.

The reactions of Cu(II) acetate with the other bioflavonoids was compared at a 1:1 mole ratio (Table 2).

The results indicate that the reactions of **1** and **2** with Cu(II) acetate are similar. The IR spectrum of the solid product that was repeatedly treated with water and alcohol to remove starting materials exhibited new strong bands with maxima at 1570-1560 cm^{-1} (ν_{as}) and 1440 cm^{-1} (ν_s) for the asymmetric and symmetric, respectively, stretching vibrations of carboxylate. Comparison of the spectrum of the reaction product with those of pure DHQ and Cu(II) acetate and their solid mixture indicates that the reaction product is a complex of DHQ and Cu(II) acetate. The absorption maxima corresponding to ν_{as} of carboxylate (1570-1550 cm^{-1}) for this product are shifted by about 50 cm^{-1} to low-frequency compared with ν_{as} of the carboxylate (1620-1615 cm^{-1}, 1590 cm^{-1}) in starting Cu(II) acetate.

According to the literature, the most probable complexation mechanism of **2** and **1** involves reaction of Cu^{2+} with reactive groups in the 3,4-positions of the chromone [10], although reaction at the 4,5-positions is also possible [11] (Scheme 1).



Scheme 1.

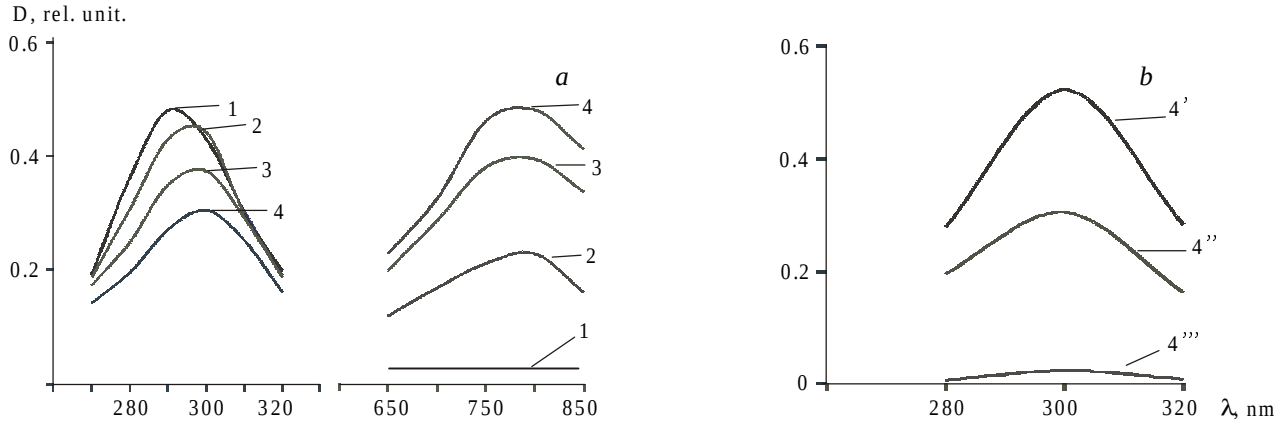


Fig. 1. UV absorption spectra of aqueous DHQ and Cu^{2+}: C_{DHQ} = const = $3.28 \cdot 10^{-5}$, τ = 0; DHQ (1), [DHQ]:[Cu^{2+}] = 1:1 (2), 1:3 (3), 1:6 (4) (a). [DHQ]:[Cu^{2+}] = 1:6 (4′, τ = 0; 4″, τ = 7 days, 4‴, τ = 2 months) (b).

The resulting flavonolate complexes can act as catalysts for oxidation processes, in particular, oxidative destruction of the heterocyclic ring and oxidation of the *o*-phenol in the 3′,4′-position to *o*-diquinones [12] (ring B in Scheme 1).

The decrease in the pH value during the reaction is consistent with the formation of type **4** and **5** structures (Table 2).

Rutin has no reactive group in the 3-position and probably forms complexes with Cu(II) acetate by a different mechanism.

Electronic spectroscopy provided information about the structure and composition of Cu flavonolate complexes. The literature indicates that changes in the visible spectrum (370-500 nm) depend more on the nature of the Cu salt, complexants present, metal salts used to maintain ionic strength, and other factors than those in the UV spectrum [9-12]. As a rule, changes in the range 250-270 nm are insignificant (region affected by ring A) whereas those in the visible range (350-500 nm) reflect changes in the chromone structure and ring B of the flavonoid [12].

Starting DHQ has one broad absorption band at λ_{max} = 290 ± 2 nm in the analyzed ranges. Its position (±2 nm) in aqueous solution depends on the pH and ionization state of the OH in the 3-position. The UV spectrum of DHQ ($3.28 \cdot 10^{-5}$ M) and $Cu(OAc)_2$ exhibits a bathochromic shift of this band to λ_{max} = 300 nm (Fig. 1*a* and *b*). The band at λ_{max} = 300 nm weakens if the Cu fraction is increased (Fig. 1*a*) and the reaction time is lengthened (Fig. 1*b*) whereas the absorption band of Cu(II) acetate aqua complexes (λ_{max} = 780 nm) decreases if the DHQ fraction is increased (Fig. 1*a*). Such a situation is probably due to a decrease in the concentration of DHQ complexes of $Cu(OAc)_2$ owing to their low solubility in water and precipitation. It should also be noted that the visible spectrum, which characterizes the electronic structure of ring B, remains unchanged.

TABLE 3. Electronic Absorption Spectra at 350-500 nm of Aqueous Mixture of Rutin and Cu(II) Acetate

Rutin conc., $M\times10^5$, $C_{rut.}$ (init.)	Cu(II) acetate conc., $M\times10^5$, $C_{cu(II)}$ (init.)	$K = C_{Cu(II)}/C_{rut.}$	Band position, λ_{max}, nm	D, rel. units at λ_{max}	Molar extinction coefficient $\varepsilon\times10^{-5}$, L/mol·cm at λ_{max},
			A		
4.0	1.31	3.28	398.0	0.415	0.104
4.0	24.0	6.0	400.0-404.0	0.469	0.117
4.0	32.0	8.0	408.2	0.505	0.126
4.0	64.0	16.0	412.4-416.7	0.575	0.144
4.0	96.0	24.0	412.4-416.7	0.609	0.152
4.0	262.4	65.0	412.2-416.7	0.644	0.161
			B		
2.8	16.0	5.71	400.0-404.0	0.331	0.118
3.3	21.9	6.56	404.0-408.2	0.413	0.124
2.8	23.6	8.43	408.2-412.4	0.374	0.133
2.8	28.8	10.28	408.2	0.375	0.134
2.0	131.2	65.60	412.4-416.7	0.319	0.160
2.0	262.4	131.20	412.4-416.7	0.329	0.163

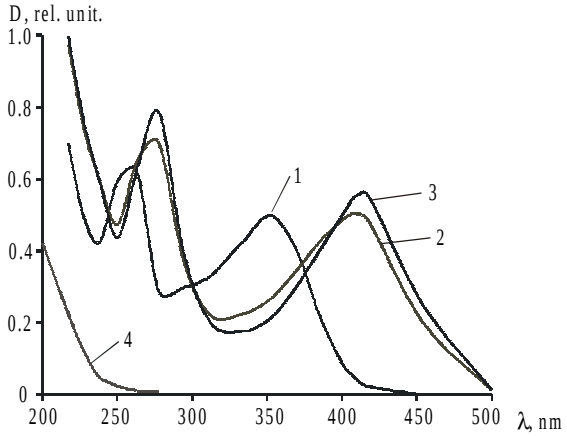


Fig. 2. Electronic absorption spectra of aqueous rutin ($C_{0,rut} = 4\cdot10^{-5}$ M) and Cu(II) acetate. Initial rutin (1), $K = C_{0,Cu(II)}/C_{0,rut} = 8.0$ (2), K = 16.0 (3), Cu(II) acetate, $C_0 = 3.2\cdot10^{-4}$ M without rutin (4).

In general, the UV spectral results confirm the structures **4** or **5** for the DHQ complexes with $Cu(OAc)_2$ in solution.

Changes in the electronic spectra of Cu complexes with rutin and Q are more complicated.

Complexation of Cu by Q has been studied either with supporting electrolytes to maintain constant ionic strength (perchlorates, nitrates, etc.) [10] or with additional complexants to improve the solubility of the products [12].

We investigated electronic spectra of aqueous solutions of Q and rutin without irrelevant compounds (doubly distilled water at pH 6.4).

Under these conditions, complexation of Cu(II) acetate by Q ($2.8\cdot10^{-4}$ M) at a 1:1 mole ratio causes almost immediately complete disappearance of the band at $\lambda_{max} = 370$ nm. This is accompanied by the development of a dark brown precipitate.

In contrast with this, the absorption band at $\lambda_{max} = 350$ in the electronic spectrum of a mixture of rutin and Cu(II) acetate undergoes a bathochromic shift from 47 to 65 nm depending on the mole ratio of the components (Fig. 2).

Analysis of the spectra showed that the position of this band depends smoothly on the mole ratio of the Cu(II) and rutin concentrations (Table 3). As the ratio ($K = C_{Cu(II)}/C_{rut}$) increases, the wavelength of the band reaches the limiting value 415 nm at K = 16. The wavelength of the absorption maximum remains constant if the rutin:Cu(II) acetate ratio is increased further (Table 3). As the wavelength increases, the optical density and molar extinction coefficient increase similarly. The molar extinction coefficient is greatest for K = 24.

TABLE 4. Calculated Complex Compositions in Aqueous Rutin and Cu(II) Acetate (by Electronic Spectroscopy at 350-500 nm)

Initial conc. rutin, $M \cdot 10^5$	$K = C_{Cu(II)}/C_{rut.}$	Fraction free rutin in complex α, %*	Fraction rutin in complexes β, % of composition**			
			1:4	1:8	1:16	1:20+1:24
			I	II	III	IV+V
2.8	2.9	40.0	60.0	-	-	-
4.0	3.3	18.0	82.0	-	-	-
6.0	2.7	33.0	67.0	-	-	-
2.8	5.7	-	57.0	43.0	-	-
4.0	6.0	-	50.0	50.0	-	-
2.8	8.4	-	90.0	10.0	-	-
4.0	8.0	-	-	100.0	-	-
2.8	10.3	-	43.0	57.0	-	-
4.0	16.0	-	-	-	100.0	-
4.0	24.0	-	-	-	-	100.0
5.0	40.0	-	-	-	-	100.0
2.0	65.0	-	-	-	-	100.0
4.0	65.0	-	-	-	-	100.0
2.0	131.0	-	-	-	-	100.0

*$\alpha=\{([P_{0,init}]-[P])/[P_{0,init}])\}\cdot 100\%$; $\beta=([P]/[P_{0,init}])\cdot 100\%$, where [P] and $[P_{0,init}]$ are the number of moles of rutin in the complex and inital state, respectively; complex composition expressed in molar ratio of reagents in the complex [rutin]:[Cu(II) acetate].

The bathochromic shift of the rutin bands in this spectral region is probably indicative of the formation of Cu complexes with the 3′,4′-*o*-phenols of ring B.

It is known that Cu(II) acetate, which is a dimer $[Cu_2(OCOCH_3)_4]\cdot 2H_2O$ in the crystal, partially dissociates [13]. The waters in the crystalline hydrate can be replaced successively by other ligands, for example, amines or phosphine oxides, without substantial structural changes. Each Cu atom has typical distorted octahedral coordination [14].

It has also been noted that Cu(II) acetate can form polynuclear complexes with ligands that coordinate through oxygen atoms, for example, with pyrocatechol and tartrate [14, 15]. The situation is most probably analogous for the reaction of rutin and $Cu(OAc)_2$.

Thus, two types of complexes can form in the mixture of rutin and $Cu(OAc)_2$. These are complexes of Cu(II) acetate of general formula $[Cu_n(OCOCH_3)_m(H_2O)_q]\cdot kH_2O$ and complexes that are newly formed from rutin and Cu(II) acetate or rutin and the Cu(II) acetate complexes.

Designating rutin as component P and the Cu complexes as component M, the ratio of components determining the composition of the newly formed complexes can be expressed as

$$[P]:x[M] \text{ or } [P]:x\cdot n[Cu(CH_3COO)_2],$$

where x is the number of moles of M that reacts with one mole of rutin and n is the number of moles of Cu(II) acetate incorporated into M.

A formal description of the newly formed complexes assumes that the complex contains one rutin molecule and one M structural unit for $x = 1$. For $x = 2$, two M structural units react with one rutin molecule. Both flavonolate complexes with a 3′,4′-*o*-phenol in ring B that have one or two hydroxyls bound to one Cu in M and complexes resulting from substitution of one ligand in M by rutin correspond to such a formal approach.

However, the existence of complexes with $x > 2$ can be proposed because the electronic spectra undergo substantial changes at very large excesses of Cu(II) acetate (K = 4-16).

Spectra of a mixture with a mole ratio rutin:Cu(II) acetate ≈ 1:3 indicate that the mixture contains free and uncomplexed rutin in addition to the complex (Table 4).

Variational fitting of the quantity n at this mole ratio and mole composition was used to analyze quantitatively the spectra of the reaction mixtures. It shows that n is 4.

Using the derived value $n = 4$ and the rutin:Cu(II) acetate mole ratio, we calculated the mole composition of various complexes in all mixtures (Table 4).

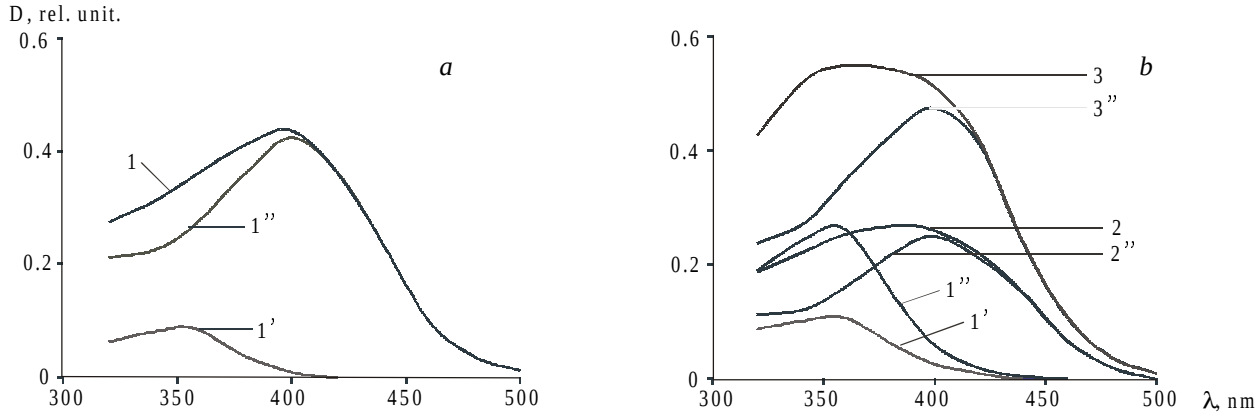


Fig. 3. Electronic absorption spectra of rutin and Cu(II) acetate with graphical band separation: reaction mixture (1, 2, 3); free rutin absorption bands: uncomplexed (1′, 2′, 3′), after graphical separation (1″, 2″, 3″). $C_{0,rut} = 4.0 \cdot 10^{-5}$ M, K = 3.3 (1, 1′, 1″) (a); $C_{0,rut} = 2.8 \cdot 10^{-5}$ M, K = 2.8 (2, 2′, 2″), $C_{0,rut} = 6.0 \cdot 10^{-5}$ M, K = 4.0 (3, 3′, 3″) (b).

It can be seen that a certain λ_{max} corresponds to each type of complex.

Figure 3*a* and *b* shows graphically the separation of the absorption bands [16] in spectra of certain reaction mixtures.

Thus, various types of complexes that are determined by the rutin:Cu^{2+} ratio form characteristically for mixtures of rutin and Cu(II) acetate.

It should be noted that, in general, reactions of flavonoids and $Cu(OAc)_2$ produce under the studied conditions flavonolate complexes of various structure determined by the concentration, temperature, and flavonoid:Cu(II) acetate mole ratio.

EXPERIMENTAL

DHQ (98% pure) was obtained from larch wood and supplied by OOO "Rosbioprom" (Sarov, Nizhegorodskaya Oblast).

DHQ, rutin, and Q were repeatedly recrystallized from a mixture of alcohol and water and dried to constant mass.

The DHQ concentration was determined spectrophotocolorimetrically using a cyanidine chloride test [18] and analytically using UV spectra in the range λ_{max} = 290 nm and a calibration curve. Cyanidine chloride was prepared by treating an aliquot of DHQ solution (5 mL) with a mixture of conc. HCl (ultrahigh purity), glacial acetic acid (ultrahigh purity), water (3:3:1 vol%), and zinc dust (analytically pure, 0.2 g) at 50°C for 1 min. The calibration curve and routine analyses of DHQ were made at 550 nm on a SF-46 spectrophotometer.

Uni- and bivalent Cu were determined spectrophotocolorimetrically using the reaction of univalent Cu with bicinchonic acid that gives a colored complex [17]. Aqueous solutions were analyzed for Cu(I) content by placing a sample (5 mL) in a 50-cc flask containing universal buffer (acetate—phosphate—borate) at pH 6 [19], adding bicinchonic acid solution (2 mL, 0.1% in 2% KOH), and adjusting the buffer to the mark. Precipitates were analyzed by dissolving the substance (0.015 g) in bicinchonic acid (2 mL), placing the solution in a 50-cc flask, and adding universal buffer at pH 6 to the mark. The solution was stirred and analyzed 20 min later at λ_{max} = 560 nm. The total Cu content was determined from a calibration curve that was constructed using portions of a standard Cu solution. The content of Cu(II) was determined analogously by adding hydroxylamine hydrochloride solution (5 mL) to a flask and reducing bivalent Cu to univalent.

Electronic spectra were recorded in the range 200-500 nm in quartz cuvettes on a Specord M-40 (Karl Zeiss, DDR) UV-Vis spectrophotometer. Spectra were recorded using doubly distilled water and solutions of metal salts as references. The cuvette thickness was d = 1 cm.

IR spectra were obtained on a Perkin—Elmer 180 spectrometer in the range 3200-500 cm^{-1} as KBr pellets (0.1-0.3% analyte) and in thin layers of mineral oil.

The pH values of buffer solutions were found using a microprocessor pH 320 SET pH-meter (Wissenschaftlich Technische Werkstatten GmbH).

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