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Spectrophotometric Studies on Flavonoid-Copper(II) Complexes in Methanol Solution

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Complex formation of flavonoids (rutin, quercetin, flavocummelin and flavonol) with copper(II) was studied by spectrophotometry. These flavonoids have some possible chelating sites (such as 3-hydroxy-4-keto, 5-hydroxy-4-keto and 3,4-*o*-dihydroxy groups denoted as site a, b, and c, respectively) depending on the number and position of hydroxyl substituents in their molecules.

Absorption spectra of the flavonoid-copper(II) systems with the changes of Cu^{2+} concentrations were observed, and the molar ratio and continuous variation plots indicated the existence of only 1:1 complex in the flavonol-, flavocummelin-, and rutin-copper(II) systems, and both 1:1 and 1:2 types in the quercetin-copper(II) system. A comparison of the magnitudes of bathochromic shift was compared to consider the sites used for the complex formation. The 1:1 complex formation in the flavonol-copper(II) system appears to take place at site a whereas that of the flavocummelin- and the rutin-copper(II) systems at site b. In the case of quercetin, the initial complex formation seems to occur at site a and the subsequent complex formation at site c.

In order to compare the relative stability of the flavonoid-copper(II) complexes, the free Cu^{2+} concentrations equilibrated in the mixed solutions of each flavonoid and copper(II) were determined by polarography. The relative stability decreased in the order of quercetin, flavonol, rutin, and flavocummelin.

Keywords——quercetin; rutin; flavonol; flavocummelin; spectrophotometric study; complex formation; flavonoid complexes; copper(II) complexes; flavonoid-copper(II) complexes

Certain flavonoids have extensively been used as colorimetric reagents for the determination of metal ions.[2] While a number of studies have been made for the analytical uses of flavonoids, however, stoichiometry of complexes formed between flavonoids and metal ions has not been elucidated satisfactorily. The diversity of the position and the number of hydroxyl substituents, especially in naturally occurring flavonoids, makes the feature of the complex formation complicated. Recently, aluminum(III) complexes of hydroxyflavones have been studied by spectroscopy for the comparison of chelating ability among some hydroxyflavone.[3] In these studies, only a few papers[4] have dealt with the formation of complexes involving copper(II).

1) Location: *1432–1, Horinouchi, Hachioji, Tokyo 192–03, Japan.*
2) M. Katyal, *Talanta*, **15**, 96 (1968).
3) *a*) L.J. Porter and K.R. Markham, *J. Chem. Soc. C*, **1969**, 344; *b*) L.J. Porter and K.R. Markham, *ibid.*, **1970**, 1309.
4) *a*) I.E. Makasheva and M.T. Golovkia, *Zh. Obshch. Khim.*, **43**, 1640 (1973); *b*) M. Thompson and C.R. Williams, *Anal. Chim. Acta*, **85**, 375 (1976).

Participation of flavonoids and copper(II) in biochemical reactions has become of interest in recent years. For example, quercetinase was recently found to be a copper(II)-containing enzyme,[5] by which quercetin in microorganisms is decomposed. Inhibition effect of flavonoids on the oxidation of ascorbic acid is generally known,[6] and the fact that the presence of some flavonoids was found to decrease the catalytic activity of copper(II) on the oxidation of ascorbic acid[7] lead us to the further study on the interaction of flavonoids with copper(II).

The present paper deals with spectrophotometric studies on the complex formation between some flavonoids (flavonol, flavocummelin, quercetin, and rutin) and copper(II) in methanol solution. The stoichiometry of the complex formation will be discussed in relation to the structure of these flavonoids.

Experimental

Visible absorption spectra were recorded on a Hitachi double-beam spectrophotometer Model 356. DC polarograms were recorded on a Yanagimoto pen-recording polarograph Model P-8 using a dropping mercury electrode in a usual manner. The temperature was 25°.

Quercetin, rutin and flavonol were obtained commercially. Flavocummelin was synthesized and kindly supplied by Mr. Hoshino of this College. All other chemicals were of reagent grade and used without further purification. A stock Cu^{2+} solution (0.10 M) was prepared by dissolving $CuSO_4$ in redistilled water and standardized by titration with EDTA. This solution was diluted with MeOH to make Cu^{2+} solutions in the desired concentration before each use. Stock solution of flavonoids (1.0×10^{-3} M) were prepared by dissolving each flavonoid in MeOH.

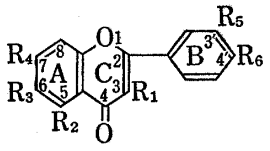
The test solutions for the absorption measurement were prepared by mixing 0.4 ml of the stock flavonoid solution and 0.4 ml of different concentrations of Cu^{2+} solution (1.0×10^{-4} to 1.0×10^{-2} M) and then methanol was added to make the final volume 10 ml, so that the amount of water does not exceed 0.4%.

In the polarographic measurement, 50% MeOH–H_2O solutions containing 1.0×10^{-3} M Cu^{2+} and 5.0×10^{-4} M flavonoids were used. All the solutions contained 0.1 M $NaClO_4$ as a supporting electrolyte.

Results and Discussion

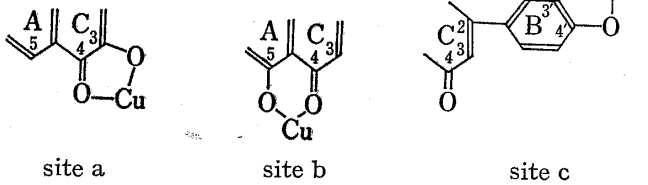
The structural formulae of the flavonoids studied are shown in Chart 1, together with the possible chelating sites with metal ions. Flavonol (3-hydroxy-flavone) contains only one

Flavonoids



flavonol	R_1=OH, R_2,R_3,R_4,R_5,R_6=H
flavocummelin	R_2=OH, R_1,R_5=H, R_3=glucoside
	R_4=OCH_3, R_6=*o*-rhamnoside
quercetin	R_1,R_2,R_4,R_5,R_6=OH, R_3=H
rutin	R_2,R_4,$R_5$$R_6$=OH, R_3=H
	R_1=*o*-rhamnoglucoside

Complex-forming sites



site a site b site c

Chart 1

5) T. Oka, F.J. Simpson, and H.G. Krishnamury, *Can. J. Microbiol.*, **18**, 493 (1972).
6) *a*) W. Heimann and B. Heinrich, *Fette, Seifen, Anstrichm.*, **61**, 1024 (1959); *b*) K.A. Harper, A.D. Morten, and E.J. Rolfe, *J. Food Technol.*, **4**, 255 (1969); *c*) A. Letan, *J. Food Sci.*, **31**, 395 (1966).
7) K. Takamura and M. Ito, *Chem. Pharm. Bull.* (Tokyo), **25**, 3218 (1977).

site of 3-hydroxy-4-keto grouping (denoted as site a). Flavocummelin also contains only one site of 5-hydroxy-4-keto grouping (site b). Quercetin and rutin have more than two chelating sites. The former has three possible sites, site a, site b, and 3′, 4′-dihydroxy grouping (site c), whereas the latter in which the chelating at site a is sterically hindered by *o*-rhamnoglucoside, has two possible sites, site b and c. The complex formation of copper(II) with the flavonoids at these sites was studied.

Absorption Spectra

Absorption spectra of methanol solutions of 4.0×10^{-5} M flavonoids with different concentrations of Cu^{2+} are shown in Fig. 1 and 2. The absorbance of flavonoids decreased and a

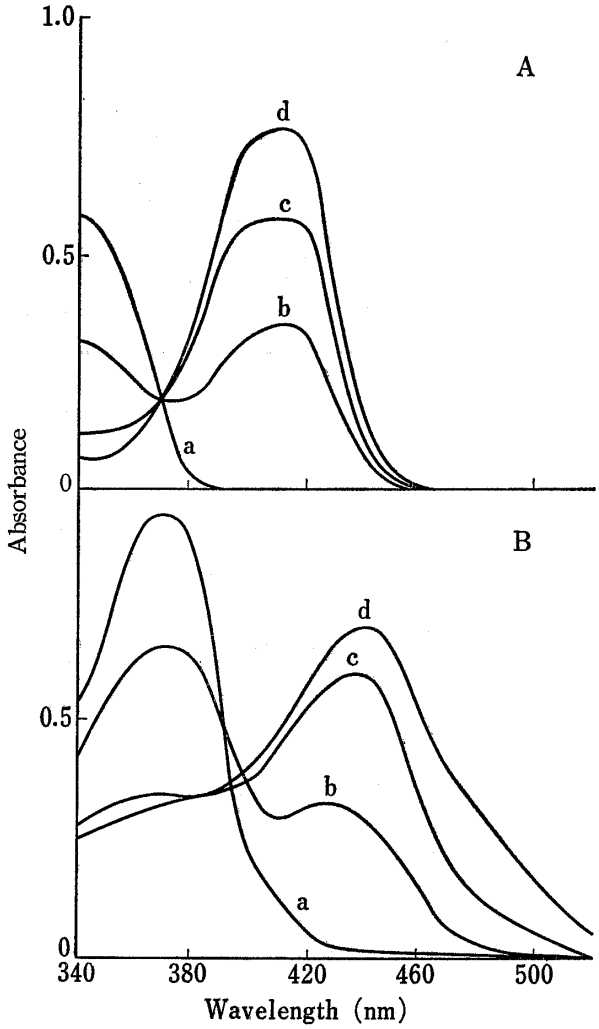


Fig. 1. Absorption Spectra of 4.0×10^{-5} M Flavonol (A) and Quercetin (B) in the Presence of Various Concentrations of Cu^{2+} in Methanol Solution

Concentration of Cu^{2+}: (a) 0, (b) 1.2×10^{-5}, (c) 4.0×10^{-5}, (d) 1.6×10^{-4} M.

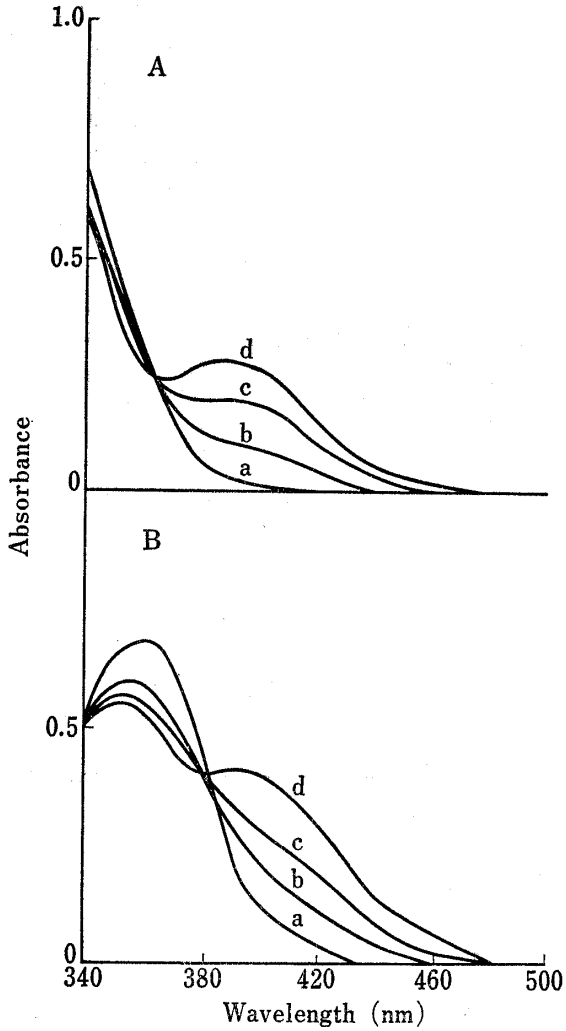


Fig. 2. Absorption Spectra of 4.0×10^{-5} M Flavocummelin (A) and Rutin (B) in the Presence of Various Concentrations of Cu^{2+} in Methanol Solution

Concentration of Cu^{2+}: (a) 0, (b) 1.0×10^{-5}, (c) 4.0×10^{-5}, (d) 1.6×10^{-4} M.

new absorption band which appeared at longer wavelength increased with the amount of Cu^{2+} added. This spectral change may indicate a complex formation.

In the flavonol-copper(II) system (Fig. 1, A), the addition of Cu^{2+} resulted in lowering of the absorbance at 341 nm and the appearance of a new peak at 418 nm, and appearance of an isosbestic point, may indicate the presence of only one complex species in this system.

The absorption spectra obtained for the quercetin-copper(II) system (Fig. 1, B) are complicated compared with those of the flavonol-copper(II) system. The addition of Cu^{2+} decreased the absorbance at 370 nm and at the same time the absorption maximum of a new band shifted to a longer wavelength in the range from 410 to 460 nm, with the increasing the amount of Cu^{2+}, and finally a shoulder appeared at around 485 nm. This fact indicates the existence of more than two complex species in the quercetin-copper(II) system.

The absorption spectra obtained for the flavocummelin-copper(II) and the rutin-copper-(II) systems are shown in Fig. 2. Addition of Cu^{2+}, resulted in the appearance of new peaks in a longer wavelength side than the original peaks of these flavonoids (the former system at 390 nm and the latter at 405 nm). The spectra cross at the isosbestic point, indicating the formation of only one complex in both systems. Even at a higher concentration of Cu^{2+}, the increase in the height of the new peaks is relatively small, and decrease in the original peaks of flavonoids' is also slight. Such a fact suggests that both flavocummelin and rutin do not form the complexes with copper(II) easily compared to flavonol and quercetin.

The ultraviolet (UV) spectrum of the flavone-copper(II) system was almost identical to that of flavone itself, there being no appreciable interaction between flavone and copper(II). Thus, the hydroxyl

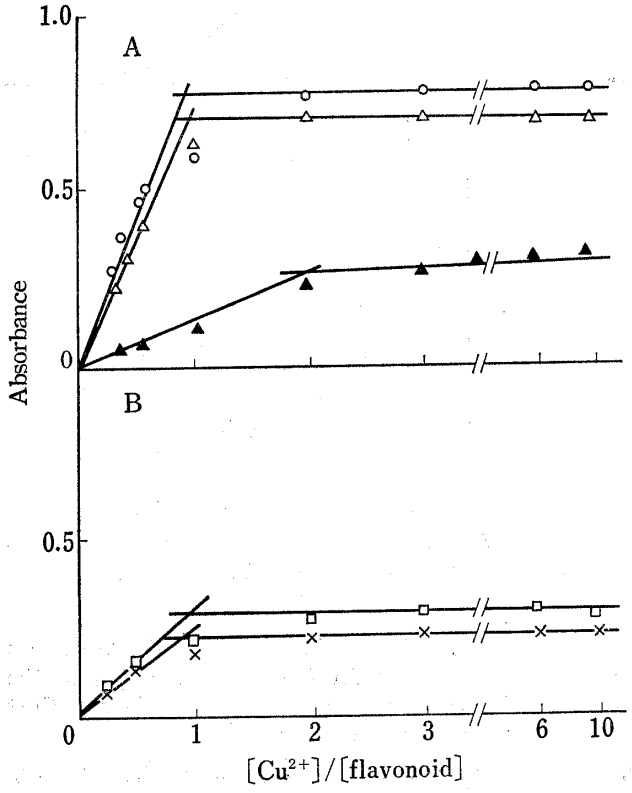


Fig. 3 Molar Ratio Plots applied to the Flavonoid-Copper(II) Systems with the Concentration of Flavonoid Constant at 4.0×10^{-5} M

○, flavonol (418 nm),
△, quercetin (442 nm),
▲, quercetin (490 nm),
□, rutin (420 nm),
×, flavocummelin (390 nm).

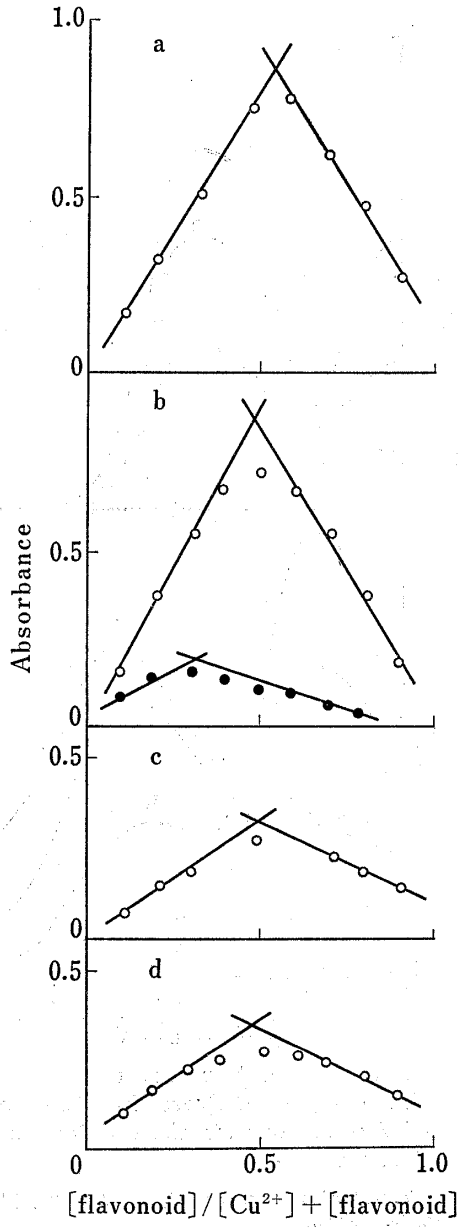


Fig. 4. Continuous Variation Plots applied to the Flavonoid-Copper(II) Systems
$[Cu^{2+}]+[\text{flavonoid}]=10.\times10^{-4}$ M

a : flavonol (418 nm),
b : quercetin (442 nm: ○, 490 nm: ●),
c : flavocummelin (390 nm),
d : rutin (420 nm).

groups in the A, B and C rings of flavonoid molecule are essential for the complex formation with copper(II).

Determination of the Composition of Complexes

In general, polyphenols such as the flavonoids used in this experiment are liable to be oxidized in the presence of copper(II). However, the absorption spectra obtained for the flavonoid-copper(II) systems remained practically unchanged for several hours under the present experimental conditions, indicating that the decomposition of the flavonoids is negligible unless the concentration of Cu^{2+} exceeds 10 times that of the flavonoids.

The molar ratio and continuous variation plots were applied to determine the composition of the complexes formed in four flavonoid-copper(II) systems. The concentration of flavonoid was kept at 4.0×10^{-5} M in the molar ratio method and, the total concentration of flavonoid and Cu^{2+} was maintained at 1.0×10^{-4} M in the continuous variation method. Their results are shown in Fig. 3 and 4.

Both plots for the flavonol-copper(II) system using the absorbance at 418 nm indicate the presence of only 1:1 complex of flavonol and copper(II). No evidence for the formation of other complexes, such as 1:2 complex of flavonol and copper(II), is provided from Fig. 3, in which the absorbance at 418 nm remains constant irrespective of the copper(II) concentration, even when a large excess is present.

As stated above, the absorption band due to the complex formation in the quercetin-copper(II) system has two peaks (Fig. 1B), indicating the existence of at least two kinds of complex species. Then the plot were made at 442 and 490 nm (an overlap of the main peak is negligible at 490 nm), from which the complex formation of both 1:1 and 1:2 of quercetin and copper(II) was proved.

It is obvious from Fig. 3B, 4c and 4d that only 1:1 type complexes are present for flavocummelin-copper(II) and rutin-copper(II) systems.

Since the formation of higher order complexes such as 2:1 of flavonoid to copper(II) is expected with increasing pH of the solution,[3b] the experiments similar to those described above were made for the flavonoid-copper(II) systems in methanol solution containing 0.1 M sodium acetate. In this medium, flavonol gave an absorption maximum at 343 nm. The presence of copper(II) shifted the maximum to 410 nm, suggesting a complex formation. The molar ratio plot at 410 nm, as well as the continuous variation plot at 410 nm, indicated the existence of a complex of a 2:1 type, as was predicted.

No characterized spectrum was obtained in the quercetin-copper(II) system because of the catalytic oxidation of quercetin by copper(II) in an alkaline medium.[8] Difficulties also arose in the case of the flavocummelin-copper(II) and rutin-copper(II) systems, *i.e.*, well-defined spectra could not be observed by interference of some fluorescent precipitates which was produced gradually after the addition of Cu^{2+} to the flavonoid solutions. Further details of such the phenomena will be reported later.

Consideration of the Structure and Stability of Complexes

The spectrophotometric data for the flavonoid-copper(II) complexes described above are listed in Table I. The magnitude of the bathochromic shift due to the complex formation is found to be related to the complexing site of the ligand at which metal ions are actually linked.[3a,b,9] Based on this fact, comparison of the magnitude of the shift (denoted at $\Delta\lambda$ in Table I) was made to consider the site for the complex formation.

8) *a*) A. Nishinaga, T. Tojo, and T. Matsuura, *J. Chem. Soc.* (*C*), **1974**, 896; *b*) N. Delaporte and J.J. Macheix, *Anal. Chim. Acta*, **59**, 279 (1972).

9) *a*) G.M. Saxena and T.R. Seshadry, *Proc. Indian Acad. Sci.*, **46a**, 218 (1957); *b*) V.A. Nazarenko and V.P. Antonovich, *Zh. Anal. Khim.*, **22**, 1812 (1967) [*Chem. Abstr.*, **68**, 6893*u* (1968)]; *c*) U. Bilinska-Kozicka, *Rocz. Chem.*, **41**, 1215 (1967) [*Chem. Abstr.*, 68, 53983*h* (1968)].

Table I. Spectrophotometric Data for Flavonoid-Copper(II) Complexes Obtained in Methanol Solutions

	λ_{max} (nm)		$\Delta\lambda$ (nm)	Composition Flavonoid: Copper(II)
	Flavonoid	Flavonoid-Copper(II)		
Flavonol	341	418	77	1 : 1
	343[a]	410[a]	67[a]	2 : 1[a]
Quercetin	370	442	72	1 : 1
		485	115	1 : 2
Flavocummelin	325	390	65	1 : 1
Rutin	359	405	46	1 : 1

a) Data obtained in methanol solution containing 0.1 M AcONa.

The value of 77 nm as $\Delta\lambda$ in flavonol corresponds to that for the 1:1 complexation at site a, because site a is the only possible site for the complexation in the molecule of flavonol. The 2:1 complex formed in 0.1 M sodium acetate solution might also be possible through coordination at each site a in two flavonol molecules.

Quercetin has three complex-forming sites (sites a, b, and c). From the fact that the $\Delta\lambda$ value (72 nm) corresponding to the 1:1 complex is comparable to that for the flavonol-copper(II) complex, it seems most likely that the initial complex formation takes place at site a. The subsequent coordination step is reasonably assumed to occur at site c, because site b is no longer capable of chelation when site a is occupied.

As the value of $\Delta\lambda$ for 1:1 complex between flavocummelin and copper(II), 65 nm is obtained. This is obviously the case for the chelation at site b.

In the case of rutin-copper(II) complex, one cannot readily decide on either of the sites (b and c) that participates in the complex formation. However, a complex formed at site b is expected to be more stable than that formed at site c from the findings by Porter, *et al.*[3b] To consider the reason why the difference in $\Delta\lambda$ is observed between flavocummelin- and rutin-copper(II) complexes, even if they are formed through the chelation at site b, pendulin and penduletin seem to be useful compounds; both compounds are flavonoid derivatives having the same substituents except that the former has O-glucosyl while the latter has a hydroxyl group at 4′-position, and the values of $\Delta\lambda$ for their aluminum complexes (1:1) were reported as 75 and 56 nm, respectively.[10] In analogy with this fact, the lower value of $\Delta\lambda$ for rutin-copper(II) complex compared to flavocummelin-copper(II) complex can be interpreted as a result of the substituent effect at 4′-position.

It is difficult to determine the stability constants of flavonoid-copper(II) complexes exactly, because data on the dissociation constants of flavonoids were not obtained in methanol solution. The relative stability of the complexes was compared. Concentrations of free Cu^{2+} equilibrated in the solutions containing 1.0×10^{-3} M of Cu^{2+} and 5.0×10^{-4} M of each flavonoid were obtained by polarography. The 50% methanol containing 0.1 M sodium perchlorate was used as a base electrolyte solution. In such a solution, copper(II) gave a well-defined reduction wave ($E_{1/2}=+0.02$ V *vs.* SCE) due to the reaction

$$Cu^{2+} + 2e \longrightarrow Cu$$

and the diffusion current (i_d) obtained at -0.8 V was found to be proportional to the Cu^{2+} concentration. This potential was chosen by considering the following facts: Some of the flavonoids used give cathodic waves at a more negative potentials than -1.0 V, while they

10) T.J. Mabry, K.R. Markham, and M.B. Thomas, "The Systematic Identification of Flavonoids," Springer-Verlag, New York, 1970, pp. 145—146.

are an adsorbed on the electrode surface at more positive potential than −0.7 V and, as a result of which the polarogram of copper(II) is deformed. The results are as follows:

Flavonoid	None	Flavocummelin	Rutin	Flavonol	Quercetin
$i_d(\mu A)$	3.95	3.90	3.75	3.40	3.30

From the results one may compare the effect of flavocummelin, rutin, flavonol, and quercetin on the free Cu^{2+} concentration, which becomes more marked in that order. This order readily corresponds to the relative stability of the flavonoid-copper(II) complexes, and it agrees well with that obtained for the flavonoid-aluminum(III) complexes.[3a,b,9]

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