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Effects of Metal Ions and Flavonoids on the Oxidation of Ascorbic Acid[1]

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Effects of metal ions and flavonoids on the oxidation of ascorbic acid (I) have been investigated polarographically in the acetate buffer solution of pH 5.4. Since I gives a diffusion-controlled anodic wave due to a two-electron oxidation to form dehydroascorbic acid, the rate of the oxidation of I can be followed by measuring the change in the limiting current of I with time.

The rate of the oxidation of I was enhanced by the presence of some metal ions. Among them, the effect of copper(II) was especially pronounced. Such the fact can be explained on the basis of the formation of a coordinative linkage between metal and ascorbate ions and the reducibility of metal ions.

Contrary to this, flavonoids exhibited an inhibition effect on the copper(II)-catalyzed oxidation of I. The effect became more marked in the order of 3-hydroxyflavone<rutin< quercetin, but no inhibition was observed for flavone. From the experimental result that the complex forming tendency of copper(II) with flavonoids increase in the order of flavone <3-hydroxyflavone<rutin<quercetin, a decrease in the concentration of free copper(II) as a result of the complex formation between flavonoids and copper(II), was found to be primarily responsible for the inhibition effect of flavonoids.

Keywords——ascorbic acid; quercetin; rutin; flavone; 3-hydroxyflavone; polarography; copper(II)-catalyzed oxidation; flavonoids complexes; copper(II) complexes; inhibition effect of flavonoids

The copper (II)-catalyzed oxidation of ascorbic acid (I) by oxygen has attracted our attention in recent years. I is well-known as a reducing agent in biological redox systems and sometimes copper-containing oxidase enzymes (*e.g.*, ceruloplasmin, ascorbic oxidase, *etc.*) play an important part in the oxidation of I. In order to give a clue to the elucidation of such the effects of copper (II) in biochemical reactions, it seems worthwhile to study the copper (II)-catalyzed oxidation of I *in vitro* comparing with the effects of other metal ions. However, only a few papers[3] have dealt with this problem. Onishi, *et al.*[3c] compared the catalytic effects among fifteen metals involving copper (II) by following the decreasing amount of I during the oxidation by spectrophotometric measurement in the presence of iron (III)-1,10-phenanthroline, but in their procedure special care seems not to be taken to prevent the reaction mixtures from contamination by oxygen.

As the reason why I remains stable in living plants even in the presence of copper-containing enzymes, it has been suggested that flavonoids in plants would inhibit the enzyme action for the ascorbic acid oxidation.[4] Some processes, such as chelation with copper (II) or capture of ascorbic acid radicals, have so far been discussed to be responsible for the inhibition effect of flavonoids, but the mechanism of protection for I by them is still uncertain.

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[2] Location: *Horinouchi 1432-1, Hachioji, Tokyo, 192-03, Japan.*

[3] *a*) M. Dezelic and J. Grujic-Vasic, *Bull. Soc. Chimistes Repub. Populaire Bosnie et Herzegovine* 3, 23 (1954); *b*) B.K. Gupta, *Pharmastudent*, 17, 28 (1973); *c*) I. Onishi and T. Hara, *Bull. Chem. Soc. Jpn.* 37(9), 1314 (1964).

[4] *a*) W. Heimann and B. Heinrich, *Fette, Seifen, Anstrichmittel*, 61, 1024 (1959); *b*) G. Stenlid and G. Samorodova-Bianci, *Lantbrhogsk. Annlr.*, 35, 837 (1969); *c*) D. Nomura and Y. Oda, *Hakko Kogaku Zasshi*, 6, 309 (1962); *d*) K.A. Harper, A.D. Morton, and E.J. Rolfe, *J. Food Technol.*, 4, 255 (1969).

The present paper is concerned with the comparison of the catalytic effects of some metal ions on the ascorbic acid oxidation and the discussion of the factors which may contribute to the inhibition of flavonoids for copper (II)-catalyzed oxidation of I.

Experimental

Reagents and Preparation of Solutions——Acetate buffer solution of pH 5.4 was used as the base electrolyte in the present work. A known amount of reagent grade *l*-ascorbic acid (Wako Pure Chemical Industries, Ltd.) was dissolved in 0.1 M $HClO_4$ solution to prepare a stock solution because of its stability in strongly acidic medium. The concentration of I in the test solution was adjusted by pipetting the stock solution into the base electrolyte solution.

Flavone, 3-hydroxyflavone, quercetin and rutin were obtained commercially. Because of the extremely low solubility of those flavonoids in water, the use of mixed solvent of isopropyl alcohol and water (1 : 1) is convenient for the study of the effects of the flavonoids on the oxidation of I, so that the solution of the flavonoids was prepared by dissolving them in absolute isopropyl alcohol and then adding the required amount of the base electrolyte solution.

All other chemicals were reagent grade and used without further purification.

Apparatus——Direct current (D.C.) polarograms were recorded on a Yanagimoto pen-recording polarograph Model P8 in the usual manner. All potentials in this paper are relative to a saturated calomel electrode (SCE). Ultraviolet (UV) and visible absorption spectra were recorded on a Hitachi spectrophotometer Model 139 using 10 mm-quartz cell.

Procedure——The initial concentrations of I in the test solutions were determined polarographically by measuring the anodic current at +0.2 V. Before each run to record a polarogram, pure nitrogen gas was bubbled through the test solution to remove the dissolved oxygen. Nitrogen gas flowed continuously above the test solution to prevent recontamination of the solution by air during the measurement.

The rate of the oxidation of I in the presence of metal ions with or without flavonoids was followed by measuring the change in the anodic current of I with time. The oxidation was initiated by adding 0.2 ml of the stock solution of I into the air-saturated reaction mixture with the aid of a pipet within 5 sec. The anodic currents were recorded at +0.2 V at appropriate time intervals to obtain the current-time curves. Air was supplied by bubbling into the solution at constant flow rate during the reaction, except when the anodic current was recorded.

All the measurements were carried out at 25°.

Results and Discussion

Changes in the Polarogram of Ascorbic Acid due to Oxidation

D. C. polarogram of 1.0×10^{-3} M I obtained in the base electrolyte solution is shown in Fig. 1 (curve a). I gives a diffusion-controlled anodic wave in the positive potential region due to a two-electron oxidation form dehydroascorbic acid in the lower pH range than 8.[5]

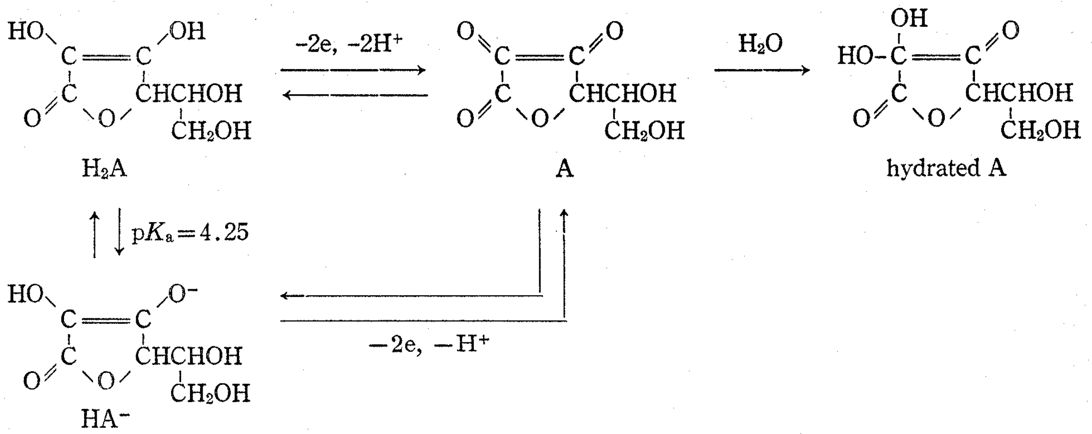

Chart 1

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[5] *a*) S. Ono, M. Takagi, and T. Wase, *Bull. Chem. Soc. Jpn.* **31**, 356 (1958); *b*) D.M.H. Kern, *J. Chem. Soc.*, **1954**, 1011; *c*) S.P. Perone and W.J. Kretlow, *Anal. Chem.*, **38**, 1763 (1966); *d*) I.M. Kolthoff and J.J. Lingane, "Polarography," Vol. II, Interscience Publishers, Inc., New York, 1952, pp. 727—729.

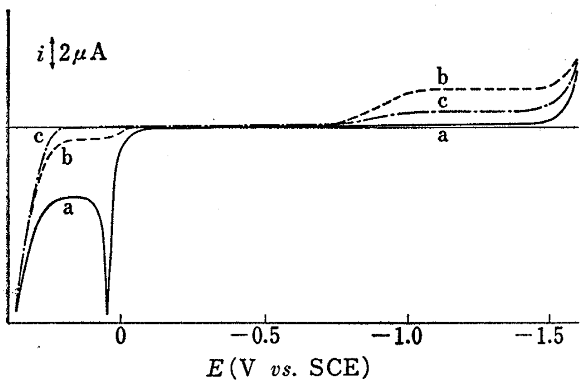


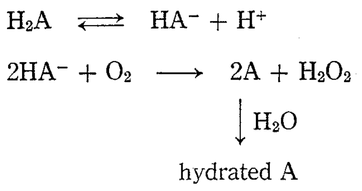
Fig. 1. Polarograms of 1.0×10^{-3} M I Obtained in Aqueous Acetate Buffer of pH 5.4

a, before oxidation; b, after oxidation for 6 hr; c, after oxidation for 20 min in the presence of 1.0×10^{-5} M copper (II).

The electrode processes are represented as given in Chart 1, where H_2A and HA^- denote the undissociated and the monoionic species of I respectively, and A denotes dehydroascorbic acid. K_a is the first dissociation constant of H_2A. The oxidation product A readily changes to hydrated A through an irreversible hydration.[5c]

The limiting current of the anodic wave is independent of pH, while the half-wave potential ($E_{1/2}$) shifts to the negative potential side with increasing pH of the solution. A plot of $E_{1/2}$ vs. pH gives a straight line, and the slope ($\Delta E/\Delta$pH) is *ca.* 0.06 and 0.03 V/pH at the pH ranges lower than 4 and higher than 5, respectively. This fact apparently indicates the electro-oxidation mechanism given in Chart 1 correct, so that the limiting current corresponds to the total concentration of H_2A and HA^-.

The oxidation of I results in a marked decrease in the limiting current and an appearance of a new cathodic wave ($E_{1/2}=-0.95$ V). The result was shown as curve b in Fig. 1, which was recorded after I was oxidized by air bubbling for 6 hr in the base electrolyte solution. The new wave was found to be due to the reduction of hydrogen peroxide by the comparison of curve b with the polarogram of hydrogen peroxide obtained in the same base solution. Contrary to this, no change in the polarogram was found in strongly acidic media even through the prolonged oxidation. The decrease in the limiting current due to the oxidation of I becomes more rapidly as the pH of the solution rises. Those facts suggest that the oxidation of I proceeds through the following reaction sequence.



$$\begin{array}{l} H_2A \rightleftharpoons HA^- + H^+ \\ 2HA^- + O_2 \longrightarrow 2A + H_2O_2 \\ \qquad\qquad\qquad \downarrow H_2O \\ \qquad\qquad\quad \text{hydrated A} \end{array}$$

Similar results were also obtained in the mixed solvent of 50% isopropyl alcohol–H_2O.

Comparison of Catalytic Effects of Various Metal Ions on the Oxidation of I

The rate of the oxidation of I is enhanced by the presence of small amounts of some metal ions. The best example of such a case is shown in Fig. 1 as curve c, in which the disappearance of the anodic wave of I is seen by air bubbling only for 20 min in the presence of 2.0×10^{-5} M copper (II). A comparison of the effects of metal ions was made by the measurement of the change in the concentration of I with time. The results are given in Fig. 2. Among the metal ions, copper (II) exhibited an extremely pronounced effect so that the difficulty arose in the trace of the decrease in the concentration of I by the addition of more than 1.0×10^{-3} M copper (II) by the present technique.

It is known that the oxidation of I obeys first-order kinetics with respect to the concentration of I in a particular pH range under saturation of oxygen.[2c,6] A plot of log C_0/C vs. time (C_0 and C are the initial and the residual concentrations of I, respectively) using the data in Fig. 2 was found to be linear and the first-order rate constants (k) were calculated from 2.303 times the slope of the plots. The results are given in Table I indicating that the catalytic activity of metal ions decreases in the following order:

$$Cu^{2+} \gg Zn^{2+}, Co^{2+} > Pb^{2+}, Cd^{2+} > Ni^{2+}$$

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[6] M.M. Taqui Khan and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 4176 (1967).

Table I. The Rate Constants of the Oxidation of I in the Presence of Various Metal Ions obtained from the Data in Fig. 2

Metal ion	Rate constant $k \times 10^3$, min^{-1}
—	2.76
Cu^{2+}	106
Zn^{2+}	7.48
Co^{2+}	7.29
Cd^{2+}	5.75
Pb^{2+}	5.57
Ni^{2+}	4.61

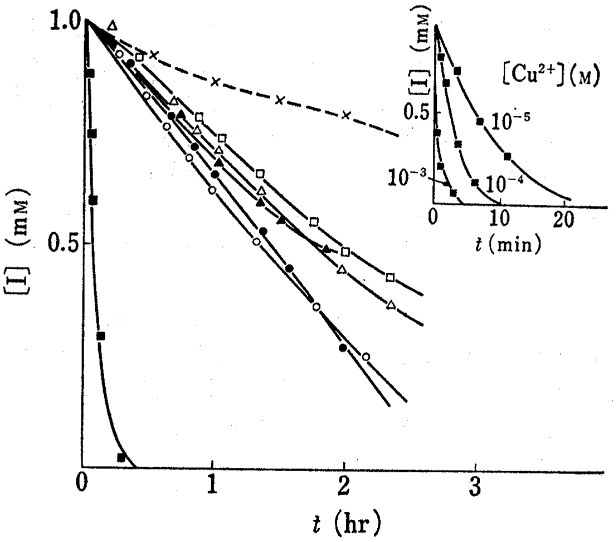

Fig. 2. Time Dependence of Concentration of I during the Oxidation in the Presence of Various Metal Ions

Initial concentration of I: 1.0×10^{-3} M. Concentration of metal ion added: 1.0×10^{-5} M for Cu^{2+} and 1.0×10^{-2} M for other metal ions.
--×--, without metal ion;
—□—, Ni^{2+}; —△—, Cd^{2+}; —▲—, Pb^{2+};
—●—, Co^{2+}; —○—, Zn^{2+}; —■—, Cu^{2+}.

This fact leads to the question of what factors may contribute to the marked tendency of copper (II) to catalyze the oxidation of I. According to Harper, *et al.*,[4d] the following sequence can be given as a possible mechanism for the copper (II)-catalyzed oxidation of I.

$$H_2A \xrightleftharpoons{K_a} HA^- + H^+ \tag{1}$$

$$HA^- + Cu^{2+} \xrightarrow{K_f} [HA\,Cu]^+ \tag{2}$$

$$[HA\,Cu]^+ \xrightarrow{k} [Cu\,HA\cdot]^+ \tag{3}$$

$$[Cu\,HA\cdot]^+ \xrightarrow{\text{fast}} HA\cdot + Cu^+ \tag{4}$$

$$HA\cdot + O_2 \xrightarrow{\text{fast}} HAO_2\cdot \tag{5}$$

$$HAO_2\cdot + H^+ \xrightarrow{\text{fast}} A + H_2O_2 \tag{6}$$

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + HO^- + HO\cdot \tag{7}$$

where K_a and K_f are the first dissociation constant of H_2A and the formation constant of the intermediate complex between copper (II) and ascorbate anion, respectively, and k is the rate constant for the electron transfer from ascorbate anion to copper (II).

Since the complex formed by the reaction (2) makes it easy to produce the free radical of I which readily reacts with oxygen molecule, the rate of the copper (II)-catalyzed oxidation is much faster than that of conventional oxidation. According to the above reaction pathways, copper (I) produced through the dissociation of the intermediate complex by an intramolecular electron transfer causes hydrogen peroxide to decompose, resulting in the reoxidation of itself to copper (II). Actually, in the present experiment, the decrease in the height of the reduction wave of hydrogen peroxide was evident in the presence of copper (II) (see Fig. 1, curve c), indicating the turnover catalyst in the reaction sequence.

Provided that the above reaction sequence can be extended to the catalytic oxidation of I by other metal ions, the following factors must be considered when a comparison of the catalytic activity of metal ions is made:

1) The ease to form a coordinative linkage between metal and ascorbate ions,
2) Tendency of a valence state lowering of metal ion.

Since I is successively decomposed in the presence of metal ions, the quantitative treatment of the equilibrium of reaction (2) seems rather difficult. Then we are forced to infer the tendency to form the intermediate complex from the analogy with a general rule for the complex formation such as Irving's rule, *i.e.*, $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$. Mellor and Maley also reported the order of $Pd^{2+} > Cu^{2+} > Ni^{2+} > Pb^{2+} > Co^{2+} \cdot Zn^{2+} > Cd^{2+} > Mg^{2+}$ for the stability of those metal chelates with salicylaldehyde. Anyhow, it appears quite likely that among metal ions copper (II) has an appreciably high affinity for I in the present reaction system.

As a matter which takes part in the valence state lowering of metal ions, the reducibility of metal ions was compared in terms of their half-wave potentials in the same base electrolyte solution. As tabulated in Table II, the reducibility of metal ions decreases in the order of $Cu^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+}$.

TABLE II. The Half-Wave Potentials of Metal Ions Obtained in Aqueous Acetate Buffer Solution of pH 5.4

Metal ion	Cu^{2+}	Pb^{2+}	Cd^{2+}	Zn^{2+}	Ni^{2+}	Co^{2+}
$E_{1/2}$ V *vs.* SCE	−0.025	−0.43	−0.61	−1.01	−1.02	−1.21

Both the factors stated above seem to be responsible to some extent for the extremely high activity of copper (II) as a catalyst for the oxidation of I. Catalytic effects of some metal ions have discussed by Onishi, *et al.* only in relation to the stability of complexes involving those metals and I,[3c)] however, it seems still necessary to consider the participation of the reducibility of metal ions.

Effects of Flavonoids on the Copper (II)-Catalyzed Oxidation of I

In order to realize the effects of flavonoids on the copper (II)-catalyzed oxidation of I, the concentration change of I with time in the reaction mixtures containing some flavonoids was followed polarographically. The results are shown in Fig. 3 and the rate constants are given in Table III. On comparing those data, the inhibition effect is evident for quercetin, rutin and 3-hydroxyflavone, of which the effect becomes more marked in the order of 3-hydroxyflavone<rutin<quercetin. On the other hand, flavone shows no inhibition effect but slightly enhances the reaction rate. In the reaction mixture containing flavone, the formation of phenol groups was found by the test using ferric chloride reagent. This fact suggests that hydroxylation to the flavone ring with the consumption of the proton liberated from I occurs subsequently to the oxidation of I, resulting in making the oxidation more rapidly.[7)] Other flavonoids having phenol groups in their molecules are less possible for further hydroxylation.

Then the experiments stated below were done to find what is the dominating factor for the inhibition effects of 3-hydroxyflavone, rutin and quercetin. Fig. 4 shows the polarograms of 1.0×10^{-3} M copper (II) in the presence of 5.0×10^{-4} M flavonoids. Flavone gives no effect on the reduction wave of copper (II), but addition of 3-hydroxyflavone, rutin and quercetin to the solution gives rise to a decrease in the wave height, suggesting a decrease in the concentration of free copper (II) as a result of the interaction between those flavonoids and copper (II). The decreasing effect of flavonoids on the limiting current of copper (II) becomes more marked in the order of 3-hydroxyflavone≤rutin<quercetin. The order is the same as that of the inhibition effect of them on the copper (II)-catalyzed oxidation of I.

On addition of those flavonoids to the copper (II) solution, characteristic colors were observed (3-hydroxyflavone: yellowish green, rutin: yellowish brown, quercetin: brown) and

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[7)] S. Udenfriend, C.T. Clark, J. Avelrod, and B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).

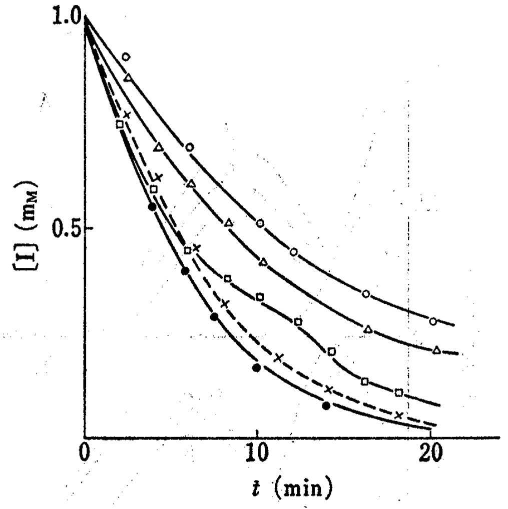

Fig. 3. Time Dependence of Concentration of I during the Copper(II)-Catalyzed Oxidation in the Presence of 1.0×10^{-3} M Flavonoids

Initial concentration of I: 1.0×10^{-3} M.
Concentration of Cu^{2+} added: 1.0×10^{-5} M.
—×—, without of flavonoid;
—○—, quercetin;
—△—, rutin;
—□—, 3-hydroxyflavone;
—●—, flavone.

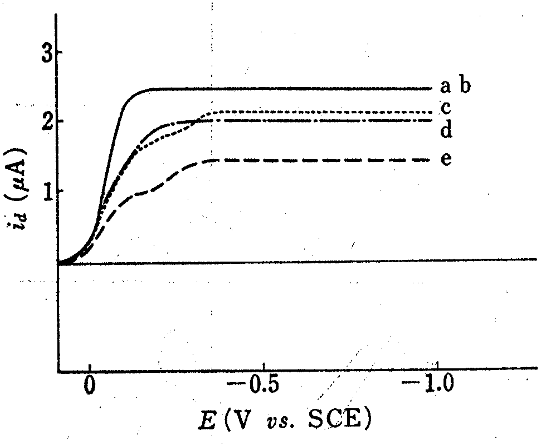

Fig. 4. Effect of Flavonoids on Polarograms of 1.0×10^{-3} M Copper(II) Obtained in 50% Isopropyl Alcohol Solution Containing Acetate Buffer of pH 5.4

Flavonoids: a, without flavonoid; b, flavone; c, 3-hydroxyflavone; d, rutin; e, quercetin.
Concentration of flavonoid added: 5.0×10^{-4} M.

Table III. The Rate Constants for the Copper(II)-Catalyzed Oxidation of I in the Presence of Flavonoids Obtained from the Data in Fig. 4

Cu^{2+}+flavonoid	Rate constant $k\times10^{2}$, min^{-1}
—	13.8
Flavone	14.7
3-Hydroxyflavone	11.3
Quercetin	8.39
Rutin	6.45

sometimes reaction products were precipitated from the solution, but flavone gave neither color change nor precipitate. Rutin and quercetin are well-known examples of polyhydroxy phenols using as a colorimetric reagent for the determination of some metal ions based on the complex formation reaction. From those facts the complex formation of copper (II) with flavonoids is expected to occur except flavone.[8]

Further confirmation of the complex formation was attained by spectrophotometric measurements in the present experiment. UV and visible spectra of 4.0×10^{-5} M flavonoids in the presence of 4.0×10^{-5} M copper (II) are shown in Fig. 5. UV spectrum of copper (II)-flavone mixture is essentially identical to that of flavone itself, whereas UV spectra obtained for copper (II)-3-hydroxyflavone, -rutin and -quercetin mixtures differ from those for each flavonoid. In the visible region, the absorbance at the wavelength of absorption maximum is lowered by the presence of copper (II), and a new peak appears at the longer wavelength region showing a bathochromic shift.

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[8] L. Hörhamer and R. Hänsel, *Arch. Pharm.*, **285**, 438 (1952); K.A. Harper, *J. Food Technol.*, **4**, 405 (1959); I.E. Makasheva and M.T. Golovkina, *Zh. Obshch. Khim.*, **43**, 1640 (1973).

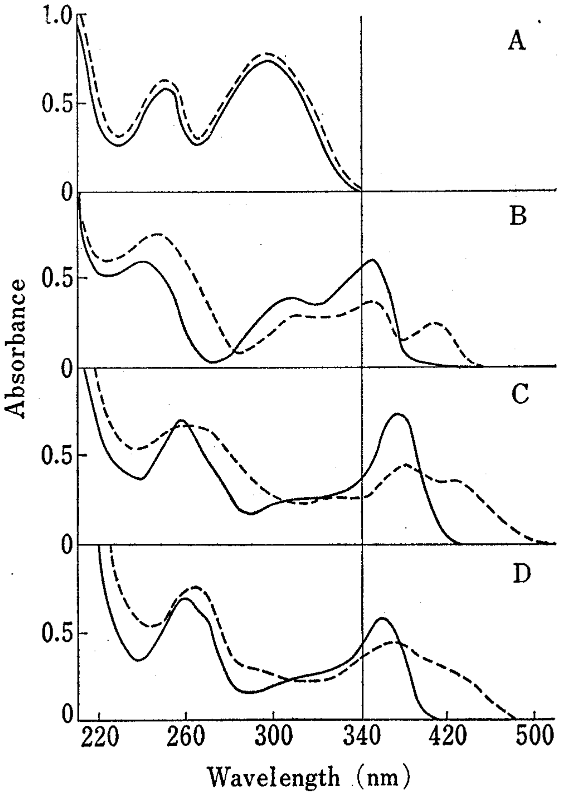


Fig. 5. UV and Visible Absorption Spectra of 4.0×10^{-5} M Flavonoids in the Absence (——) and in the Presence of 4.0×10^{-5} M Copper (II) (---) in 50% Isopropyl Alcohol Solution Containing Acetate Buffer of pH 5.4

A: flavone, B: 3-hydroxyflavone, C: quercetin, D: rutin.

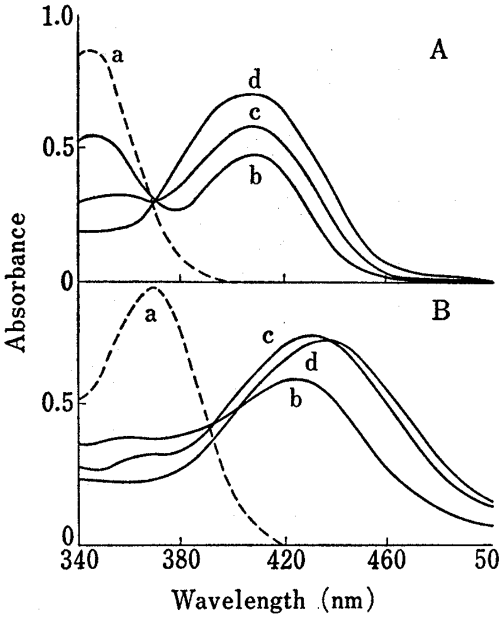
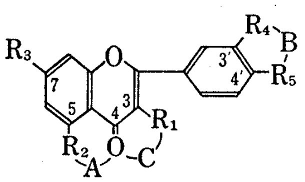


Fig. 6. Visible Absorption Spectra of 5.0×10^{-5} M 3-hydroxyflavone (A) and 5.0×0^{-5} M Quercetin (B) in the Presence of Various Concentrations of Copper (II)

Concentration of Cu^{2+} added: a, 0; b, 5.0×10^{-4} M; c, 1.0×10^{-3} M; d, 2.0×10^{-3} M.

From Fig. 5, the complex formation of copper (II) with 3-hydroxyflavone, rutin and quercetin is evident under the experimental conditions. In the case of quercetin, the presence of a large excess of copper (II) leads to an increase in the absorbance at 425 nm and a shift of the absorption maximum to the longer wavelength (Fig. 6, B). Similar trends were also observed for rutin. If only a 1: 1 complex exists even with an excess of copper (II), the wavelength of absorption maximum must remain unchanged. Copper (II)-3-hydroxyflavone system is an example of such a case (Fig. 6, A). In copper (II)-quercetin and -rutin mixtures, some other complicated species may be involved besides 1: 1 complex.



flavone: $R_1=R_2=R_3=R_4=R_5=H$
3-hydroxyflavone: $R_1=OH$, $R_2=R_3=R_4=R_5=H$
quercetin: $R_1=R_2=R_3=R_4=R_5=OH$
rutin: $R_2=R_3=R_4=R_5=OH$
R_1=o-rutinoside

Chart 2

To explain all the results stated above, one may relate the tendency of the complex formation of the flavonoids used in this experiment to their structure. As is obvious in Chart 2, flavone has no hydroxyl group, whereas in other flavonoids some hydroxyl groups are favorably located for the chelate ring formation. Among them, 3-hydroxyflavone has only one chelating site, while in both the cases of quercetin and rutin there lie some more sites to be capable of the chelate formation. Actually, the formation of 2: 1 complex was found for Al(III)-quercetin and Al(III)-rutin by Porter, *et al.*, and the chelation was proved at the sites C and B for quercetin and A and B for rutin.[9] The chelation at the site A is found to be somewhat more difficult than at the site C because 5-hydroxy and 4-keto groups do not lie in the same plane.

Such a fact readily leads to the consideration that the complex forming tendency of copper (II) with the flavonoids will increase in the order of flavone$<$3-hydroxyflavone$<$rutin $<$quercetin. The order is essentially the same as that of the decrease in the polarographic wave height due to the reduction of free copper (II) equilibrated in the copper (II)-flavonoids systems. Then it can reasonably concluded that the predominant factor in the inhibition effect of flavonoids on the copper (II)-catalyzed oxidation of I is their complex forming character which results in the decrease in the free copper (II) concentration.

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9) L.J. Porter and K.R. Markham, *J. Chem. Soc.*, **1970**, 344; *idem, ibid.*, **1970**, 1310.