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CHELATING AND FREE RADICAL SCAVENGING MECHANISMS OF INHIBITORY ACTION OF RUTIN AND QUERCETIN IN LIPID PEROXIDATION

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Abstract—Inhibitory effects of flavonoids rutin and quercetin on ferrous ion-dependent lipid peroxidation of lecithin liposomes and NADPH- and CCl_4-dependent lipid peroxidation in rat liver microsomes were studied to elucidate the chelating and free radical scavenging activities of these compounds. The intercation of rutin with superoxide ion and ferrous ions and the reaction of quercetin with lipid peroxy radicals were also studied. Both flavonoids were significantly more effective inhibitors of iron ion-dependent lipid peroxidation systems due to chelating iron ions with the formation of inert iron complexes unable to initiate lipid peroxidation. At the same time, iron complexes of flavonoids retained their free radical scavenging activities. The chelating mechanism of inhibition was more important for rutin than for quercetin. The mutual effect of rutin and ascorbic acid on non-enzymatic lipid peroxidation was also studied. It was concluded that rutin and quercetin are able to suppress free radical processes at three stages: the formation of superoxide ion, the generation of hydroxyl (or crypto-hydroxyl) radicals in the Fenton reaction and the formation of lipid peroxy radicals.

Flavonoids are widely distributed vegetable pigments possessing P vitamin activity. They are expected to be promising potential drugs for combating "free radical" pathologies such as ischaemia, anaemia, arthritis, asbestosis, and so on because, being generally non-toxic natural compounds, they possess both antiradical (anti-oxidative) and chelating properties. As phenolic compounds, flavonoids can scavenge free hydroxyl and peroxy radicals (Reactions 1 and 2); on the other hand, as metal-chelating agents, they may extract iron ions and, by this, depress the superoxide-driven Fenton reaction (Steps 3 and 4) which is currently considered as the most important route to active oxygen radicals.

$$ROO\cdot + Fl\text{–}OH \rightarrow ROOH + Fl\text{–}O\cdot \quad (1)$$

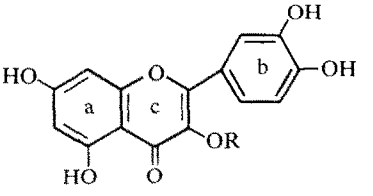
$$HO\cdot + Fl\text{–}OH \rightarrow H_2O + Fl\text{–}O\cdot \quad (2)$$

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (3)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^- \quad (4)$$

Flavonoids may also react with superoxide ion via a one-electron transfer mechanism or a "concerted" mechanism [1] (with $O_2^{\cdot-}$ abstracting simultaneously a hydrogen atom and a proton from the hydroxy groups).

Antioxidative effects of flavonoids on the CCl_4- and NADPH-dependent lipid peroxidation in rat liver microsomes and mitochondria [2–4], the lysis of human erythrocytes [5], the autoxidation of linoleic acid and methyl linoleate [6], the lipid peroxidation in human erythrocytes [7] and illuminated spinach chloroplasts [8] have already been studied. Recently, antioxidative properties of flavonoids was studied on the basis of their inhibiting effects on CCl_4-dependent chemiluminescence in rat liver [9]. There are also some data concerning chelating properties of flavonoids [10]. On the other hand, some flavonoids may apparently manifest pro-oxidant action [7, 11]. In this work we were chiefly concerned with elucidating the mechanism of antiradical action of two practically important flavonoids, rutin and quercetin using various non-enzymatic and enzymatic lipid peroxidation systems. The results obtained are discussed in connection with the possibility of using these drugs for treating "free radical" pathologies.



Quercetin R = H
Rutin R = D-Glucose-L-Rhamnose

Fig. 1. The molecular structures of rutin and quercetin.

MATERIALS AND METHODS

Chemicals. Rutin, ascorbic acid and egg lecithin were of U.S.S.R. production. Quercetin and soybean lipoxygenase were from the Sigma Chemical Co. (St. Louis, MO), linoleic acid was from P.-L. Biochemical Inc. (Milwaukee, WI) cytochrome *c* and NADPH were from Reanal (Hungary).

Preparation of lecithin liposomes. Lecithin lipo-

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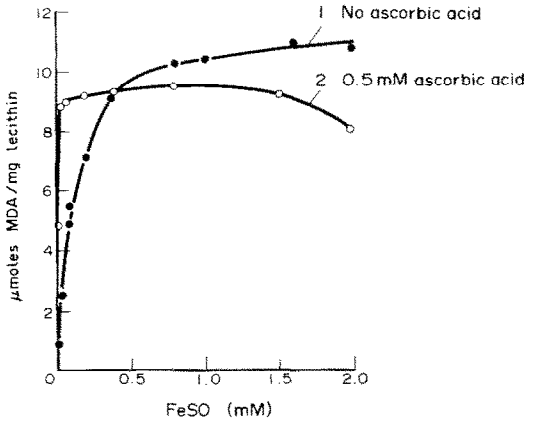

Fig. 2. Ferrous ion-dependent lipid peroxidation of lecithin liposomes as a function of ferrous ion concentration. (1) Without ascorbic acid. (2) 0.5 mM ascorbic acid was added. Liposomes were incubated with $FeSO_4$ at 25° for 60 min in phosphate buffer (pH 7.4).

somes were prepared by the method of Fukuzawa *et al.* [12]. Egg lecithin (25 mg) was dissolved in chloroform (0.2 ml), evaporated to dryness under nitrogen and dispersed in 0.05 M phosphate buffer (5.8 ml), pH 7.4. The suspension was subjected to ultrasonic treatment in a MSE Soniprep 150 and was stored at 0°.

Preparation of rat liver microsomes. Young adult Wistar rats (130–180 g) were fasted over 24 hr. The livers were removed under ether anesthesia and washed with 0.9% NaCl. When the livers were clear, (1:3) homogenates were prepared and centrifugated at 12000 *g* for 20 min. The supernatant obtained was centrifuged at 105000 *g* for 60 min. The microsomes were stored at −20°.

* Abbreviations used: RLM, rat liver microsomes: MDA, malonaldehyde; DMSO, dimethylsulfoxide; DMF, dimethylformamide; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene.

Non-enzymatic lipid peroxidation of lecithin liposomes. Liposomes (0.03 mg lecithin/ml) were incubated at 25° for 60 min with $FeSO_4$ (5 μM–2 mM), ascorbic acid (0–2 mM) and rutin (0–1 mM) in phosphate buffer (0.05 M), pH 7.4 (3 ml final solution). Lipid peroxidation was terminated by adding 0.5 ml of 0.5 M trichloroacetic acid and 0.2 ml 0.1 M EDTA. After this, 1 ml 0.8% (w/v) solution of 2-thiobarbituric acid was added, and the solution was heated at 100° for 15 min. After centrifugation of precipitated proteins, the malonaldehyde content was determined by measuring the absorbance of the adduct at 532 nm ($\varepsilon = 1.56 \times 10^5$/M/cm) [13].

Carbon tetrachloride-dependent lipid peroxidation of rat liver microsomes. Rat liver microsomes (RLM)* (1.2–1.3 mg protein/ml) were incubated at 37° for 7.5 min with CCl_4 (3.4 mM), NADPH (1 mM), NaCl (20 mM), and EDTA (0.6 mM) in phosphate buffer (0.005 M), pH 7.4 (5 ml of final solution). Carbon tetrachloride was added in ethanol solution [final concentration of ethanol 2% (w/v)]. Subsequent treatment of reaction mixture and determination of malonaldehyde (MDA) was carried out as described above.

NADPH-dependent lipid peroxidation of rat liver microsomes. RLM (1.2–1.3 mg protein/ml) were incubated at 37° for 7.5 min with NADPH (0.3 mM), NaCl (20 mM), $FeSO_4$ (10 μM) in phosphate buffer (0.05 M), pH 7.4 (5 ml of final solution). Subsequent treatment and determination of MDA are described above.

In all experiments with RLM rutin and quercetin were added in DMSO solution [final concentration of DMSO 2% (w/v)].

Demethylation of aminopyrine by rat liver microsomes. RLM (4–5 mg protein/ml) were incubated at 37° for 15 min with aminopyrine (0.04–0.4 mM), NADPH (2 mM) and $MgCl_2$ (16 mM) in Tris–HCl buffer (0.04 M), pH 7.6 (1 ml of final solution). The formaldehyde formed was measured using the Nash reaction [14].

Reaction of superoxide ion with rutin. The super-

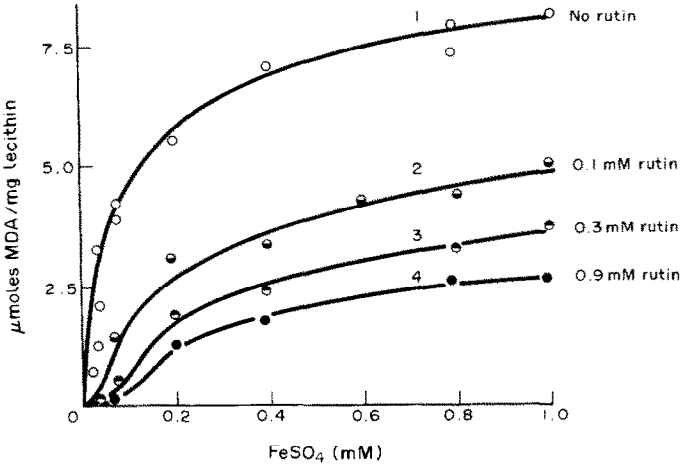

Fig. 3. Effect of rutin on ferrous ion-dependent lipid peroxidation of lecithin liposomes. (1) Without rutin. (2) 0.1 mM rutin. (3) 0.3 mM rutin. (4) 0.9 mM rutin.

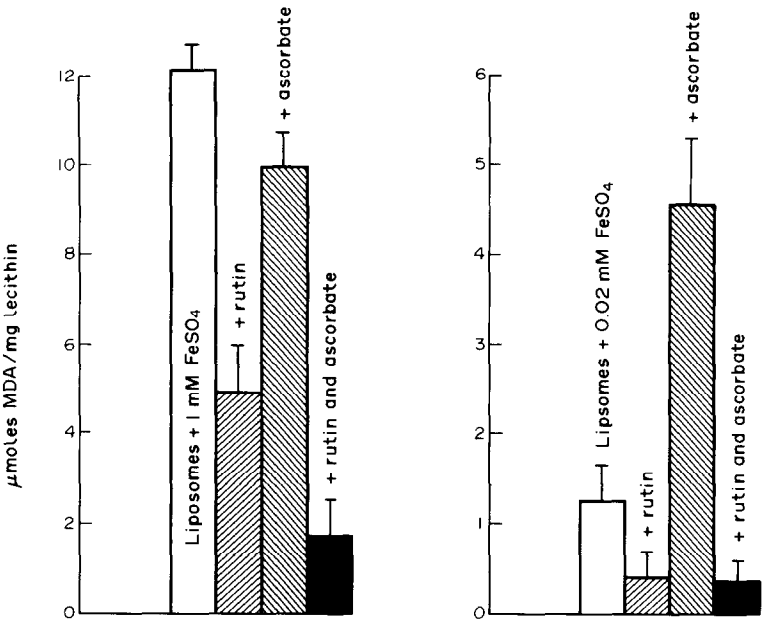

Fig. 4. Effects of rutin and ascorbate on ferrous ion-dependent lipid peroxidation of lecithin liposomes. Left: 0.05 mM rutin and 0.075 mM ascorbic acid. Right: 0.05 mM rutin and 0.5 mM ascorbic acid.

oxide ion was obtained by the electrochemical reduction of dioxygen in acetonitrile with tetrabutylammonium perchlorate as the supporting electrolyte [15]. After electrolysis, the superoxide concentration was equal to 1–10 mM. Rutin was dissolved in DMF (0.5–1 mM). The reaction between the superoxide ion and rutin was completed immediately after mixing the solutions in the cell of a spectrophotometer.

Reaction of lipid peroxy radicals with quercetin and rutin. The hydroperoxide of linoleic acid was obtained by the oxidation of linoleic acid catalyzed by soybean lipoxygenase [16]. Linoleic acid hydroperoxide (6 μM) was incubated at 37° with quercetin or rutin (15 μM), cytochrome *c* (0.4 mg protein/ml) and EDTA (0.1 mM) in 0.05 M phosphate buffer, pH 7.4 (3.0 ml of final solution). The reaction was followed spectrophotometrically.

Optical spectra were recorded on spectrophotometers Cary 219 and Specord M 40.

RESULTS

Non-enzymatic lipid peroxidation of lecithin liposomes

The ferrous ion-dependent lipid peroxidation of lecithin liposomes increased with increasing ferrous ion concentration within the range of 0.02–2.0 mM (Fig. 2, Curve 1), the rate of lipid peroxidation being levelled off over 1 mM $FeSO_4$. From Fig. 2, ascorbic acid had a double effect on lipid peroxidation: it was a pro-oxidant at low iron ion concentrations (0.02–0.4 mM) and an antioxidant at high iron ion concentrations (more than 0.4 mM).

These findings indicate that simultaneous application of flavonoids and ascorbic acid in the ferrous ion-dependent lipid peroxidation must lead to a complicated effect. Indeed, we found that rutin alone decreased lipid peroxidation of lecithin liposomes in a concentration-dependent manner (Fig. 3). But mutual effect of rutin and ascorbic acid depended on ferrous ion concentration (Fig. 4). It is seen that both compounds inhibited lipid peroxidation of lecithin liposomes at high iron ion concentrations (1 mM). However, as it should be expected, ascorbic acid acted as a pro-oxidant and rutin acted as an antioxidant at low iron ion concentrations (0.02 mM).

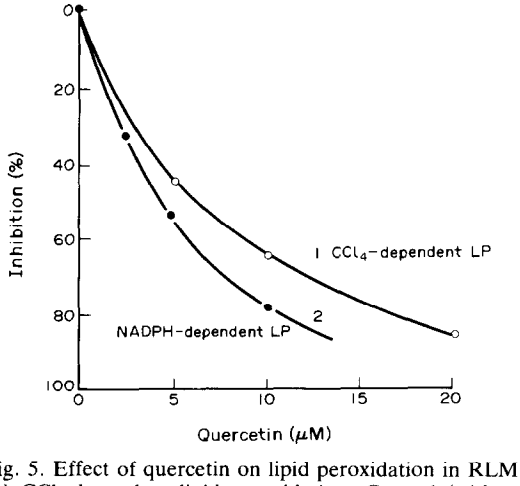

Fig. 5. Effect of quercetin on lipid peroxidation in RLM. (1) CCl_4-dependent lipid peroxidation. Control (without quercetin) 0.8 nM MDA/mg protein/min. (2) NADPH-dependent lipid peroxidation. Control 2 nM MDA/mg protein/min. RLM were incubated with NADPH, CCl_4, NaCl, and EDTA at 37° for 7.5 min in phosphate buffer (pH 7.4).

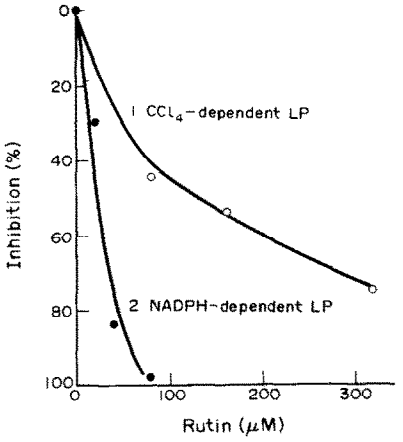

Fig. 6. Effect of rutin in lipid peroxidation in RLM. (1) CCl_4-dependent lipid peroxidation. Control (without rutin) 0.8 nM MDA/mg protein/min. (2) NADPH-dependent lipid peroxidation. Control 2 nM MDA/mg protein/min. RLM were incubated with NADPH, CCl_4, NaCl, and EDTA at 37° for 7.5 min in phosphate buffer (pH 7.4).

NADPH- and CCl_4-dependent lipid peroxidation in rat liver microsomes

The effects of rutin and quercetin on the NADPH- and CCl_4-dependent lipid peroxidation in RLM were studied and were compared with that of a classical antioxidant, 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) (Figs 5 and 6, Table 1). It is seen that lipid peroxidation was inhibited by these flavonoids in both cases, quercetin being a more powerful inhibitor. It is also evident that rutin and quercetin inhibit more strongly the NADPH-dependent lipid peroxidation, and by this their behavior differs completely from that of BHT because I_{50} values (doses of inhibitor required to inhibit activity by 50%) for the latter were the same in both lipid peroxidation systems (Table 1).

Both flavonoids did not affect the activity of cytochrome P-450 within the whole interval of the concentrations used as the amount of formaldehyde formed during the oxidation of aminopyrine by RLM did not change in the presence of flavonoids. Therefore, the inhibition of CCl_4-dependent lipid peroxidation by rutin or quercetin is explained exclusively by their antiradical action.

The interaction of rutin with superoxide ion

Mixing solutions of electrogenerated superoxide ion and rutin resulted in the immediate disappearance of an absorption maximum of rutin at 360 nm and the appearance of a new maximum at 454 nm (Fig. 7). Analogous spectra were also obtained after treatment of rutin with alkali and ascorbate anion (data are not shown).

Antiradical activity of flavonoids

To estimate the antiradical activity of flavonoids, their interaction with free radicals generated in the decomposition of linoleic acid hydroperoxide in the presence of cytochrome *c* was studied. It was found that quercetin and (more slowly) rutin decomposed

Table 1. I_{50} Values for free radical inhibitors in lipid peroxidation systems

Inhibitor	I_{50} (μM) NADPH-dependent lipid peroxidation	I_{50} (μM) CCl_4-dependent lipid peroxidation
Quercetin	4.5	6.0
Rutin	16	116
BHT	1.25	1.3

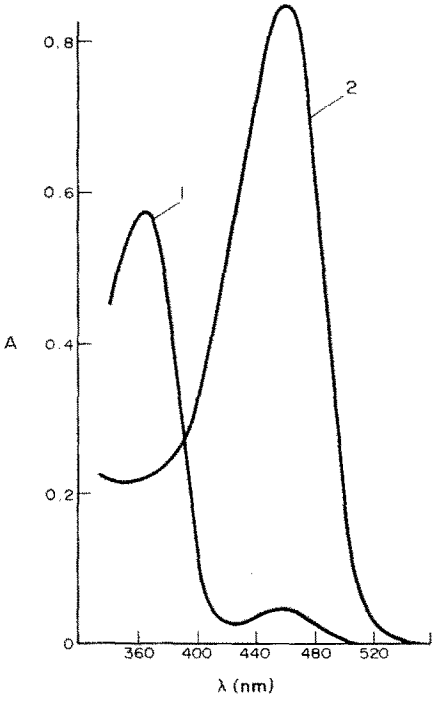

Fig. 7. Absorption spectra of rutin (1) and a product forming in the reaction of electrogenerated superoxide ion with rutin in DMF (2). (1) 0.04 mM rutin. (2) 0.24 mM rutin + 0.24 mM $O_2^{\cdot -}$.

to form the products absorbed in the short-wave region (Fig. 8). The following mechanism of this process was proposed

$$\mathrm{ROOH} + \mathrm{cyt}\cdot c^{2+} \rightarrow \mathrm{RO}\cdot + \mathrm{HO}^- + \mathrm{cyt}\cdot c^{3+} \quad (5)$$

$$\mathrm{RO}\cdot + \mathrm{ROOH} \rightarrow \mathrm{ROH} + \mathrm{ROO}\cdot \quad (6)$$

$$\mathrm{RO}\cdot + \mathrm{Fl{-}OH} \rightarrow \mathrm{ROH} + \mathrm{Fl{-}O}\cdot \quad (7)$$

$$\mathrm{ROO}\cdot + \mathrm{Fl{-}OH} \rightarrow \mathrm{ROOH} + \mathrm{Fl{-}O}\cdot \quad (1)$$

Chelating activity of rutin

The addition of $FeSO_4$ to rutin in phosphate buffer (pH 7.4) resulted in the shift of a maximum at 360 nm to the long-wave region. This shift is clearly seen in differential spectra (Fig. 9). The intensity of a maximum recorded against the $FeSO_4$ solution was the same as that of an original spectrum of rutin

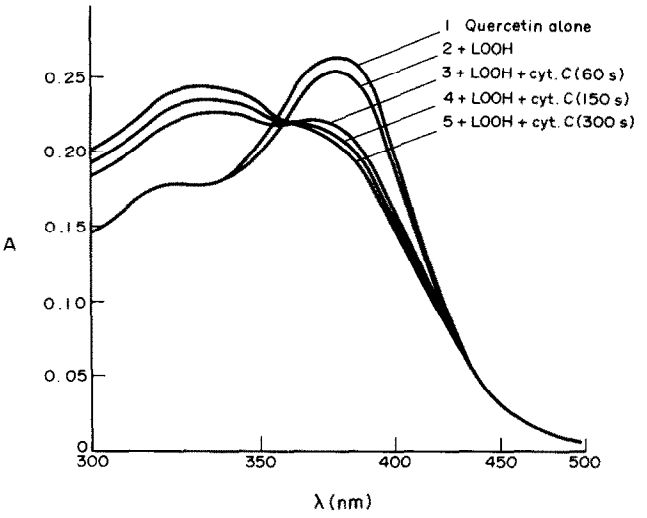

Fig. 8. Oxidation of quercetin by the linoleic acid hydroperoxide–cytochrome *c* system. (1) Absorption spectrum of quercetin in the absence of hydroperoxide. (2) Absorption spectrum after hydroperoxide adding. (3), (4) and (5) are absorption spectra 60, 150 and 300 sec after cytochrome *c* addition. Linoleic acid hydroperoxide was incubated with quercetin, cytochrome *c* and EDTA at 37° in phosphate buffer (pH 7.4).

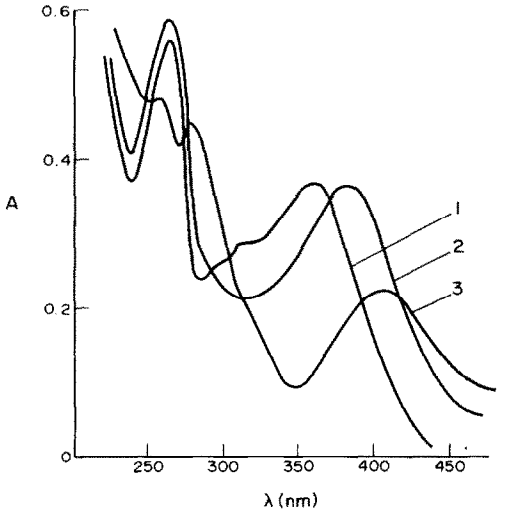

Fig. 9. Absorption spectra of rutin and ferrous ion–rutin complex (phosphate buffer, pH 7.4). (1) Absorption spectrum of rutin (22.5 μM). (2) Differential spectrum of ferrous-ion-rutin complex against the $FeSO_4$ solution. (3) Differential spectrum of ferrous ion–rutin complex against the rutin solution.

(Curve 2). But the most interesting spectrum of the iron–rutin complex was obtained when recorded against the rutin solution (Curve 3). This spectrum contained new maxima at 420, 279 and 258 nm.

The iron–rutin complex was sufficiently stable, and its spectrum did not change during the 8 hr or longer. The rate of its formation depended on the reagent concentrations: the equilibrium was achieved in 1–5 min at concentrations $\geqslant$0.1 mM and in several hours at concentrations about 10 μM. Using the technique of saturation concentrations [17], it was calculated that the iron–rutin complex has a 1:2 stoichiometry in 0.05 phosphate buffer (pH 7.4).

DISCUSSION

The above results show that flavonoids rutin and quercetin possess indeed a unique ability to inhibit free radical processes in cells at three different stages: an initiation (by the interaction with superoxide ion), the formation of hydroxyl or "crypto-hydroxyl" radicals (by chelating iron ions) and lipid peroxidation (by reacting with lipid peroxy radicals). We believe that the reaction of O_2^- with rutin proceeds via a one-electron transfer mechanism as in the case of anthracyclines [18, 19]. Indeed, the same product with a maximum at 454 nm was formed in the reaction of rutin with superoxide ion and ascorbate anion. This product cannot be an anion because ascorbate anion is unable to deprotonize phenolic hydroxyls being much more strong acid than phenols ($pK_a(AH_2) = 4.10$, $pK_a(ArOH) = ca$ 10 [20]). Therefore, it must be formed as a result of electron transfer from O_2^- and ascorbate anion to rutin.

Scavenging of superoxide ion (generated by xanthine oxidase or applied as potassium superoxide) by quercetin was demonstrated earlier [21] as the suppression by this flavonoid of the ESR spectrum of a spin-adduct of O_2^- with 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). In addition, the destruction of quercetin by the superoxide ion was observed spectrophotometrically. It seems to be very probable that the reaction of superoxide ion with quercetin proceeds by the same mechanism as with rutin.

The ability of rutin to form the complexes with iron ions has been shown some time ago [22]. It was found that the ferrous ion–rutin complex is sufficiently stable at physiological pH values, and there-

fore, its formation may be of an importance in the iron ion-dependent lipid peroxidation. Indeed, if the iron–rutin complex was used instead of ferrous salt, no MDA was formed in the peroxidation of lecithin liposomes. The absence of pro-oxidant effect of the iron–rutin complex was recently also shown in the Fenton reaction and the lysis of erythrocytes induced by asbestos fibers [23].

The inhibitory action of rutin and quercetin on lipid peroxidation was clearly demonstrated in all the peroxidation systems studied. This action is explained by both chelating and antioxidative properties of flavonoids, as inhibition was observed at flavonoid concentrations much smaller than the concentrations of ferrous ions. The addition of ascorbate did not change the mechanism of the inhibitory effect of rutin, but mutual effects of rutin and ascorbate depended on ascorbate and ferrous ion concentrations (Fig. 4). It is explained by the competition between pro-oxidant (at low ascorbate and ferrous ion concentrations) and antioxidant (at high ascorbate and ferrous ion concentrations) effects of ascorbic acid on lipid peroxidation expressed by Reactions 8 and 9, respectively

$$AH_2 + Fe^{3+} \rightarrow AH\cdot + Fe^{2+} + H^+ \quad (8)$$

$$AH_2 + ROO\cdot \rightarrow AH\cdot + ROOH \quad (9)$$

The estimate of chelating and antioxidant contributions in the total effect of flavonoids on free radical processes can be made from comparison of microsomal NADPH- and carbon tetrachloride-dependent lipid peroxidation. It is known that microsomal NADPH-dependent lipid peroxidation is catalyzed by NADPH cytochrome P-450 reductase and proceeds in the presence of iron ions [24]. On the other hand, the activation of carbon tetrachloride takes place on cytochrome P-450 and does not need iron ions [25]. Therefore, one may expect that the chelating action of flavonoids must be more prominent in the case of NADPH-dependent lipid peroxidation.

As seen from Figs 5 and 6, it is true for both flavonoids studied. If the difference in inhibitory activity of flavonoids in both lipid peroxidation systems can be considered as a measure of their chelating properties, then it is obvious that the chelating mechanism of inhibition is more important for rutin than for quercetin. A stronger total inhibitory effect of quercetin in both peroxidation systems is possibly due to its additional active phenolic hydroxyl. It is also interesting to compare the inhibitory effects of rutin and quercetin with that of BHT which does not have chelating properties. It is seen from Table 1 that this antioxidant is equally effective in both peroxidation systems. Therefore, stronger inhibitory effects of rutin and quercetin on NADPH-dependent lipid peroxidation in RLM are really explained by their chelating properties.

Thus, the ability of rutin and quercetin to react with superoxide ion and lipid peroxy radicals and to form iron complexes which are unable to catalyze the formation of active oxygen radicals but retain their antioxidative function indeed indicates the possibility of the use of these flavonoids for combating free radical pathologies. Their therapeutic significance may be much more than that of such a "strong" synthetic antioxidant as BHT, because they (especially, rutin) are non-toxic and may inhibit free radical processes at various stages.

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