Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties

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The flavonoids constitute a large group of polyphenolic phytochemicals with antioxidant properties *in vitro*. The interactions of four structurally related flavonoids (quercetin, kaempferol, rutin and luteolin) with Cu^{2+} ions were investigated in terms of the extent to which they undergo complex formation through chelation or modification through oxidation, as well as in their structural dependence. The ortho 3′,4′-dihydroxy substitution in the B ring is shown to be important for Cu^{2+}-chelate formation, thereby influencing the antioxidant activity. The presence of a 3-hydroxy group in the flavonoid structure enhances the oxidation of quercetin and kaempferol, whereas luteolin and rutin, each lacking the 3-hydroxy group, do not oxidize as readily in the presence of Cu^{2+} ions. The results also demonstrate that the reactivities of the flavonoids in protecting low-density lipoprotein (LDL) against Cu^{2+} ion-induced oxidation are dependent on their structural properties in terms of the response of the particular flavonoid to Cu^{2+} ions, whether chelation or oxidation, their partitioning abilities between the aqueous compartment and the lipophilic environment within the LDL particle, and their hydrogen-donating antioxidant properties.

INTRODUCTION

Previous studies *in vitro* have demonstrated the antioxidant activities of the flavonoids as hydrogen-donating free radical scavengers and their structural dependence [1,2]. The ability of flavonoids to inhibit the oxidation of low-density lipoproteins (LDLs) demonstrates their potential as chain-breaking antioxidants [3–8] and this might involve a role in the sparing of α-tocopherol [9,10]. Other studies suggest that polyphenols might inhibit free radical formation and the propagation of free radical reactions through the chelation of transition-metal ions, particularly those of iron and copper [11–16]. Many studies have demonstrated the antioxidant effects of quercetin in lipid systems in the presence of a range of pro-oxidants [9,17,18]. In transition-metal-free lipid systems, the phenolics act as antioxidants by virtue of their ability to act as hydrogen donors, leading to the formation of aryloxyl radicals; compounds containing the *o*-dihydroxycatecholate structure are the most effective. In the presence of transition metal ions, however, it is not clear to what relative extents radical scavenging or metal chelation might contribute to the antioxidative effects.

The purpose of the work described here is to examine the structure–antioxidant-activity relationships of a group of structurally related flavonoids: quercetin, rutin, luteolin and kaempferol (Figure 1), through their abilities to interact with Cu^{2+} ions and consequently to determine their influence on copper-mediated LDL oxidation. The results show that the reactivities in enhancing the resistance of LDL to oxidation are dependent on their interactions with Cu^{2+} ions, whether chelation or oxidation, on their relative abilities to partition into lipid, as well as their hydrogen-donating antioxidant properties.

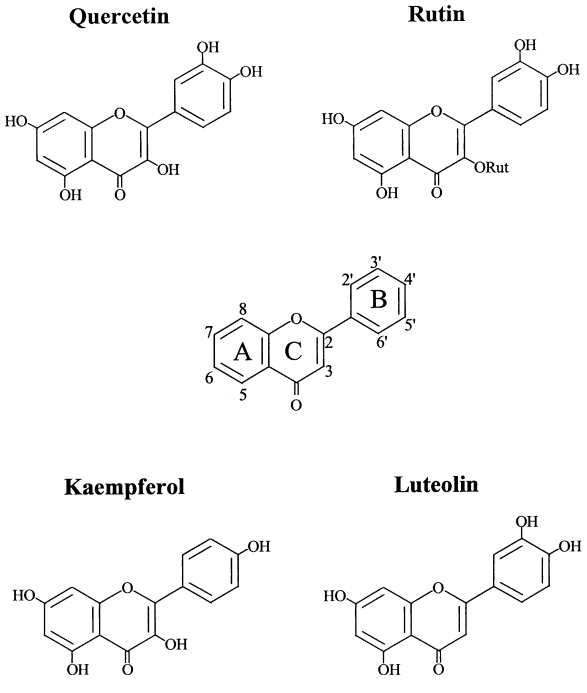

Figure 1 Structural features of flavones and flavonols

MATERIALS AND METHODS

Materials

Quercetin and rutin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Luteolin and kaempferol were purchased from Extrasynthèse (Genay, France). All other chemicals used were of analytical grade.

Copper interaction studies

Stock solutions of each flavonoid (1 mM) were prepared in methanol. Then 25 μM solutions were prepared in a cuvette

Abbreviations used: BHT, butylated hydroxytoluene; DTPA, diethyltriamine penta-acetic acid; $E_p/2$, half-oxidation potential; LDL, low-density lipoprotein; REM, relative electrophoretic mobility.

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containing PBS (10 mM, pH 7.4) and the absorption spectra were recorded between 200 and 800 nm. Scans with 12.5, 25.0, 37.5 or 50.0 μM $CuSO_4$ were taken after 10 s and compared with flavonoid alone. The effect of a 2.5-fold EDTA concentration (125 μM) was examined on the flavonoid–copper complex. Diethyltriamine penta-acetic acid (DTPA) (125 μM) was used as a comparison.

LDL isolation and preparation

Blood was collected from healthy volunteers by venepuncture into vials containing acid citrate dextrose and 100 μM EDTA. LDL was isolated by using a modified discontinuous ultracentrifugation method [19]. Isolated LDL was then sterilized by passing it through a 0.2 μm filter (Flowpore; ICN Pharmaceuticals, U.S.A.). For each set of oxidation experiments, LDL was dialysed for 6 h at 4 °C with PBS containing EDTA (10 μM). LDL protein concentrations were estimated by using a modified Lowry method [20].

LDL oxidation

The relative effectiveness of two flavonols (kaempferol and quercetin) and two flavones (luteolin and rutin) at delaying lipid peroxidation was evaluated by examining their inhibitory effects on the oxidative modification of LDL *in vitro* over a range of flavonoid concentrations (0.1–2.0 μM).

LDL oxidation was performed with a modification of the method described by Esterbauer et al. [21]. Incubations were performed with 62.5 μg/ml LDL protein supplemented with methanolic solutions of each flavonoid individually or controls with the same volume (2 μl) of methanol. Oxidation was initiated by the addition of Cu^{2+} at a final concentration of 1.66 μM. All incubations were performed at 30 °C.

The kinetics of the oxidation (assessed by conjugated diene formation) were determined by continuously monitoring the absorbance at 234 nm on a Beckman DU 7500 photodiode array spectrophotometer equipped with Peltier temperature control. Results from the conjugated diene measurements are expressed in terms of lag phase (defined as the intercept at the abscissa in the diene–time plot) [21] and rate of oxidation during the propagation phase.

The extent to which each flavonoid inhibited an increase in the net negative surface charge of apolipoprotein B_{100} was also monitored. For these measurements, oxidation of the LDL was terminated at 4 h with 20 μM butylated hydroxytoluene (BHT) in methanol, and 10 μl samples were applied to Beckman precast agarose gels. Electrophoresis was performed at pH 8.6 for 45 min (100 V). Relative electrophoretic mobility (REM) was calculated by determining the ratio of the distance from the origin to the midpoint of the oxidized LDL band in relation to that for the native, untreated LDL. These values were compared with the REM of LDL incubated with Cu^{2+} in the absence of flavonoid. Incubations with each flavonoid in the absence of Cu^{2+} were also performed as controls.

To investigate the influence of copper chelation by the polyphenols, LDL oxidation (62.5 μg of LDL protein/ml) was also conducted with haem protein as the pro-oxidant in the form of metmyoglobin [22]. Higher concentrations of haem protein are required to produce similar oxidation kinetics to that of Cu^{2+} ions [22]. The kinetics of the oxidation of LDL were determined by continuously monitoring the absorbance at 234 nm on a Beckman DU 7500 spectrophotometer equipped with Peltier temperature control (37 °C). Results are expressed in terms of the time of extension in the lag phase to oxidation compared with control.

Partition coefficient determination

Partition coefficients were measured by using an automated continuous flow method (filter probe method) [23] and the shake-flask method. The two phases used in the determination were Mops buffer (50 mM, pH 7.4, prepared with Milli-Q water) and octan-1-ol, each of which was pre-equilibrated with the other phase before use (the solubility of water in octan-1-ol is 2.3 M) [24]. All solutions were stored and manipulated at 25 $\pm$ 0.5 °C. Flavonoid solutions (100 μM) were prepared in the aqueous phase (typically 40 ml) to give an absorbance of 1.5–2.0 at the pre-selected wavelength of approx. 280 nm. On commencement of the analysis, absorbance measurements were automatically recorded at preselected time intervals, usually 1 s. When the absorbance readings had stabilized (changing by less than 0.002 absorbance unit over a minimum of 10 min), a suitable volume of octan-1-ol was added to the aqueous phase. Absorbance readings were subsequently recorded until the system had reached equilibrium again, at which point a further aliquot of octan-1-ol was added. The cycle was repeated for at least five additions of octan-1-ol. At each stage of octan-1-ol addition the corresponding partition coefficient was calculated from the formula:

$$K_{\text{part}} = [(A_0 - A_1)/A_1](V_w/V_0)$$

where A_0 is initial absorbance, A_1 is absorbance at equilibrium after addition of octan-1-ol, V_w is the volume of Mops buffer and V_0 is the total volume of octan-1-ol added to the glass vessel.

Statistical methods

Results are expressed as means $\pm$ S.D.; significant differences ($P < 0.05$) were determined by unpaired t test after a significant ($P < 0.05$) one-way analysis of variance.

RESULTS

Flavonoid interactions with copper Cu^{2+} ions

The direct interactions of the flavonoids with Cu^{2+} ions at pH 7.4 were assessed by UV/visible spectroscopy. The effect of stepwise increments of $CuSO_4$ concentration on the spectral characteristics of each flavonoid is described in terms of shifts in band I (320–385 nm) and band II (250–285 nm), which relate to B and A ring absorptions respectively. The results are shown in Table 1.

Interactions of Cu^{2+} ions with luteolin (25 μM) at 1:1 and 2:1 copper-to-flavonoid ratios produced bathochromic shifts in band I of 23 and 40 nm respectively from 369 nm. No changes in the position of band II were observed at the 1:1 ratio but a small (5 nm) red shift was produced at the 2:1 ratio. On addition of 125 μM EDTA the original spectrum was recovered (Figure 2a). A similar effect was observed with 125 μM DTPA.

Under similar conditions, band I of the rutin spectrum demonstrated a 22 nm bathochromic shift in the presence of a concentration ratio of 1:1 Cu^{2+} to flavonoid (25 μM), which was associated with a small increase in absorbance. Further addition of Cu^{2+} ions increased the intensity of the absorbance of band I coincidentally with a small bathochromic shift of a further 6 nm. Band II at 270 nm demonstrated a slight hypsochromic shift (2 nm) with 1:1 Cu^{2+} to flavonoid. This was accompanied by a small increase in absorbance. Further addition of Cu^{2+} ions up to a 2:1 Cu^{2+}-to-flavonoid ratio resulted in additional smaller increases in the absorbance of band II at 268 nm. On treatment with either 125 μM EDTA or DTPA, the spectrum returned to its original position and absorbance (Figure 2b).

Table 1 Spectral shifts of each flavonoid

The concentration of each flavonoid was 25 μM. Data are representative of two or three experiments.

		Peak position (nm)				
Band	Flavonoid	Control	Cu^{2+}-to-flavonoid molar ratio ...	0.5:1	1:1	1.5:1	2:1
I	Rutin	370		373	392	397	398
	Luteolin	369		378	392	405	409
	Quercetin	378		379	408	441	449
	Kaempferol	370		340, 405	339, 409	338, 409	339, 409
II	Rutin	270		270	268	268	268
	Luteolin	269		269	269	271	274
	Quercetin	269		270	271	274	274
	Kaempferol	270		271	273	275	274, 258

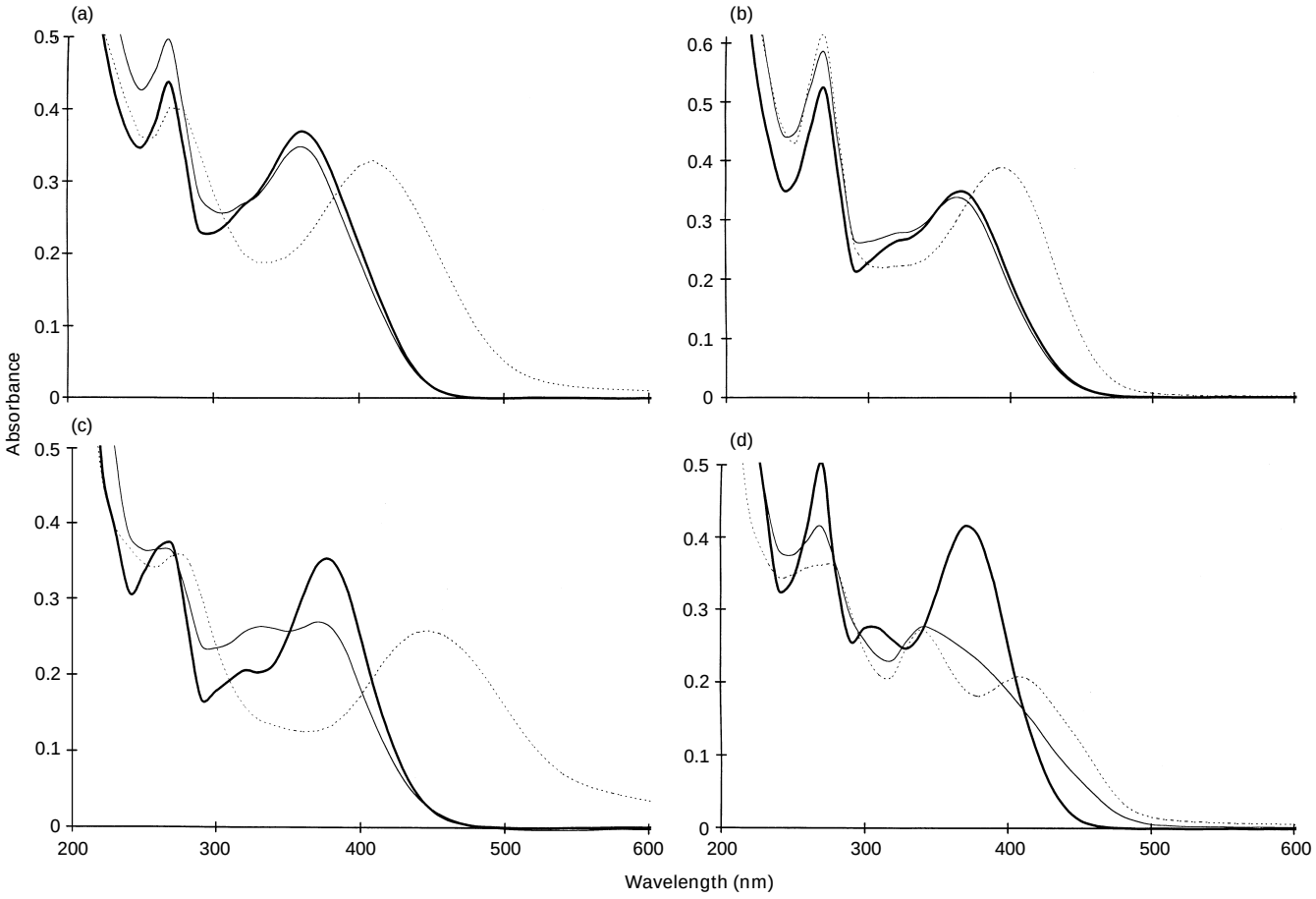


Figure 2 Effect of EDTA on Cu^{2+} chelate formation with (a) luteolin, (b) rutin, (c) quercetin and (d) kaempferol

Thick solid line, flavonoid (25 μM); dotted line, flavonoid (25 μM) plus Cu^{2+} ions (50 μM); thin solid line, flavonoid (25 μM) plus Cu^{2+} ions (50 μM) after addition of EDTA (125 μM).

With quercetin (25 μM), the presence of an equimolar concentration of Cu^{2+} ions caused a greater red shift in band I than with luteolin and rutin (Figure 2c). On doubling the Cu^{2+} ion concentration, the shift in band I increased by a further 41 nm. The small shoulder at 321 nm disappeared in the presence of Cu^{2+} ions. The addition of either EDTA or DTPA (125 μM) to chelate the Cu^{2+} ions did not regenerate the original spectrum (Figure 2c), unlike the situations for luteolin and rutin.

Spectral shifts for kaempferol were more complex. Band I (370 nm) split into two peaks at 339 and 408 nm in the presence of an equimolar Cu^{2+} ion concentration. These peaks persisted in the presence of 2:1 Cu^{2+}-to-flavonoid ratio. A 20% decrease in

Table 2 Partition coefficients of flavonoids in octan-1-ol/water mixtures

Results are expressed as means ± S.D. for $n = 5$ determinations for each compound.

Compound	K_{part}
Quercetin	1.2 ± 0.13
Kaempferol	69.5 ± 1.32
Rutin	0.37 ± 0.06
Luteolin	22.2 ± 2.5

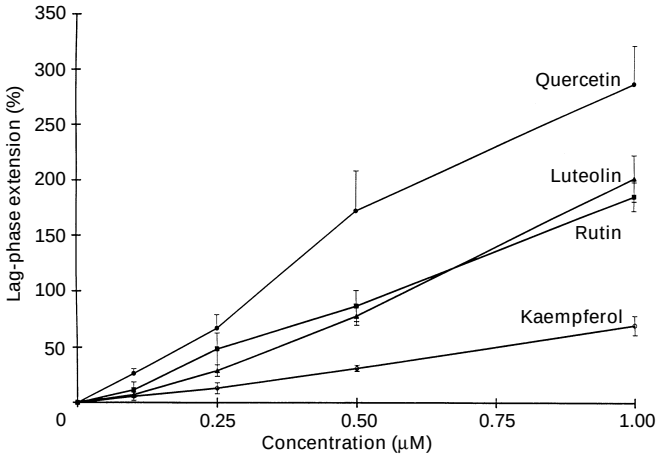


Figure 3 Effect of quercetin, kaempferol, rutin or luteolin on lag-phase extension

LDL (62.5 μg/ml) was incubated with $CuSO_4$ (1.66 μM) with concentrations of quercetin (●), kaempferol (○), rutin (■) or luteolin (▲) ranging between 0.1 and 1.0 μM.

absorbance at 270 nm (band II) was observed in the presence of equimolar Cu^{2+} ion concentrations. Doubling the concentration of Cu^{2+} ions led to band II's splitting into two equal peaks at 258 and 274 nm. Neither EDTA nor DTPA (125 μM) addition regenerated the original spectrum (Figure 2d).

Partition coefficients

Determination of the relative solubilities of the flavonoids was undertaken by investigating their partition coefficients in octanol/water mixtures. Rutin (quercetin 3-rutinoside) has the lowest K_{part}, 0.37 (Table 2), with a greater solubility in the aqueous environment, presumably due to the covalently bound disaccharide. The partition coefficient of quercetin, 1.2, suggests an approximately equal distribution between aqueous and lipophilic compartments. Both luteolin and kaempferol possess higher K_{part} values, 22.2 and 69.5 respectively, demonstrating their greater solubilities in the hydrophobic environment. In both cases the compounds possess one fewer hydroxy function than quercetin.

Inhibition of LDL oxidation

The extent to which the structurally related polyphenols act as antioxidants in preventing LDL oxidation was assessed by investigating the suppression of lipid peroxidation, the decrease in the rate of oxidation during the propagation phase and their

Table 3 Effect of quercetin, kaempferol, rutin or luteolin on propagation rate of LDL oxidation medicated by Cu^{2+} ions (1.66 μM)

The concentration of LDL was 62.5 μg of LDL protein/ml. Results are expressed as means ± S.D. The average propagation rate of conjugated diene formation in the control system was 0.028 ± 0.003 absorbance units/min. * $P < 0.05$.

	Propagation rate (% of control)			
Compound Concentration (μM) ...	0.10	0.25	0.50	1.00
Quercetin	102.9 ± 1.3	103.8 ± 0.4	95.3 ± 9.4	67.9 ± 5.1*
Kaempferol	104.2 ± 9.9	104.0 ± 9.9	109.8 ± 7.8	115.0 ± 12.2
Rutin	95.9 ± 17.6	96.3 ± 13.3	86.0 ± 12.7	69.8 ± 15.9*
Luteolin	100.0 ± 6.6	98.9 ± 3.1	94.9 ± 11.4	94.1 ± 6.5

effects in preventing apolipoprotein B_{100} modification. The effects of the polyphenols at concentration ratios of Cu^{2+} to flavonoid of greater than 1 were investigated in terms of their ability to enhance the resistance of LDL to oxidation, by measuring the lag phase to oxidation. Both the flavones and flavonols extended the lag phase in a dose-dependent manner. Quercetin was the most effective, prolonging the lag phase by more than 287 ± 35 % at 1 μM from 62 to 178 min (Figure 3). Kaempferol (structurally similar to quercetin but lacking the 3′-hydroxy group) was the weakest of the four compounds, extending the lag phase by 70 ± 9 % at 1 μM. Luteolin and rutin were of intermediate efficacies.

Both quercetin and rutin significantly decreased the propagation rate at 1 μM ($P < 0.05$; Table 3) but not at the lower concentrations. In contrast, luteolin and kaempferol, which lack either the 3-hydroxy or 3′-hydroxy group respectively, had no significant effect on the rate of the propagation phase. Interestingly, a plot of partition coefficient against inhibition of propagation rate at 1 μM for each of the four compounds yielded a significant correlation ($r = 0.97$; $P < 0.02$).

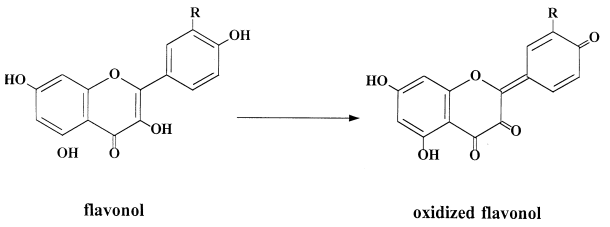
The ability of the phenolics to inhibit the alteration in the surface charge of the apolipoprotein B_{100} when LDL was incubated with Cu^{2+} ions was monitored by observing the effects on electrophoretic mobility by agarose-gel electrophoresis. The pattern of activities for the four structurally related flavones was generally consistent with that seen for the lag-phase measurements.

To investigate the antioxidant properties of the flavonoids when metal-chelating effects and reducing reactions between Cu^{2+} ions and the flavonoids were eliminated, oxidation of LDL by metmyoglobin (5 μM) was also undertaken. By monitoring the increased formation of conjugated dienes, a control LDL lag phase of 88 ± 8 min was observed. Luteolin (0.1 μM) extended the lag phase by 65.5 ± 14.7 %, close to that of quercetin (40.4 ± 11.4 %; $P = 0.092$) and significantly greater than either rutin (29.0 ± 8.6 %; $P < 0.05$) or kaempferol (17.4 ± 8.7 %, $P < 0.05$). This compares with lag-phase extensions of approx. 5–10 % for kaempferol, rutin and luteolin at 0.1 μM, and a 26 % extension for quercetin when Cu^{2+} ions (1.66 μM) were used as pro-oxidant.

DISCUSSION

Interaction of the polyphenols with copper ions

The flavones luteolin and rutin both contain the *o*-3′,4′-dihydroxy structure in the B ring but have differences at the 3-position. Luteolin lacks the free 3-hydroxy group in the C ring, whereas rutin has the 3-hydroxy group replaced by the disaccharide



Scheme 1 Structure of flavonols and their oxidized forms

R = OH, quercetin; R = H, kaempferol.

glucorhamnoside. On interaction with Cu^{2+} ions, new peaks are assumed to be characteristic of a copper–flavone chelate; the reversibility of the reaction confirms this. Chelation probably occurs through the catechol structure.

The spectroscopic studies indicate that quercetin is capable of chelating Cu^{2+} ions; however, on titrating out the bound Cu^{2+} ions, a new spectrum appears, indicating that quercetin has not reverted to its original form before Cu^{2+} ion interaction but that it has become oxidized. In view of the structural similarities of quercetin to its 3-rutinoside (rutin), these observations suggest that the oxidation of the free 3-hydroxy group (the only structural difference between them) has occurred during the Cu^{2+} ion–quercetin interaction, and that the changes in band I suggest an additional effect on the hydroxy groups of the B ring. Likely structural assignments of the oxidized quercetin are shown in Scheme 1.

Major spectral changes also occur on Cu^{2+} ion binding to kaempferol, although the peak perceived to be characteristic of a Cu^{2+} chelate is much less pronounced than for the other compounds studied. This observation suggests that the major contributor to Cu^{2+} ion chelation in this family of flavones is the catechol structure (absent from kaempferol) rather than the 4-oxo group in the C ring with either of its adjacent hydroxy groups, which are present in the kaempferol structure. This is consistent with the observed effects of the addition of EDTA, decreasing the small new red-shifted peak but sustaining the marked change in the spectrum of kaempferol, including the band I spectral profile (i.e. the B ring), suggesting that oxidation of the molecule has occurred, probably at the 3-hydroxy and the 4′-hydroxy groups, as for quercetin (Scheme 1).

These observations conform with the reported reducing properties as depicted by the half-oxidation potentials ($E_p/2$) [15], in that quercetin is the most effective reducing agent with a much lower $E_p/2$ (i.e. 0.03 V) than kaempferol (0.12 V), closely similar to those of luteolin and rutin (0.18 V) [15]. However, the flavone apigenin, structurally similar to kaempferol but lacking the 3-hydroxy group in the C ring has a value of > 1 V, supporting the notion that the 3-hydroxy group might be the point of oxidation of the molecule on interaction with Cu^{2+} ions, with consequent 4′-hydroxy group oxidation. The contribution of the 3-hydroxy group to the redox cycling properties of the flavonols is expounded in [15]; on measurement of the $E_p/2$ values, those authors suggest that the 3-hydroxy group should be blocked (in the presence of a C2–C3 double bond in the C ring) to render a compound that is unable to redox cycle.

Inhibition of LDL oxidation by the polyphenols

In the study reported here, all the flavonoids (quercetin, rutin, luteolin and kaempferol) demonstrated increasing abilities to prevent or intercept Cu^{2+}-ion-induced (1.66 μM) peroxidation of LDL as measured by their capacity to extend the lag phase to oxidation over the concentration range 0.1–1.0 μM. The phenolics that had the greatest propensity for Cu^{2+} ion chelation in the chemical system, namely quercetin, rutin and luteolin, were the most effective in inhibiting LDL oxidation in the Cu^{2+} ion system, the sequence of reactivities being similar. Kaempferol, lacking the catechol structure, was less effective at intercepting LDL oxidation and showed minimal responses to Cu^{2+} ion chelation in the chemical system, but also was susceptible to maximal chemical modification on interaction with Cu^{2+} ions, again suggesting oxidation. Whereas luteolin and kaempferol did not affect the rate of propagation of LDL oxidation at 1 μM concentration, the less hydrophobic compounds quercetin and rutin significantly decreased this rate at the same concentration. This might be indicative of Cu^{2+} ion chelation under these conditions and can be interpreted as the greater accessibility of rutin and quercetin to the Cu^{2+} ions during the course of the LDL oxidation, as indicated by their partition coefficients; in contrast, the higher lipophilicities of luteolin and kaempferol might be expected to limit their access to the Cu^{2+} ions. This is supported by a significant correlation between partitioning and the inhibition of propagation rate.

The direct interaction of Cu^{2+} ions with the flavonoids and its influence in retarding LDL oxidation is supported by the observation of the inhibitory effects of the flavonoids on LDL oxidation induced by metmyoglobin, to preclude the question of flavonoid–copper interactions. From the ratio of lag phase extension in the haem protein oxidation and lag phase extension in the presence of Cu^{2+} ions, it is evident that quercetin and rutin have a greater capacity to delay oxidation in the Cu^{2+} ion system, whereas luteolin is the most effective in the haem protein oxidation system. Kaempferol, in contrast, responds poorly in both systems. This emphasizes the importance of the catechol arrangement in the B ring structure, which kaempferol lacks, in the radical scavenging process [1,15,25]. The comparative effects of the flavonoids under the influence of the two pro-oxidants also highlights the role of partitioning and the interplay between radical scavenging and metal chelation. In considering the Cu^{2+} ion system, rutin is more soluble in aqueous media and thus has a greater tendency to form Cu^{2+} complexes than does luteolin, which is more lipophilic. However, rutin would have less accessibility to lipid peroxyl radicals within the LDL particle. The two flavonoids therefore probably inhibit oxidation through broadly different mechanisms. In contrast, in the haem protein oxidation system the chelation is less important than peroxyl scavenging activity: luteolin, the more lipophilic compound, is more effective.

The importance of partitioning and Cu^{2+} ion interaction has been observed previously with the more hydrophilic members of the catechin/gallate family of flavanols in connection with LDL oxidation [8]. Furthermore differences in the antioxidant abilities of quercetin and rutin in the inhibition of the autoxidation of rat cerebral membranes and iron-induced peroxidation of linoleic acid were concluded to be dictated by structural features and their propensity to interact and to penetrate lipid bilayers [26].

We thank the Ministry of Agriculture, Fisheries and Food for financial support.

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Received 5 September 1997/18 November 1997; accepted 20 November 1997