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Thermodynamic versus Kinetic Control of Antioxidant Synergism between β-Carotene and (Iso)flavonoids and Their Glycosides in Liposomes

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Antioxidant synergism (or antagonism) between plant (iso)flavonoids (daidzein, baicalein, and quercetin) or their glycosides (puerarin, baicalin, and rutin) and β-carotene in phosphatidylcholine liposomes (pH 7.4) with oxidation initiated thermally by the lipophilic free radical initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and followed by the formation of conjugated dienes did not depend simply on the bond dissociation enthalpy (BDE) of the phenol O−H bond or the HOMO/LUMO energy gap based on density functional theory (DFT) calculations. Rate of regeneration of β-carotene from the β-carotene radical cation as the one-electron oxidation product of the lipid phase antioxidant by the monoanion form of the (iso)flavonoids in homogeneous (1:9 v/v methanol/chloroform) solution, as studied by laser flash photolysis and occurring on a microsecond time scale with biphasic kinetics, was in better agreement with the observed nonadditive antioxidative effects. However, correcting the observed (pseudo)-first-order rate constant for β-carotene regeneration for water/lipid distribution of the (iso)flavonoids provided an almost correct ordering of the (iso)flavonoids, according to the nonadditive effects with β-carotene on lipid oxidation.

KEYWORDS: β-Carotene; flavonoid; isoflavonoid; antioxidant synergism

INTRODUCTION

The health benefits of fruits and vegetables are often ascribed to a high content of antioxidants (*1*). Antioxidants, moreover, protect important nutrients in foods and beverages against oxidative degradation and against formation of toxic oxidation products, in effect providing more safe foods (*2*). Antioxidant interaction seems, however, important for optimal protection of food and their nutrients (*3*, *4*). Supplementation with single antioxidants such as β-carotene in high doses has, moreover, been found to have adverse effects on human health (*5*), suggesting that positive health effects of antioxidants depend on a balance between individual compounds as found in many foods of vegetable origin (*6*, *7*). Such natural mixtures of antioxidants may show synergistic effects with protective effects being higher than expected by the simple summation of their individual effects (*3*, *8*).

Carotenoids are, besides being quenchers of singlet oxygen protecting against light induced oxidation, also efficient radical scavengers (*9*). Radical scavenging alone does, however, not constitute a good chain-breaking antioxidant, but there is increasing experimental evidence for a synergistic interaction between carotenoids and chain-breaking antioxidants such as tocopherols and tocotrienols (*10*). Such observations of synergistic effects have recently been supported by density functional theory (DFT) calculations and establishment of a novel two-dimensional electron donor−acceptor map (DAM) for compounds regulating biological oxidations (*10*−*13*).

Plant polyphenols have also been classified according to their potential as regulators of biological oxidation in concerted action with carotenoids and vitamin antioxidants, providing a theoretical basis for exploring antioxidant synergism experimentally (*14*). The mechanism of interaction between plant polyphenols including their glycosides and carotenoids as antioxidants has, however, been the subject of only a few experimental studies (*3*, *15*). The antioxidant synergism observed between isoflavonoids and β-carotene, which seems to depend on a regeneration of the carotenoid radical cation formed in the lipid phase by the isoflavonoid in lipid/water interfaces, may play an important role in the regulation of biological oxidation. Accordingly, we have initiated more systematic studies in model membrane systems to establish whether this interaction resulting in antioxidant synergism is thermodynamically or kinetically controlled and to what degree the interaction depends on the hydrophobic/hydrophilic balance of the polyphenol.

Quercetin, daidzein, and baicalein were selected as important (iso)flavonoids of structural diversity together with their glycosides, that is, rutin, puerarin and baicalin, to obtain a variation in lipid/water distribution. Theoretical calculations of molecular properties were combined with a real-time kinetic study of antioxidant free radical interaction using laser flash photolysis and with experimental evaluation of antioxidant synergy in a liposome system.

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MATERIALS AND METHODS

Chemicals and Sample Preparation. Daidzein, puerarin, and naringenin (>90%) were purchased from Shaanxi Huike Botanical Development Co., Ltd. (Xi'an, China) and were recrystallized before use. Baicalein, baicalin, and rutin (>95%) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Quercetin (>95%) was purchased from Fluka Biochemika (Buchs, Switzerland) and used as received. 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN, >95%) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). β-Carotene (>95%), tetramethylammonium hydroxide (>97%), and natural soybean phospholipid mixture (L-α-phosphatidylcholine (PC) content ~23%) were purchased from Sigma-Aldrich. Sodium chloride, sodium hydroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, acetic acid, boric acid, and phosphoric acid were supplied by Beijing Chemical Works (Beijing, China). Methanol (HPLC grade, Caledon Laboratories Ltd., Ontario, Canada), ethanol, and 1-octanol (AR; both from Beijing Chemical Works) were used as received, whereas chloroform (AR; Beijing Chemical Works) was purified by distillation before use. To increase the solubility in aqueous solution, all six selected plant (iso)flavonoids predissolved in dimethyl sulfoxide (DMSO) or ethanol (AR; both from Beijing Chemical Works) were added to ion-exchanged water from a Milli-Q Academic water purification system (Millipore Corp., Billerica, MA). For laser flash photolysis, conjugated bases of the six selected (iso)flavonoids in methanol/chloroform binary solvents (1:9 v/v) were prepared by adding 1 equiv of tetramethylammonium hydroxide.

Molar Extinction Coefficient, Log_{10} Distribution Coefficient, and pK_a. For all measurements, degassed solutions were used. To determine the molar extinction coefficient (ε), all six (iso)flavonoid samples weighed with an accuracy of 0.01 mg using an AB-135S balance (Mettler Toledo, Urdorf, Switzerland) were predissolved in DMSO and then diluted with ion-exchanged water. UV−visible spectra of the preparations with varying (iso)flavonoid concentrations were recorded with a standard quartz cuvette (optical path length = 1 cm) on a Cary50 spectrophotometer (Varian Inc., Palo Alto, CA), and ε was determined on the basis of the Lambert−Beer law by linear regression fitting to the data of absorbance versus absolute compound concentration ($(1.0-5.0) \times 10^{-5}$ M).

UV−visible absorption spectra of all six (iso)flavonoids in 1-octanol were measured before and after the extraction with aqueous phase (0.01 M phosphate buffer solution at pH 7.4), and the $\log_{10}$ distribution coefficient ($\log D_{7.4}$) was obtained using the relationship

$$\log D = \log \frac{\sum [\text{sample}]_{\text{org}}}{\sum [\text{sample}]_{\text{aq}}} = \log \frac{[\text{sample}]}{[\text{sample}]_0 - [\text{sample}]}$$

where subscripts "org" and "aq" stand for the organic and aqueous phases, respectively, and $[\text{sample}]_0$ and [sample] for the concentrations of (iso)flavonoid in the organic phase before and after the aqueous extraction, respectively.

To determine pK_a, all six (iso)flavonoids (~5.0×10^{-5} M) in Britton−Robinson buffer (0.1 M) were prepared over a pH range of 5.0−12.0 with the ionic strength controlled at 0.1 M using NaCl. During the spectroscopic and pH measurements samples were regulated at 25 °C by the use of a water-flow type thermostat (RTE-100, Neslab Instruments Inc., Newington, NH). The pK_a values were determined by fitting the data of specific absorbance against pH to the relationship derived from the acid−base equilibrium

$$\text{Abs} = \sum_{i=0}^{n} \left[A_i \frac{[\text{H}^+]^{n-i} \prod_{j=1}^{i} K_{aj}}{\sum_{i=0}^{n} ([\text{H}^+]^{n-i} \prod_{j=1}^{i} K_{aj})} \right]$$

where K_{aj} are dissociation constants and A_i are fitting parameters related to the molar absorptivity at specific wavelength for neutral or conjugated bases of polyphenols.

Naringenin (5,7,4′-trihydroxyflavanone) was included in the determination as a standard for validation of the method for determination of the pK_a values due to the very prominent spectral differences between the various acid/base forms of naringenin (see the Supporting Information).

Antilipoxidation Activity and Synergistic/Antagonistic Interaction with β-Carotene. Liposome was prepared following refs *16* and *17* with certain modifications. Briefly, soybean L-α-phosphatidylcholine (0.75 mM) and/or β-carotene (7.5 μM) in chloroform was mixed with AMVN (32 mM) in methanol, and the volume ratio of chloroform and methanol was 2:1. Solvents were removed under reduced pressure (0.01 MPa) with protection by high-purity nitrogen by using a rotary evaporator at the bath temperature of 22 °C. Afterward, high-purity nitrogen was introduced to re-establish the atmospheric pressure. The flask was covered with aluminum foil and kept in an ice−water bath under vacuum for 1.5 h.

The lipid residues with or without β-carotene were rehydrated with 0.01 M phosphate buffer solution (pH 7.4) under sonication, then 25 μL of ethanol solution of (iso)flavonoid (3.0×10^{-4} M) was added to 5.00 mL of liposome suspension, and the ethanol vehicle was 0.5% v/v. The preparation was passed through hydrophilic polyethersulfone membranes (200 nm pore size) 20 times using Acrodisc syringe filters (Pall Corp., East Hills, NY) and then incubated for 90 min under room temperature and reduced light and oxygen partial pressure. The final concentration of lecithin was 0.15 mM, and that of AMVN was 3.2 mM. The final β-carotene or (iso)flavonoid concentrations were 1% of the molar fractions of lecithin.

Following ref *18*, the time evolution profiles of AMVN-induced lipid peroxidation were traced by the absorbance of conjugated diene at the characteristic wavelength of 234 nm, and 3.0 mL of the suspension in a sealed 1 cm quartz cuvette was thermostated at 43 °C during the measurements. Liposomal preparation without antioxidant was used as a reference. The induction period (IP, in min) was determined as the time elapsed to the intersection of the tangent of the propagation phase and that of the induction phase and was corrected by subtracting the IP of the reference. The interaction between (iso)flavonoid and β-carotene with equal concentration was studied, and the synergistic effect could be confirmed when the relationship $IP_{(\text{iso})\text{flavonoid}+\beta\text{-carotene}} > IP_{(\text{iso})\text{flavonoid}} + IP_{\beta\text{-carotene}}$ was satisfied.

Laser Flash Photolysis. The experimental setup has been described in detail elsewhere (*19*). Briefly, the pump laser pulse with 10 ns duration at 355 nm was supplied by a Nd:YAG laser operating at a repetition rate of 10 Hz (Quanta-Ray Pro-Series, Spectra Physics Lasers Inc., Mountain View, CA), and the pulse energy to excite the sample was ~2.8 mJ. β-Carotene (12.5 μM) and (iso)flavonoid monoanion (50 μM) dissolved in the methanol/chloroform binary solvent (1:9, v/v) were kept in a quartz cuvette (optical path length = 1 cm) and stirred by the use of a magnetic stirrer. Near-infrared kinetics probed at 950 nm for β-carotene radical cation ($\beta\text{-Car}^{\bullet+}$) was detected with an avalanched photodiode (model C5460, Hamamatsu Photonics, Hamamatsu, Japan) attached to a TriVista spectrograph (Princeton Instruments, Trenton, NJ), and the kinetics traces were stored and averaged with a digital storage oscilloscope (bandwidth = 600 MHz; LeCroy WaveSurfer 64Xs, Chestnut Ridge, NY) connected to a personal computer. For kinetics analyses, the time evolution profiles of optical density change (ΔOD) upon pulsed excitation were fit to a three-exponential decay function

$$\Delta\text{OD} = -A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \exp(-k_3 t)$$

where A is the amplitude parameter and k is the pseudo-first-order rate constant. The program of least-squares curve fitting was coded on Matlab 5.3 (Mathworks Inc., Natick, MA), and the fitting goodness was evaluated by the use of χ^2 statistics.

Quantum Chemical Calculations. The molecular geometries of all six plant (iso)flavonoids and their radicals were optimized with the B3LYP density functional theory in conjunction with the 6-31G(d,p) basis set by use of the Gaussian 03 package (*20*). Gas-phase bond dissociation enthalpy (BDE) and deprotonation enthalpy (DE) were calculated as the enthalpy difference of the processes $\text{ArOH} \rightarrow \text{ArO}^{\bullet} + \text{H}^{\bullet}$ and $\text{ArOH} \rightarrow \text{ArO}^{-} + \text{H}^{+}$, respectively (*3*).

RESULTS

Quercetin, baicalein, and daidzein have, together with their glycosides rutin, baicalin, and puerarin, the form in which they appear in various plants, been characterized with respect to UV−visible absorption spectra for aqueous solutions at various pH values. From the molecular structures given in **Figure 1**, it may

Table 1. Molar Absorptivities (ε in L mol^{-1} cm^{-1} at λ_{max} Given), 1-Octanol/Water Distribution Coefficients at pH 7.4 (log$D_{7.4}$), Dissociation Constants of Phenol Groups (pK_a) at Ionic Strength 0.1 M and 25 °C, Energy (Gap) of Frontier Orbitals (E_{HOMO}, E_{LUMO}, and $E_{HOMO}-E_{LUMO}$ in eV), and Trolox Equivalent Antioxidation Capacities (TEAC) of (Iso)flavonoid Samples

	daidzein	puerarin	baicalein	baicalin	quercetin	rutin
ε (band I)	2.6×10^4 (248 nm)	2.3×10^4 (249 nm)	2.4×10^4 (274 nm)	2.2×10^4 (276 nm)	2.3×10^4 (255 nm)	2.1×10^4 (256 nm)
ε (band II)	1.0×10^4 (300 nm)	0.8×10^4 (305 nm)	1.6×10^4 (324 nm)	1.4×10^4 (317 nm)	2.4×10^4 (370 nm)	1.8×10^4 (358 nm)
log$D_{7.4}$	0.75	−0.31	0.84	0.55	0.90	<−2
pK_{a1}	7.3 ± 0.1	7.1 ± 0.1	5.4 ± 0.1	7.6 ± 0.1	5.7 ± 0.1	6.2 ± 0.1
pK_{a2}	9.2 ± 0.1	9.5 ± 0.1	9.8 ± 0.1	10.1 ± 0.1	7.0 ± 0.1	7.6 ± 0.1
pK_{a3}			11.3 ± 0.2		9.6 ± 0.1	10.1 ± 0.1
pK_{a4}					11.8 ± 0.2	11.3 ± 0.2
E_{HOMO}	−5.65	−5.57	−5.74	−5.52	−5.48	−5.45
E_{LUMO}	−1.37	−1.29	−1.95	−1.93	−1.84	−1.55
$E_{HOMO} - E_{LUMO}$	4.28	4.28	3.79	3.59	3.64	3.90
TEAC	1.9	2.1 (*21*)	2.0 (*26*)	1.1 (*26*)	4.8 (*27*)	2.6 (*27*)

Table 2. DFT-Calculated AC-to-B Dihedral Angles (α, in Degrees) for (Iso)flavonoid Neutral Molecules (F) and Their Phenolate Anions and Deprotonation Enthalpies (DE, in kJ mol^{-1}) in Gas Phase for Hydroxyl Groups of (Iso)flavonoids[a]

	daidzein		puerarin		baicalein		baicalin[b]		quercetin		rutin	
	α	DE	α	DE	α	DE	α	DE	α	DE	α	DE
F	36.4		36.7		0.0		20.1		0.0		21.4	
3′-O$^-$									0.0	1442.9	22.4	1428.8
4′-O$^-$	27.6	1498.5	27.7	1501.1					0.0	1410.6	13.2	1416.4
5-O$^-$					0.0	1498.4	8.2	1624.2	0.0	1498.4	24.3	1495.0
6-O$^-$					0.0	1464.7	6.1	1593.8				
7-O$^-$	33.6	1448.7	34.1	1445.6	0.0	1467.9			0.0	1437.6	24.9	1440.0

[a] All calculations are on the basis of B3LYP method and 6-31G(d,p) basis set. [b] In calculation of baicalin phenolate anions, the 6″-carboxyl is in its anionic form.

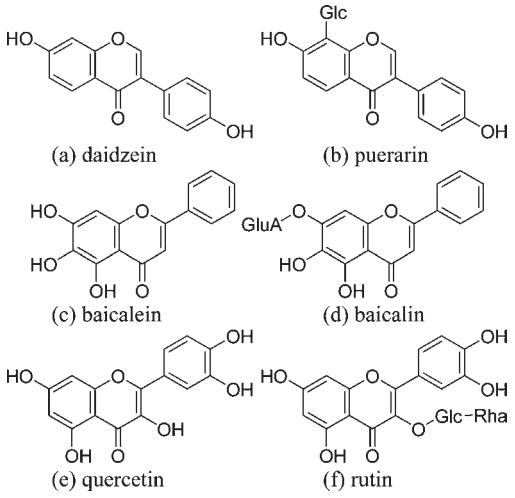

Figure 1. Molecular structures of daidzein (**a**), puerarin (**b**), baicalein (**c**), baicalin (**d**), quercetin (**e**), and rutin (**f**), where Glc =D-glucose, GluA = D-glucuronic acid, and Rha =L-6-deoxymannose (L-rhamnose).

be seen that daidzein and its *C*-glycoside puerarin both have two phenol functionalities and that both the absorption spectra (see the Supporting Information) and the pK_a values, as calculated from the absorption spectra and presented in **Table 1**, are very similar. This is in contrast to the baicalein/baicalin couple for which the aglycone has three phenol groups and the glycoside only two, resulting in marked differences in the pH dependence of the absorption spectra and the pK_a values, with the aglycone being significantly more acidic. A similar pattern is seen for quercetin/rutin, although less pronounced because the number of phenolic groups is the same and the glycoside bond is formed with the enol group of quercetin. Some uncertainties appear in the literature concerning pK_a values of (iso)flavonoids (*3*, *21*−*23*). Naringenin was selected as a standard compound for method validation, and the pK_a values obtained (pK_{a1} = 7.0 ± 0.1, pK_{a2} = 9.6 ± 0.1, and pK_{a3} = 11.4 ± 0.2 assigned to 7-OH, 4′-OH, and 5-OH, respectively) are in acceptable agreement with previous values reported especially for pK_{a1}. Notably, pK_{a2} and pK_{a3} are sensitive to oxidation reactions under the alkaline conditions prevailing for their determination, and it is accordingly mandatory to determine values for these constants under anaerobic conditions as in the present study. Quercetin and rutin were found to have lower pK_{a1} values than previously reported (*22*). However, these lower values are supported by the deprotonation enthalpies (DE) calculated (**Table 2**). The ordering of the lowest DE for the (iso)flavonoids studied is quercetin < rutin < puerarin < daidzein < baicalein < baicalin, which is in agreement with the ordering of the pK_{a1} values except for baicalein, which is more acidic than expected on the basis of the DE values. These theoretical calculations support accordingly the experimental determined pK_a values. For baicalein, specific hydrogen bonding effects may stabilize the 6-phenolate anion in aqueous solution for the *vicinal*-triphenol in the A-ring. The compounds were further characterized by their 1-octanol/water distribution coefficients at pH 7.4 (log$D_{7.4}$).

On the basis of DFT calculations, the bond dissociation enthalpy (BDE) of the individual phenolic groups in the six compounds were further estimated together with the dipole moment, μ, and the AC-to-B dihedral angle, α, and the results are presented in **Figure 2** as optimized molecular structures, together with their numerical values in **Table 3**. In addition, the energy gaps, $E_{HOMO} - E_{LUMO}$, also derived from the DFT calculations, are given in **Table 1**, which also includes literature values for the capacity of the individual compounds as antioxidants as determined by reduction of the ABTS$^{\bullet+}$ radical and quantified as Trolox equivalent antioxidant capacity (TEAC) (*24*, *25*). According to the BDE, the ease by which the compounds may donate a hydrogen atom follows the order rutin ≈ quercetin > baicalein > baicalin > puerarin > daidzein. The energy gap $E_{HOMO} - E_{LUMO}$ relates to the ease by which an electron is donated by the

Table 3. DFT-Calculated AC-to-B Dihedral Angles (α, in Degrees) and Dipole Moments (μ, in Debye) for (Iso)flavonoid Neutral Molecules (F) and Their Phenoxyl Radicals and Bond Dissociation Enthalpies (BDE, in kJ mol^{-1}) in Gas Phase for Hydroxyl Groups of (Iso)flavonoids[a]

	daidzein			puerarin			baicalein			baicalin			quercetin			rutin		
	α	μ	BDE	α	μ	BDE	α	μ	BDE	α	μ	BDE	α	μ	BDE	α	μ	BDE
F	36.4	3.16		36.7	5.41		0.0	2.92		20.1	6.59		0.0	0.40		21.4	6.21	
3′-O•													0.0	4.39	340.4	26.2	4.95	339.4
4′-O•	30.8	8.10	368.4	30.9	10.77	367.4							0.0	4.53	329.5	18.6	6.71	332.7
5-O•							0.0	5.37	402.8	20.7	9.01	394.1	0.0	3.58	430.5	21.2	3.28	479.0
6-O•							0.0	7.04	336.6	19.8	10.28	361.4						
7-O•	35.7	3.39	386.9	36.0	2.03	402.2	0.0	5.66	385.9				0.0	7.00	393.4	16.9	11.62	392.6

[a] All calculations are on the basis of B3LYP method and 6-31G(d,p) basis set.

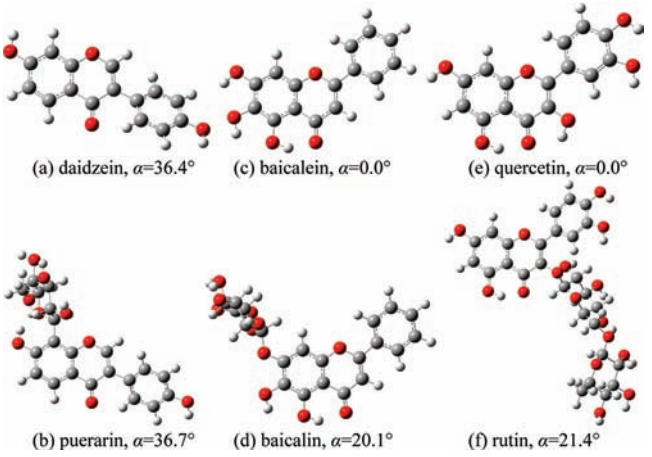

Figure 2. DFT-optimized molecular geometries of neutral daidzein (**a**), puerarin (**b**), baicalein (**c**), baicalin (**d**), quercetin (**e**), and rutin (**f**) using B3LYP method and 6-31G(d,p) basis set, where α denotes the AC-to-B dihedral angle.

compound, and the ordering is seen to be different from the ordering of the BDE: baicalin > quercetin > baicalein > rutin > daidzein = puerarin. The isoflavonoid daidzein and its glycoside puerarin are both seen to be low ranking according to both hydrogen atom transfer tendency (BDE) and electron donation (energy gap) and are accordingly expected to be poor antioxidants.

Notably, the BDE of the 4′-OH in daidzein is hardly affected by glycosation as the value for BDE of puerarin is similar to the value of daidzein. This is in contrast to baicalein, for which glycosation increases the BDE of the 6-OH, making baicalin a poorer hydrogen atom donor than baicalein. This difference between the two (iso)flavonoid/glycoside couples is clearly reflected in the respective TEAC values, which for daidzein/puerarin are very similar (*21*), whereas for baicalein/baicalin, the glycoside (baicalin) with the higher BDE has lower TEAC value (*26*). Similarly, the value of the AC-to-B dihedral angle for daidzein/puerarin is not affected by glycosation as seen for both the native molecule and for the phenoxyl radicals, whereas glycosation of the planar baicalein to yield baicalin induces a significant deviation from planarity. For the quercetin/rutin couple, similar effects are noted, as glycosation of the planar quercetin induces deviation from planarity with a concomitant decrease in TEAC value (*27*). Apparently, planarity facilitates conjugation in the (iso)flavonoids as compared to their glycosides, in effect making the (iso)flavonoids better antioxidants than their glycosides as reflected in the TEAC values.

The antioxidant efficiency for the six compounds was determined in L-α-phosphatidylcholine (PC) liposomes, in which oxidation was initiated thermally in the lipid phase by the free radical

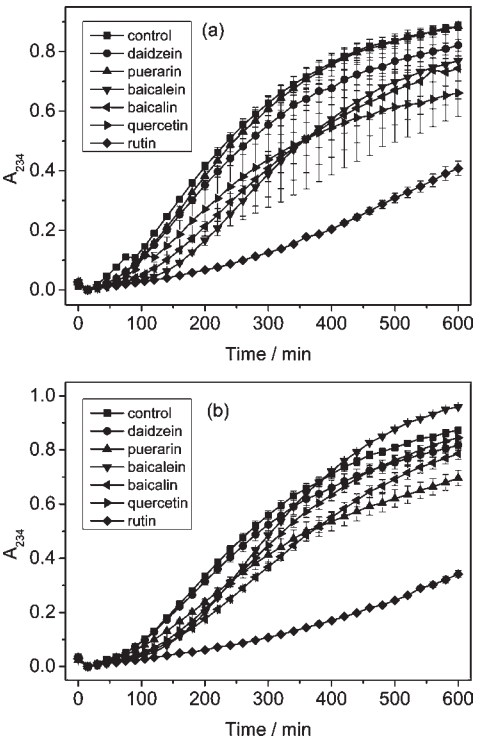

Figure 3. Time evolution profiles of conjugated diene absorption at 234 nm arising from the lipoxidation initiated by AMVN inside membrane in L-α-phosphatidylcholine (PC) liposomes without β-carotene (**a**) and with β-carotene added (**b**). Final concentrations: [PC] = 1.5 × 10^{-4} M, [AMVN] = 3.2 mM, [antioxidant] = 1% molar fraction of PC. Statistics: mean ± SD, n = 3.

initiator AMVN, and oxidation monitored as the appearance of conjugated dienes as indicative of formation of lipid hydroperoxides as a primary lipid oxidation product (*18*). The efficiencies of the individual phenolic compounds and β-carotene were quantified as the induction period (IP) for the appearance of conjugated dienes (see **Figure 3**) (*26*). The values for IP as determined for liposomes with an aqueous phase of pH 7.4 are presented in **Table 4**, showing the following ordering of the investigated compounds as antioxidants: rutin > baicalein > baicalin > quercetin > daidzein > puerarin, an ordering that does not simply reflect the BDE or the $E_{HOMO} - E_{LUMO}$ energy gap, indicating kinetic control of reaction. In the presence of β-carotene, the ordering is very similar, rutin > baicalein > baicalin > quercetin > puerarin > daidzein (> β-carotene), as only the positions of puerarin and daidzein are reversed and β-carotene itself is less efficient alone than together with any of the (iso)flavonoids. However, β-carotene enhances the efficiency of some of the (iso)flavonoids as evidenced by the calculated

Table 4. Induction Periods (IP) Determined by Spectrophotometric Measurements of Conjugated Diene in Soybean L-α-Phosphatidylcholine Liposomes with or without β-Carotene with Free Radical Initiation by AMVN in the Lipid Phase and the Synergism between (Iso)flavonoids and β-Carotene (See **Figure 4**)[a]

	induction period/min				
sample	without β-carotene	with β-carotene	synergism/%	f	k_{eff}/s^{-1}
control	0.0	20.3 ± 1.1			
daidzein	11.8 ± 1.7	22.8 ± 3.6	−(29 ± 22)	0.15	4.7×10^2
puerarin	9.3 ± 1.5	43.5 ± 1.7	+(47 ± 19)	0.67	2.4×10^3
baicalein	77.1 ± 2.5	77.5 ± 1.0	−(20 ± 5)	0.13	9.7×10^2
baicalin	58.5 ± 3.1	76.4 ± 4.2	−(3 ± 10)	0.22	1.1×10^3
quercetin	22.8 ± 3.6	65.3 ± 2.3	+(52 ± 22)	0.11	1.2×10^3
rutin	199.6 ± 4.8	290.7 ± 5.3	+(32 ± 6)	0.99	1.1×10^5

[a] f is the fraction of antioxidant present in the aqueous phase and $k_{eff}(=k_2 f)$ the rate constant for regeneration of β-carotene from β-Car$^{\bullet+}$ corrected for distribution.

antioxidant synergism according to

$$\text{antioxidant synergism} = \frac{IP_{\beta\text{-carotene} + \text{(iso)flavonoid}} - (IP_{\beta\text{-carotene}} + IP_{\text{(iso)flavonoid}})}{IP_{\beta\text{-carotene}} + IP_{\text{(iso)flavonoid}}}$$

and presented in **Table 4**. Notably, β-carotene, being moderately efficient as an antioxidant itself, enhances especially the effect of quercetin and puerarin as antioxidants, whereas the effect of β-carotene on daidzein and baicalein is clearly antagonistic.

Previously it was demonstrated that the synergy between β-carotene and puerarin in liposomes occurs only for oxidation initiated by lipophilic initiators as also used in the present study (*3*). It was suggested that the carotenoid radical cation formed by electron donation from the carotenoid to lipid or initiator radicals in the lipid phase as part of the antioxidant action was regenerated by puerarin in the lipid/water interface. This regeneration reaction seems to be crucial for the interaction of β-carotene with (iso)flavonoids in general as antioxidants and was accordingly studied using real-time kinetic methods as previously described (*3*). β-Carotene forms, when excited in an electron-accepting solvent such as chloroform, the radical cation.

$$\beta\text{-Car} \xrightarrow{h\nu} \beta\text{-Car}* \rightarrow \beta\text{-Car}^{\bullet+} + e^-(\text{solvent})$$

Using laser flash techniques, the regeneration of β-carotene from the radical cation by (iso)flavonoids was followed by transient absorption spectroscopy in the near-infrared region, where β-Car$^{\bullet+}$ absorbs. The (iso)flavonoids did not react with β-Car$^{\bullet+}$, but upon addition of 1 equiv of hydroxyl ions, the more reductive monoanion form of the (iso)flavonoids was formed, which each reacted readily. The formation and decay of β-Car$^{\bullet+}$ in the absence and presence of (iso)flavonoid monoanion as shown in **Figure 4** for conditions of excess (iso)flavonoid monoanion could be accounted for by a three-exponential expression.

$$\Delta OD = -A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \exp(-k_3 t)$$

The formation of the β-carotene radical cation corresponding to the first term is fast and almost independent of the nature of the (iso)flavonoid present. The decay curves have two components, both in the microsecond time regime. The rate constants and the amplitudes obtained from the nonlinear regression analysis are found in **Table 5**. Clearly, the reaction between β-Car$^{\bullet+}$ and the (iso)flavonoid monoanion depends on the nature of the (iso)flavonoid. On the basis of a comparison with the rate constants previously obtained for the dianion of puerarin and daidzein with

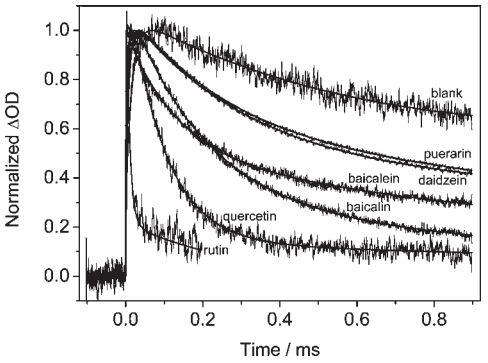

Figure 4. Decay kinetics of β-Car$^{\bullet+}$ in methanol/chloroform binary solvent (1:9, v/v) with the addition of conjugated bases of the six selected (iso)flavonoids (50 μM). Excitation and probing wavelengths were 355 and 950 nm, respectively. Smooth lines are three-exponential fitting curves. Each curve is a mean of two experiments.

Table 5. Pseudo-First-Order Decay Rate Constants and Amplitudes of β-Car$^{\bullet+}$ in Methanol/Chloroform Binary Solvent (1:9, v/v) in the Presence of 50 μM Conjugated Bases of the Six Selected (Iso)flavonoids

sample	k_2/s^{-1}	k_3/s^{-1}	A_2	A_3
blank	$(1.29 \pm 0.28) \times 10^3$		0.055	
daidzein	$(3.16 \pm 0.04) \times 10^3$	$(1.53 \pm 0.17) \times 10^2$	0.082	0.056
puerarin	$(3.60 \pm 0.04) \times 10^3$	$(3.03 \pm 0.11) \times 10^2$	0.077	0.075
baicalein	$(7.46 \pm 0.11) \times 10^3$	$(5.83 \pm 0.11) \times 10^2$	0.021	0.020
baicalin	$(4.98 \pm 0.05) \times 10^3$	$(6.68 \pm 0.31) \times 10^2$	0.039	0.013
quercetin	$(1.07 \pm 0.01) \times 10^4$	$(4.14 \pm 0.39) \times 10^2$	0.016	0.002
rutin	$(1.14 \pm 0.05) \times 10^5$	$(3.70 \pm 0.34) \times 10^2$	0.0078	0.0015

β-Car$^{\bullet+}$, the fast component will be considered as the rate constant for the primary reaction between the (iso)flavonoid monoanion and β-Car$^{\bullet+}$, whereas the slower component may be due to a parallel reaction with components in equilibrium with the monoanion such as the neutral form. The fast component has, moreover, in each case the larger amplitude. The reactivity of the six (iso)flavonoid monoanions spans more than one decade according to the numerical value of k_2. Notably, these rate constants are pseudo-first-order rate constants and depend on the concentration of excess (iso)flavonoid monoanion (*15*), but will allow for a comparison among the six compounds.

DISCUSSION

Antioxidant efficiency of the six (iso)flavonoids and antioxidant synergism (or antagonism) with β-carotene in phosphatidylcholine liposomes as a model for membranal systems did not correlate in a simple way with the BDE of the O−H bond of the (iso)flavonoids or their HOMO/LUMO energy gap as obtained from the DFT calculations as indicators of tendency for hydrogen atom transfer or electron donation, respectively. The rate constants for regeneration of β-carotene from β-Car$^{\bullet+}$, as formed under oxidative stress by electron transfer or through breakdown of carotenoid/radical adducts, by the monoanions of the (iso)flavonoids as studied by laser flash photolysis and applying to the reaction

$$\beta\text{-Car}^{\bullet+} + R-O^- \rightarrow \beta\text{-Car} + R-O^{\bullet}$$

show a somewhat better correlation with the nonadditive effect observed for combination of β-carotene and (iso)flavonoid monoanions as antioxidants. However, puerarin, which regenerates β-carotene slowly, shows at the same time a significant synergism with β-carotene in contrast to daidzein, which with a

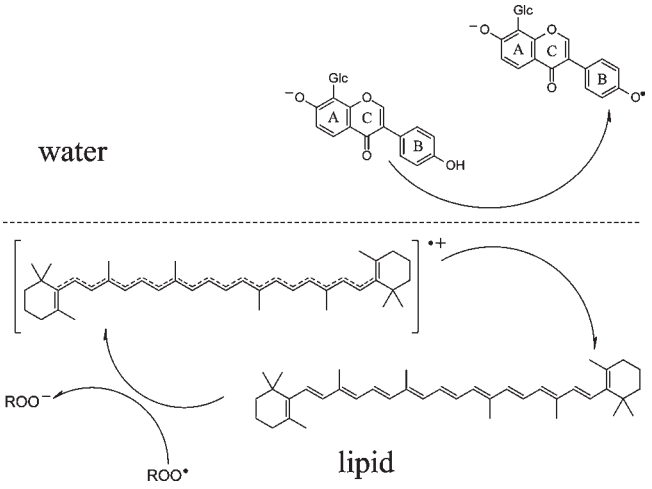

Figure 5. Puerarin orientation near the water/lipid interface with the more reducing 4′-hydroxyl group in the hydrophobic part of the molecule embedded in the lipid phase may facilitate regeneration of β-carotene from the β-carotene radical cation slightly more hydrophilic than β-carotene. The B-ring radical of the oxidized puerarin will, according to spin density calculations, delocalize in the full A−C−B ring system: [ACB]• (*29*). ROO• is a peroxyl radical of an unsaturated lipid.

similar rate constant shows a clear antagonism. Other factors clearly have to be taken into account, considering that β-carotene acts as an antioxidant in the lipid phase by reactions with lipid radicals.

$$\mathrm{LOO^{\bullet}} + \beta\text{-Car} \rightarrow \mathrm{LOO^{-}} + \beta\text{-Car}^{\bullet+}$$

Other lipid-soluble antioxidants such as baicalin (Φ-OH) may simply react in parallel reactions

$$\mathrm{LOO^{\bullet}} + \Phi\text{-OH} \rightarrow \mathrm{LOOH} + \Phi\text{-O}^{\bullet}$$
$$\mathrm{LOO^{\bullet}} + \Phi\text{-O}^{-} \rightarrow \mathrm{LOO^{-}} + \Phi\text{-O}^{\bullet}$$

resulting in additive antioxidative effects. A regeneration of β-carotene should occur with contributions from the water phase, and the distribution between water and lipid becomes important, for which the distribution coefficient applies

$$D = \frac{\sum[\text{antioxidant (lipid)}]}{\sum[\text{antioxidant (water)}]} = \frac{c-x}{x} = \frac{1-f}{f}$$

and in which $f = x/c$ is the fraction of the antioxidant in the water phase and $1-f$ accordingly the fraction present in the lipid phase.

Because the rate of regeneration of β-carotene depends on the concentration of the other antioxidant in the aqueous phase near the water/lipid interface, the lipid/water distribution may be corrected for according to

$$\text{rate} = k_2[\beta\text{-Car}^{\bullet+}]c_{\text{antioxidant}}f = k_{\text{eff}}[\beta\text{-Car}^{\bullet+}]c_{\text{antioxidant}}$$

The rate constant determined in homogeneous methanol/chloroform solution, k_2, should accordingly be corrected to be valid in the water phase of a heterogeneous system by

$$k_{\text{eff}} = k_2 f$$

Such a correction has been done and the value of k_{eff} added to **Table 4**. The ordering of k_{eff} as seen in **Table 4** is rutin > puerarin > quercetin > baicalin > baicalein > daidzein, which is similar to the ordering for nonadditive effects seen as antioxidant synergism/antagonism: quercetin ≈ puerarin > rutin > baicalin > baicalein > daidzein. The suggested mechanism of β-carotene regeneration leading to antioxidant synergism with (iso)flavonoids such as puerarin is depicted in **Figure 5**. A more quantitative agreement is, however, not to be expected, because the rate for the β-carotene regeneration reaction is determined in homogeneous methanol/chloroform solution, whereas the synergism according to the proposed mechanism depends on regeneration at the lipid/water interface. As for the observed antagonism, the decrease in antioxidative efficiency is suggested to be the result of adduct formation between β-carotene radicals and the more lipophilic (iso)flavonoids in the lipid phase, in effect decreasing the active antioxidant concentration (*28*).

For antioxidant regeneration in heterogeneous systems leading to antioxidant synergism, kinetic factors are accordingly important provided that the regenerating antioxidant is more reducing than the antioxidant to be regenerated and active as primary antioxidant in the lipid phase. β-Carotene is according to the electron donor/acceptor classification of regulators of biological oxidation among the best radical scavengers as both a good donor and a good acceptor (*12*). However, it is not only a chain-breaking antioxidant but, due to its capacity to react with oxygen, generating peroxyl radical, also chain-carrying depending on oxygen pressure. In the liposome system, the chain-breaking function is dominant and becomes further enhanced by regeneration by a(n) (iso)flavonoid belonging to the group of good electron donors (*14*). Notably, the important conclusion of the present investigation is that when distribution phenomena are taken into account, a more accurate kinetic description of the important regeneration mechanism for β-carotene by (iso)flavonoids can be obtained.

ABBREVIATIONS USED

DFT, density functional theory; BDE, bond dissociation enthalpy; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); β-Car, β-carotene; DMSO, dimethyl sulfoxide; TEAC, Trolox equivalent antioxodation capacity; PC, L-α-phosphatidylcholine.

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Supporting Information Available: Validation procedure for determination of pK_a values and UV-vis absorption spectra of various (iso)flavonoids and glycosides. This material is available free of charge via the Internet at http://pubs.acs.org.

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