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Different antioxidant effects of polyphenols on lipid peroxidation and hydroxyl radicals in the NADPH-, Fe-ascorbate- and Fe-microsomal systems

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Abstract

Antioxidant and pro-oxidant effects of 14 naturally occurring polyphenols (PP) on rat liver microsomal lipid peroxidation (LP) and hydroxyl radical (•OH) production were studied in NADPH-dependent, 50 μM Fe^{2+}–500 μM ascorbate (Fe–AA) or 50 μM Fe^{2+} system, respectively. LP determined by the thiobarbituric acid method was inhibited in the NADPH system by flavonols and *trans*-resveratrol that were more effective than other flavonoids and derivatives of benzoic and cinnamic acid and were mostly more efficient than in the Fe–AA system. Inhibition of LP in the Fe system was higher by one order of magnitude than in the Fe–AA system. •OH production in the NADPH system, measured by formaldehyde production, was decreased by myricetin, fisetin and quercetin, but increased by kaempferol, morin and *trans*-resveratrol, indicating that •OH played a minor role in LP, which all of these PP inhibited. None of these PP at up to 40 μM concentration quenched •OH in the Fe–AA system. All tested PP, except *trans*-resveratrol and gentisic acid, spectrally interacted with Fe^{2+} or Fe^{3+}, indicating formation of complexes or oxidation of PP. In contrast to the NADPH system we found no correlation between Fe^{2+} chelation and inhibition of Fe–AA- or Fe-dependent LP indicating that iron chelation did not play a significant role in the two latter systems. It is concluded that the inhibition of LP by PP was apparently due to their hydrogen donating properties rather than chelation of iron.

Keywords: Antioxidants; Polyphenols; Vitamins; Lipid peroxidation; Hydroxyl radical; Iron ions

1. Introduction

Epidemiological and animal studies and *in vitro* experiments revealed that polyphenols (PP) present in certain kinds of fruit and vegetables possess antioxidant properties including ROS quenching and inhibition of lipid peroxidation (LP), and exert anticarcinogenic, antimutagenic, antitumoral, antibacterial, antiviral and anti-inflammatory effects [1,2].

Under physiological conditions of the organism, various reactive oxygen species (ROS, e.g. $O_2^{\bullet -}$, H_2O_2, •OH) are formed and decomposed by enzymatic and non-enzymatic reactions. Increased levels of ROS can initiate LP, damage proteins and DNA, thereby causing acute tissue damage or development of various diseases. Fe and other transient metals participate in ROS formation and LP. The •OH radical is very reactive (rate constant for •OH reaction with linoleate is 8×10^9 M^{-1} s^{-1}) [3], but the role of •OH and other ROS in LP initiation [4–6] is not clear [7–9]. •OH may be formed in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$). Fe ions can initiate LP directly [6,10] or *via* complexes between oxygen and various Fe redox states. They include the ferryl ion $(FeO)^{2+}$ or $FeOH^{3+}$ [11,12], perferryl ion ($Fe^{2+}O_2$ or Fe^{3+}:O_2^-) [5,13–15] or $Fe^{4+}O$ [11,12]. The molar ratio between Fe^{2+} and Fe^{3+} is of significance according to some authors [8,12,16]. Fe^{2+} decompose LP intermediate hydroperoxides (ROOH) to RO• (alkoxyl) radicals and Fe^{3+} to less potently oxidizing ROO• (peroxyl) radicals [17].

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Abbreviations: •OH, hydroxyl radical; AA, ascorbic acid; CYP, cytochrome P-450; Ep/2, half peak oxidation potential; LP, lipid peroxidation; MDA, malondialdehyde; O_2^-, superoxide anion radical; PP, polyphenols; RO•, alkoxyl radical; ROO•, peroxyl radical; TBARS, thiobarbituric acid-reactive substances.

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Fe occurs in the organism mostly bound to hemoglobin or tissue ferritin (68 and 27% of total iron, respectively), the physiological concentration of Fe in the plasma is 10–25 μM [18]. It can be released from these complexes by ROS, AA or lipid peroxides [4]. Free Fe can react with AA. After daily intake of 100 mg AA, an about 57 μM concentration was found in the serum [19], while the concentration in the nervous tissue was about 1 mM [20].

Antioxidant properties of PP were reported by various authors [21–31], but their true physiological effects are difficult to estimate for the use of various systems (microsomes, liposomes, LDL, hepatocytes), problems with some IC_{50} being outside the solubility range of some very hydrophobic PP, complications with the use of some artificial radicals whose actual reactions with antioxidant remain uncertain, limited series of PP in one study, etc. Variability of IC_{50} of the same PP in different studies and relatively small differences in IC_{50} of different PP may limit validity of published structure–activity interpretations. Also, in different systems of ROS generation used in literature (the Fenton system, $H_2O_2 + Fe^{3+}$:EDTA [32], UV photolysis of H_2O_2 [33] or pulse radiolysis [34]), different ROS may predominate and are generally analyzed only indirectly, since they escape direct identification. That complicates the interpretation of the relationship between ROS and LP and the site of PP action. Various authors consider chelation of Fe as the main mechanism of PP action [35–38], while others consider ROS quenching to dominate in the antioxidant effects [39].

In an attempt to resolve some of these uncertainties (1) we followed the antioxidant effects (IC_{50}) of PP on microsomal LP in three systems resembling different situations in the organism. In an NADPH (CYP-dependent) system, the antioxidant effect may be related to CYP inhibition or direct quenching of ROS or LP radicals, and it could be decreased by PP metabolism. In the simple Fe^{2+} or Fe^{3+} system, Fe chelation by PP was expected to play a relatively higher role than in the two other systems. Our 50 μM Fe^{2+} and 500 μM AA system complies with omnipresence of ascorbate in blood and soft tissues. The aim was to establish IC_{50} in these systems and to compare them with the differing data in the literature. The true absolute IC_{50} values are important for evaluating the antioxidant efficiency of PP and its physiological relevance, possibly different in different compartments of the organism. (2) Mechanisms of antioxidant effects of the 14 PP under study were investigated by comparing the IC_{50} values for inhibition of LP with those for $^{\bullet}OH$ inhibition and with quantitatively estimated PP chelation of Fe^{2+} and Fe^{3+}, respectively. (3) Moreover, we wanted to contribute to understanding of the apparent controversy between the pro-oxidant effects of the Fe–AA complex and the accepted antioxidant properties of ascorbate by analyzing their various concentrations and their ratios in relation to pro-oxidant or antioxidant outcome. It seemed of particular interest for physiologically widely different concentrations of AA in blood and tissue and for different Fe concentrations in various disease states.

2. Materials and methods

2.1. Compounds

All polyphenols and most other chemicals of the highest purity were obtained from Sigma-Aldrich. BSA was purchased from Lachema and glucose-6-phosphate dehydrogenase was from Boehringer. All other reagents were of analytical grade.

2.2. Preparation of microsomes

Untreated rat liver microsomes were prepared by differential centrifugation [40], protein was estimated using BSA as a standard [41]. Total CYP content (0.6–0.7 nmol/mg protein) was estimated after Omura and Sato [42].

2.3. Inhibition of LP and $^{\bullet}OH$ production

The reaction mixtures for LP or $^{\bullet}OH$ production were incubated in glass test tubes under air in a shaking water bath for 60 min at 37°. The final concentrations in the samples were: phosphate buffer, 0.066 M (pH 7.4) in the Fe–AA and NADPH systems, but 0.001 M (pH 7.4) in the Fe system (higher concentration of phosphate buffer strongly inhibited LP (our unpublished data)), microsomal protein (1 mg/mL), and NADPH-generating system: 1 mM NADP, 10 mM glucose-6-phosphate, 10 mM $MgCl_2$, and glucose-6-phosphate dehydrogenase, EC 1.1.1.49, 0.5 U/mL. Alternatively, 50 μM Fe^{2+} + 500 μM AA, or 50 μM Fe^{2+}, freshly prepared before use, were used instead of the NADPH-generating system.

The formation of thiobarbituric acid-reactive substances (TBARS) was quantified by malondialdehyde (MDA) standards [43]. This method was previously revealed to be quite efficient, simple, quick and shows a good correlation with other methods used to evaluate LP [20]. The quantification of $^{\bullet}OH$ was based upon measurement of formaldehyde production from DMSO according to Khan *et al.* [44].

2.4. Checking of the solubility of polyphenols

Since some PP are weakly soluble and may decompose in water, their stock solutions in methanol (kept at −80°) were used, methanol was evaporated under N_2 and PP were dissolved in Milli-Q water. Up to 100 μM levels were reached after 10 min sonication at 25–35° in derivatives of acids, flavanols and resveratrol, but 55–75° was required to dissolve more hydrophobic flavonols. Their dissolution in incubation mixtures was checked by extraction of aliquots into ethylacetate and spectrophotometric measuring of PP concentration (Table 1).

Table 1
Molar extinction coefficients of PP in ethylacetate and water, respectively

Compound	Ethylacetate ε ($L\ mol^{-1}\ cm^{-1}$)/λ (nm)	H_2O ε ($L\ mol^{-1}\ cm^{-1}$)/λ (nm)
Myricetin	19568/370	8377/370
Morin	9100/355	6970/355
Quercetin	19336/370	16015/375
Rutin	16660/355	15038/350
Kaempferol	13600/345	13338/360
Fisetin	19814/350	Not dissolved
Naringenin	16280/285	15002/290
(+)-Catechin	4698/280	3440/280
(−)-Epicatechin	4410/280	3370/280
Gallic acid	9510/270	7972/265
Gentisic acid	4909/340	4628/320
Caffeic acid	15824/325	13226/315
p-Coumaric acid	16578/300	18158/290
trans-Resveratrol	20216/305	19406/305

One millimolar PP was dissolved in methanol, evaporated under N_2 and redissolved in Milli-Q water or ethylacetate. UV-Vis spectra of 10–100 μM PP were immediately measured.

2.5. Interaction of PP with iron ions

Interactions of PP with Fe ions in water were estimated from changes in UV-Vis polyphenol absorption spectra (220–600 nm) recorded after addition of freshly prepared water solution of $FeSO_4$ or $FeCl_3$. Since the complexes may not be formed immediately, spectral changes were measured immediately after iron ion addition and also after 1 hr (22°, daylight) and 24 hr later (4°, dark), respectively after incubation of PP with Fe ions. Reversal of the spectral changes by EDTA (a strong iron chelator) was used to confirm the chelation capacity of PP. The molar ratios of PP to Fe ions and EDTA, respectively, were (μM) 50:25:25, 50:50:50 or 50:100:100. The spectra were recorded with Spekord M400 spectrophotometer.

3. Results

The results revealed different antioxidant efficiency of PP against microsomal LP and •OH in the Fe^{2+}-depedent, Fe^{2+}–AA-dependent and NADPH-dependent systems, a different role of Fe-chelation in each of these systems, and revealed concentrations which govern the antioxidant or pro-oxidant action in the Fe^{2+}–AA system and suggested that in these systems •OH radicals played a negligible role in LP initiation.

3.1. LP and •OH production in the Fe and Fe–AA-dependent system

The 50 μM Fe^{2+}–500 μM AA system offered a suitable choice for being pro-oxidant, using levels reached *in vivo*. Fe^{2+} or Fe^{3+} ions alone did not stimulate LP in microsomes (data not shown), however, they increased •OH production in relation to 0–150 μM Fe levels (Fig. 1).

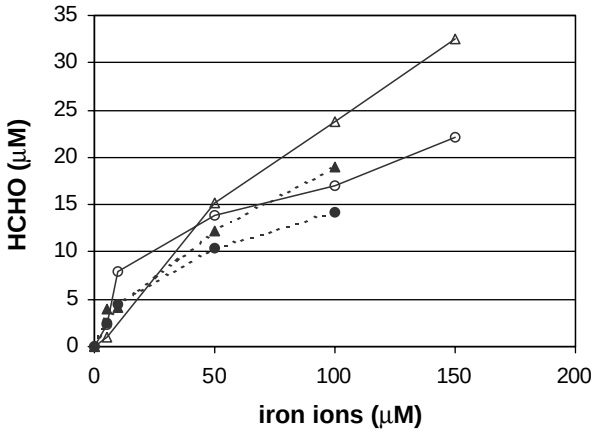


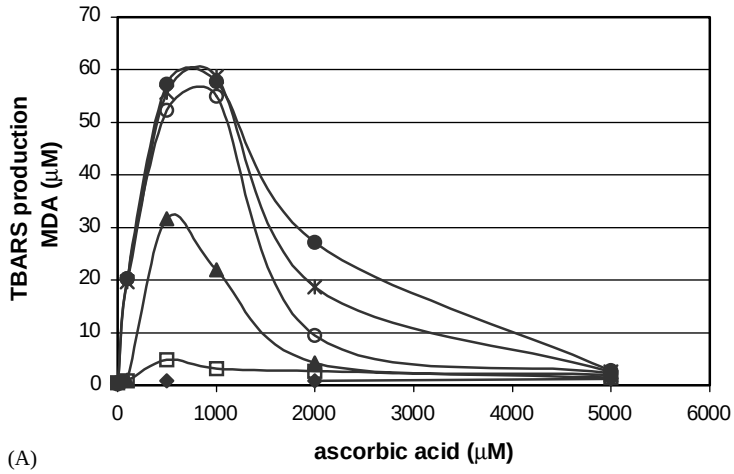
Fig. 1. Production of •OH by Fe^{3+} (○) or Fe^{2+} (△) ions expressed as concentration of formaldehyde (μM), the influence of microsomes present (- - -) or absent (—) in the incubation mixture. 0–150 μM iron ions with 66 mM phosphate buffer were incubated in presence or absence of microsomes for 60 min at 37°. •OH production was measured as described in Section 2.

The effect was slightly higher with Fe^{2+} than Fe^{3+} ions, and it was the same with or without microsomes. Likewise, AA alone did not stimulate LP, but 100–5000 μM AA stimulated •OH production in a saturated manner with the half-maximum concentration about 500 μM (Fig. 2A and B). Addition of Fe^{2+} to AA induced LP proportionally to 0–150 μM Fe^{2+}, and also up to 500–1000 μM AA, but LP decreased gradually to zero levels at 2000–5000 μM AA, even at 150 μM Fe. •OH production also increased in proportion to Fe levels, about 2.5-fold in the whole AA concentration range. In contrast to LP, the enhancement induced by AA upon the Fe^{2+}-catalyzed •OH production was 10-fold or higher up to 5000 μM AA.

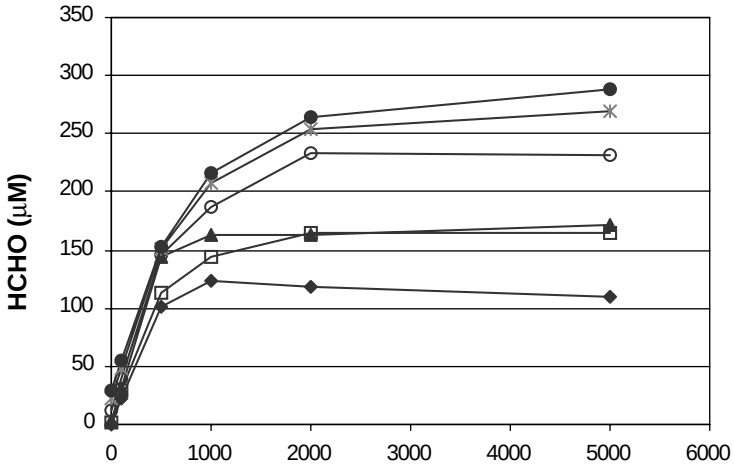
3.2. Effect of PP on Fe-, Fe–AA- or NADPH-dependent LP and •OH production

NADPH-enriched microsomes are a powerful enzyme oxidation system, where catalysis by CYP should be responsible for both of LP and •OH production. The LP was similar, but •OH production was up to 10-fold lower in the NADPH system than in the Fe–AA system.

In the Fe–AA-dependent and NADPH-dependent microsomal LP, flavonoids and *trans*-resveratrol were markedly more effective antioxidants than derivatives of gallic and cinnamic acid (Table 2). The order of flavonoid efficiency in the inhibition of LP in our Fe–AA system assayed by IC_{50} was resveratrol ⩾ kaempferol > epicatechin > morin > catechin > quercetin > myricetin > fisetin. In contrast, their efficiency in the NADPH system decrease in the order: quercetin > *trans*-resveratrol > fisetin > myricetin > morin > kaempferol > epicatechin > catechin. Flavonols were more effective than flavanols, while flavanone (naringenin) was inefficient at biologically relevant concentrations. In the eight most effective flavonoids the range of IC_{50} was similar, but their relative efficiencies were



(A)



(B)

Fig. 2. The effect of ascorbic acid and iron ions on LP in rat liver microsomes expressed as TBARS production (μM) (A) or production of •OH without microsomes expressed as formaldehyde production (μM) (B); concentration of Fe^{2+}(μM): 0 μM (◆), 5 μM (□), 15 μM (▲), 50 μM (○), 100 μM (✳) or 150 μM (●) Fe^{2+} were incubated with 0–5000 μM AA in the absence or presence of microsomes for 60 min at 37°. LP and •OH production were measured as reported in Section 2.

different in the NADPH- and Fe–AA systems, respectively. Several of our IC_{50} values for specific PP were outside the rather broad data range in the literature. However, the validity of our data is supported by 2- to 10-fold repetition of the assays. In the Fe^{2+}-dependent microsomal LP, flavanols and *trans*-resveratrol were most efficient, caffeic acid and gallic acid inhibited LP similarly like flavonols. Fe^{2+}-dependent LP was inhibited more effectively comparing with NADPH- or Fe–AA systems and their IC_{50} values were generally similar: 4–5 μM for gallic acid, caffeic acid, resveratrol, fisetin, quercetin, catechin, kaempferol and myricetin, 9 μM for morin. IC_{50} was not reached up to 40 μM concentration for naringenin.

The Fe^{2+}–AA-dependent production of •OH was not influenced by any of the tested PP up to 40 μM level or by the presence of microsomes. The NADPH-dependent production of •OH, which required the presence of microsomes, was efficiently inhibited by myricetin, fisetin and quercetin, other PP were ineffective, whereas kaempferol, morin and *trans*-resveratrol enhanced •OH production (Table 2).

3.3. Estimation of the role of iron chelation in the antioxidant effects of PP

Iron chelation or oxidation of PP can be assessed from their UV-Vis spectra. The λ_{max} increase: red shift in UV-Vis polyphenol spectra after addition of iron ions corresponds to Fe chelation [26,35,45,46]. It can be reversed by subsequent addition of EDTA. The reported molar ratios between PP and iron were 1:1, 2:1 or 3:1 [35,46–49]. Thus, we measured their interaction in molar ratios of 2:1, 1:1 and 1:2. In contrast to Ref. [38] we observed no absorbance bands around 550 nm for gallic acid, caffeic acid and (+)-catechin. Oxidation of PP can be demonstrated spectrally by the decrease of λ_{max} absorbance, blue shift and isosbestic points production. Oxidation was demonstrated previously electrochemically in kaempferol and quercetin

Table 2
Inhibition of LP, •OH scavenging in NADPH-dependent, Fe^{2+}–AA-dependent or Fe-dependent systems, respectively

Compound	OH substitution	Inhibition of LP IC_{50} (μM)			•OH quenching IC_{50} (μM)		Chelation (yes/no) or oxidation (ox) by Fe ions	
		NADPH-dependent system	Fe^{2+}–AA-dependent system	Fe^{2+}-dependent system	NADPH-dependent system	Fe^{2+}–AA-dependent system	Fe^{2+}	Fe^{3+}
Myricetin	3, 5, 7, 3′, 4′, 5′	7.5 [5]	E 43.4; [10]	4.2	9.9	>40	Yes	ox
Quercetin	3, 5, 7, 3′, 4′	1.7 [6]	29.5 [9]	4.8	24.1	>40	Yes	ox
Rutin	5, 7, 3′, 4′	>30 [21]	>40 [10]		>40	>40	Yes	Yes
Fisetin	3, 7, 3′, 4′	6.9 [6]	E 61.1 [16]	5.5	22.1	>40	Yes	Yes
Kaempferol	3, 5, 7, 4′	13.9 [11]	5.3 [6]	3.4	S: 145.0%	>40	Yes	Yes
Morin	3, 5, 7, 2′, 4′	8.8 [1.3]	13.4 [8.9]	7.5	S: 174.7%	>40	Yes	Yes
Naringenin	5, 7, 4′	>30 [1137]	E 37.2 [918]	>40	>40	>40	Yes	Yes
(+)-Catechin	3, 5, 7, 3′, 4′	22.2	16.8	3.0	>40	>40	Yes	Yes
(−)-Epicatechin	3, 5, 7, 3′, 4′	15.9	12.5	1.6	>40	>40	Yes	Yes
trans-Resveratrol	3, 5, 4′	6.1	2.9	3.6	S: 131.5%	>40	No	No
Gallic acid	3, 4, 5	>40	>80	6.7	>40	>40	Yes	Yes
Gentisic acid	2, 5	>30	>80		>40	>40	No	ox
Caffeic acid	3, 4	>30	E 51.4; {438}	4.5	>40	>40	Yes	Yes
p-Coumaric acid	4	>25	>80		>40	>40	u.i.	Yes
α-Tocopherol	–	3.9	>20	>12	>40	>40	–	–
Ascorbic acid	–	>40	–		>40	–	–	–

Classes of antioxidants: flavonols (myricetin, quercetin, rutin, fisetin, kaempferol, morin), flavanone (naringenin), flavanols ((+)-catechin, (−)-epicatechin), stilbene (*trans*-resveratrol), derivatives of acids (gallic acid, gentisic acid, caffeic acid, *p*-coumaric acid) and vitamins (α-tocopherol, ascorbic acid). PP were incubated for 60 min at 37° with 50 μM Fe^{2+} + 500 μM AA or NADPH-generating system, 66 mM phosphate buffer (pH 7.4) (Fe–AA- or NADPH-dependent system) or 1 mM phosphate buffer (pH 7.4) (Fe-dependent system) and rat liver microsomes as described in Section 2. The efficiency of PP expressed as IC_{50} (μM) represents means of at least five independent experiments in NADPH- and Fe^{2+}–AA-dependent systems and up to three experiments with the Fe system, all of them in duplicate samples of at least four concentrations of the antioxidant. Results were compared with van Acker *et al*. [26] expressed as "[ ]" (mouse liver microsomes, 10 μM $FeSO_4$ + 50 μM AA, Tris/KCl 50/150 mM, pH 7.4) or with Zhou and Zheng [22] (mouse liver microsomes, 10 μM $FeSO_4$ + 100 μM AA, 0.2 mM PBS, pH 7.4) expressed as { }. Symbol "S" means stimulation of •OH production by PP expressed in % (100%: value without the PP; up to 40 μM PP were used); "E": extrapolated value; u.i.: unknown interaction.

[50], by enzymatic oxidation of fisetin, quercetin [51,52], and caffeic acid by polyphenoloxidase [53].

In our study, all PP except *trans*-resveratrol and gentisic acid (interaction only with Fe^{3+}) interacted with Fe^{2+} and Fe^{3+}, but the interactions varied widely. The greatest UV-Vis spectral changes were observed in flavonols, small changes were detected with flavanols and phenolic acid derivatives. The Fe concentration- and the time-related red shift in the spectra of most PP, induced by Fe^{2+} or Fe^{3+}, indicated iron chelation (Fig. 3A). The Fe^{2+}-induced decrease of *p*-coumaric acid absorbance did not show any Fe^{2+}-complex or oxidation with no blue shift or isosbestic point production. Relative spectral interaction of PP with Fe^{2+} evaluated by the red shift were: quercetin > myricetin > fisetin > morin > kaempferol > rutin (Fig. 4A and B). In the PP interacting with Fe^{3+} (Fig. 4C and D) the order was fisetin > quercetin > morin > rutin > caffeic acid > gallic acid > coumaric acid > myricetin. The other PP reacted less and *trans*-resveratrol did not react at all (Fig. 4B and D). Myricetin (Fig. 5A), quercetin, and possibly also gentisic acid were oxidized by Fe^{3+}, as shown by decreased λ_{max} absorbance, blue shift and isosbestic point production. It can obviously influence their chelation.

Fe chelation interactions were confirmed by regeneration of the UV-Vis spectra of most PP by adding equimolar EDTA (Fig. 3B). The regeneration of fisetin and morin spectra after reaction with Fe^{3+} required 100 μM EDTA, but after interaction of Fe^{3+} with quercetin or myricetin, their spectra could not be regenerated (Fig. 5B), confirming an Fe-induced chemical change suggested above. The spectrum of the Fe–EDTA complex prevented elucidation of the *p*-coumaric acid interaction (decreased λ_{max} OD) for absorbing in the same region.

Increasing Fe concentrations induced greater λ_{max} changes in all PP that interacted with Fe ions (Fig. 4A and C). Spectral changes without Fe addition occurred in fisetin, quercetin and kaempferol (oxidation after 1 hr) and myricetin (changes in spectrum after 24 hr), all other PP were stable. The observed significant effect of light on the spectrum of *trans*-resveratrol is known to be due to a change from *trans*- to *cis*-configuration.

4. Discussion

IC_{50} values and relative PP efficiency in the Fe, Fe–AA and NADPH microsomal systems were different from values found in the literature. The IC_{50} values of 14 PP against LP in the Fe^{2+} system were lower than in the Fe–AA system, but the latter (higher) values are more biologically relevant, since AA is omnipresent in tissues. The IC_{50} for inhibition of LP in the Fe–AA system correlated

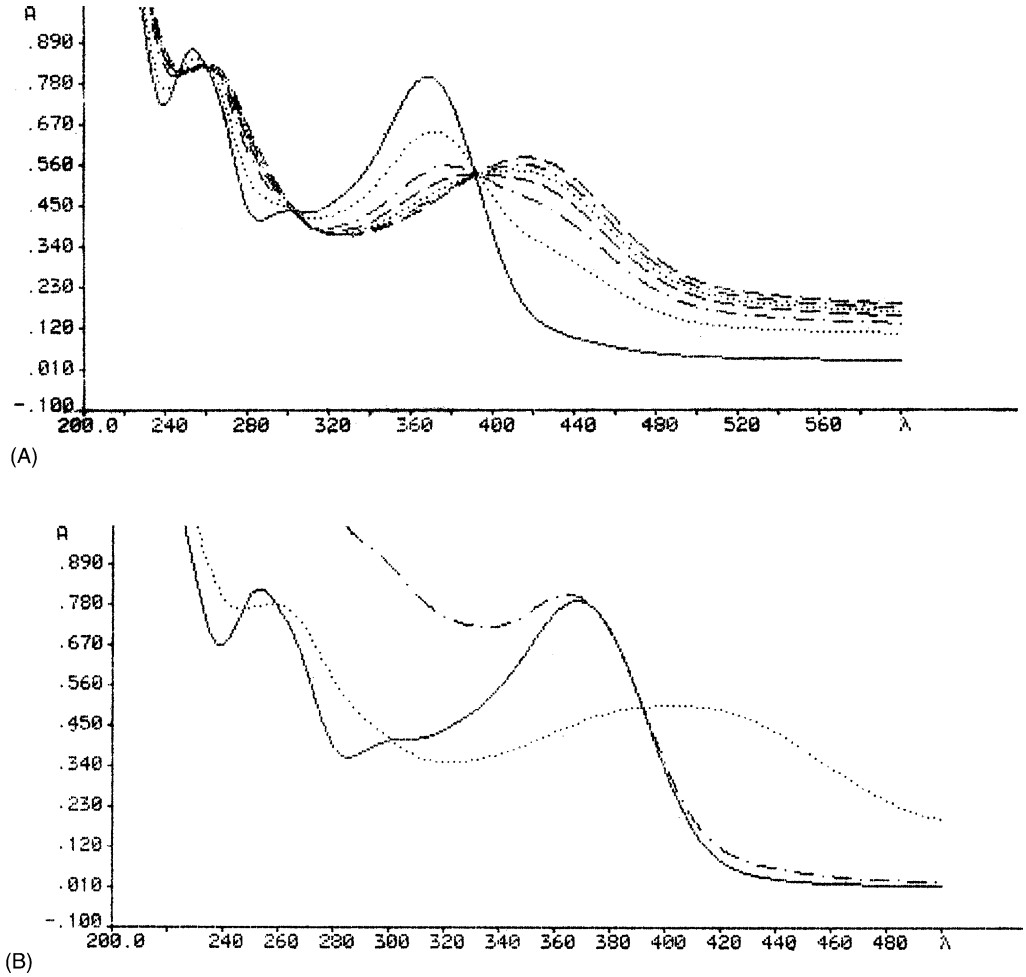


Fig. 3. (A) Chelation of Fe^{2+} with 50 μM myricetin, effect of time on 50 μM PP chelation of Fe^{2+} (100 μM): —, myricetin only; · · ·, 0 and 30 min; –·–, 10 and 45 min; – –, 20 and 60 min represent time after addition of 100 μM $FeSO_4$ (0, 10, 20, 30, 45, 60 min). Fifty micromolar PP dissolved in water was incubated with 100 μM Fe^{2+} at 22°, time dependent changes of UV-Vis spectrum were assayed as reported in Section 2. (B) Regeneration of UV-Vis spectrum by addition of EDTA. —, 50 μM myricetin; · · ·, 50 μM myricetin + 100 μM Fe^{2+}; –·–, 50 μM myricetin + 100 μM Fe^{2+} + 100 μM EDTA. Fifty micromolar myricetin dissolved in water was incubated with 100 μM Fe^{2+} for approximately 1 min at 22°, the regeneration of myricetin UV-Vis spectrum was measured immediately after addition of EDTA.

($r = 0.856$) with data of van Acker *et al*. [26], although their Fe and AA concentrations (10/50 μM) were lower than ours (50/500 μM), mutually supporting the validity of the data. However, individual IC_{50} values markedly differed (van Acker/our data, μM): (+)-catechin 3.8/16.8), myricetin 8.9/43.4, quercetin 8.1/29.5, rutin 9.5/over 40, fisetin 18.2/61. The higher Fe–AA concentrations apparently caused our higher IC_{50} values, but they correspond to the 100–850 μM concentration of AA in tissues. IC_{50} values differing even more were found by other authors. Some IC_{50} values can be reached *in vivo*. The IC_{50} values in the NADPH system were almost identical in both studies and also significantly correlated ($r = 0.97$), although their assays [26] included doxorubicin. In contrast to that study, however, the IC_{50} values in our NADPH system did not correlate with those in Fe–AA system.

A similar range of IC_{50} values, but different relative efficiency of individual PP in our series suggests different antioxidant mechanisms in the NADPH- and Fe–AA systems. The antioxidant action of a large series of structurally different PP against LP in the Fe–AA system was reported to be due to their Fe-chelation capacity [26], but our IC_{50} values did not correlate with the quantitatively assayed chelation potency of our PP (Scheme 1). Moreover, the published IC_{50} values [26] of our structurally similar PP did not correlate with Fe chelation either (rather a negative correlation was apparent). However, our IC_{50} values in the NADPH system correlated with quantitatively assayed Fe chelation ($r = 0.93$), suggesting that Fe chelation may be an important antioxidant mechanism in this system. The PP could chelate the CYP heme iron, which occurs in the Type II substrates. The similar IC_{50} values of resveratrol in all three systems may reflect the fact that it is not an Fe chelator, while different IC_{50} values of the other PP in the NADPH and Fe–AA systems support a different role of Fe chelation in each of them.

Although our PP efficiently inhibited LP, they inhibited $^{\bullet}OH$ less in the NADPH system and not at all in the Fe–AA

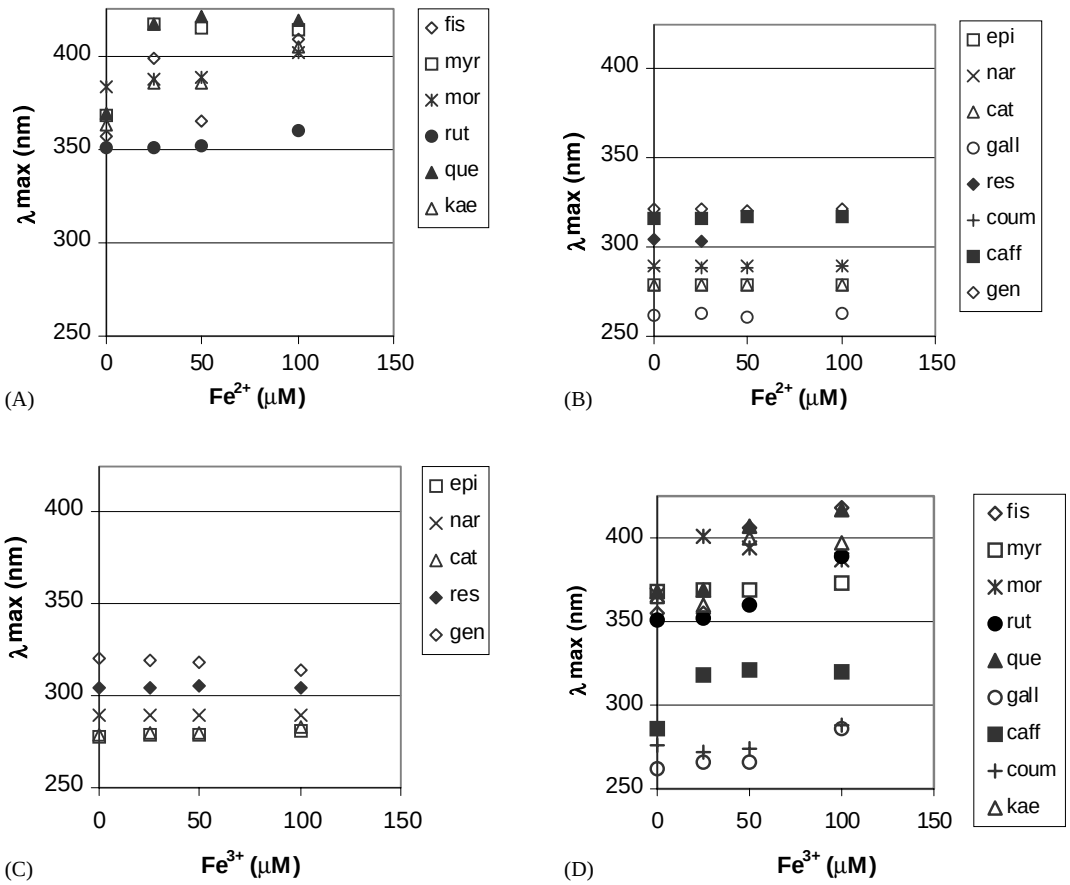


Fig. 4. The red shift after addition of Fe (25, 50 or 100 μM). (A) Strongly interacting PP with Fe^{2+}, (B) weakly interacting PP with Fe^{2+}, (C) strongly interacting PP with Fe^{3+}, (D) weakly interacting PP with Fe^{3+}. Fifty micromolar PP dissolved in water was incubated with 25, 50 or 100 μM iron ions for 1 min at 22° and UV-Vis spectrum was measured. fis, fisetin; myr, myricetin; mor, morin; rut, rutin; que, quercetin; kae, kaempferol; epi, epicatechin; nar, naringenin; cat, catechin; gall, gallic acid; res, *trans*-resveratrol; coum, *p*-coumaric acid; caff, caffeic acid; gen, gentisic acid.

system, apparently due to higher •OH levels than with NADPH. Our •OH formation assayed by HCHO formed from DMSO in the Khan's system [44] was higher than that detected by ESR. The formation of HCHO from AA in the presence of Fe^{2+} and O_2 [54], suggests that our detection of •OH by HCHO may exaggerate real values. However, HCHO formation from DMSO by Fe^{2+} or AA alone and their combination indicated that in the Fe–AA system, only about 20% HCHO could originate from AA. Since the PP did not inhibit •OH in the Fe–AA system, •OH apparently played a negligible role in LP. The inhibition of LP was therefore probably due to direct quenching of LP radicals.

Our IC_{50} values in the NADPH system correlated with the half peak oxidation potentials (Ep/2) in the literature [26] ($r = 0.764$), suggesting another possible mechanism of the PP antioxidant action. It, however, depends on the same electron properties of flavonoid molecules, which are responsible for the donation of hydrogen or an electron, and the stability of the PP• radical formed during its antioxidant action, i.e. on the same common mechanism. In any case, lack of correlation between IC_{50} values in the NADPH and Fe^{2+}–AA systems, apparently different roles of Fe^{2+} chelation, and different effects on •OH formation, indicate that mechanisms of LP and its inhibition in the two systems are different.

The stimulation of •OH production by kaempferol, morin and resveratrol in the NADPH system, where they markedly inhibited LP, may be due to their participation in O_2 reduction to O_2^-, H_2O_2 and •OH catalyzed by CYP enzymes, or by stimulation of the CYP futile cycle reported with hydroquinone and benzoquinone [55,56], which inhibited LP as well. It presents another example of •OH/LP discrepancy. The notion that PP decrease LP by inhibiting CYP enzymes [57,58] is not supported by our data which show that the potency to inhibit LP did not correlate with CYP2C8, CYP3A4/4 and CYP3A1/2 catalysis [59].

Other interactions of PP can mediate their action. In the PP–Fe complex, chelation of Fe may prevent its prooxidant action, but (in analogy with EDTA) it may decrease its redox potentials and the PP are then more active in the conversion of O_2^- to H_2O_2. Catecholic–Fe^{2+} complexes have much lower redox potentials compared with that of Fe^{3+}/Fe^{2+}, complexation with iron may also decrease the redox potential of the semiquinone/catechol redox pair [48], with cytoprotective consequences. Therefore, PP–Fe complexes may protect biomolecules

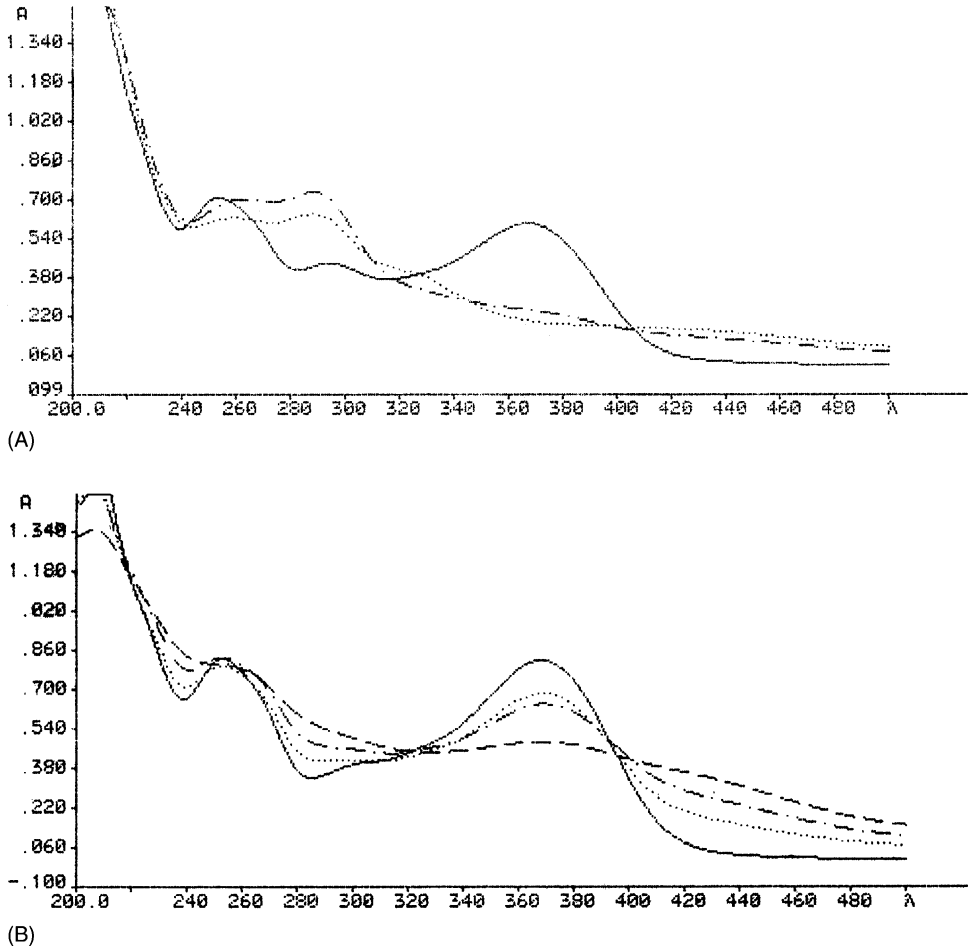
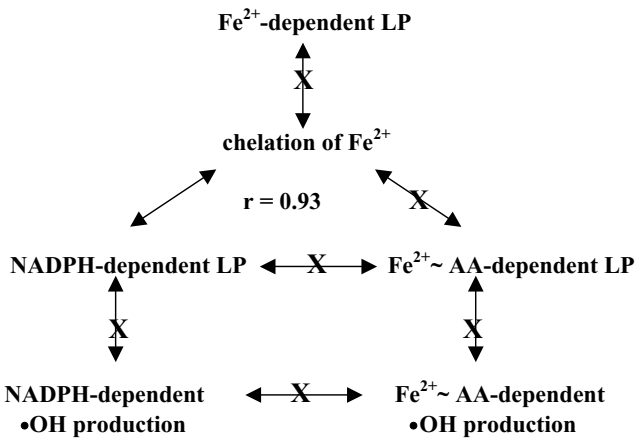


Fig. 5. (A) Oxidation of PP by Fe^{3+} demonstrated by changes in UV-Vis spectra immediately after addition of Fe^{3+}. —, 50 μM myricetin; · · ·, 50 μM myricetin + 25 μM Fe^{3+}; –·–, 50 μM myricetin + 50 μM Fe^{3+}; – –, 50 μM myricetin + 100 μM Fe^{3+}. Myricetin dissolved in water was incubated with 25, 50 or 100 μM Fe^{3+} for approximately 1 min at 22° and the UV-Vis spectrum was measured. (B) No regeneration by EDTA of PP spectrum after reaction with Fe^{3+}. —, 50 μM myricetin; · · ·, 50 μM myricetin + 25 μM Fe^{3+}, incubation 60 min (22°, daylight); –·–, 50 μM myricetin + 25 μM Fe^{3+}, incubation 60 min (22°, daylight) + 25 μM EDTA, measured immediately. Fifty micromolar PP dissolved in water was incubated for 60 min at 22° with 25 μM Fe^{3+}, the UV-Vis spectrum was measured immediately after EDTA addition.



Scheme 1. Correlation between inhibiting effects of PP on LP (IC_{50}), •OH scavenging and Fe^{2+} chelation in the Fe-, Fe–AA- and NADPH-dependent systems. PP were incubated for 60 min at 37° with 50 μM Fe^{2+}–500 μM AA or the NADPH-generating system in 66 mM phosphate buffer (pH 7.4) or in 1 mM phosphate buffer (pH 7.4) (Fe-dependent system) and rat liver microsomes. LP and •OH production were assayed as described in Section 2. Chelation was expressed as λ_{max} changes of UV-Vis spectrum of PP after 1 min incubation with Fe^{2+} ions at 22°. X: no correlation.

and the cell by different mechanisms, where inhibition of LP seems a more important mechanism than certain steps of reductive activation of oxygen. Auto-oxidation of PP apparently occurs at the same time and although it may result in their inactivation, this phenomenon apparently also indicates their simultaneous antioxidant action. The strong antioxidants, quercetin and myricetin, were oxidized in water by Fe^{3+}. Quercetin, fisetin and kaempferol were also rapidly oxidized in aerobic water without Fe as well as myricetin, although more slowly. Quercetin, fisetin, myricetin and morin were the most powerful LP antioxidants in NADPH-dependent LP; and myricetin, fisetin and quercetin, in descending order, were the most effective flavonoids against •OH production.

Comparing the efficiency of flavonoids to inhibit LP in the NADPH-, Fe–AA- and Fe-dependent systems, respectively, (Table 3) has shown that different structures may be essential in the individual systems. In our opinion, the overall antioxidant capacity of many PP depends on several modes of PP interaction, which may participate to differing degrees, depending on the pro-oxidant system used, and

Table 3
The structure–activity relationship of flavonoids in inhibition of NADPH-, Fe–AA- and Fe-dependent LP, respectively

Effect of (the presence of)	Presence of the compared structure increases (↑) or decreases (↓) the antioxidant effect				
	van Acker *et al*. [26]		This study		
	NADPH-dependent	Fe–AA-dependent	NADPH-dependent	Fe–AA-dependent	Fe-dependent
2,3-Double bond and 4-keto group (quercetin) (absent in catechin (−), epicatechin (−))	↑	↓	↑	↓	↓
3-OH group (quercetin) (absent in rutin)	↑	No effect	↑	↑	Not measured
3′,4′-OH group (quercetin) vs. 4′-OH group (kaempferol)	↑	↓	↑	↓	No effect
3′,4′,5′-OH group (myricetin) vs. 3′,4′-OH group (quercetin)	No effect	No effect	↓	↓	No effect
2′,4′-OH group (morin) vs. 3′,4′-OH group (quercetin)	Not measured	Not measured	↑	↓	↓

The data of our study were compared with van Acker *et al*. [26] (mouse liver microsomes, 10 μM $FeSO_4$ + 50 μM AA or NADPH-generating system + 35 μM doxorubicin, Tris/KCl 50/150 mM, pH 7.4). PP were incubated for 60 min at 37° with 50 μM Fe^{2+} + 500 μM AA or the NADPH-generating system, 66 mM phosphate buffer (pH 7.4) (Fe–AA- or NADPH-dependent system) or 1 mM phosphate buffer (pH 7.4) (Fe-dependent system) and rat liver microsomes as described in Section 2. The presence of the compared structure caused a higher (↑) or lower (↓) antioxidant effect as compared with a polyphenol where the structure is absent.

consequently also differently under different biochemical conditions (circumstances) in the organism. A ''generalization'' for all circumstances seems to lead to unnecessary contradictions in the structure–activity relationships.

The strong pro-oxidant effects of AA seem to be contradictory to its accepted role as a physiological antioxidant. Under physiological AA levels (100–850 μM in blood and tissues), even 5 μM Fe caused strong pro-oxidant action. AA can transfer e^- or $H^•$ to O_2^-, $^•OH$ [60] $ROO^•$, $RO^•$ or conjugated dienes or e^- to transient metals [61] indicating, why AA can have antioxidant or pro-oxidant effects. The pro-oxidant effect of iron is also influenced by assay conditions. We observed that in 0.006 M or higher phosphate concentration (pH 7.4) Fe^{2+} or Fe^{3+} alone did not stimulate LP, and we also confirmed the data of Fukumoto and Mazza [62] that AA alone did not stimulate LP. Therefore, our results showing that under identical incubation conditions, $^•OH$ were effectively formed by Fe^{2+}, Fe^{3+} or AA alone, indicated that $^•OH$ were not responsible for LP. The significantly higher $^•OH$ production in the Fe–AA system than by Fe or AA alone is apparently due to more efficient Fe reduction. The pro-oxidant effect of the Fe–AA system is probably due to reduction of Fe^{3+} to Fe^{2+}, which then catalyzes the Fenton reaction and also decomposes ROOH. Since Fe^{2+} reduces O_2 to O_2^- and AA can be the source of $H^•$ to form H_2O_2, the requirements for $^•OH$ production are fulfilled. However, the fact that in the presence of 10–150 μM Fe^{2+}, 5000 μM AA stimulated $^•OH$ production, but LP decreased to zero, indicates that either Fe^{3+} reduction is not sufficient for the pro-oxidant effect, or the quenching of radicals by AA prevailed. Hsieh and Hseih [63] reported that at pH above 6.8, AA did not reduce Fe^{3+} efficiently, supporting the data of Afanas'ev *et al*. [35] that equimolar levels of Fe and AA were not pro-oxidant. However, in our conditions of 10 μM Fe^{2+} and at least 500 μM AA and in 50–150 μM Fe^{2+} and at least 100 μM AA (molar ratio of 0.67–50, dependent on absolute Fe concentration), LP was significantly stimulated, indicating that the pro-oxidant effects on LP at pH 7.4 prevailed. In $^•OH$ production, all ratios of 10–150 μM Fe^{2+} and 100–5000 μM AA were pro-oxidant. At 5000 μM AA concentration, its direct antioxidant action in LP prevailed, although $^•OH$ formation was high, but this is of little biological significance, as 5000 μM is not a physiological AA level. In fact, under physiological AA concentrations the presence of at least 5 μM Fe appeared to exert only pro-oxidant action on both LP and $^•OH$.

In conclusion, our results showed that the antioxidant efficiency of flavonoids against LP (1) differed in the NADPH system from the Fe–AA-, and Fe system and that the relation to structure in the respective systems was different, (2) did not correlate with $^•OH$ radical quenching, (3) did not correlate with quantitatively estimated Fe chelation by flavonoids in the Fe–AA- and Fe systems, but correlated in the NADPH system, (4) was apparently due to direct quenching of lipid radicals especially in the Fe–AA- and Fe systems and (5) that a 5-μM Fe level was sufficient for the pro-oxidant effect at physiological AA concentrations.

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