Protein Protection by Antioxidants: Development of a Convenient Assay and Structure-Activity Relationships of Natural Polyphenols

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In this work, a convenient test of antioxidant activity was developed, with BChE-contaminated HSA as the target of AAPH-induced oxidation and its esterase activity as the marker of protein integrity or degradation. The method is relatively simple, of low cost, and convenient to use. Its application to natural polyphenols showed that quercetin (**1**), verbascoside (**2**), chlorogenic acid (**3**), caffeic acid (**4**), 1,3,6,7-tetrahydroxyxanthone (**5**), and mangiferin (**6**), are good antioxidants ($IC_{50} < 9$ μM). 1,5-Dihydroxy-3-methoxyxanthone (**7**), flemichin D (**8**), and cordigone (**9**) showed modest activities (*ca.* 50 μM $< IC_{50} <$ 350 μM), whereas danthrone (**10**) was inactive. Complementary experiments with two of the more active antioxidants, namely quercetin (**1**) and chlorogenic acid (**3**) showed that both antioxidants were better radical scavengers than chain-breaking antioxidants. The relative adiabatic oxidation potential (ΔH_{ox}), the relative H-bond dissociation energy (ΔH_{abs}), and the first oxidation potential measured by cyclic voltammetry were found to be related to the radical-scavenging activity of these antioxidants.

1. Introduction[1]). – The increased interest in the bioactivity of natural polyphenols is due to their potential efficacy against oxidative stress. Studies in great number have implicated oxygen-reactive species, in particular free radicals, in the pathological mechanisms of several diseases including atherosclerosis, cancer, and *Alzheimer*'s disease, and also in aging [1]. Some natural polyphenols have been reported to quench oxygen-reactive species [2], inhibit the *in vitro* oxidation of low-density lipoproteins [3][4], and show hepatoprotective [5], antiaggregatory [6], and cancer chemotherapeutic and chemopreventive properties [7–9].

Polyphenolic compounds are largely present in the human diet (*e.g.*, in fruits, berries, vegetables, red wine, and tea) [10], and are also used as food additives, *e.g.*, to prevent lipid oxidation. The bioavailability of most of these natural antioxidants is still controversial, but pharmacokinetic studies have demonstrated that repeated intake of quercetin-containing foods will lead to accumulation of quercetin in plasma [11]. Although some 'antioxidants' that inhibit lipid peroxidation may also accelerate oxidative damage to nonlipidic biomolecules such as DNA or carbohydrates [12], very few studies have investigated the antioxidant or pro-oxidant effects of natural polyphenols on proteins. Only quercetin is known to bind to DNA and bovine serum albumin and facilitate the fragmentation of both [13][14]. It was therefore of interest to

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[1]) *Abbreviations:* AAPH: 2,2′-azobis(2-amidinopropane) dihydrochloride; BCECF: 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; HSA: human serum albumin; PCR: polymerase chain reaction.

test the antioxidant activity of a series of natural polyphenolic compounds against protein oxidation caused by peroxyl radicals.

Various methods can quantify protein oxidation, *e.g.*, the detection of carbonyl groups [15][16], or a decrease in native fluorescence [17–19]. Other assays are specific for damage to amino acids [20][21]. Protein fragmentation or aggregation can be measured by SDS-polyacrylamide gel electrophoresis [22–25]. Unfortunately, the color of natural polyphenols interferes with some spectrophotometric assays (*e.g.*, derivatization to form yellow 2,4-dinitrophenylhydrazones) and with the decrease in intrinsic fluorescence of the oxidized protein. Moreover, all these assays quantify structural damages to proteins.

The assay proposed here allows to test colored antioxidants and is based on a functional test, namely the decrease in hydrolytic activity of an esterase damaged by free radicals. The isolated cholinesterase (also known as butyrylcholinesterase; BChE; EC 3.1.1.8) was used at the beginning, but this enzyme appeared too sensitive to experimental conditions and was inhibited by some antioxidants. Adding human serum albumin (HSA) avoided enzymatic inhibition, but the extent of oxidation was erratic. As it is known that albumin may be contaminated with BChE [26], the work was continued with commercially available contaminated albumin. A highly reproducible system was obtained, and costs were lowered.

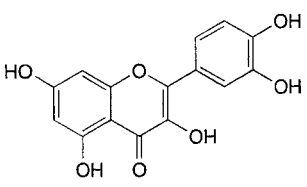
The compounds studied were a flavonoid (quercetin (**1**), three hydroxylated cinnamic acids (verbascoside (**2**), chlorogenic acid (**3**), and caffeic acid (**4**)), three xanthones (1,3,6,7-tetrahydroxyxanthone (**5**), mangiferin (**6**), and 1,5-dihydroxy-3-methoxyxanthone (**7**)), a benzo-γ-pyrone derivative (flemichin D (**8**)), a furan derivative (cordigone (**9**)), and a anthraquinone (danthrone (**10**)) (*Fig. 1*). These compounds are known to be inhibitors of lipid peroxidation in rat brain synaptosomes [27]. To test selectively their free-radical-scavenging properties without implicating their metal-ion-chelating capacities, we used 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator, rather than a $FeCl_2/FeCl_3$ system.

The antioxidant activities of the polyphenolic compounds were compared to their relative adiabatic oxidation potential (ΔH_{ox}), their relative H-bond dissociation energy (ΔH_{abs}), and their first oxidation potential (Epa_1) measured by cyclic voltammetry [27].

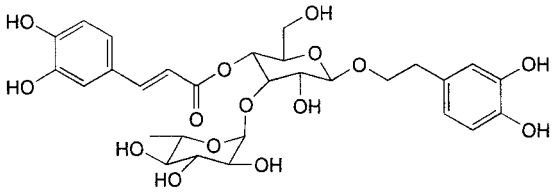
2. Results and Discussion. – 2.1. *Preliminary Investigations.* 2.1.1. *Experiments with Isolated Horse BChE.* The experiments with isolated BChE appeared to be problematic. The enzyme is heat-sensitive, and its activity was also influenced by the nature of the cosolvent required to dissolve the antioxidants. In these assays, the only usable cosolvent proved to be DMSO. The most severe restriction was the inhibition of BChE by antioxidants. Indeed, when examining the protective effect of ascorbic acid (**12**) and trolox (**11**) on BChE activity after AAPH-induced oxidation, no IC_{50} value could be determined due to enzyme inhibition at high antioxidant concentrations (*Fig. 2*).

Quercetin (**1**) proved an even more potent inhibitor of BChE (50% and 80% inhibition at 1 and 10 μM, respectively).

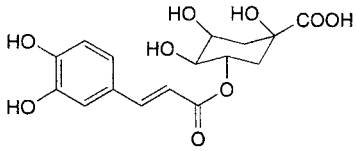
Since three reference antioxidants inhibited BChE, the use of isolated BChE did not appear appropriate to test natural compounds, nor would their litte amounts



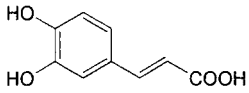
Quercetin (**1**)



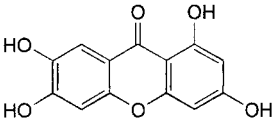
Verbascoside (**2**)



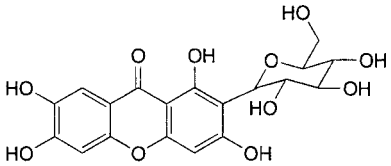
Chlorogenic acid (**3**)



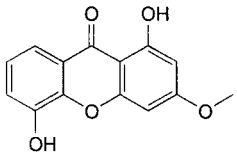
Caffeic acid (**4**)



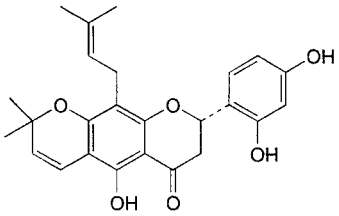
1,3,6,7-tetrahydroxyxanthone (**5**)



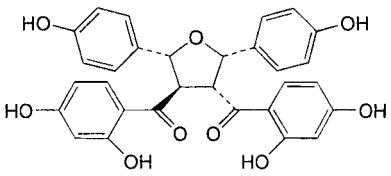
Mangiferin (**6**)



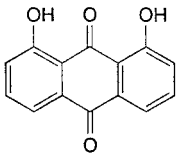
1,5-dihydroxy-3-methoxyxanthone (**7**)



Flemichin D (**8**)

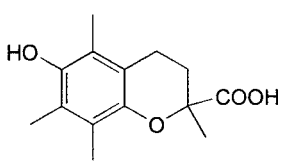


Cordigone (**9**)

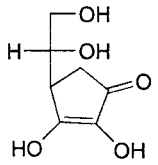


Danthrone (**10**)

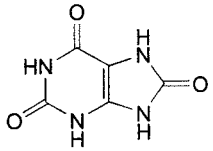
Fig. 1. *Structures of the natural polyphenols and other compounds examined*



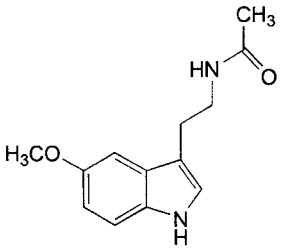
Trolox (**11**)



Ascorbic acid (**12**)



Uric acid (**13**)



Melatonin (**14**)

Fig. 1 (cont.)

available have permitted a complete study of enzyme inhibition and protein protection against oxidation.

2.1.2. *Addition of Albumin to BChE.* Albumin is often used (*e.g.*, during enzyme isolations, immunoassays, and PCR) to protect delicate molecules by acting as a surrogate macromolecule that binds to surfaces [28]. Thus, the addition of 2 μM HSA was tested, and many positive effects were observed: *1*) First, albumin appears to render BChE heat-resistant, since the activity of the latter decreased linearly without albumin, whereas it was stable over a period of 500 min in the presence of albumin. *2*) The activity of BChE was increased (*i.e.*, the half-life of moxisylyte was shorter) in the presence of albumin, probably because albumin prevented the enzyme from being adsorbed on the surface of recipients. *3*) The most important effect of albumin was to protect BChE from inhibition by some test compounds. Indeed, when 2 μM albumin was added to the reaction mixture, the half-life of moxisylyte in the presence of 10 μM quercetin (**1**; $t_{1/2} = 11.6 \pm 1.4$ min) was the same as without **1** ($t_{1/2} = 11.33 \pm 0.84$ min), whereas it was increased more than threefold in the absence of albumin. It is interesting to note that protection against enzymatic inhibition was achieved simply by mixing the two protein solutions.

Nevertheless, BChE inhibition was detectable at higher quercetin (**1**) concentrations. This could be prevented by increasing the concentration of albumin to 50 μM. However, even under such conditions, nonreproducible results were obtained for loss of enzymatic activity caused by AAPH and for antioxidant activities (results not shown).

Protein–protein interactions are complex phenomena, the stoichiometry and kinetics of macromolecular associations being difficult to control. Since preliminary assays with commercially available, BChE-contaminated albumin gave reliable results

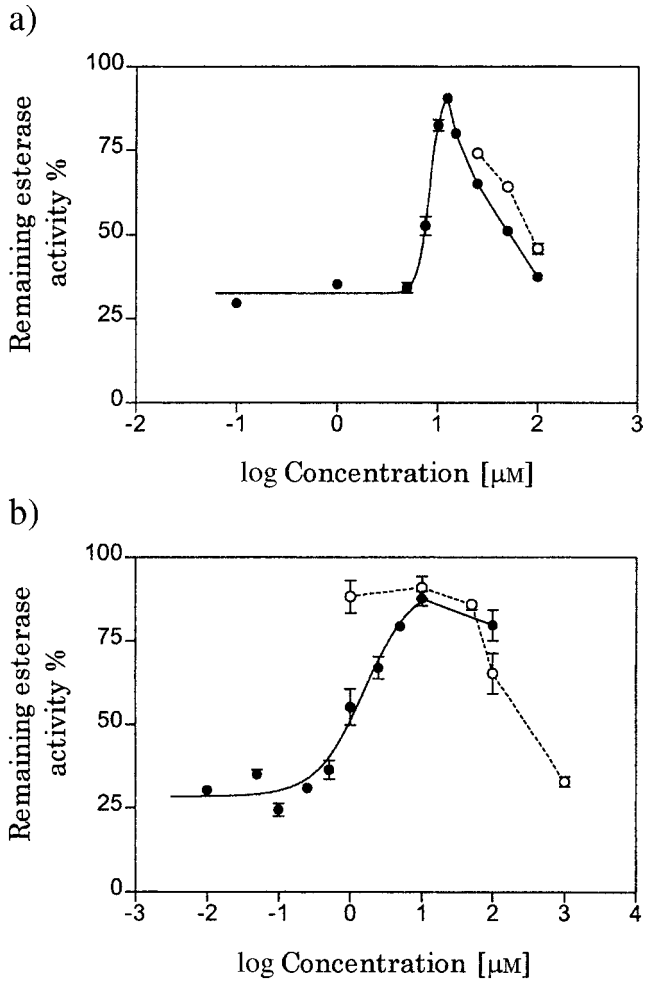


Fig. 2. *Effect of log concentration of ascorbic acid* (a) *or trolox* (b) *on BChE activity obtained without oxidation* (○) *and with 0.4 mM AAPH* (final conc.) (●). Incubation: 60 min at 37 ± 0.1°. Data are expressed as mean ± SD for duplicate experiments.

with high day-to-day reproducibility, this material was used to develop an antioxidant assay.

2.2. *Assays with BChE-Contaminated HSA.* 2.2.1. *Hydrolytic Activity.* The albumin used (*Sigma A-1887*) was an essentially fatty-acid-free HSA prepared from fraction V according to the method of *Cohn et al.* [29], fatty acids being removed by charcoal treatment [30]. The esterase activity of different batches of this albumin varies considerably [26]. A batch having esterase activity similar to that of fraction V powder was used here. A plot of relative BChE activity *vs.* log of the quercetin (**1**) concentration is shown in *Fig. 3*.

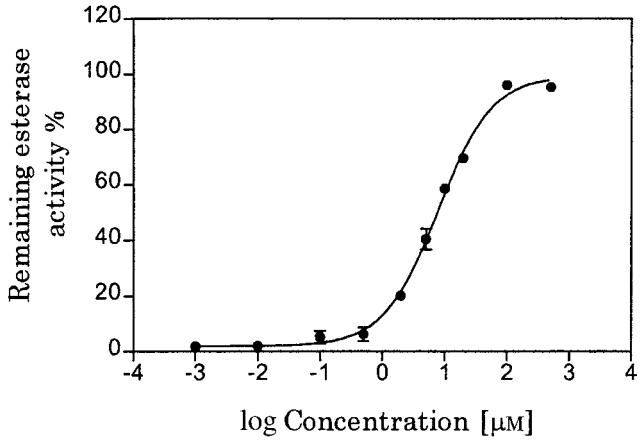


Fig. 3. *Effect of log concentration of quercetin* (**1**) *on esterase activity obtained with HSA contaminated with BChE* (*Sigma A 1887*; *batch 118F9311*). Oxidation conditions: 120 min at 45 ± 0.1° in presence of 1 mM AAPH (final conc.). Data are expressed as means ± SD for duplicate experiments.

2.2.2. *Effect of AAPH on Esterase Activity.* Incubation of BChE-contaminated HSA with the H_2O-soluble free-radical generator AAPH resulted in a time-dependent decrease of the initial rates of enzymatic activity, as shown in *Fig. 4*.

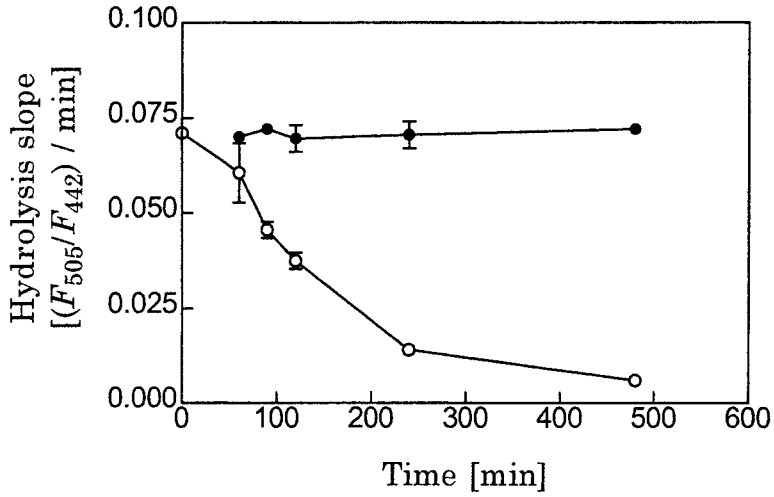


Fig. 4. *Esterase activity as a function of the duration of oxidation.* ○: Initial rates of moxisylyte hydrolysis after incubation of 50 μM contaminated HSA at 45° with 1 mM AAPH (pH 7.4 ± 0.1); ●: controls without AAPH. Data are expressed as the average of two experiments.

AAPH was chosen as a free-radical generator because it produces two C-centered free radicals at a constant rate by thermal decomposition. Under aerobic conditions, these radicals react rapidly with molecular O_2 to yield peroxyl radicals. In our assay conditions, AAPH decomposed unimolecularly with first-order kinetics, in agreement with the literature [14]. The linearity of free-radical generation extended for much

longer periods than the duration of our experiments. The decrease in the initial rates of hydrolysis followed apparent first-order kinetics (*Fig. 4*). Under the present conditions, however, the hydrolytic reaction itself followed pseudo-first-order kinetics for up to 2 h, allowing the decrease in esterase activity to be used as a marker of BChE oxidation.

Interestingly, the BChE-contaminated HSA was more sensitive to AAPH-induced oxidation than a mixture of BChE and pure albumin (results not shown). This difference might be due to the different origins of BChE (human *vs.* equine) although the two enzymes have 89% amino acid identity. Alternatively, different molecular forms present in the two preparations may be the reason for this discrepancy. Indeed, it is well-known that human plasma cholinesterase is a highly polymorphic enzyme existing in different molecular forms (monomeric, dimeric, and tetrameric forms) and subject to complex genetic polymorphism [31].

2.2.3. *Influence of Substrate, Organic Cosolvent, and Incubation Time.* To assess the influence of the substrate on the IC_{50} values of antioxidants, three esters (moxisylyte, acetylcholine, and 2-butoxyethyl nicotinate) were examined. No major differences were observed (results not shown). Moxisylyte was chosen as substrate owing to its good solubility and stability in solution, and to its rapid hydrolysis by BChE.

The influence of MeOH was tested at two concentrations (1 and 5%). The IC_{50} values obtained for trolox (**11**) were 14 ± 2.2, 17 ± 2.9, and 16 ± 1.8 μM with 0, 1, and 5% MeOH, respectively. These differences were not statistically significant. Since MeOH at high concentrations shortened the time interval during which hydrolysis followed pseudo-first-order kinetics [32], MeOH at a low concentration of 1% was used as a co-solvent in all assays.

The optimal duration of pre-incubation of antioxidants with BChE before addition of AAPH was defined using quercetin (**1**), verbascoside (**2**), chlorogenic acid (**3**), caffeic acid (**4**), and mangiferin (**6**; data not shown). Considering that the same protection was obtained after 15, 30, and 60 min, a pre-incubation time of 15 min was chosen in all assays.

2.2.4. *Interference with the Fluorescence Indicator.* Preliminary results showed that AAPH interferes with the fluorescence signal of the pH indicator BCECF used to monitor proton release. In fact, the fluorescence at 505 nm decreased as a function of time depending on AAPH concentration and duration of incubation at 45° (*i.e.*, depending on the number of free radicals generated). This decrease seemed to be due to the oxidation of BCECF, but not to a decrease in pH, as, indeed, verified potentiometrically. Moreover, *Fig. 5* shows that the degradation of BCECF was prevented by the presence of an antioxidant or HSA. Since HSA was used in all assays at the concentration of 50 μM, no detectable BCECF degradation could be observed. Therefore, the pH indicator BCECF could be used safely under the present conditions.

Another potential problem was the fluorescence yield. Although the fluorescence of the pH indicator BCECF was stable and independent of pH at 442 nm, some colored antioxidants modified its fluorescence at this wavelength. This made it impossible to monitor the fluorescence ratio, and consequently measuring fluorescence at 505 nm was the sole possibility. *Table 1* compares the IC_{50} values of antioxidants measured by the fluorescence ratio and by the fluorescence at 505 nm. No statistically significant difference was found. To avoid any potential problem, the fluorescence of colored antioxidants was monitored at 505 nm only, and the hydrolysis slope was divided by the

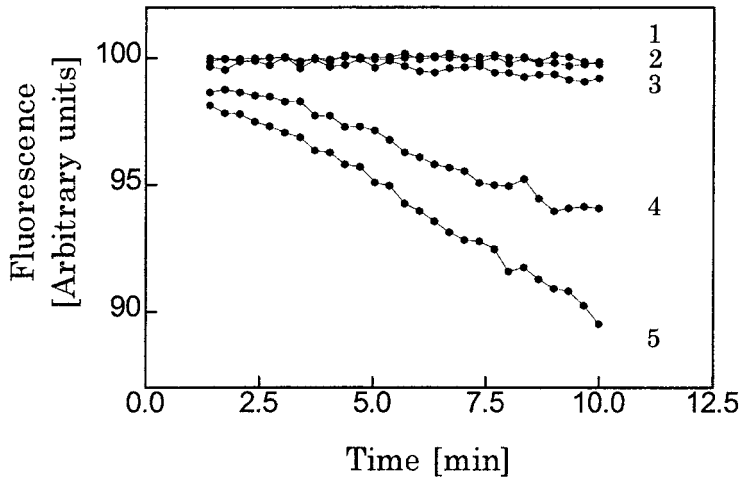


Fig. 5. *Decrease in BCECF fluorescence as a function of time in the presence of AAPH* 1 *mM and ascorbic acid* (**12**) *500 μM* (*1*), *AAPH 1 mM and HSA 50 μM* (*2*), *AAPH 100 μM* (*3*), *AAPH 500 μM* (*4*), *and AAPH 1 mM* (*5*). The pH indicator BCECF (96 nM) was added to the oxidation mixture after incubation during 2 h at 45 ± 0.1° in a phosphate buffer of pH 7.4 ± 0.1.

Table 1. *Protection by Antioxidants against Loss of BChE Activity: Comparison between* IC_{50} *Values Obtained by the Fluorescence Ratio and by Fluorescence at 505 nm*[a])

Compound	IC_{50} [μM]	
	Fluorescence ratio (F_{505}/F_{442})	Fluorescence at 505 nm
Trolox (**11**)	14 ± 2.2	13 ± 2.4
Ascorbic acid (**12**)	18 ± 3.0	23 ± 2.9
Uric acid (**13**)	10 ± 1.8	11 ± 1.3
Caffeic acid (**15**)	3.9 ± 0.74	5.9 ± 0.64

[a]) Conditions: AAPH 1 mM, 120 min, 45 ± 0.1°, pH 7.4 ± 0.1. Data are expressed as mean of two experiments.

Y-intercept of the linear regression to compensate for changes in dye concentration or illumination intensity.

2.3. *Inhibition of BChE Oxidation by Natural Polyphenols and Structure-Activity Relationships.* Natural polyphenols and other compounds (*Fig. 1*) were examined for their potency to inhibit the decrease in esterase activity of BChE caused by free radical oxidation (*Table 2*).

Of the ten compounds investigated, six (**1**–**6**) showed a good protective capacity under these conditions ($IC_{50} < 9$ μM), three other compounds (**7**–**9**) were less effective in this system (*ca.* 50 μM $< IC_{50} <$ *ca.* 350 μM), and one (**10**) was completely inactive in its solubility range. This demonstrates the efficacy of polyphenolic compounds as peroxyl radical scavengers in H_2O. Clearly, the catechols **1**–**6** were more active than the monophenols, **7**–**10** against AAPH-induced oxidation.

Previous studies have revealed a correlation between oxidation potential and antioxidant activity against lipid peroxidation for tocopherols and analogues [33], flavonoids [34], and caffeic acid esters [35]. To determine whether such a correlation

Table 2. *Activities and Physicochemical Properties of Antioxidants*

Compounds	Inhibition of BChE oxidation		Epa_1[a])	ΔH_{ox}[b])	ΔH_{abs}[b])
	IC_{50} [μM][c])	Percent inhibition[d])			
Quercetin (**1**)	7.6 ± 0.39	96.1 ± 0.57	0.11	4.26	− 1.06
Verbascoside (**2**)	2.3 ± 0.25	95 ± 5.2	0.19	12.22	− 0.70
Chlorogenic acid (**3**)	3.8 ± 0.47	98 ± 2.9	0.23	15.27	− 0.70
Caffeic acid (**4**)	3.9 ± 0.74	96 ± 9.3	0.23	16.67	− 0.52
1,3,6,7-Tetrahydroxyxanthone (**5**)	9 ± 1.3	97 ± 4.5	0.28	16.76	2.95
Mangiferin (**6**)	9 ± 1.6	97 ± 5.0	0.32	15.66	2.90
1,5-Dihydroxy-3-methoxyxanthone (**7**)	350 ± 40	21 ± 5.1	0.60	17.52	5.24
Flemichin D (**8**)	66 ± 9.9	59 ± 8.8	0.56	2.68	7.44
Cordigone (**9**)	300 ± 43	24.5 ± 0.57	0.64	16.56	5.57
Danthrone (**10**)	(inactive)	0	0.96	25.00	12.27
Trolox (**11**)	14 ± 2.2	102 ± 3.0	0.11	0.00	0.00
Ascorbic acid (**12**)	18 ± 3.0	101 ± 4.7	–	14.49	− 7.06
Uric acid (**13**)	10 ± 1.8	103 ± 4.5	–	11.86	2.71
Melatonin (**14**)	37 ± 3.5	75 ± 8.0	> 1.4	− 6.64	8.05

[a]) First oxidation potential (in V) measured by cyclic voltammetry [27]. [b]) Relative adiabatic oxidation potential (ΔH_{ox}) and relative H-bond dissociation energy (ΔH_{abs}) in kcal mol^{-1}. [c]) Concentration inhibiting oxidation by 50%. Values are expressed as the average of two experiments. [d]) Percent inhibition at a concentration of 100 μM. Values are expressed as the average of two experiments.

exists with BChE oxidation, the antioxidant activities of polyphenols were compared with their relative oxidizability. Since the primary antioxidant mechanism for a radical scavenger is to reduce radicals while itself undergoing oxidation, we examined three physicochemical parameters related to this mechanism. These parameters were the relative adiabatic oxidation potential (ΔH_{ox}) and the relative H-bond dissociation energy (ΔH_{abs}) as obtained from quantum-mechanical calculations, and the first oxidation potential as measured by cyclic voltammetry (Epa_1) [27].

No correlation was found between ΔH_{ox} and Epa_1. However, the good correlation between ΔH_{abs} and Epa_1 (*Eqn. 1*) suggests that, for this series of compounds, the first oxidation potential measured in protic solvents reflects the stability of oxy radicals.

$$Epa_1 = 0.06\ (\pm 0.013) \cdot \Delta H_{abs} + 0.19\ (\pm 0.07) \qquad (1)$$

$$n = 11;\ r^2 = 0.92;\ s = 0.08;\ F = 107$$

where n = the number of compounds; r^2 = the squared correlation coefficient; s = the standard deviation of regression; F = the *Fischer* test of significance. The 95% confidence limits are given in parentheses. The equation indicates that the experimental Epa_1 and the theoretical ΔH_{abs} contain the same information. As a consequence, ΔH_{abs} rather than Epa_1 was used in the subsequent search for structure-activity relationships.

Interesting conclusions emerge when comparing antioxidant activities with the two theoretical parameters ΔH_{abs} and ΔH_{ox}, as shown in *Fig. 6*,*a* and *b*. Taken together, these parameters appear as promising predictors of antioxidant activity against BCHE inactivation, but they also suggest that the mechanism of antioxidant action may differ among the compounds examined.

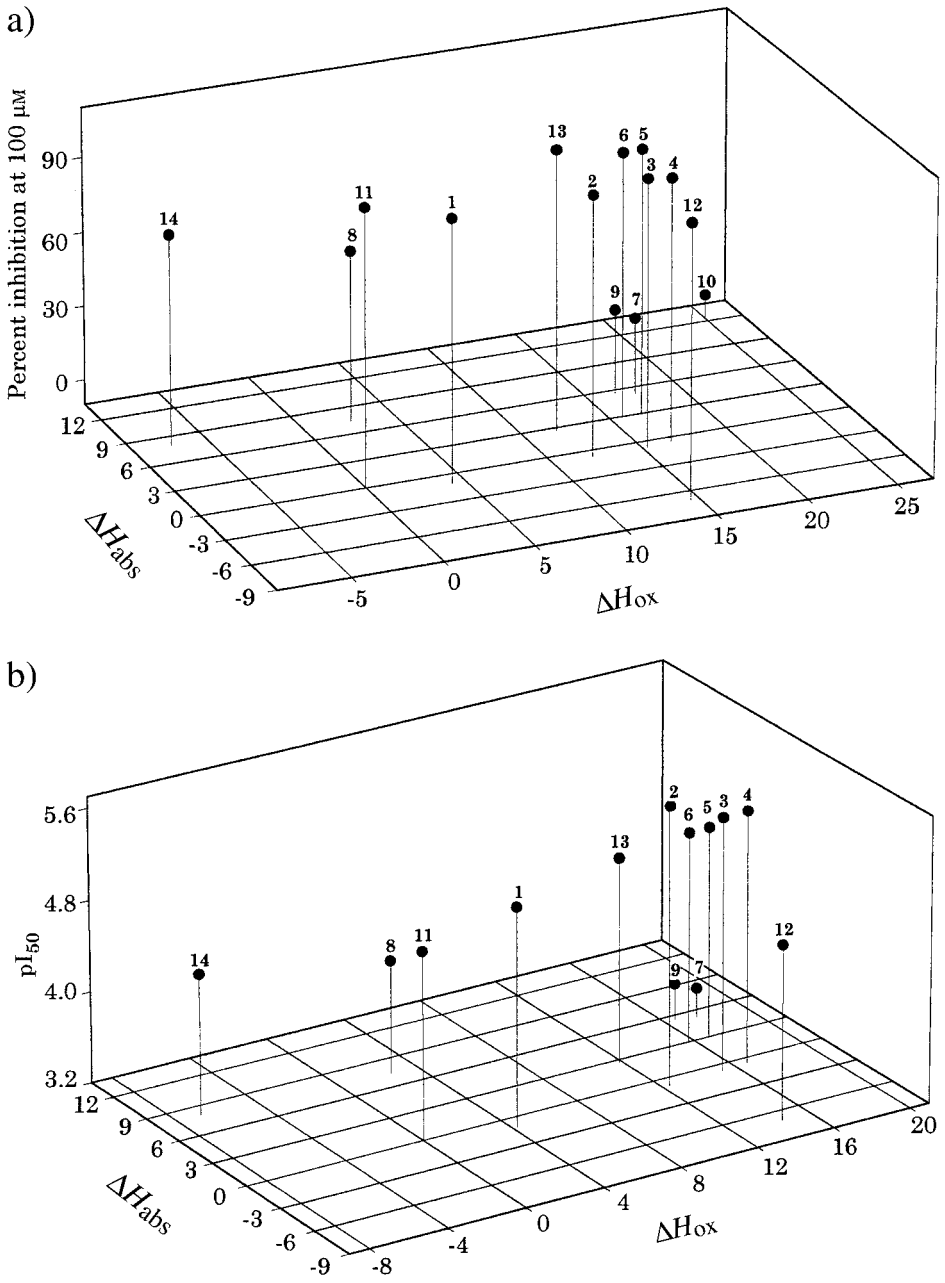


Fig. 6. *Percent inhibition at 100* μM (*a*) *and* IC_{50} (*b*) *of antioxidants as a function of their relative O–H bond dissociation energy* (ΔH_{abs}) *and their relative adiabatic potential* (ΔH_{ox}). For uric acid (**13**) and melatonin (**14**), the relative N–H bond dissociation energy was used. The theoretical parameters for ascorbic acid (**12**) and uric acid (**13**) were −7.06 and 2.71 kcal mol^{-1}, respectively, for the relative bond dissociation energy, and 13.79 and 8.86 kcal mol^{-1}, respectively for their relative adiabatic potential.

Antioxidants, which possess a O–H (or N–H) bond reactive toward homolysis (low H-bond dissociation energy: $\Delta H_{abs} < ca.$ 3 kcal mol^{-1}), belong to the group of efficient antioxidants operating *via* a H-atom-transfer reaction. Compounds lacking a breakable O–H (or N–H) bond may remain active antioxidants *via* a single-electron transfer (SET), if their relative adiabatic ionization potential (ΔH_{ox}) is low, as seen for flemichin D (**8**) and melatonin (**14**). They are inactive or poorly active, when their oxidation is difficult ($\Delta H_{abs} < 3$ kcal mol^{-1} and high ΔH_{ox}) as for 1,5-dihydroxy-3-methoxyxanthone (**7**), cordigone (**9**), and danthrone (**10**). The theoretical parameters indicate that both mechanisms might be operative for quercetin (**1**) and trolox (**11**) [36].

Furthermore, oxidizability may not to be the only factor involved, since the antioxidant activities of quercetin (**1**) and trolox (**11**) are lower than expected from the theoretical parameters. Indeed, complementary studies showed that these two compounds, and only these two, bind to human serum albumin. This suggests that binding to proteins may be an additional factor to consider when characterizing a good protein antioxidant [14].

2.4. *Mechanism of Antioxidant Effects.* To differentiate between the scavenging and chain-breaking effects of the antioxidants under study, we examined their ability to stop the oxidation reaction once it was initiated by adding the antioxidant after a given delay. This was done by measuring the remainder of hydrolytic activity as a function of oxidation time.

First, the remainder of esterase activity in the absence of any antioxidant was found to be $82 \pm 2.9\%$, $65 \pm 1.0\%$, and $50 \pm 6.5\%$ after 60-, 90-, and 120-min oxidation time, respectively. Should an antioxidant possess dual radical-scavenging and chain-breaking activity, its addition 60 or 90 min after the beginning of BCHE oxidation would preserve 82 or 65% of hydrolytic activity, respectively, after a total oxidation time of 120 min. Adding an antioxidant without chain-braking properties would leave 50% of esterase activity after 120 min.

For this experiment, 100 μM of quercetin (**1**), chlorogenic acid (**3**), or trolox (**11**) were added either 0, 60, or 90 min after the initiation of BCHE oxidation. When added at 0 min (*i.e.*, before AAPH), each of these compounds completely protected BCHE esterase activity, proving their good radical-scavenging capacity. When the antioxidants were added 60 or 90 min after the initiation of oxidation, the remaining esterase-like activity was 60–70% or 50%, respectively. This indicated that the compounds can scavenge the radicals generated in solution, but cannot completely block the chain reaction initiated on BCHE. The results for trolox are illustrated in *Fig. 7.*

In a second series of experiments, the percentage of remaining esterase activity was determined as a function of the amount of free radicals generated. The objective of these experiments was to investigate whether the protection of proteins by natural polyphenols remains complete until the antioxidant is entirely consumed by free radicals. Such a 'lag phase' corresponding to the total consumption of antioxidant was observed in some lipid-peroxidation systems [19].

The kinetics of AAPH decomposition indicated that 30, 60, and 120 μM of free radicals were generated from 1 mM AAPH after 2, 4, and 8 h, respectively, under our oxidation conditions ($45 \pm 0.1°$, pH 7.4) [14]. Studies with 100 μM of quercetin (**1**), caffeic acid (**4**), or trolox (**11**) added before the initiation of the oxidation reaction,

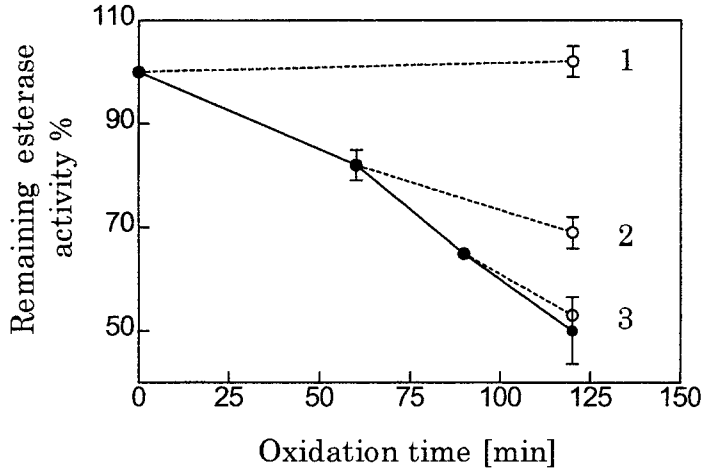


Fig. 7. *Example of protection by trolox* (**11**). Decrease in esterase activity as a function of time, in the absence of antioxidant (●). BChE esterase activity remaining after 120 min, when trolox was added before the initiation of oxidation (*1*), when trolox was added 60 min after the initiation of oxidation (*2*), and when trolox was added 90 min after the initiation of oxidation (*3*).

showed that only **1** completely protected BCHE (*Fig. 8*). *Fig. 8* indicates that, except for **1**, 60 μM of free radicals generated in 4 h were not completely destroyed by 100 μM **4**, or **11**. In other words, damage to the esterase active site was detectable before **4** or **11** was totally consumed. Thus, the functional damage to BCHE does not display a 'lag phase', as also seen in the oxidation of bovine serum albumin monitored by tryptophan oxidation, fragmentation, and conformational changes [19].

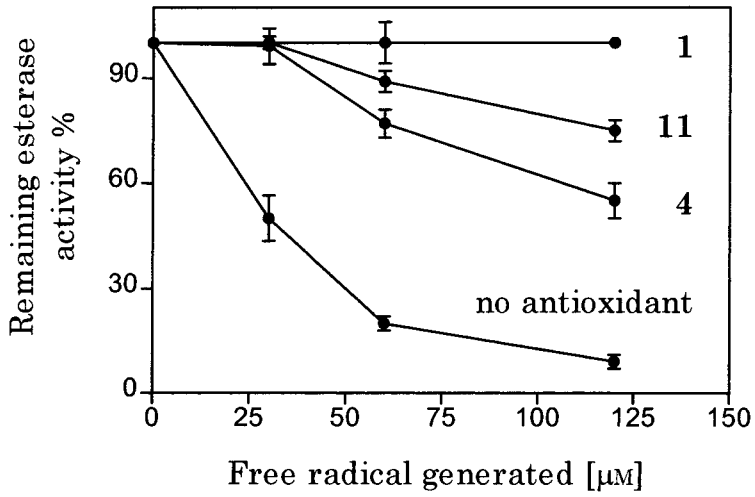


Fig. 8. *Percentage of esterase activity remainder and protection by 100* μM of *antioxidant* (quercetin (**1**), caffeic acid (**4**), and trolox (**11**)) *as a function of free radical generated*, i.e., *30* μM *after 2 h*, *60* μM *after 4 h*, *and 120* μM *after 8 h of oxidation at 45° C, pH 7.4*. Data are expressed as the mean of two experiments.

In a previous study, the same series of natural polyphenols were investigated for their capacity to inhibit lipid peroxidation caused by the oxidation system $FeCl_2/FeCl_3$ [27]. The free-radical generator AAPH was chosen here to assess antioxidant activities independently of metal-ion-chelating capacities. The activities presented here are not correlated with results of protection against lipid peroxidation, demonstrating the importance of other molecular properties such as metal-ion-chelating capacity and lipophilicity in inhibiting lipid peroxidation.

3. Conclusions. – The assay of antioxidant activity developed here appears to be convenient, sensitive, and accurate. Its application to a series of natural polyphenols and other antioxidants allowed us to gain insights into their mechanism of action, and to obtain IC_{50} values that proved of interest in structure-activity relations.

Experimental Part

Chemicals and Reagents. Essentially fatty-acid-free HSA (*A-1887*; lot *118F9311*) and essentially fatty-acid-free and globulin-free HSA (*A-3782*; lot *94H9318*), highly purified cholinesterase (BChE) from horse serum, moxisylyte hydrochloride and quercetin were obtained from *Sigma Chemical Co.* (St Louis, MO, USA). Essentially fatty-acid-free and globulin-free HSA (*05418*), dimethyl sulfoxide (DMSO), chlorogenic acid (**3**), uric acid (**13**), melatonin (**14**), acetylcholine chloride, and phosphate salts for the buffers were purchased from *Fluka* (CH-Buchs). 2-Butoxyethyl nicotinate was kindly donated by Dr. *Karl Thomae* (D-Biberach an der Riss). BCECF (=2′,7′-bis(ethoxycarbonyl)fluorescein-5(6)-carboxylic acid) came from *Calbiochem* (La Jolla, CA, USA). AAPH (=2,2′-Azobis(2-amidinopropane) dihydrochloride) was from *Wako Chemicals* (D-Neuss). Trolox (=6-hydroxy-2,5,7,8-tetraethylchroman-2-carboxylic acid; **11**) and caffeic acid (**4**) were purchased from *Aldrich-Chemie* (D-Steinheim). Ascorbic acid (**12**) was acquired from *Merck* (D-Darmstadt). Danthrone (**10**) was obtained from *Roth* (D-Karlsruhe). The other natural products were isolated as previously described: verbascoside (**2**) from the root bark of *Sesamum angolense* (Pedaliaceae) [37]; 1,3,6,7-tetrahydroxyxanthone (**5**) and mangiferin (**6**) from *Gentiana rhodentha* (Gentianaceae) whole plant [38]; 1,5-dihydroxy-3-methoxyxanthone (**7**) from the roots of *Chironia krebsii* (Gentianaceae) [39]; flemichin D (**8**) from the roots of *Eriosema tuberosum* (Leguminosae) [40]; cordigone (**9**) from *Cordia goetzei* (Boraginaceae) stem bark [41].

All chemicals were of anal. grade, and the solns. were prepared with demineralized and purified H_2O obtained with the system *Seralpur Pro 90C* (*Seral*, *Renggli*, CH-Rotkreuz). MeOH was of HPLC-grade and obtained from *Romil Chemicals* (UK-Loughborough).

Equipment. Protein oxidation was carried out in a thermostated bath *Heto Maxishake* (*Kleiner*, CH-Wahlen). Fluorescence was monitored with a *Perkin-Elmer* luminescence spectrometer *LS 50B* (*Perkin-Elmer*, UK) for assays with BChE-contaminated HSA and a *SAFAS flx* spectrofluorimeter (*SAFAS*, Monaco) for assays on BChE with or without albumin. All measurements were made with standard 1-cm, sealed quartz cells (*111 QS*, *Hellma Swiss*, CH-Basel). The cells were maintained at $37 \pm 0.2°$ with a water-bath circulator (*Haake D8* and *D1*, *Digitana*, CH-Lausanne). A *Metrohm 654* pH-meter (CH-Buchs) was used for all pH measurements.

Oxidation Induced by the Free Radical Generator AAPH. The concentrations of BChE and BChE-contaminated albumin (*Sigma A-1887*) stock solns. were adjusted to obtain 50% hydrolysis of moxisylyte after 10–12 min when incubated without AAPH. The albumins added to BChE (*Sigma A-3782* and *Fluka 05418*) were tested separately to verify their absence of activity toward moxisylyte.

For the oxidation assays on BChE with or without 2 μM albumin (*Sigma A-3782*), the final solns. consisted in 100 μl of BChE 0.05 mg/ml (in 10 mM phosphate buffer of pH 8.0), 0 or 100 μl of albumin 75 μM (*Sigma A-3782* in 7.4 mM phosphate buffer of pH 7.4), 40 μl of antioxidant in DMSO, and completed to a volume of 3.87 ml with phosphate buffer (pH 7.4 ± 0.1; 7.4 mM; ionic strength 0.173 adjusted with KCl). These solns. were incubated at $37 \pm 0.1°$ for 15 min. Oxidation was then initiated by the addition of 40 μl AAPH (0.4 mM final conc.), and incubation was continued at 37° for another 60 min under continuous shaking.

A somewhat different procedure was used for the inactivation of BChE-contaminated HSA (50 μM *Sigma A-1887*) or when using a mixture of BChE and albumin from another source (50 μM *Fluka 05418*). Here, the antioxidants were dissolved in MeOH, the phosphate buffer was more diluted (pH 7.4 ± 0.1; 6.1 mM;

ionic strength 0.173 adjusted with KCl), because of the buffer capacity of albumin, the incubation temp. was $45 \pm 0.1°$, and the oxidation time was 120 min in the presence of 1 mM AAPH (final conc.).

Controls in the absence of antioxidants (maximal oxidation) or AAPH (no oxidation) were carried out by adding the co-solvent or phosphate buffer, resp., to the protein soln.

Kinetic Studies of Esterase Activity. Immediately after incubation, 20 µl of the pH indicator BCECF (96 nM) was added to the protein sample, and the enzymatic reaction was initiated by adding the moxisylyte soln. (67 µl). The concentration of the ester substrate in the final reaction mixture was fixed at 1 mM in all assays. Hydrolysis was monitored at $37 \pm 0.2°$ by a continuous spectrofluorimetric assay as described in [42].

The slopes of initial hydrolysis were determined by linear regression from the plots representing the decay of the ratio (F_{505}/F_{442}) *vs.* time, where (F_{505}/F_{442}) is the fluorescence ratio of BCECF (excitation at 505 and 442 nm, emission at 533 nm, slits of 10 nm × 15 nm).

The protective activity of the antioxidants tested was calculated as the percentage of esterase activity (initial rates of moxisylyte hydrolysis) obtained in the absence of oxidation (assumed to be 100%). IC_{50} Values were calculated by curve fitting according to classical sigmoidal dose-response equation using Prism V 3.0 (*GraphPad Software*, San Diego, USA). The IC_{50} values of the antioxidants were determined in duplicate from at least five concentrations.

Quantum-Mechanical Calculations. As already demonstrated in the case of vitamin E analogues [36], melatonin, and carvedilol [43], semi-empirical calculations yield useful parameters to characterize antioxidants, namely their adiabatic ionization potential and O–H bond dissociation energy. These parameters were calculated for the natural polyphenols according to the following strategy:

1) In each case, the most stable conformation of the polyphenol was determined by systematic search for the more rigid compounds and by high-temp. molecular dynamics [44] for the flexible compounds **1**, **8**, and **9**. In the case of verbacoside (**2**) and chlorogenic acid (**3**), model catechol moieties were calculated, namely the methyl ester of the caffeic acid and 4-(2-methoxyethyl)benzene-1,2-diol. All the calculations were performed twice, first with the Tripos force-field [45], then with the AM1 Hamiltonian [46] using the Sybyl 6.4 [47] and Spartan 5.0 [48] softwares running on *Silicon Graphics* workstations (*O2 R5000* and *Origin 2000 R10000*).

2) The compounds were then oxidized by removing one electron, and the geometry of the corresponding radical cation was optimized at the semi-empirical level using the UHF approximation. Trolox (**11**) was used as the reference compound, allowing the relative adiabatic potential ΔH_{ox} to be calculated by *Eqns. 2* and *3* (*Table 2*).

$$\mathrm{ROH} + \mathrm{Trolox}^{\bullet+} \rightleftharpoons \mathrm{ROH}^{\bullet+} + \mathrm{Trolox} \tag{2}$$

$$\Delta H_{ox} = \Delta H_f(\mathrm{Trolox}) + \Delta H_f(\mathrm{ROH}^{\bullet+}) - \Delta H_f(\mathrm{Trolox}^{\bullet+}) - \Delta H_f(\mathrm{ROH}) \tag{3}$$

3) O–H Bonds were then cleaved homolytically, the geometry of the corresponding radical was optimized at the semi-empirical level using the UHF approximation, and the more stable radical was retained for each compound. The bond dissociation energy relative to Trolox was calculated by *Eqns. 4* and *5* (*Table 2*).

$$\mathrm{ROH} + \mathrm{Trolox}^{\bullet} \rightleftharpoons \mathrm{RO}^{\bullet} + \mathrm{Trolox} \tag{4}$$

$$\Delta H_{abs} = \Delta H_f(\mathrm{Trolox}) + \Delta H_f(\mathrm{RO}^{\bullet}) - \Delta H_f(\mathrm{Trolox}^{\bullet}) - \Delta H_f(\mathrm{ROH}) \tag{5}$$

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