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QUARTERLY

Antioxidant activity: what do we measure?

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Inhibition of oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by free radicals generated by decomposition of 2,2′-azobis(2-amidopropane) (ABAP) by antioxidants and biological material was studied. A correlation was found between the ability of various substances to delay the onset of ABTS oxidation and their rapid reduction of the $ABTS^{+\bullet}$ cation radical, and between the ability to reduce the maximal rate of ABTS oxidation and slow reduction of $ABTS^{+\bullet}$. The length of the lag period of ABTS oxidation was found to be independent of ABTS concentration. Similar decrease of peroxynitrite-induced $ABTS^{+\bullet}$ formation by antioxidants was observed when the antioxidants were added before and after peroxynitrite. All these findings indicate that the main effect of antioxidants in this system is reduction of $ABTS^{+\bullet}$ and not prevention of its formation. Reduction of oxidation products rather than inhibition of their formation may be the predominant mode of action of antioxidants in various assays of antioxidant activity.

Recently, we proposed a simple spectrophotometric method for the determination of antioxidant activity of biological fluids. In this method, free radicals generated by homolytic decomposition of 2,2′-azobis(2-amidopropane) (ABAP) oxidize 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to a green cation radical ($ABTS^{+\bullet}$); antioxidants present in the sample inhibit this oxidation (Bartosz *et al.,* 1998a, b). An obvious interpretation of the effect of antioxidants was that these compounds react with peroxyl radicals generated from ABAP and prevent their reactions with ABTS; therefore, we called this test the estimation of the "peroxyl radical-trapping capacity". However, when attempting to get an insight into the mechanism of this reaction, we found that some substances delay the onset of ABTS oxidation (a lag time in the reaction is observed), others decrease the

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Abbreviations: ABAP, 2,2′-azobis(2-amidopropane); ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl; Tempamine, 4-amine-2,2,6,6-tetramethylpiperidine-1-oxyl.

maximal rate of ABTS oxidation without introducing the lag, while there are also substances which affect the reaction in both ways. Analysis of the mechanism of the influence of various compounds on ABTS oxidation in our system leads to a conclusion that most if not all of the effects of antioxidants are due to the reaction of antioxidants with $ABTS^{+\bullet}$ rather than with the primary radicals. This conclusion is apparently applicable also to other assays employing ABTS and also to other assays of antioxidant activity.

MATERIALS AND METHODS

ABAP was purchased from Polysciences (Warrington, PA, U.S.A.; Cat. No. 08963). ABTS was from Sigma-Aldrich (St. Louis, MO, U.S.A., Cat. No. A 1888). Other reagents were from Sigma-Aldrich; analytical grade sodium phosphates were from POCh (Gliwice, Poland). Peroxynitrite was synthesized by ozonation of azide solution (Pryor *et al.*, 1995).

The assay of "peroxyl radical-trapping capacity" was executed as described before (Bartosz *et al.*, 1998a, b). Briefly, the sample to be assayed was introduced into a spectrophotometric cuvette containing 150 μM ABTS and 0.1 M sodium phosphate buffer, pH 7.0, prewarmed to 37°C. The reaction was initiated by the addition of a stock (200 mM) ABAP solution to a final concentration of 20 mM. Final reaction volume was 3.0 ml. Absorbance increase at 414 nm was monitored in a thermostated recording spectrophotometer at 37°C for at least 10 min. From the kinetic curves, the lag time and the maximal rate of absorbance increase were read.

Oxidation of ABTS by peroxynitrite was studied by the additon to 150 μM ABTS solution in 0.1 M phosphate, pH 7.0, of a stock solution of peroxynitrite to a final concentration of 100 μM. After rapid mixing, absorbance of samples at 414 nm was measured.

RESULTS AND DISCUSSION

Coincubation of ABAP and ABTS results in the appearance of a green colour of $ABTS^{+\bullet}$. The typical course of ABTS oxidation in reference samples (containing no antioxidant) and in samples containing Trolox and blood plasma is shown in Fig. 1. A simple interpretation of this reaction would be as shown in Scheme 1: radicals generated by thermal decomposition of ABAP oxidize ABTS to the green cation radical $ABTS^{+\bullet}$. ABTS is not oxidized under aerobic conditions (not shown) which indicates that peroxyl (and perhaps alkoxyl) radicals are the oxidizing factors. Antioxidants present in the sample prevent this reaction by reacting with peroxyl radicals. Antioxidants react with these radicals faster

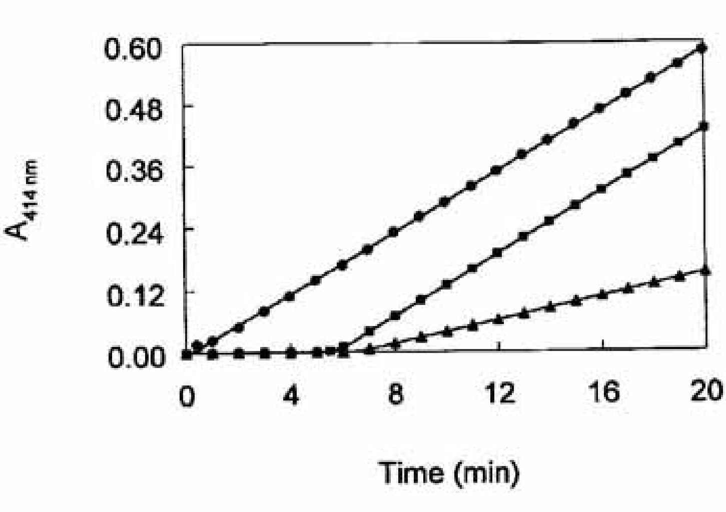
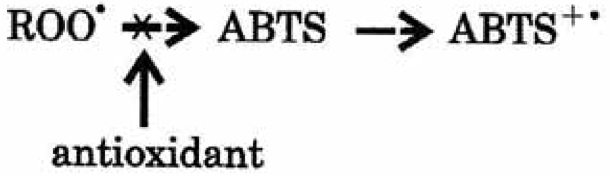


Figure 1. ABTS oxidation by ABAP ●, in the absence of antioxidants; ■, in the presence of 2 μM Trolox, and ▲, in the presence of blood plasma (20 μl/3 ml).

than with ABTS so they are consumed first; only when they are used up, ABTS oxidation commences. Thus, in the presence of antioxidants there is a lag time of ABTS oxidation. Therefore, the length of the lag is a measure of the content of antioxidants reacting (mainly) with peroxyl radicals in the sample ("peroxyl radical trapping capacity" of the material studied). However, the actual course of reactions does not seem to follow this scheme.



Scheme 1.

Such antioxidants as Trolox, ascorbate, reduced glutathione and urate delay the onset of ABTS oxidation (Fig. 2) and do not affect the maximal rate of ABTS oxidation (Bartosz *et al.*, 1998b). On the other hand, blood plasma (Fig. 1) as well as beverages studied such as beer or wine both introduce the lag and decrease the maximal rate of ABTS oxidation. From among amino acids, tryptophan (Fig. 3) and to a lesser degree tyrosine are able to reduce the maximal rate of ABTS oxidation (though not introducing the lag) (Bartosz *et al.*, 1998b). These observations suggest that while the length of the lag of the ABTS oxidation by free radicals generated upon the ABAP decomposition is a measure of the content of antioxidants in the sample, the maximal rate of ABTS oxidation is influenced by reactions of components of doubtful antioxidant significance. Amino acids such as tryptophan and tyrosine may have a beneficial antioxidant effect in some situations (e.g., tyrosine was found to protect the yeast *Saccharomyces cerevisiae* from oxidative stress (Lupo *et al.*, 1997)). Proteins can be considered as sacrificial antioxidants but this idea can hardly

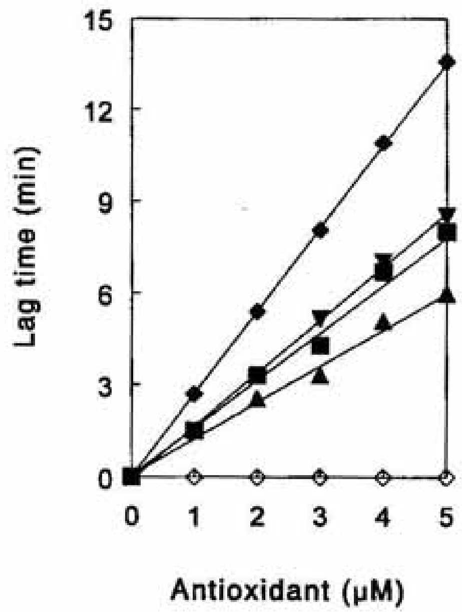


Figure 2. Dependence of lag time on antioxidant concentration in the sample; ◆, Trolox; ▼, urate; ■, ascorbate; ▲, glutathione; ◇, Tempo.

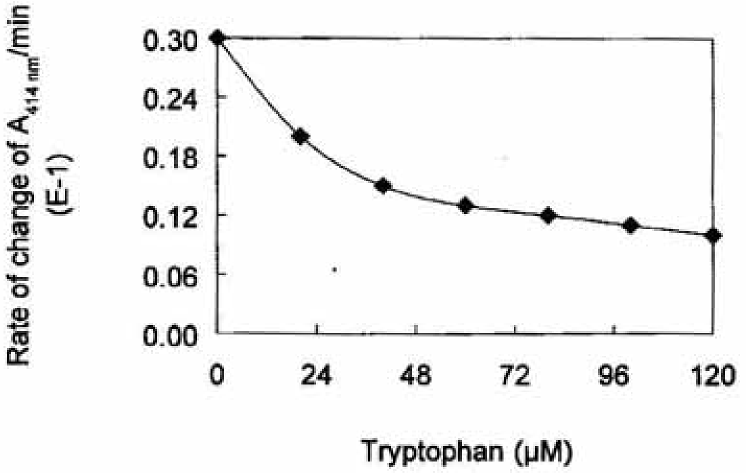


Figure 3. Dependence of the maximal rate of ABTS oxidation on tryptophan concentration in the sample.

be applied to proteins which are functionally important; free-radical damage to tryptophan and tyrosine residues may inactivate such proteins. Therefore, reactions of protein components should not be included in an assay of antioxidant capacity of biological samples and assays based on lag time of ABTS oxidation should be recommended, *vis-a-vis* assays based on the maximal rate of ABTS oxidation or fixed time-point assays influenced by both these parameters. The "peroxyl radical-trapping capacity" of blood plasma determined on the basis of the lag time in the ABAP–ABTS assay (640 ± 132 μM) was lower than usually reported but did not leave space for an "unidentified antioxidant" reported to contribute to the antioxidant capacity of human plasma (Aejmelaeus *et al.*, 1996; 1997).

Not all acknowledged antioxidants were found to introduce the lag. ABTS oxidation was not delayed in the presence of nitroxyls such as 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) or 4-amine-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempamine) while these compounds decreased the maximal rate of ABTS oxidation. Still other antioxidants (adrenaline and quercetin) both introduced the lag and decreased the maximal rate of ABTS oxidation (Table 1). We observed a correlation between the ability of various substances to introduce the lag in ABTS oxidation in the ABAP–ABTS assay and their ability to reduce preformed $ABTS^{+\bullet}$. These substances which introduced the lag reduced $ABTS^{+\bullet}$ rapidly; upon addition of such substances (as shown for Trolox in Fig. 4a) the reduction was instantaneous in the time-scale of the kinetic assay employed. On the other hand, substances which decreased the maximal rate of ABTS oxidation reduced ABTS slowly (as shown for Tempo in Fig. 4b). Substances which both introduced the lag time and decreased the maximal rate of ABTS oxidation showed two phases of $ABTS^{+\bullet}$ reduction, a rapid one followed by a slow one. These results suggest that the course of reactions in the ABAP–ABTS assay corresponds rather to Scheme 2: ABTS is first oxidized to $ABTS^{+\bullet}$ and in the second step $ABTS^{+\bullet}$ is reduced by reductants present in the sample. In reality, both patterns presented in Schemes 1 and 2 can contribute to the net outcome of reactions. However, the following arguments speak for a dominant contribution of the reaction pattern presented in Scheme 2.

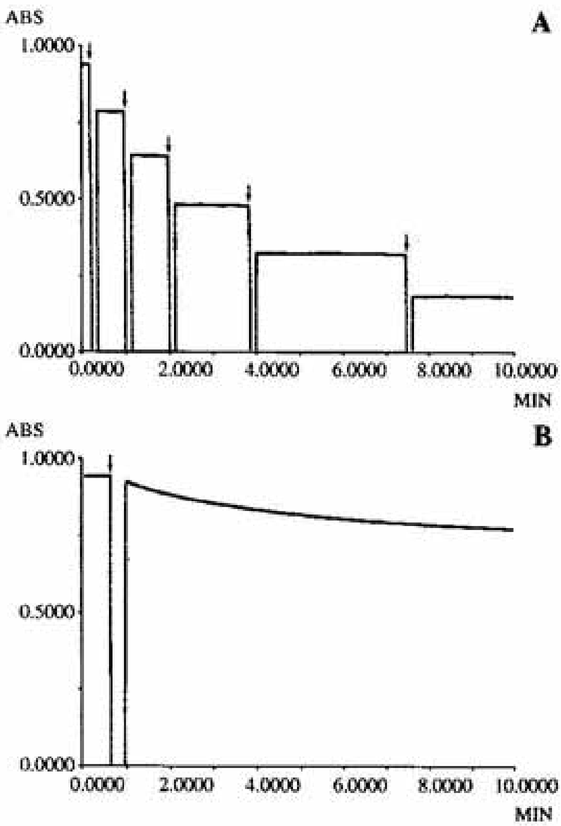


Figure 4. Reduction of preformed $ABTS^{+\bullet}$ by consecutive portions of Trolox (2 μM final) (a) and by 20 μM Tempo (b).

$$ROO^{\bullet} \rightarrow ABTS \rightarrow ABTS^{+\bullet} \rightarrow ABTS$$

$$\uparrow$$

antioxidant

Scheme 2.

Firstly, the length of the lag time was found to be independent of ABTS concentration (Fig. 5). Let us assume that the lag time corresponds to the time in which the reaction rate of peroxyl radicals with an antioxidant is

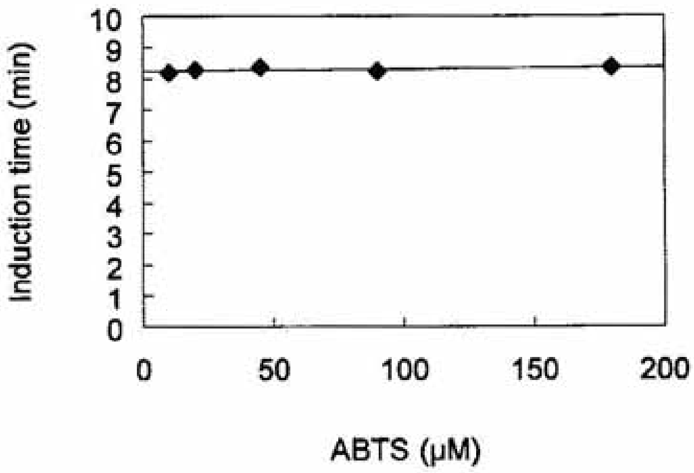


Figure 5. Effect of ABTS concentration on the length of the induction period of ABTS oxidation in the presence of 4 μM Trolox.

higher than the rate of reaction of these radicals with ABTS, $v_{ant} > v_{ABTS}$. Since appropriate rates are products of the corresponding rate constants times concentrations, $v_{ant} = k_{antioxidant} \times [antioxidant]$ and $v_{ABTS} = k_{ABTS} \times [ABTS]$. In such a case changing the concentration of ABTS should affect the length of the lag time which was not the case.

Secondly, diminution of ABTS oxidation by peroxynitrite is independent of the sequence of additions of peroxynitrite and antioxidants. Reaction of peroxynitrite with all the antioxidants studied attenuated ABTS oxidation; however, the reduction of ABTS oxidation did not differ much when antioxidants were added before or after addition of peroxynitrite (Fig. 6). Peroxynitrite reacts with ABTS in a time scale of seconds (Bartosz, 1996) so the reactions of antioxidants added about 1 minute after peroxynitrite represent reduction of preformed $ABTS^{+\bullet}$.

Similar conclusions have been reached by Romay and coworkers who suggested a possibility of assaying antioxidants reacting rapidly and slowly with $ABTS^{+\bullet}$ in s decoloration assay (Romay *et al.*, 1996).

Reactions of $ABTS^{+\bullet}$ with various components of biological systems have been reported by other authors. $ABTS^{+\bullet}$ was found to be reduced by oxalate, glyoxylate and malonate especially in the presence of Mn^{2+} ions (Collins *et al.*, 1998), and to react with hydroperoxides in addition to antioxidants (Aliaga & Lissi, 1998).

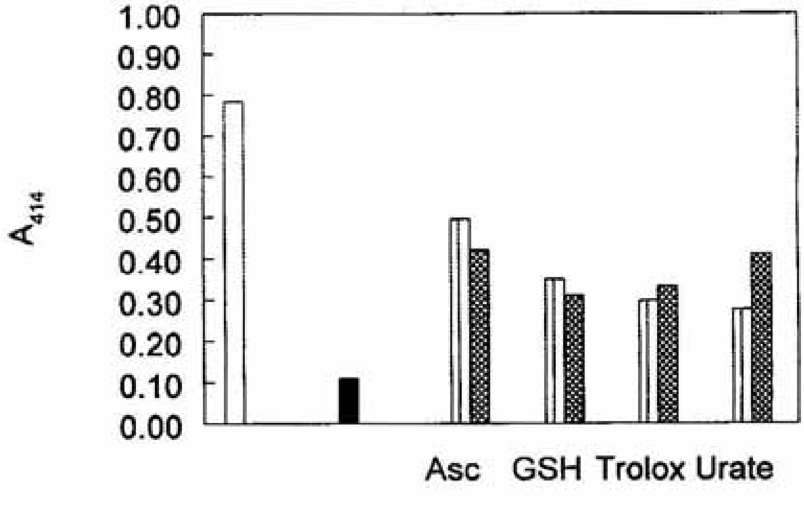


Figure 6. Attenuation of ABTS oxidation by peroxynitrite (100 μM) by antioxidants added before or after the addition of peroxynitrite.

Asc, ascorbate; □, control; ■, peroxynitrite; striped bars, antioxidant added before peroxynitrite; netted bars, antioxidant added after peroxynitrite.

Table 1. Effect of various substances on the lag time and maximal rate of ABTS oxidation

Substances	Introduces lag time	Affects ABTS oxidation rate	Reduces $ABTS^{+\bullet}$ rapidly	Reduces $ABTS^{+\bullet}$ slowly
Ascorbate	+	–	+	–
GSH	+	–	+	–
Cysteine	+	–	+	–
Trolox	+	–	+	–
Urate	+	–	+	–
Adrenaline	+	+	+	+
Quercetin	+	+	+	+
Tempamine	–	+	–	+
Tempo	–	+	–	+
Most amino acids	–	–	–	–
Tryptophan	–	+	–	+
Tyrosine	–	+	–	+
Blood plasma	+	+	+	+
Urine	+	+	+	+
Seminal plasma	+	+	+	+
Beverages	+	+	+	+

These results point to the necessity of careful interpretation of the estimations of "antioxidant activity" employing $ABTS^{+\bullet}$ as indicator. The possibility must be considered that the "antioxidant effect" measured is mostly due to the reduction of $ABTS^{+\bullet}$ rather than to the prevention of its formation. What is potentially even more embarassing, this reaction pattern can be shared by other reversible redox indicators. Preliminary experiments (Stepien *et al.*, in preparation) point to a comparable extent of inhibition of peroxynitrite-induced dihydrorhodamine 123 oxidation by antioxidants added before and after peroxynitrite. The coincidence of the results of determination of antioxidant activity of blood plasma by methods apparently based on the prevention of oxidation of indicator substances (Ghiselli *et al.*, 1995; Lönnrot *et al.*, 1996; Uotila *et al.*, 1994; Wayner *et al.*, 1985) and by the FRAP assay based on determination of the ability of plasma to reduce ferric ion (Benzie & Strain, 1996) can also be another facet of the same problem.

The fact that a considerable if not dominant fraction of the antioxidant action in some assays of antioxidant activity is due to the reduction of oxidized indicator rather than to the prevention of its formation does not invalidate these assays provided their interpretation does not go too far. Moreover, this fact suggests that the mode of action of antioxidants *in vivo* may also be due vastly to reduction or chemical repair of important targets rather than to prevention of their damage as far as reversible redox reactions are concerned.

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