paper on methodology

Spectrophotometric assay for total peroxyl radical-trapping antioxidant potential in human serum

Miia Valkonen and Timo Kuusi[1]

Department of Medicine, University of Helsinki, 00290 Helsinki 29, Finland

Abstract Antioxidants prevent modification of low density lipoprotein (LDL) by free radicals and possibly also atheroma formation. The capacity of human serum to resist attacks by free radicals is measured by the total peroxyl radical-trapping potential (TRAP). Its measurement has thus far required equipment not available in many clinical laboratories such as a thermostated oxygen electrode cell or a luminometer. To develop a simpler method we used a free radical probe, dichlorofluorescin-diacetate (DCFH-DA), described before in studies of respiratory burst in inflammatory cells. Its oxidation by radicals from thermal decomposition of 2,2′-diazobis(2-amidinopropane)dihydrochloride (AAPH) converts this compound to highly fluorescent dichlorofluorescein (DCF). The DCF also has absorbance at 504 nm thus enabling the determination of TRAP either fluorometrically or spectrophotometrically. Increasing the concentration of AAPH enables the measurement of DCF formation and its inhibition by serum samples at room temperature. The intra- and interassay coefficients of variation of this assay are 3.4% and 4.6%, respectively. The mean value for serum TRAP of healthy subjects is 1155 μmol/l (n = 38). The TRAP in human serum can be increased by adding various antioxidant substances to the assay in vitro or by dietary supplementation of healthy subjects with vitamin E in vivo ($P < 0.025$). An increase was also found in serum vitamin E levels ($P < 0.0001$) and in the length of the time human LDL is able to resist oxidation ($P < 0.05$). Thus the determination of TRAP by this method, which requires only commercially available chemicals, can be used for the evaluation of phenomena associated with lipid accumulation in human artery wall.—**Valkonen, M., and T. Kuusi.** Spectrophotometric assay for total peroxyl radical-trapping antioxidant potential in human serum. *J. Lipid Res.* 1997. **38:** 823–833.

Supplementary key words dichlorofluorescin-diacetate • free radicals • 2,2′-diazobis(2-amidinopropane)dihydrochloride lipid-peroxidation • LDL • vitamin E supplementation • Trolox®

Atherosclerosis is an inflammatory process, where modification of low density lipoprotein (LDL) by oxygen free radicals from damaged tissue or inflammatory cells is thought to play a central role (1, 2). The LDL modification is inhibited by several antioxidant substances present in human serum and tissues, and only a failure of this antioxidant barrier allows LDL lipid peroxidation to take place (3–5).

LDL lipids are attacked by the lipophilic radicals, mainly formed by free radicals of the aqueous phase, that the water-soluble antioxidants have failed to neutralize especially after they have been consumed (6, 7). Thus, both aqueous and lipid phase antioxidants are needed to protect LDL from lipid peroxidation (8). In the lipid phase the first line of defense is offered by vitamin E, which is located on the surface of LDL particles with its chromanol ring facing the aqueous phase (9–12). This location enables the cooperation with aqueous antioxidants, such as ascorbic acid and bilirubin, that can protect LDL from lipid peroxidation also by regenerating vitamin E from tocopheryl radical (13–16).

The cooperation of antioxidants in human serum provides greater protection against attacks by free radicals than any antioxidant alone (6, 17). This has been shown by in vivo studies where dietary antioxidant supplementation leads to a much larger increase in the overall antioxidant capacity of serum than the increase in the serum concentration of the dietary antioxidant would suggest (18). Therefore, efforts have been made to determine the total peroxyl radical-trapping potential (TRAP), which is the combined capacity of all antioxidants to neutralize free radicals in serum (19, 20). The methods for TRAP also enable comparison of the effects of the biologically important antioxidants in vitro (6). In addition, in vivo dietary antioxidant supplementation (18) and some physiological (21) and disease states (21–25), have been reported to cause changes in TRAP.

Abbreviations: AAPH, 2,2′-diazobis(2-amidinopropane) dihydrochloride; DCFH-DA, dichlorofluorescin-diacetate; TRAP, total peroxyl radical-trapping potential; LDL, low density lipoprotein; DCF, fluorescent dichlorofluorescein; PBS, phosphate-buffered saline; SOD, superoxide dismutase.

[1]To whom correspondence should be addressed.

The introduction of water-soluble azo-compounds (26) has enabled the peroxidation of lipids at a constant rate in a test tube. The azo-compound, 2,2′-diazobis (2-amidinopropane)dihydrochloride (AAPH), which in aqueous milieu generates free radicals at a constant rate, has especially been widely used as a free radical source for the determination of TRAP. The measurement of serum TRAP is based on the determination of the length of time that a subject's serum is able to resist artificially induced peroxidation. Wayner et al. (27) followed the peroxidation by monitoring oxygen consumption in a thermostated oxygen electrode cell during peroxidation of linoleate by free radicals, whereas Metsä-Ketelä (28) used a centrifugal luminometer to automate the TRAP determination. This equipment is, however, not common in most clinical laboratories. A thermostated fluorescence method of quantitating the oxygen-radical absorbing capacity, ORAC, of serum antioxidants has been introduced by Cao, Alessio, and Cutler (29).

We used 2,7-dichlorofluorescin-diacetate (DCFH-DA) to follow the formation of free radicals during the decomposition of AAPH. This compound has previously been used for the determination of peroxide concentration in various solutions (30) and as a respiratory burst indicator of inflammatory cells in flow-cytometry (31, 32). Cellular esterases first hydrolyze DCFH-DA to DCFH, which is then oxidized by free radicals to the fluorescent DCF. In this TRAP assay the cellular esterases are replaced by those of serum. Cells are replaced by the AAPH as a source of free radicals. Thus, the use of DCFH-DA is extended to determine the antioxidant defense of human serum and to compare the antioxidant capacity of different sera.

After the antioxidants have been consumed, the free radicals oxidize the nonfluorescent DCFH to fluorescent dichlorofluorescein (DCF). Notably, the oxidation product, DCF, also appeared to have absorbance peaking at 504 nm, allowing its determination by spectrophotometry. The effects of exogenous esterases on TRAP was studied in vitro. Furthermore, the influence of various antioxidants on TRAP was studied in vivo by giving vitamin E to healthy young subjects and in vitro by adding α-tocopherol, β-carotene, ascorbic acid, uric acid, bilirubin, and the antioxidant enzymes, superoxide dismutase and catalase, to human serum.

MATERIAL AND METHODS

Reagents

Sodium urate, porcine carboxyl esterases (EC 3.1.1.1.), (210 U/mg), superoxide dismutase (EC 1.15.1.1.), catalase (EC 1.11.1.6.) and ± α-tocopherol were from Sigma Chemicals (St. Louis, MO). Bilirubin was a standard of Bioclin Co (BV 1295, no. 00013) and β-carotene was obtained from Merck (Darmstadt, Germany). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich Chemical Co (Milwaukee, WI). L-Ascorbic acid was from BDH Chemicals; AAPH (2,2′-diazobis (2-amidinopropane) dihydrochloride) from Polysciences Europe (Germany) and 2,7-dichlorofluorescein-diacetate (DCFH-DA) was obtained from Serva (Germany). Sodium urate, ascorbic acid, superoxide dismutase, AAPH, and DCFH-DA were dissolved in phosphate-buffered saline (PBS, 1 mol/l). β-Carotene and α-tocopherol were dissolved in isopropanol. AAPH and DCFH-DA were kept at −20°C. Trolox® was dissolved in ethanol and stored at 4°C for 2 months.

Subjects

Blood was taken from healthy subjects (25–40 years, no medication, lipid disorder, thyroid disease, diabetes, or any other disease to our knowledge), after an overnight fast (10–12 h), into tubes kept in ice. Serum or plasma containing 1 mg/ml of ethylenediamine tetraacetate (Na_2EDTA 1 mg/ml) was separated by centrifugation for 10 min at $2300/g_{av}$ and at 4°C. If not used immediately, samples were stored at −80°C and used within 2 months. The effect of α-tocopherol on TRAP was studied in six male and five female subjects, who were given 100 mg of α-tocopherol (DO E, Orion Co., Finland) t.i.d. for 1 week. The subjects were advised to continue their habitual diet during the study. Blood was sampled before (day 0) and after supplementation (on days 8 and 21).

Chemical determinations

Serum cholesterol, triglycerides, and HDL were determined enzymatically in a Cobas Mira-S Centrifugal Analyzer (Roche Inc, Basel, Switzerland) using commercially available kits (Roche, Cat. No: Chol 0736643, TG 0736805, HDL 0720674). Serum uric acid was measured using commercially available reagents (No. 0736813). Ascorbic acid was determined according to the spectrophotometric method of Denson and Bowers (33) at 520 nm. Serum α-tocopherol concentrations were determined by high performance liquid chromatography according to the method of Schäfer Elinder and Walldius (34). Lipid-standardized α-tocopherol values were calculated by dividing α-tocopherol concentration by the sum of serum total cholesterol and triacylglycerol concentrations (35). Serum sulfhydryl groups were determined by the method described by Ellman (36).

LDL preparation and oxidation

LDL was isolated by rate-zonal ultracentrifugation in a density gradient essentially as follows. The density of serum was adjusted to 1.5 g/ml with solid NaBr, followed by pipetting 1.0 ml into 6-ml tubes (Nalgene Co. Ultra tube Cat. No. 3400-1610) and overlayering successively with 2.0 ml of 1.21 g/ml and 1.063 g/ml solutions. Finally, 1.0 ml of H_2O was added. All solutions contained mg/ml of EDTA. Ultracentrifugation was carried out at 270,000 g_{av} and at 4°C for 1.5 h in a Beckman L8-70 ultracentrifuge using a Beckman T1 50.4 rotor. The distinct LDL band was carefully separated and EDTA was removed using small dextran-sulfate affinity columns (Liposorber LA-15, Kaneka Co., Osaka, Japan). LDL-oxidation was initiated by adding freshly prepared $CuSO_4$ solution to the final concentration of 2.66 μmol/l. The formation of conjugated dienes was monitored at 234 nm in a motorized 6-cuvette cell-equipped Shimadzu spectrophotometer (UV-1201) connected to a microcomputer through a RS232 cable (37).

Determination of TRAP

Free radicals were formed during thermal decomposition of AAPH in H_2O and followed by measuring the conversion of DCFH-DA to the highly fluorescent DCF. The DCF formation was measured in an automated Transcon 102 FN fluorometer analyzer (Orion Co., Finland) at room temperature using excitation and emission wavelengths of 480 nm and 526 nm, respectively. In addition, the DCF formation was followed spectrophotometrically at 504 nm in a Shimadzu spectrophotometer.

Serum was mixed with PBS to a final dilution of 1%, followed by addition of DCFH-DA to a final concentration of 14 μmol/l. The reaction was started by adding AAPH to a final concentration of 56 mmol/l. It was stored at −20°C, thawed, and kept in ice until added to the incubation. Trolox®, 8.4 μmol/l, was used as an internal standard and it was added during the propagation phase when the absorbance at 504 nm had increased to 0.25–0.45. In some experiments antioxidants such as ascorbic acid, uric acid, bilirubin, catalase, SOD, α-tocopherol, and β-carotene were added to the incubation as described in detail in Results and in the legends to the figures.

DCF fluorescence or absorbance formation contains four phases. The first lag phase is due to the antioxidants in the sample. After their consumption by free radicals from AAPH, the reaction proceeds to the first propagation phase. The second lag phase, which interrupts this propagation, is due to the addition of the internal standard, Trolox®, to the incubation ($T_{Trolox®}$), and in accordance, the second propagation of the reaction follows the consumption of the Trolox®. To calculate TRAP reproducibly, the determination of the length of these phases was computerized. Tangents of the curve due to the lag phases were calculated from the minimum value of the derivates during these periods, whereas the tangents of the propagation phases were calculated from the maximum of the derivates. The duration of the two lag phases was calculated using the intercepts of these tangent lines.

The statistical analysis was done using the Systat statistical package. Values are given as means ± SD. The values before and after α-tocopherol supplementation were tested for significance with the analysis of variance for repeated measurements and were also compared by paired *t*-testing.

RESULTS

Incubation of the nonfluorescent DCFH-DA with the hydrophilic AAPH led to an increase of the DCF fluorescence with an excitation and emission maximum of 480 nm and 526 nm, respectively (**Fig. 1**). The reaction

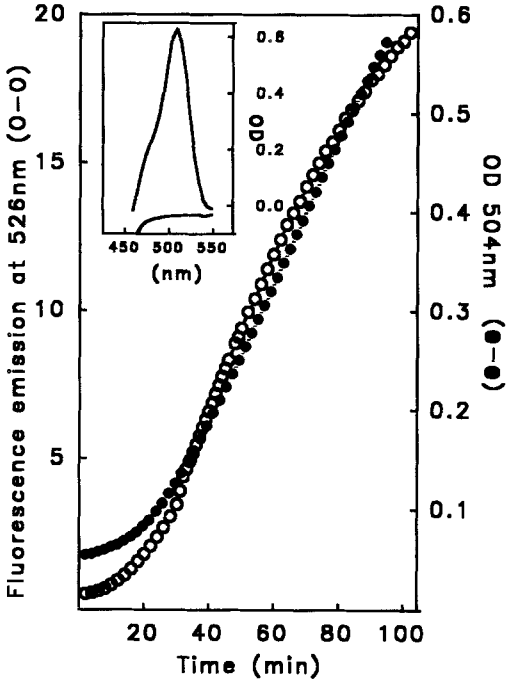

Fig. 1. Oxidation of DCF in the presence of AAPH and serum according to formation of fluorescence (○) and absorbance (●); oxidation of the same serum sample in fluorometry and spectrophotometry. The reaction mixture contained: 1% serum, 56 mmol/l AAPH, and 14 μmol/l DCFH-DA and resulted in lag times of 19 and 17 min, respectively. The inset shows the absorbance spectrum of DCF between 425 and 575 nm. The peak at 504 nm disappeared by omitting either DCFH-DA or AAPH from the reaction mixture (represented by the lower line).

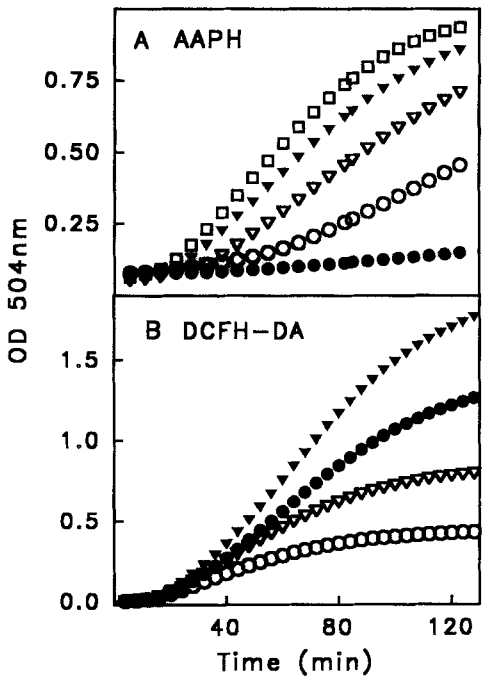

Fig. 2. Optimization of the assay with respect to AAPH and DCFH-DA. Increasing concentrations of AAPH, (4, 16, 32, 46, and 56 mmol/l) were added to the reaction mixture containing 1% serum and 14 μmol/l DCFH-DA. This resulted in the lag phases of 96 min (●), 38 min (○), 22 min (▽), 14 min (▼), and 14 min (□), respectively (Fig. 2A). The signal heights induced by increasing concentrations of DCFH-DA, 7 mmol/l (○), 14 mmol/l (▽), 28 mmol/l (●), and 42 μmol/l (▼) are indicated in Fig. 2B. In addition to DCFH-DA the reaction mixture contained 1% of serum and 56 mmol/l of AAPH. The lag phase of each of the curves is 24 min.

products of DCFH-DA oxidation (DCF) were also studied spectrophotometrically. The absorbance spectrum between 204 nm and 1200 nm revealed an increase in absorbance peaking at 504 nm (insert in Fig. 1). Essentially, a parallel increase in both DCF absorbance and fluorescence could be recorded when the TRAP of the same serum sample was determined (closed vs. open symbols in Fig. 1). After initiation of the free radical formation, the absorbance of DCF at 504 nm first increases slowly (closed symbols in Fig. 1). After all antioxidant capacity is lost, the radical formation and thus the absorbance or the fluorescence of DCF increases rapidly.

Higher concentrations of AAPH resulted in shorter lag times (T-Serum) in TRAP determination, **Fig. 2A.** The rate of increase of DCF absorbance at 504 nm, i.e., the slope of the curve also increased. However, above the concentration of 48 mmol/l of AAPH (Fig. 2A) no further increase in the rate of the reaction was obtained. On the basis of this experiment, the AAPH concentration of 56 mmol/l was chosen for further use. Using increasing amounts of DCFH-DA resulted in higher absorbancies, Fig. 2B. Thus, the DCF formation increased depending on the amount of DCFH-DA added with no apparent saturation. The concentration of 14 μmol/l was chosen for further experiments as adequate signal height was obtained with this concentration, Fig. 2B.

The water-soluble vitamin-E analogue, Trolox®, was used as an internal standard in the reaction (**Fig. 3**). Its addition to the incubation during the propagation phase with rapid increase of DCFH-DA absorbance induced a second lag time, $T_{Trolox®}$. The length of this time before the absorbance increases again depends on the amount of Trolox® added (**Fig. 4A**). Lag-phases of similar duration were obtained irrespective of the order of adding Trolox® to the same serum sample before oxidation was initiated or during uninhibited peroxidation (Fig. 3). The Trolox® concentration of 8.4 μmol/l was used as an internal standard in further experiments.

Each Trolox® molecule is able to neutralize two molecules of peroxyl radicals (38). The TRAP can thus be calculated (27):

$$\text{TRAP} = (T_{serum}/T_{Trolox®}) \times \text{serum dilution factor} \times 2 \times 8.4\ \mu\text{mol/l}$$

Values are expressed as μmoles of peroxyl radicals trapped by one liter of serum. The intra- and interassay coefficients of variation of the assay are 3.4% (n = 30) and 4.6% (n = 17, consecutive assays), respectively. No difference in TRAP could be detected between EDTA-anticoagulated plasma and serum taken from the same individuals. Storage at −80°C for 2 months had no influence on the determination of TRAP as judged from experiments with sera analyzed fresh or after storage

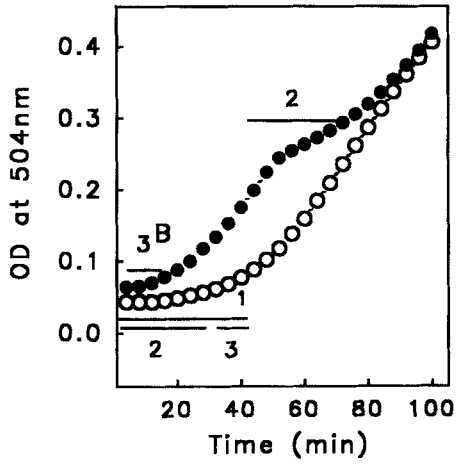

Fig. 3. Effect of Trolox® on TRAP determination. Trolox® (11.4 μmol/l) was added before (○), (line 1) or during (●), (line 2) the propagation phase. Line 3 represents the lag phase of the same serum sample without Trolox®. Note that the sum of lines 2 and 3 equals line 1.

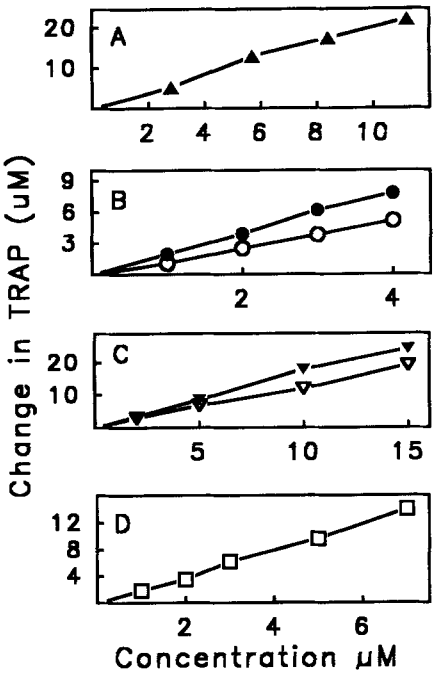

Fig. 4. The stoichiometric values of antioxidants. Increasing concentrations of Trolox® (4A,▲), β-carotene and α-tocopherol (4B ○, ●), urate and ascorbate (4C, ▽, ▼) and bilirubin (4D, □) were added before AAPH (56 mmol/l) to the reaction mixture containing 1% of serum and 14 µmol/l DCFH-DA. The increase in experimental TRAP value produced by addition of antioxidants is plotted. The slopes are 2.030, 1.27, 1.985, 1.26, 1.674, and 1.964 for Trolox®, β-carotene, α-tocopherol, urate, ascorbate, and bilirubin, respectively ($r > 0.9$, $P < 0.001$).

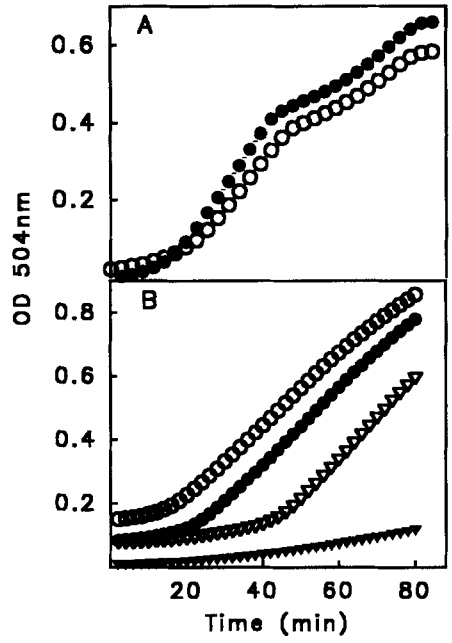

Fig. 5. Effect of storage of samples and serum concentration on TRAP. A: Effect of storing samples. The TRAP of the same serum sample determined fresh (○) and after storage at −80°C up to 2 months (●). The reaction mixture contained 1% serum, 14 µmol/l DCFH-DA, 56 mmol/l AAPH, and 8.4 µmol/l of added Trolox®. The lag time, T-Trolox® and the corresponding TRAP exp values are 15 min, 19.5 min, and 1292 µmol/l for the fresh samples and 16.5 min, 23 min, and 1205 µmol/l for the stored samples, respectively. B: Determination of TRAP using serum concentrations of 0%(▼), 1%(○), 1.6%(●), 4.2%(▽). The reaction mixture contained 14 µmol/l DCFH-DA and 56 mmol/l AAPH. The lag times produced by increasing serum concentrations are 14 min, 22 min, and 42 min for 1%, 1.6%, and 4.2% serum, respectively.

(**Fig. 5A**). Increasing amounts of serum caused prolongation of the lag phase depending on the sample volume, Fig. 5B. Finally, the results were similar by either spectrophotometry or fluorometry, **Fig. 6** ($r = 0.975$, $P < 0.001$, n = 24).

When no serum was added and DCFH-DA was incubated with AAPH alone, no major oxidation of DCFH-DA to DCF took place (Fig. 1, inset). This is evidently due to esterases required to first convert the DCFH-DA to DCFH. The maximal conversion was obtained with the intrinsic esterase activity in serum samples as adding various amounts of exogenous esterase (0–40 IU) to the incubation containing 1% serum, AAPH, and DCFH-DA did not influence the length of lag time of the reaction (data not shown). Thus, exogenous esterases were not used in further determinations of TRAP.

In vitro studies with antioxidants

The influence of urate, ascorbate, and bilirubin on TRAP was studied after adding them to the reaction mixture in concentrations ranging between 0 and 15 µmol/l (Fig. 4C and 4D). α-Tocopherol and β-carotene were added in concentrations ranging between 0 and 4

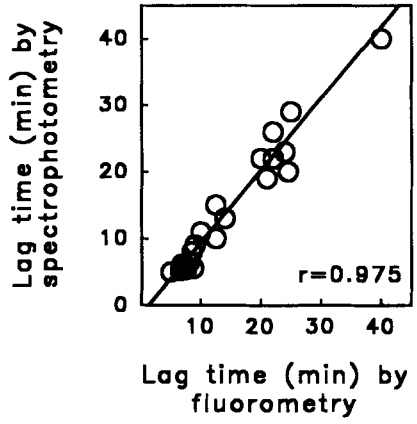

Fig. 6. Correlation between spectrophotometry and fluorometry. Lag times of the same serum samples obtained by the two methods ($r = 0.975$, n = 24 sample pairs, $P < 0.001$). The reaction mixture contained 1% serum in PBS, 14 µmol/l DCFH-DA, and 56 mmol/l AAPH. Increasing amounts of urate (0–10 µmol/l) or ascorbate (0–10 µmol/l) were added to ten of the sample pairs before the reaction was initiated.

TABLE 1. Serum antioxidants, lipoprotein profile, experimental and calculated TRAP values, and the lag time of LDL oxidation in healthy subjects (n = 11) before (Pre) and after 7 days treatment with 300 mg/day α-tocopherol on day 8 (Post 1) and after 2 weeks (Post 2)

	Pre	Post 1	Post 2	*P* Value (ANOVA)
Antioxidant				
Urate (μmol/l)	297 ± 89	276 ± 72	281 ± 86	NS
Ascorbate (μmol/l)	35 ± 17	31 ± 13	34 ± 13	NS
SH groups (μmol/l)	349 ± 74	382 ± 58	365 ± 33	NS
α-Tocopherol (μmol/l)	29.3 ± 6.0	58.9 ± 11.5	29 ± 6.2	<0.001
α-Tocopherol/Chol + TG	5.1 ± 0.5	10.3 ± 1.6	5.0 ± 0.5	<0.001
Lipids				
Chol (mmol/l)	4.8 ± 0.7	4.9 ± 0.8	4.7 ± 0.9	NS
HDL (mmol/l)	1.6 ± 0.3	1.6 ± 0.3	1.6 ± 0.4	NS
LDL (mmol/l)	2.8 ± 0.7	2.8 ± 0.8	2.8 ± 0.7	NS
TG (mmol/l)	0.9 ± 0.5	0.9 ± 0.4	0.9 ± 0.3	NS
TRAP and oxidation				
TRAPEx (μmol/l)	871 ± 326	1090 ± 315	1152 ± 417	<0.025
TRAPcalc (μmol/l)	655 ± 110	691 ± 104	637 ± 119	<0.01
LDL lag time (min)	182 ± 32	228 ± 57	165 ± 43	=0.001

Values are means ± SD; NS, not significant; TG, triacylglycerol; Chol, cholesterol; α-tocopherol/Chol + TG, lipid-standardized α-tocopherol.

μmol/l (Fig. 4B). All these antioxidants caused a prolongation of the lag phase when added to the incubation before AAPH. The water-soluble antioxidants urate, ascorbate, and bilirubin bound to albumin (which does not influence the present assay as such) could also be added to the incubation during the reaction, whereas the lipid-soluble substances had to be added before AAPH to achieve adequate mixing. Similar to Trolox® (Fig. 3, lines 1 and 2) the water-soluble antioxidants, except ascorbate, caused similar increases of the lag phases irrespective of the order of addition (data not shown). However, the lag times achieved with all antioxidants correlated well with their concentrations, and accordingly the molar amount of free radicals trapped by one mole of each antioxidant (stoichiometric values) could be calculated (Fig. 4)(6).

The effect of antioxidant enzymes, superoxide dismutase (SOD) and catalase, were then studied. The SOD (600–7000 IU/ml) was added to the reaction mixture before AAPH or during uninhibited peroxidation. This caused no prolongation and no second lag phase. In contrast, an up to 30% more rapid increase of DCF formation took place upon addition of SOD. This effect was comparable with that obtained by adding hydrogen peroxide in a final concentration of 0.2–0.3% to the reaction during its propagation. The enhancing effects of hydrogen peroxide (but not of SOD) on DCF formation could be prevented by adding catalase (17,100 IU) before the incubation. The catalase as such (0–17,100 IU) had no effect on DCF formation.

In vivo studies with antioxidants

Blood samples for the determination of serum antioxidant concentrations and TRAP were taken from 11 healthy young subjects (6 female and 5 male) before and after supplementation with α-tocopherol 300 mg/day for one week. When the data were grouped by sex no significant differences were observed in TRAP values or in the concentrations of serum antioxidants and lipids between the sexes. However, males had significantly higher ($P < 0.01$) serum urate levels (355.5 ± 55) compared to females (227 ± 68). Supplementation with vitamin E resulted in a significant increase in serum α-tocopherol (**Table 1**) in all subjects, **Fig. 7A.** Two weeks after discontinuation of vitamin E, its serum concentration had returned to baseline level. No significant changes were observed when comparing pre- and post-

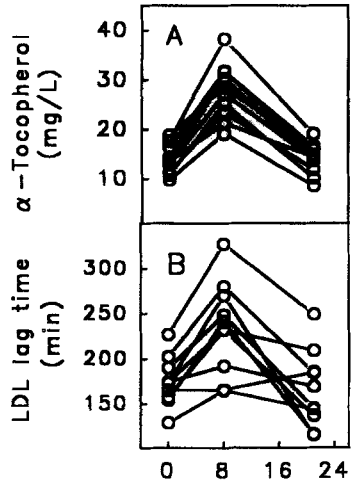

Fig. 7. Effect of antioxidants in vivo. Effect of vitamin E supplementation on A) serum vitamin E levels (mg/l) and B) on the lag time (min) of diene formation during LDL oxidation. The values on days 0, 8, and 21 of each study participant (n = 11) are presented.

TABLE 2. Molar amount of free radicals trapped by 1 mole of each antioxidant (stoichiometric values), serum concentration, and percentage contribution to calculated (TRAPcalc) and to experimental TRAP (TRAPexp) values of antioxidants before vitamin E supplementation in 11 subjects (mean)

	Stoichiometric Value	Concentration	TRAPcalc	TRAPexp
		µmol/l	µmol/l	µmol/l
Urate	1.3	297	57.8	48.2
Ascorbate	1.7	35.4	8.9	7.8
SH groups	0.44[a]	349	24.2	19.3
α-Tocopherol	2.0	29.3	9.1	7.4

[a]Value obtained from reference 26.

supplementation concentrations of antioxidant vitamins or protein sulfhydryl groups (Table 1). Serum and lipoprotein lipid levels did not change during administration of vitamin E (Table 1).

The baseline TRAP values measured in these subjects ranged from 373 to 1292 µmol/l (mean 870, SD ± 236). The calculated TRAP values were determined using the stoichiometric values (**Table 2**) and the measured serum concentrations of these antioxidants. At baseline, the calculated TRAP values ranged from 441 to 869 µmol/l, which were 25% lower than those determined experimentally. After 8 days on vitamin E the measured and the calculated TRAP values increased significantly, Table 1. However, the calculated and experimental TRAP values did not display any correlation throughout this study (Table 1). On day 8 the mean calculated TRAP was 36.6% lower than the mean measured TRAP. Two weeks after discontinuation of vitamin E, serum TRAP was still higher than before the experiment in all subjects, and notably it had not decreased at all in six subjects, **Fig. 8.**

A significant increase in the resistance of LDL to oxidation was apparent when postsupplementation lag times of conjugated diene formation were compared to baseline, Table 1, Fig. 7B. This prolongation of the length of the lag phase of LDL oxidation correlated with serum α-tocopherol levels ($r = 0.67$, n = 11, $P < 0.05$) (Fig. 7A). The corresponding TRAP (9A) and LDL oxidation (9B) are shown in detail in **Fig. 9.**

Finally, the experimental TRAP values in a normal population of healthy subjects (n = 38) were determined. The TRAP values ranged from 672 to 1680 (mean 1155, SD ± 290).

DISCUSSION

A need to quantitate the antioxidant defense of human serum protecting LDL from lipid peroxidation and the discovery of DCFH-DA in studies of respiratory burst of inflammatory cells led to the development of an assay for TRAP determination based on DCF fluorescence. However, the DCF also has specific absorbance at 504 nm. This enabled the determination of serum TRAP by simple spectrophotometry, which has not been described earlier. Thermal decomposition of AAPH has previously been performed at 37°C. However, free radicals are produced at rates comparable to those reported from studies at 37°C when a higher concentration of AAPH is used at room temperature (20, 21, 27). Regardless of AAPH concentration, comparable final TRAP values of a serum sample were obtained. This was due to the use of a common water-soluble internal standard, Trolox®, in all these assays. The lag phase of plasma was compared with that caused by a known amount of Trolox®. However, as discussed below, any other antioxidant could have been used as an internal standard for TRAP determination.

The stoichiometric values of Trolox® and its lipophilic analogue, α-tocopherol, were similar (2.0) by the present method to that in the literature (38). The data obtained using α-tocopherol or Trolox® as an internal standard showed a close correlation of 0.99 (**Fig. 10**). The prolongation of the lag time in serum samples ob-

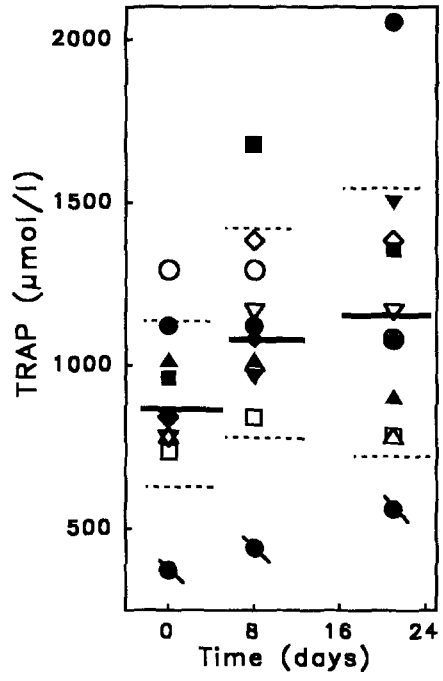

Fig. 8. Effect of antioxidants in vivo. Effect of vitamin E supplementation on TRAP. The TRAPexp (µmol/l) values on days 0, 8, and 21 of each study participant (n = 11) are presented. Solid lines (—) represent the mean TRAP values of 871, 1090, and 1152 on days 0, 8, and 21, respectively. The standard deviations of 236, 315, and 417 on days 0, 8, and 21, respectively, are marked by dashed lines (---).

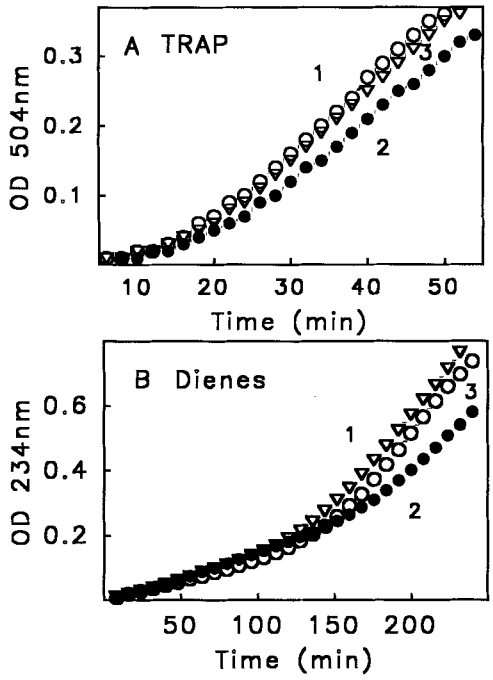

Fig. 9. Changes in mean TRAP (A) and LDL oxidation (B) during vitamin E treatment (n = 11), before (○), 2 weeks after (▽), and on day 8 (●) after treatment.

tained with ascorbate, bilirubin, and β-carotene correlated well with the concentrations of these antioxidants (Fig. 4) enabling the comparison of the intrinsic properties of various techniques to measure antioxidants, **Table 3.** The values agree well with those obtained by other authors (6, 20, 28, 29, 39). However, variation between the methods in the case of ascorbate is evident. This could be due to the differences in these assay systems and also to the fact that the stoichiometric value

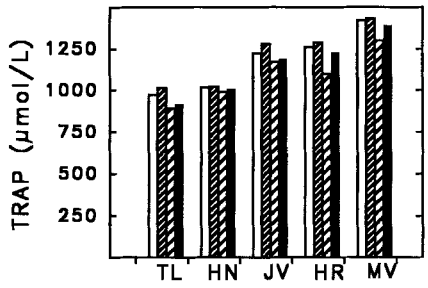

Fig. 10. Effect of various internal standards on TRAP values. The TRAP values of 5 subjects were studied. To the reaction mixture containing 1% serum in PBS and 14 μM DCFH-DA, 2 μM (final concentration) of antioxidants was added before AAPH (final concentration 56 mM). The TRAP exp (μM) values were (subject 1) 1421, 1430, 1300, 1393, (subject 2) 1018, 1021, 990, 1010, (subject 3) 973, 1014, 892, 921, (subject 4) 1224, 1280, 1170, 1190, (subject 5) 1259, 1286, 1100, 1232 using Trolox® (□), α-tocopherol (▨), urate (▨), and bilirubin (■) as internal standards.

of ascorbate seems to depend on its initial concentration in a sample and declines in higher concentrations (8). Any of the antioxidants studied (Fig. 4) could have been used as internal standard for the present method (Fig. 10). However, Trolox® has practical advantages as it is easy to handle and can be stored in ethanol at 4°C for up to two months.

Using the stoichiometric values for antioxidants after the measurement of their concentration in human samples, a theoretical TRAP value can be calculated for each individual. However, the cooperation between antioxidants cannot be taken into account resulting in 5–50% lower calculations of TRAP compared to those obtained experimentally (6, 20, 40). This probably explains the 25% gap between the calculated and measured TRAP in the present study also. Part of this discrepancy can be explained by serum bilirubin, carotenoids, and retinols, which were not included in the calculations. Bilirubin and β-carotene can probably act as efficient antioxidants (29, 41) and bilirubin may also be important in maintaining other molecules in reduced form (42). However, the contribution of bilirubin to TRAP values and its action as a chain breaking antioxidant in healthy subjects has been suggested to be negligible (3, 6, 19). Furthermore, according to the present study, the contribution of bilirubin and β-carotene to calculated TRAP in the range of normal serum levels would be 2–4% and <1%, respectively. The antioxidant enzymes SOD and catalase did not seem to act as effective chain breaking antioxidants in this assay system. In addition, unidentified major antioxidant(s) contributing to TRAP might also exist (21).

Vitamin E supplementation for a week (300 mg/day) doubled its plasma concentrations in all subjects, and increased serum TRAP by up to half. This is rather surprising as only a small fraction of total serum TRAP is due to vitamin E (6). On the basis of serum α-tocopherol determinations, only 4.7–10% of the total TRAP was due to vitamin E in healthy study subjects, which is in line with the 5% contribution reported previously (6). In addition, the lipophilic vitamin E is mainly sequestered in lipoproteins such as LDL and may not be effective enough in trapping all free radicals of the aqueous phase. However, the fact that lipid-soluble antioxidants could be studied in vitro in this method suggests that the radicals produced by AAPH in aqueous phase can also attack lipid phase and be trapped by lipid-soluble antioxidants as already shown by Cao et al. (29). Furthermore, in biological systems, the aqueous and lipophilic antioxidants cooperate (16, 43, 44). Thus, administration of a lipophilic antioxidant could affect both lipid phase and aqueous phase defense against free radicals. This type of cooperation is best known between α-tocopherol and ascorbate (17, 45).

TABLE 3. Stoichiometric values (the molar amount of free radicals trapped by 1 mole of antioxidant) studied by six authors

Antioxidant	Wayner	Cao[a]	Ghiselli	Metsä-Ketelä	Lissi	Valkonen
Trolox	2.0	2.0	2.0	2.0	2.0	2.0
Urate	1.3	1.84	1.7	2.0	1.2	1.3
Ascorbate	1.7	1.04	1.3	0.7	0.88	1.7
α-Tocopherol	NA	2.0	NA	NA	NA	2.0
β-Carotene	NA	1.28	NA	NA	NA	1.3
Bilirubin	NA	1.68	NA	NA	NA	
	Expression Used for Antioxidant Potential					
	TRAP[b] μM	ORAC[c] units/ml	TRAP μM	TRAP μM	TRAP μM	TRAP μM
Range of values	571–1284	NA	850–1870	NA	NA	672–1680
Mean values	820	1511	1152	1164	400[d]	1155
Number of cases	45	7	10	10	10	38
SD	148	363	322	135	NA	290

The values are based on the oxygen electrode method (Wayner, ref. 6), fluorescence method (Cao, ref. 29; Ghiselli, ref. 20), chemiluminescence method (Metsä-Ketelä, ref. 28; Lissi, ref. 39), and spectrophotometric method (Valkonen). NA, not available.

[a]The values obtained by Cao (1, 0.92, 0.52, 1, 0.64, 0.84) were multiplied by 2 because these are expressed as ORAC units which equals the net protection produced by 1 μM Trolox.

[b]TRAP, total peroxyl radical trapping potential.

[c]ORAC, oxygen radical absorbance capacity.

[d]Determined from a pool of 10 blood samples.

Other hitherto unknown cooperation by aqueous phase antioxidants with α-tocopherol might also exist (41). This is suggested by the finding of 37% higher TRAP values in serum after one week on vitamin E rather than by calculating from the data of vitamin E, urate, protein SH-group and ascorbate determinations. Furthermore, the increase in measured TRAP in the present study could not be explained by the increase of serum α-tocopherol concentration. This is in accordance with previous reports suggesting that the increase in measured TRAP is much larger than the molar increase in antioxidant concentrations (18). The cooperation between antioxidants might also explain the similar experimental TRAP values in males and females observed in the present study, although the higher serum urate levels in males produced a minor difference in calculated TRAP values between the sexes ($P < 0.050$). A similar difference in the calculated but not in the experimental TRAP values between the sexes has been documented previously by Mulholland and Strain (19).

An inverse correlation between vitamin E levels and mortality from ischemic heart disease has been suggested by epidemiological studies (46). Furthermore, a relatively short period and low dose supplementation with vitamin E seems to protect LDL from lipid peroxidation (47–49) and also to prevent oxidized LDL-mediated vascular injury in vitro (50). However, intervention trials with vitamin E supplements have so far failed to show any effect on the progression of the atherosclerotic lesions in vivo. In the present study, vitamin E supplementation, in addition to serum α-tocopherol concentrations and the TRAP, increased the resistance of LDL to Cu^{2+} ion-mediated oxidation. No correlation was found between the lag time of conjugated diene formation and the TRAP measurements. This is, however, not surprising as LDL oxidation was studied in vitro where the isolation of LDL from its aqueous surroundings probably accentuated the role of the lipophilic α-tocopherol. In vivo LDL will also interact with the serum water-soluble antioxidants (51).

The lag time of LDL oxidation correlated well with serum α-tocopherol concentration, Fig. 7, which is in accordance with earlier studies (52, 53). Furthermore, serum α-tocopherol concentrations have been shown to correlate with the α-tocopherol content of LDL (54), which was not determined in the present study. After discontinuation of vitamin E, serum α-tocopherol concentration as well as the LDL oxidation lag time returned to their baseline levels. Yet, the TRAP measurements in six of the subjects revealed elevated levels despite the decrease of serum α-tocopherol to its pretreatment level. This could be due to the lipophilicity of vitamin E, which, like other lipophilic antioxidants such as probucol, may be stored in cellular membranes and in adipose tissue long after discontinuation of treatment (55).

Few studies have thus far described the antioxidant defense or changes in antioxidant capacity under various clinical conditions. This is most likely due to difficulties in methodology or to expensive equipment required to determine the TRAP thus far. Furthermore, the relation of TRAP values to in vivo antioxidant poten-

tial and to prognosis of the patients remains to be clarified as high values have been found in severely diseased subjects (23). Also the importance of different antioxidant systems in vivo in protecting LDL from lipid peroxidation is not clear. This study validates a simple spectrophotometric method for determining TRAP and suggests new tools for clinical research.

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