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Original Contribution

OXYGEN-RADICAL ABSORBANCE CAPACITY ASSAY FOR ANTIOXIDANTS

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Abstract—A relatively simple but sensitive and reliable method of quantitating the oxygen-radical absorbing capacity (ORAC) of antioxidants in serum using a few μl is described. In this assay system, β-phycoerythrin (β-PE) is used as an indicator protein, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analogue) as a control standard. Results are expressed as ORAC units, where 1 ORAC unit equals the net protection produced by 1 μM Trolox. The uniqueness of this assay is that total antioxidant capacity of a sample is estimated by taking the oxidation reaction to completion. At this point all of the nonprotein antioxidants (which include α-tocopherol, vitamin C, β-carotene, uric acid, and bilirubin) and most of the albumin in the sample are oxidized by the peroxyl radical. Results are quantified by measuring the protection produced by antioxidants. This solves many problems associated with kinetics or lag-time measurements. A linear correlation of ORAC value with concentration of serum, Trolox, vitamin C, uric acid, and bovine albumin is demonstrated. The coefficient of variation within a run is found to be about 2% and from run to run about 5%. Trolox, α-tocopherol, vitamin C, β-carotene, uric acid, and bilirubin completely protect β-PE from oxidation, while bovine albumin protects β-PE only partially. On a molar basis, the relative peroxyl radical absorbance capacity of Trolox, α-tocopherol acid succinate, uric acid, bilirubin, and vitamin C is 1 : 1 : 0.92 : 0.84 : 0.52. Bovine albumin per unit weight has a lower peroxyl absorbing capacity than these antioxidants. However, the serum protein fraction, containing some lipid-soluble antioxidants, represents the major contributor to the ORAC value found in whole serum. The minimum amount of vitamin C and uric acid which could still be detectable when added to a serum supernatant fraction is 1.5 μg and 0.59 μg, respectively, which account for about 1% of the total ORAC value of the serum supernatant fraction.

Keywords—Oxygen radicals, Antioxidant, α-tocopherol, β-carotene, Vitamin C, Uric acid, Bilirubin, Free radical

INTRODUCTION

Reactive oxygen species could be important causative agents of a number of human diseases, including cancer and atherosclerosis, as well as the aging process itself.[1–5] Thus, mechanisms such as antioxidants that act to control oxidative stress state represent a major line of defense regulating general health status.[5]

Human serum contains many different antioxidants that may be important for general health maintenance. These include vitamin C, α-tocopherol, β-carotene, uric acid, bilirubin, and albumin. There are other antioxidants that appear to be less important, and perhaps others are not yet identified.[6] In addition, trace amounts of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase are found in serum to a lesser extent.[6]

Because of the many different antioxidant components in serum and the relative difficulty of measuring each antioxidant separately, a simple method to measure net or total resultant antioxidant capacity of serum would be of considerable value.[7] The available methods for this purpose at present are the fluorescence-based assay developed in Glazer's laboratory[8] and the oxygen electrode method developed by Ingold et al. known as TRAP.[9] However, the assay developed by Glazer et al. is not capable of quantitating results and is limited only to screening the free-radical-scavenging capacity of a sample.[8] The method developed by Ingold et al. is based on the time taken to prevent maximum oxygen uptake in a system containing a free radical generator, lipid, and serum or a specific antioxidant. The problem with this method is that the time taken to prevent maximum oxygen uptake cannot be measured easily and precisely and the total peroxyl radical trapping capability per mole of some antioxidants (for example, vitamin C) is dependent on their initial concentration.[10]

Our laboratory has developed a method that mea-

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sures total oxygen-radical absorbing capacity (ORAC) of serum samples that eliminate some of these problems. This ORAC assay is based largely on the work reported by Glazer's laboratory and depends on the unique properties of phycoerythrins.[8,11] These proteins function as light-harvesting components in cyanobacteria and red algae. The fluorescence quantum yield of these proteins is > 0.9 and forms the basis of a sensitive measurement of the physical and chemical integrity of the protein.

Here we report details of the ORAC assay. The uniqueness of this assay is that the reaction is driven to completion. The area under the kinetic curve is proportional to the total ORAC of the sample. To correct for small differences in analytical instrument sensitivity, reagents, and other assay conditions, the final result is expressed with reference to a known amount of an antioxidant (Trolox), a water-soluble vitamin E analogue. Sensitivity and repeatability of the assay is demonstrated in this report using human serum samples.

MATERIALS AND METHODS

Chemicals

Porphyridium cruentum β-phycoerythrin (β-PE) was obtained from Boehringer Mannhein (Germany). 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) was obtained from Polyscience (Warrington, PA). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Co. (Milwaukee, WI). D-α-tocopherol acid succinate, vitamin C, β-carotene, uric acid, bilirubin (mixed isomers, from bovine gall stones), and human albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine albumin was obtained from Miles Inc (Kankakee, IL). Acetone (spectro grade) was obtained from Kodak (Rochester, NY).

Sample preparation

Human serum samples were obtained from seven healthy adults and were stored at −80°C. For precipitation of serum proteins, 50 μl serum was diluted to 100 μl with phosphate buffer and then 400 μl of 100% saturated ammonium sulfate was added, mixed, and placed in ice water for 30 min. The samples were centrifuged at 100,000 × *g* for 10 min at 4°C in a Beckman (Fullerton, CA) Model L3-50 ultracentrifuge, and the supernatants were carefully removed and stored in ice water. The pellets were resuspended three times in 500 μl of 80% saturated ammonium sulfate and recentrifuged. All frozen serum samples were assayed within 40 d. Sample preparations were made with 7.5×10^{-2} M phosphate buffer.

Fluorescence assay

The final reaction mixture for the assay contained 1.67×10^{-8} M β-PE and 3×10^{-3} M AAPH in 7.5×10^{-2} M phosphate buffer, pH 7.0. A final volume of 2 ml was used in 10 mm wide cuvettes. Into each sample tube, 20 μl diluted serum, serum fractions, or other antioxidant solutions were also added. Samples of α-tocopherol acid succinate, β-carotene, or bilirubin were first dissolved in acetone and then added to reaction mixture. For the blank in this situation, 20 μl acetone instead of phosphate buffer was used. When the serum supernatant fraction was assayed, 80% saturated ammonium sulfate was used as a blank. AAPH was used as the peroxyl radical generator to start the reaction. Once AAPH was added, the reaction mixture was incubated at 37°C. Fluorescence was measured every 5 min at the emission of 565 nm and excitation of 540 nm using a Perkin-Elmer (Norwalk, CT) LS-5 fluorescence spectrophotometer until zero fluorescence occurred. For a standard, 20 μl of a 100 uM (1 μM in final concentration) Trolox stock solution was assayed during each run.

Quantification

The ORAC value refers to the net protection area under the quenching curve of β-PE in the presence of an antioxidant. One ORAC unit has been assigned the net protection area (*S*) provided by 1 μM Trolox in final concentration. The ORAC value (units) of a sample is calculated on the basis of a Trolox standard curve. Because of the linear correlation found between ORAC value and Trolox concentration, the results can also be calculated in the following way (Fig. 1):

$$\text{ORAC Value (U/ml)} = 50k(S_{\text{Sample}} - S_{\text{Blank}})/(S_{\text{Trolox}} - S_{\text{Blank}})$$

k: dilution factor

Blank: 20 μl phosphate buffer, or 20 μl 80% saturated ammonium sulfate, or 20 μl acetone instead of sample is added.

S refers to the area under the quenching curve of β-PE. This area is integrated by a computer connected

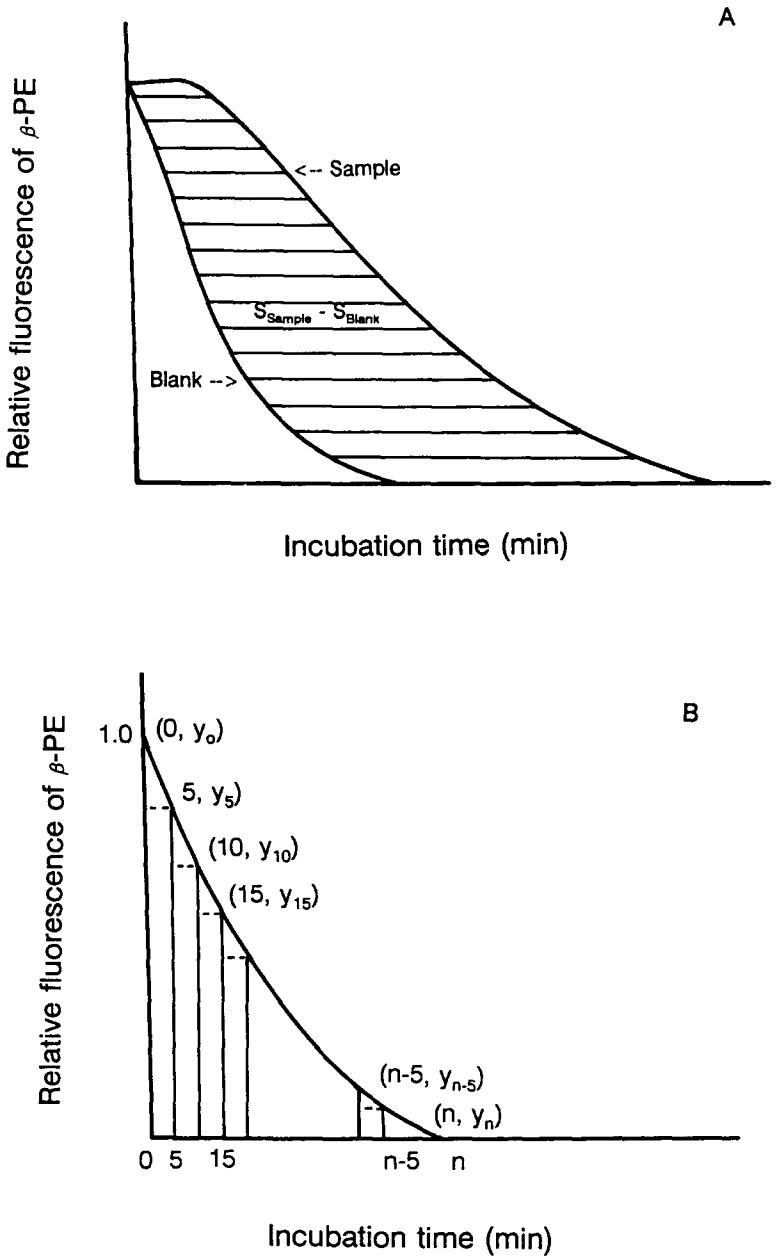

Fig. 1. Calculation of ORAC values. (A) ORAC value $\alpha(S_{Sample} - S_{Blank})/(S_{Trolox} - S_{Blank})$. (B) $S = ((y_0 + y_5) \times \frac{5}{2}) + ((y_5 + y_{10}) \times \frac{5}{2}) + ((y_{10} + y_{15}) \times \frac{5}{2}) + \cdots + ((y_{n-5} + y_n) \times \frac{5}{2}) = (0.5 + y_5 + y_{10} + \cdots + y_{n-5}) \times 5$. $y_0, y_5, y_{10}, \ldots, y_{n-5}, y_n$: the relative fluorescence of β-PE at different incubation timepoints, i.e. at 0, 5, 10, . . . , $n - 5$ and n minutes.

directly to the output of the fluorescence spectrophotometer.

RESULTS

The basis of the ORAC assay is shown schematically in Fig. 2. A linear correlation (as shown in Fig. 3) was found between the ORAC value and serum sample concentration (0–1/2000). The coefficients of variation (CV) within a run and from run to run are 2% (n = 6) and 4.9% (n = 5), respectively. The average ORAC value found for seven healthy adults was 1511 units/ml, with a CV of 24%.

When serum proteins were precipitated with ammonium sulfate, the ORAC value found in the supernatant fraction of a sample was 257 units/ml, which accounts for 14% of the total ORAC value (1806 units/ml) found in the serum. The remaining 86% was recovered in the pellet (protein fraction).

The peroxyl radical absorbing abilities of Trolox,

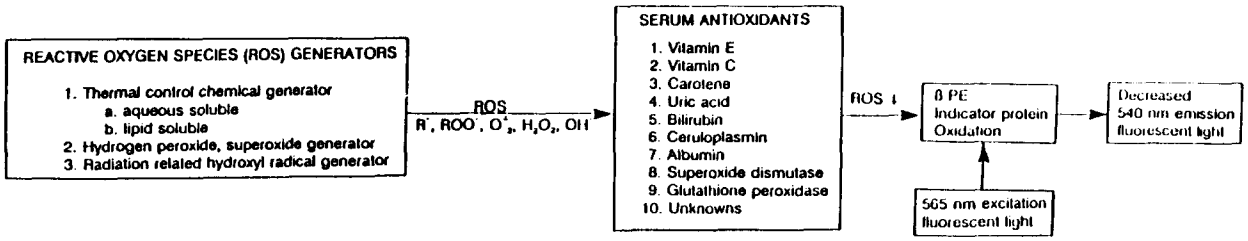

Fig. 2. The principles of the ORAC assay.

vitamin C, uric acid, and bovine albumin are shown in Figs. 4A–4D. When Trolox, vitamin C, and uric acid were added, the rate of decrease in the fluorescence of β-PE showed a plateau region, after which the fluorescence of β-PE decreased dramatically and is similar to that in the blank (no additives). The initial plateau phase was not observed with bovine albumin. The extent of the plateau became greater when a sample of higher human serum concentration or when a supernatant fraction of a serum sample was used (Figs. 4E and 4F).

A linear correlation between ORAC value and sample concentration was found using Trolox (0–3 μM), vitamin C (0–2 μM), uric acid (0–4 μM), and bovine albumin (0–50 μg/1000 ml). This is shown in Fig. 5. Based on these data, the relative peroxyl radical absorbing capacity for Trolox, uric acid, and vitamin C at 1 μM final concentration was calculated to be 1 : 0.92 : 0.52. Thus, one ORAC unit equals 1 μM Trolox, 1.08 μM uric acid, or 1.9 μM vitamin C in their final concentration. On a per unit weight basis, the relative peroxyl radical absorbing capacity for Trolox, uric acid, vitamin C, and bovine albumin is 1 : 1.44 : 0.67 : 0.01. Thus, on a per unit weight basis, one ORAC unit equals 0.5 μg Trolox, 0.36 μg uric acid, 0.75 μg vitamin C, or 48 μg bovine albumin in 2 ml total reaction mixture.

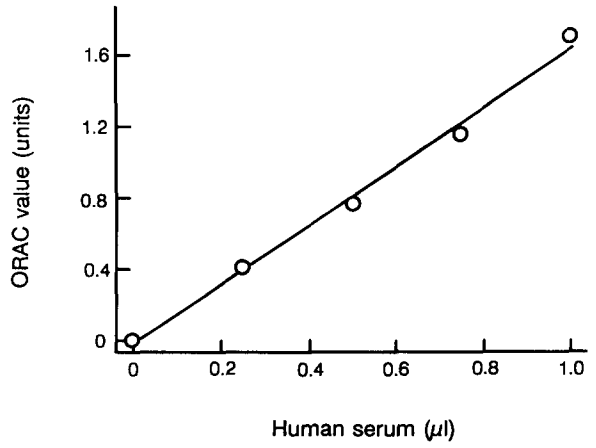

Fig. 3. Regression of ORAC value on the amount of human serum. $Y = 1.6595X - 0.02232$, $r = .9967$, $P < .001$. The reaction mixture contains 1.67×10^{-8} M β-PE, 3×10^{-3} M AAPH, and 0, 0.25, 0.5, 0.75, or 1.0 μl human serum in 2 ml 7.5×10^{-2} M phosphate buffer, pH 7.0.

The ORAC assay also can be used successfully in measuring α-tocopherol acid succinate, β-carotene, and bilirubin when they are first dissolved in acetone and then added to the reaction mixture (see Fig. 6B). The acetone itself also has some protection for β-PE. The ORAC value of α-tocopherol acid succinate equals that of Trolox in terms of the final μM concentration. The relative peroxyl radical absorbing capacity for Trolox, α-tocopherol acid succinate, β-carotene, and bilirubin at 1 μM final concentration was calculated to be 1 : 1 : 0.64 : 0.84. However, the β-carotene which we used was obtained several years ago. The quenching curves of α-tocopherol acid succinate, β-carotene, and bilirubin are also similar to those of Trolox, vitamin C, and uric acid, which showed a plateau region.

When a mixed antioxidant solution which contains 0.04 mM Trolox, 0.04 mM vitamin C, and 0.04 mM uric acid (the calculated ORAC value of this solution is 52.5 units/ml) was assayed, the experimental ORAC value was 48.6 units/ml. The difference is 7.4% of the calculated value.

When 4.5, 7.4, and 14.9 μg quantities of vitamin C sodium salt (which equal 5.9, 9.9, and 19.8 calculated ORAC units) were added to 1 ml human serum supernatant fraction, these resulted in increases of 7.5 (127% recovered), 18.7 (188% recovered), and 31.4 (158% recovered) measured ORAC units. When 5.9, 29.4, and 58.8 μg quantities of uric acid (which equal 16.4, 81.7, and 163 calculated ORAC units) were added, these resulted in increases of 18.4 (112% recovered), 91.1 (111% recovered), and 179 (109% recovered) measured ORAC units.

The minimum amounts of vitamin C (sodium salt) and uric acid added to 1 ml supernatant fraction of a human serum which could still be reliably detected by the ORAC assay are 1.5 μg and 0.59 μg, re-

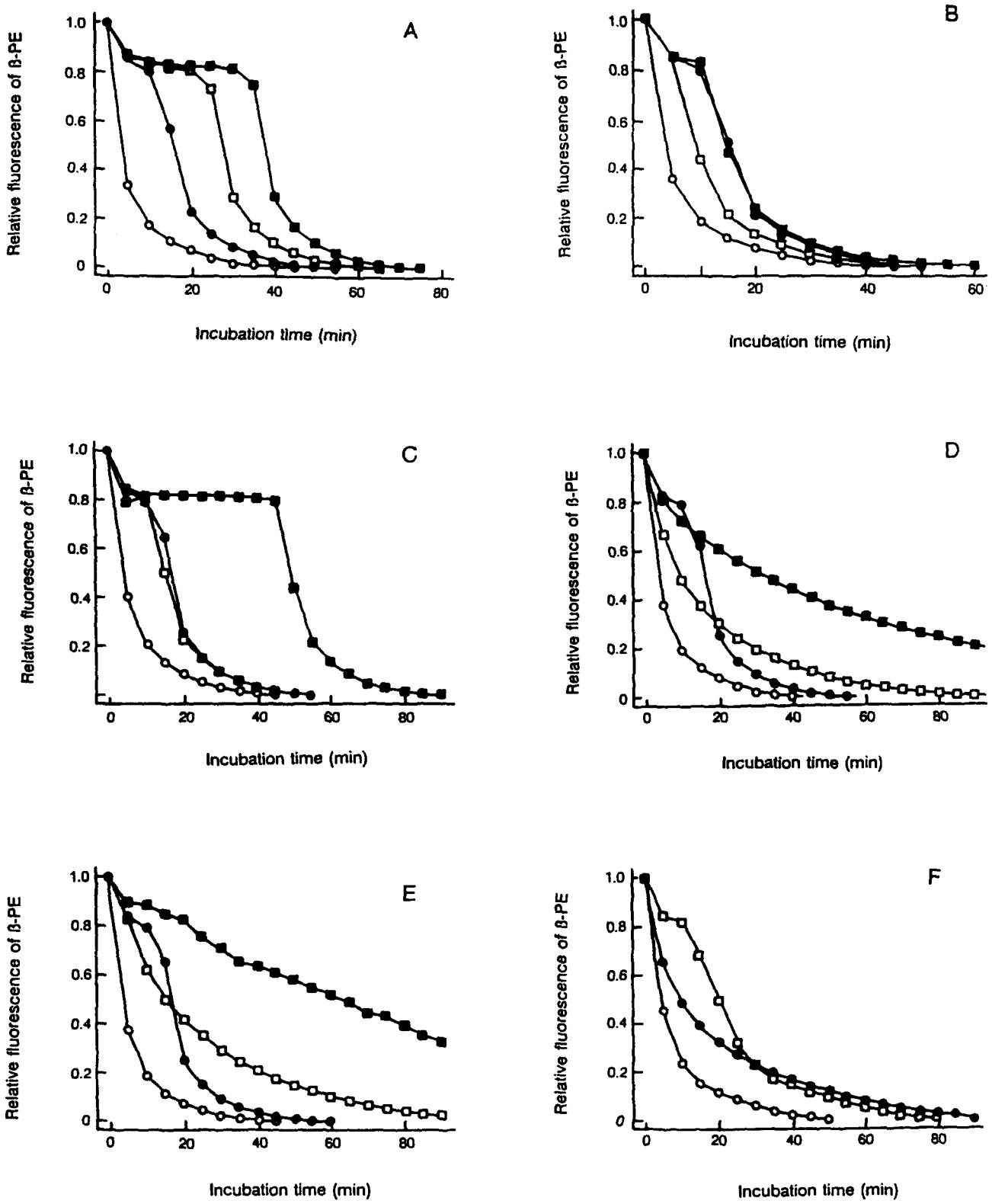

Fig. 4. Kinetics of β-PE quenching with different concentrations of antioxidants. The reaction mixture contains 1.67×10^{-8} M β-PE, 3×10^{-3} M AAPH, and (A) ○ blank, ● 1 μM Trolox, □ 2 μM Trolox, ■ 3 μM Trolox; (B) ○ blank, ● 1 μM Trolox standard, □ 1 μM vitamin C, ■ 2 μM vitamin C; (C) ○ blank, ● 1 μM Trolox standard, □ 1 μM uric acid, ■ 4 μM uric acid; (D) ○ blank, ● 1 μM Trolox standard, □ 50 μg bovine albumin, ■ 200 μg bovine albumin; (E) ○ blank, ● 1 μM Trolox standard, □ 1 μl human serum, ■ 6 μl human serum; or (F) ○ blank, □ the serum supernatant fraction separated from 5 μl human serum, ● the serum protein fraction separated from 1 μl human serum in 2 ml 7.5×10^{-2} M phosphate buffer, pH 7.0.

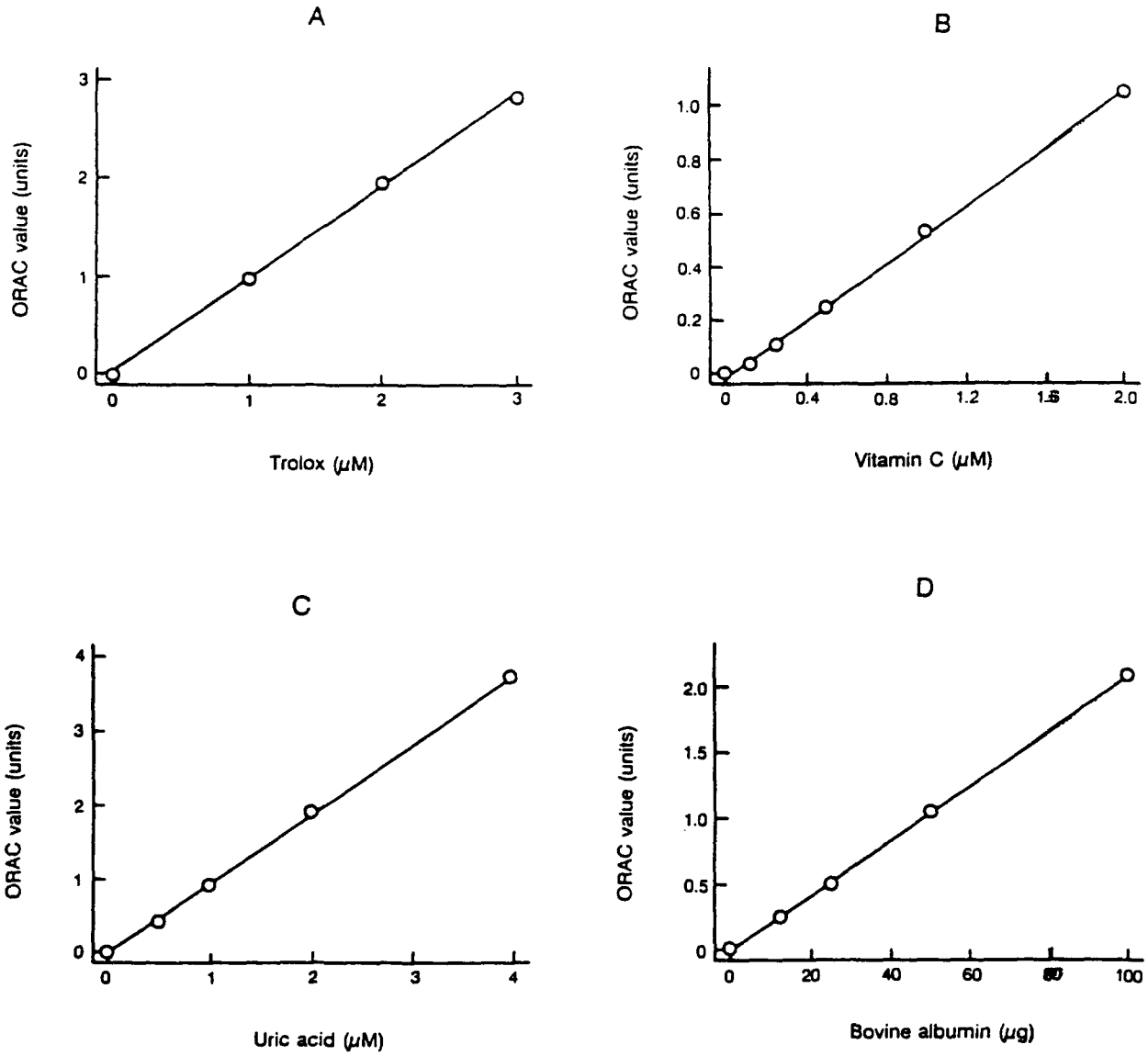

Fig. 5. Regression of ORAC value on the concentration of different antioxidants. The reaction mixture contains 1.67×10^{-8} M β-PE, 3×10^{-3} M AAPH, and different concentrations of antioxidants in 2 ml 7.5×10^{-2} M phosphate buffer, pH 7.0. (A) Trolox, $Y = 0.9493X + 0.0302$, $r = .9994$, $P < .001$; (B) vitamin C, $Y = 0.5353X - 0.0176$, $r = .9994$, $P < .001$; (C) uric acid, $Y = 0.9477X - 0.0213$, $r = .9997$, $P < .001$; (D) bovine albumin, $Y = 0.0211X - 0.0117$, $r = .9999$, $P < .001$.

spectively. This accounts for about 1% of the total $ORAC_{experimental}$ value of the human serum supernatant fraction sample.

To investigate how much antioxidant or antioxidant activity still exists in the original reaction mixture after the total destruction of β-PE, 200 μl of 1.67×10^{-7} β-PE was added to the reaction mixture again after the first assay and its fluorescence units were recorded continuously. The results are shown in Figs. 6A and 6B.

The kinetics of β-PE (which was added to the reaction mixture again after the first assay was completed) quenching in the blank were found to be similar to that of Trolox, vitamin C, uric acid, and a mixed antioxidant sample (which contained Trolox, vitamin C, and uric acid). (See Fig. 6A.) When an acetone blank was used, similar results were obtained with Trolox, α-tocopherol acid succinate, β-carotene, and bilirubin. (See Fig. 6B). This means that these antioxidants (Trolox, vitamin C, uric acid, α-tocopherol, β-carotene, and bilirubin) have been totally oxidized after the completion of the first assay.

However, the reaction mixture still has some protection for β-PE after the first assay was completed

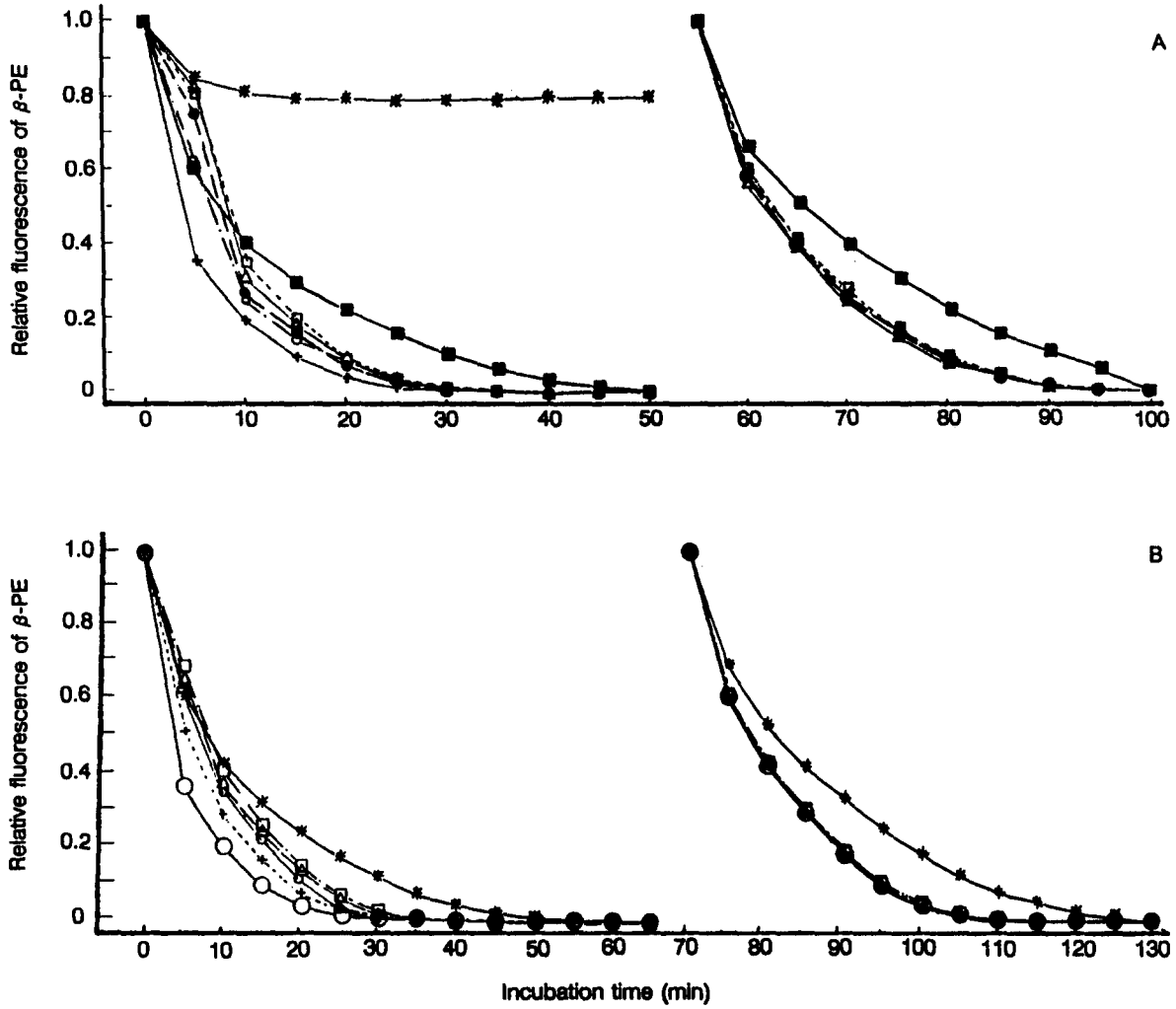

Fig. 6. Quenching curves of β-PE added to the original reaction mixture after the first assay of different types of antioxidants. The original reaction mixture contains 1.67×10^{-8} M β-PE, 3×10^{-3} M AAPH, and different types of antioxidants in 2 ml 7.5×10^{-2} M phosphate buffer, pH 7.0. After the completion of the first assay, 200 μl of 1.67×10^{-7} β-PE was added to the original reaction mixture again. (A) β-PE was added to the original reaction mixture again at 55 min; + blank, * β-PE only (no AAPH), △ 1 μM Trolox, ○ 1 μM vitamin C, □ 1 μM uric acid, ● mixed solution (0.2 μM Trolox, 0.2 μM vitamin C, 0.2 μM uric acid), ■ 0.75 μl human serum; (B) β-PE was added to the original reaction mixture again at 70 min; ○ blank 1 (phosphate buffer), + blank 2 (acetone), □ 1 μM α-tocopherol acid succinate, ○ 1 μM β-carotene, △ 1 μM bilirubin, * 25 μg human albumin.

and another 200 μl of 1.67×10^{-7} β-PE was added again when the human serum or human albumin sample was assayed. The remaining ORAC values which could be detected in the second assay were calculated to be about 10–20% and 30%, respectively, of their original values.

DISCUSSION

The ORAC assay has been found to be a simple, sensitive, and reliable method to quantitate the peroxyl radical absorbing capacity of antioxidants in serum or other biological fluids using sample volumes on the order of a few microliters. The major improvement of this assay, compared with that developed first by Glazer's laboratory, is that the reaction is driven to completion, to where all nonprotein antioxidants (which include α-tocopherol, vitamin C, β-carotene, uric acid, and bilirubin) and most of the protein (including albumin) in the sample are oxidized by the peroxyl radical. Repeatability of the assay has been improved by the adoption of an ORAC unit system (using the Trolox standard) to quantitate the results.

Glazer and his co-workers used the inhibition (%) of β-PE oxidation, which was calculated from the initial linear rate of β-PE fluorescence loss, to express

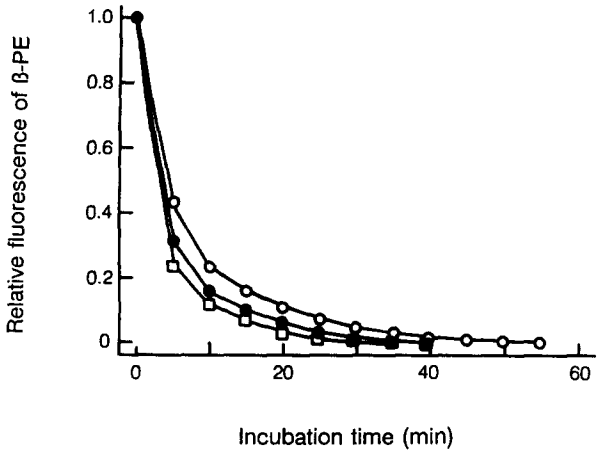

Fig. 7. Quenching curves of β-PE attacked by different concentrations of AAPH. The reaction mixture contains 1.67×10^{-8} M β-PE and ○ 2 mM AAPH; ● 3 mM AAPH; □ 4 mM AAPH in 2 ml 7.5×10^{-2} M phosphate buffer, pH 7.0.

their results.[8] This method was found to be limited in value because

1. The inhibition time of β-PE oxidation was not considered.
2. We were unable to confirm these results reporting a linear decrease in β-PE fluorescence caused by AAPH at 37°C when no antioxidant existed, when an antioxidant was completely oxidized, or when an antioxidant could only partially protect β-PE.[8,11,12] The decrease in β-PE fluorescence was not linear, as shown in Figs. 4, 6, and 7.
3. A standard reference such as Trolox could not be used in their assay system.
4. The contribution of lipid-soluble antioxidants and protein in serum to the total antioxidant capacity of the whole serum cannot be determined.

Because of these difficulties, it was emphasized in their paper that their assay served only as a screen for the detection of potentially important scavengers of peroxyl radicals.[8]

For a typical human serum sample using our assay system, oxidation of β-PE is completed in about 90 min, whereas for the blank the reaction requires about 45 min. By measuring the area under the quenching curve of β-PE, many of the problems existing in the assay developed in Glazer's laboratory are eliminated. Our results showed that the coefficient of variation within a run was only 2% ($n = 6$) and for run to run was 4.9% ($n = 5$), while it was as high as 24.2% within seven healthy adults. The linear correlation between ORAC value and antioxidant concentration was found in all samples examined (human serum, Trolox, vitamin C, uric acid, and bovine albumin). The minimum amount of vitamin C and uric acid which was added to serum supernatant fraction that could still be detectable in the ORAC assay was about 1% of the total $ORAC_{experimental}$ value of the human serum supernatant fraction.

When Trolox, uric acid, or vitamin C was added to the reaction mixture, the quenching curve of β-PE exhibited a plateau region followed by a dramatically increased loss of its fluorescence, similar to that in the blank (Figs. 4A–4C). This suggests complete protection of β-PE by these antioxidants. The decrease of β-PE fluorescence during the first 5 min of incubation was due to the rise of temperature, as we found that the fluorescence also dropped when no AAPH was added (see Fig. 6A). Bovine albumin resulted in a decreased loss of β-PE fluorescence instead of producing a plateau in the quenching curve of β-PE (Fig. 4D). This suggests that bovine albumin provides partial protection for β-PE. The relative molecular peroxyl radical absorbance capacity of Trolox, uric acid, and vitamin C was 1 : 0.92 : 0.52. Bovine albumin had the lowest peroxyl radical absorbing ability.

When α-tocopherol, β-carotene, or bilirubin exist, the kinetics of β-PE quenching are similar to that with Trolox, vitamin C, and uric acid. This means that those lipid-soluble antioxidants also provide total protection for β-PE. The relative peroxyl radical absorbing capacity for Trolox, α-tocopherol acid, β-carotene, and bilirubin at 1 μM final concentration was calculated to be 1 : 1 : 0.64 : 0.84. The successful application of this assay in measuring α-tocopherol, β-carotene, and bilirubin suggests that peroxyl radicals produced by water-soluble AAPH are also able to attack lipid-soluble antioxidants. Frei et al. also reported that exposure of plasma to aqueous peroxyl radicals could deplete the α-tocopherol in the plasma.[13]

Ingold et al. developed a method to estimate the total peroxyl radical trapping antioxidant parameter (TRAP), which also uses AAPH as a peroxyl radical generator.[9] In their system, the result was expressed as $TRAP_{experimental}$ (μM peroxyl radicals trapped/L serum), which was calculated on the basis of the stoichiometric factor of Trolox. A stoichiometric factor is defined as the moles of peroxyl radical trapped per mole of antioxidant. According to Ingold's studies, the stoichiometric factor for Trolox, vitamin C, and urate was 2, 1.7, and 1.3, respectively.[14] This means that the relative peroxyl radical absorbance capacity for these antioxidants is 1 : 0.85 : 0.65, which is different from our 1 : 0.52 : 0.92 values. However, this is not difficult to explain because they also reported that

in their TRAP experimental assay system the stoichiometric factor for vitamin C was concentration dependent[10] and the stoichiometric factor for urate was higher in the absence of other antioxidants in serum.[14]

Our results indicated that more than 100% of ORAC units was recovered after vitamin C was added to human serum. This suggests a possible synergism of vitamin C with other antioxidants in trapping peroxyl free radicals.

There are many kinds of oxygen radicals produced in the body, and the peroxyl radical may not be as biologically relevant as other prooxidants. Our goal is to measure ORAC value of serum using different types of free radical generators, as indicated in Fig. 2. This could be of value because an ability to scavenge peroxyl radicals is not necessarily the best model system to assess the ability of a compound to protect against physiologically important reactive oxygen species such as $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$,[6] and serum antioxidants are likely to be more important in protecting blood vessels from inflammatory peroxides including $O_2^{\cdot-}$, hypochlorous acid (HOCl), and peroxynitrite ($^{-}ONOO$).

We have already found that the ORAC assay system could be successfully used in evaluating the hydroxyl radical absorbing capacity of serum. The relative ORAC value of the serum from the seven human subjects was found to be 168.6 ± 14.8 (mean ± SE) units when 1.8×10^{-5} M $CuSO_4$ plus 0.3% H_2O_2 system was used as a hydroxyl radical generator. The linear correlation was also found between the relative ORAC value and the serum concentration under this system. However, the antioxidant capacity of a single antioxidant (for example, Trolox, uric acid, or vitamin C) is much more complicated in the system.

We have also found that the amount of serum sample can be substantially reduced, and the simultaneous kinetic analysis of many samples is made possible by using a fluorometry microplate reader using a 96-well plate.

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ABBREVIATIONS

AAPH—2,2′-azobis(2-amidinopropane)dihydrochloride
β-PE—β-phycoerythrin
CV—the coefficient of variation
ORAC—oxygen-radical absorbance capacity