# Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation

## The important contribution made by plasma proteins

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Plasma obtained from fasted humans has been analysed for total radical-trapping antioxidant content by subjecting it to controlled peroxidation using the thermal decomposition of water-soluble azobis (2-amidinopropane hydrochloride) at 37°C to produce peroxyl radicals at a known, steady rate. It is found that the total radical-trapping antioxidant content is rather similar for the 7 subjects that have been tested and, furthermore, it is 10–20-times larger than the effect attributable to vitamin E alone. Although it is shown that urate and ascorbate augment the contribution from vitamin E, their contributions (21–34 and 0–2%, respectively) still leave 57–73% of the total antioxidant content unaccounted for. Evidence is presented to show that this previously unrecognized large reserve of antioxidant capacity is attributable to the plasma proteins.

*Plasma auto-oxidation* *Vitamin C* *Vitamin E* *Urate* *Antioxidant* *Protein*

## 1. INTRODUCTION

There is a great interest in oxy radicals and the peroxidative damage associated with their possible role in diseases such as cancer, heart disease and rheumatoid arthritis, as well as in the degenerative processes associated with aging [1–4]. Although considerable progress has been made in identifying and understanding the mode of action of individual biological antioxidants such as vitamin C [4,5], vitamin E [6–8], uric acid [9], glutathione peroxidase [7], catalase and superoxide dismutase [10], there have been few attempts to determine the combined effect of these protective agents within intact biological fluids and tissues. That is, little is known in any quantitative sense about the total resistance to peroxidative assault of tissue containing these natural antioxidants at physiological concentrations.

Peroxidation is a chain reaction with the chain being carried by free radicals. Antioxidants reduce the rate and extent of peroxidation. They are classified into two groups [11]. Primary antioxidants reduce the rate of production of 'new' radicals which start new oxidation chains. They may act by reducing hydroperoxides (e.g., glutathione peroxidase, catalase) or by sequestering transition metal ion catalysts (e.g. transferrin). Secondary antioxidants trap radicals directly, thereby reducing the chain lengths of the oxidation and thus limiting the amplification of peroxidative damage (e.g., vitamin E, superoxide dismutase).

Previous workers have shown that blood serum, incubated with tissue homogenates (usually brain), possesses strong antioxidant properties [12–15]. Most of this antioxidant activity has been at-

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tributed to the presence in serum of the iron-binding protein, transferrin, and the ferroxidase, ceruloplasmin. In the absence of plasma, the spontaneous peroxidation of these tissue homogenates can also be prevented by the addition of the metal ion chelating agent, EDTA [15]. It is obvious from these results that serum contains powerful and effective primary antioxidants.

We have chosen to address the question: what is the nature and magnitude of the secondary antioxidant defence system in plasma when it is subjected to continuous attack by peroxyl radicals?

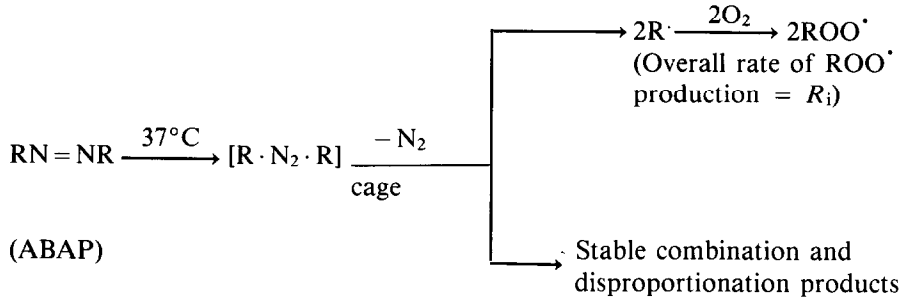
## 2. MATERIALS AND METHODS

Bovine serum albumin, transferrin, and ceruloplasmin were obtained from Sigma. Papain was a gift from Dr P. Carey (NRCC). L-Ascorbic acid (BDH Chemicals), sodium urate (Sigma), and 3,4-dihydroxybenzylamine hydrochloride (Aldrich) were all used without further purification. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was a gift from Dr J.W. Scott (Hoffman-La Roche, Nutley, NJ). ABAP [2,2′-azo-bis-(2-amidipropropane hydrochloride)] was obtained from Polysciences (Warrington, PA) and used as received.

Blood was obtained by venipuncture after an overnight fast (10–12 h). Except for serum, the blood was collected over $Na_2EDTA$ (1–2 mg/ml blood) and immediately centrifuged (10000 × g, 10 min, 4°C); the supernatant was used immediately for analysis and measurement of TRAP. Serum was obtained by allowing blood to stand for 1 h at 4°C followed by centrifugation to remove the clotted material.

Urate was measured by HPLC using a Varian Micro Pak $NH_2$-10 column eluted with a mixture of $CH_3CN$ (69%) and aqueous $NaH_2PO_4$ (0.04 M, pH 5.0) at 1.5 ml/min with UV detection at 254 nm. Samples were prepared by adding methanol (2.0 ml), the internal standard (aqueous 1 mM 3,4-dihydroxybenzylamine·HCl; 50 μl), and *n*-heptane (1.0 ml) to plasma (0.5 ml); vortex stirring followed by brief centrifugation separated the aqueous and organic layers. The *n*-heptane extract was analyzed for vitamin E [6] and the aqueous extract for urate and ascorbate.

Our experimental approach to measuring total peroxyl radical-trapping ability of plasma has taken advantage of the discovery [16,17] that the peroxidation of aqueous dispersions of oxidizable organic compounds can be readily and reproducibly initiated by the water-soluble azo compound, ABAP. ABAP decomposes thermally to yield useful quantities of peroxyl radicals at a known and constant rate, $R_i$ (eqn 1) [16,17].




(1)

After adding ABAP to the plasma we measure the length of time that oxygen uptake by peroxidizable plasma material is inhibited by the antioxidants in the plasma. This time is the induction period, $\tau_{plasma}$, and it is measured using an oxygen electrode (YSI Model 5331) and just 30 μl of plasma diluted in 3 ml of aqueous buffer. These induction periods provide for the first time a quantitative measure of the total secondary antioxidant content of a biological fluid: this quantity will be referred to hereafter as the total radical-trapping antioxidant parameter or TRAP.

TRAP, which is defined as the number of moles of peroxyl radicals trapped per liter of fluid, can be calculated from $\tau_{plasma}$ from the equation,

$$TRAP = R_i \tau_{plasma} \quad (2)$$

The value of $R_i$ is obtained by adding a known quantity of Trolox, which is a water-soluble

vitamin E analogue, to the plasma sample after the natural antioxidants have been completely consumed, i.e., well after $\tau_{plasma}$ when peroxidation is proceeding rapidly. Trolox produces a second induction period, $\tau_{Trolox}$, which yields $R_i$ via the equation,

$$R_i = n[\text{Trolox}]/\tau_{Trolox} \quad (3)$$

where $n$ is the stoichiometric factor, i.e., the number of peroxyl radicals trapped per molecule of Trolox. This number is 2.0 [16,18], as is the $n$ value for vitamin E and many other phenols [6,19].

## 3. RESULTS AND DISCUSSION

TRAP values have been measured for a number of fresh plasma samples and are given in table 1, column M. This Table also includes the measured concentrations of vitamin E, urate, and ascorbate (columns, E, U and A, respectively) in each sample.

The present results stand in interesting contrast to earlier work in which an analogous titration with peroxyl radicals for secondary antioxidants was carried out on lipids extracted from plasma

Table 1

Comparison of total radical-trapping antioxidant parameter (TRAP) with concentrations of vitamin E, urate, and ascorbate in human plasma

| Plasma sample | [Vitamin E] ($\mu$M) | [Urate] ($\mu$M) | [Ascorbate] ($\mu$M) | TRAP | | | |
|---|---|---|---|---|---|---|---|
| | | | | Measured | Calculated contribution from E+U+A | Measured − calculated | Contribution measured after gel filtration of plasma |
| | (E) | (U) | (A) | (M) | (C) | (M–C) | |
| Female | | | | | | | |
| AW,1 | 47 | 249 | 12 | 919 | 301 | 618 | 507 |
| AW,2 | 25 | 235 | 0 | 888 | 238 | 650 | 505 |
| LH | 30 | 249 | 30 | 898 | 280 | 618 | 624 |
| AM | 41 | 328 | 0 | 1005 | 344 | 661 | 576 |
| Male | | | | | | | |
| DW | 25 | 217 | 12 | 806 | 232 | 574 | n.d. |
| GB | 25 | 385 | 0 | 915 | 358 | 557 | n.d. |
| KI | 59 | 306 | 0 | 1115 | 363 | 752 | 802 |
| HL | 35 | 420 | 28 | 989 | 426 | 563 | n.d. |
| HL-serum | 35 | 420 | 28 | 1041 | 426 | 615 | n.d. |

Measured TRAP ($\mu$mol peroxyl radical trapped/L plasma) for intact plasma: 30 $\mu$l plasma in 3.0 ml of 5 mM phosphate-buffered saline (154 mM NaCl; pH 8.0) containing 3.8 mM ABAP at 37°C. The induction period, $\tau_{plasma}$, which is the length of time that oxygen uptake by peroxidizable plasma materials was inhibited by plasma antioxidants, was measured by monitoring the oxygen concentration of the air-saturated solution with an oxygen electrode. Rate of peroxyl radical generation by ABAP ($=R_i$) was determined (via eqn. 3) in each experiment by addition of 5.7 nmol Trolox to the sample shortly after the commencement of the uninhibited peroxidation, and measuring $\tau_{Trolox}$. Values of $\tau_{Trolox}$ were highly reproducible, varying by $<5\%$. Repeat determinations of TRAP (via eqn. 2) gave values that differed by $<3\%$. The calculated radical trapping capability of the vitamin E, urate, and ascorbate present in the plasma was based on their individual concentrations and stoichiometric factors, i.e., 2[vitamin E] + 0.8[urate] + 0.7[ascorbate]. The vitamin E stoichiometric factor is from [19] and the factors for urate and ascorbate were determined from eqn 4. The radical trapping capabilities of some samples of plasma were measured after passage through a Sephadex G-25 column to remove urate, ascorbate, and other low $M_r$, water-soluble compounds. The result in the last column for KI was obtained following incubation for 1 h at room temperature of 1 ml plasma with 2 ml of 5 mM phosphate-buffered saline (154 mM NaCl; pH 8.0) containing 2% *N,N*-diethyldithiocarbamate.

and red blood cells [6]. In these lipid extracts nearly all the radical-trapping capacity could be attributed to vitamin E [6]. For plasma, however, it is clear that vitamin E is responsible for only ~ 6–11% of the TRAP value (i.e., for vitamin E, $n = 2$, and so its contribution to TRAP is twice its molar concentration).

The contributions of urate and ascorbate to TRAP required the measurement of their stoichiometric factors, i.e., their $n$ values. These were determined under realistic conditions by measuring the increase in the induction period, $\delta\tau$, produced by incremental additions of urate or ascorbate, $\delta$[antioxidant], to a standard plasma sample. Plots of $\delta\tau$ vs $\delta$[antioxidant] were linear and yielded $n = 0.8$ for urate and $n = 0.7$ for ascorbate via the equation,

$$\delta\tau = n\delta[\text{antioxidant}]/R_i \quad (4)$$

The combined contributions of vitamin E, urate, and ascorbate to the TRAP values have been calculated using the corresponding $n$ values and are given in table 1, column C. Of these 3 antioxidants, urate [9,20] makes the largest contribution to TRAP. Subtraction from the measured TRAP values of the contribution due to vitamin E, urate, and ascorbate, i.e., column M–C, shows that there exists in plasma a very important, but previously unrecognized, secondary antioxidant which in quantitative terms accounts for more of the TRAP (i.e., 57–73%) than does the combined effect of the 3 known antioxidants. Most of the unknown antioxidant was shown to be associated with protein and lipoprotein material by passing the plasma through a Sephadex G-25 gel filtration column which removes urate, ascorbate, and any other low-$M_r$, water-soluble compounds (see last column in table 1 and compare with column M–C). Even more interesting is the fact that fractionation of the plasma proteins on a Sephadex G-200 column showed that secondary antioxidant activity is found in all the protein fractions particularly in the third fraction which contains albumin (see fig.1). This non-specific antioxidant activity was further confirmed by the fact that the addition of bovine serum albumin, transferrin, ceruloplasmin, or papain to a standard plasma sample produced an increase in $\tau$. There are, of course, a number of essential amino acids which contain rather labile

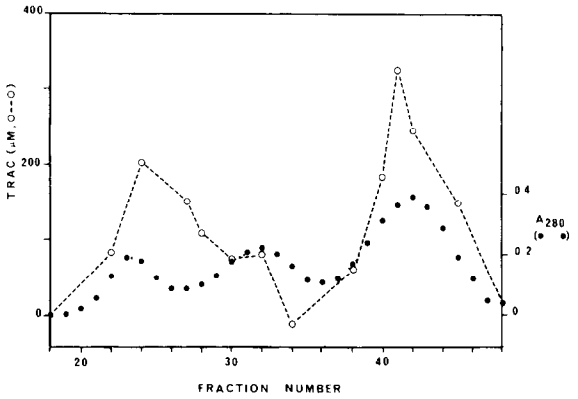



Fig.1. Total radical-trapping antioxidant content (TRAC) of individual protein fractions after gel filtration on a Sephadex G-200 column (98 × 1.6 cm) at 4°C. Plasma (0.25 ml), diluted 4-fold with 5 mM phosphate-buffered saline (PBS) (pH 8.0) was eluted with PBS at 10 mL·h$^{-1}$. The UV absorbance profile ($A_{280}$) at 280 nm (●) of the individual fractions (each 2.8 ml) is compared with the profile of the corresponding TRAC values (○). TRAC values were determined by measuring the increase of the induction period, $\tau$, obtained in the controlled peroxidation at 37°C of each fraction in the presence of a known about of a standard plasma sample, relative to the induction period, $\tau_{plasma}$, obtained for the standard plasma alone. Each fraction was made up to 3.0 ml with PBS, a sample (30 $\mu$l) of the standard plasma was added and the value of $\tau$ measured after initiating peroxidation by the addition of 30 $\mu$l ABAP (3.65 mM final concentration). The value of $\tau_{plasma}$ was determined in a separate experiment. The 3 major protein fractions include (in order of elution [25]: fraction 1 lipoproteins and macroglobulins; fraction 2, immunoglobulins, ceruloplasmin; fraction 3; transferrin, albumin.

hydrogen atoms which might allow them to function as secondary antioxidants, e.g., the OH in tyrosine, the SH in cysteine, etc.

Essentially identical TRAP values were obtained for plasma and serum (subject HL), and incubation of plasma with the superoxide dismutase inactivator, *N,N*-diethyldithiocarbamate [21], had no significant effect on TRAP (subject KI).

Although vitamin E represents only a small fraction of the TRAP it probably plays a vital role in maintaining efficient inhibition of peroxidation through its interactions with other secondary antioxidants [17,22–24]. Preliminary experiments suggest that vitamin E is depleted slowly throughout the induction period, a result consis-

tent with action by the other secondary antioxidants either to 'spare' vitamin E or to 'regenerate' it from the tocopheroxyl radical [17,22-24].

Experiments are underway to identify the chemical nature of the secondary antioxidants in proteins and to unravel the interplay between the various secondary and primary antioxidants.

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## REFERENCES

[1] Ames, B.N. (1983) Science 221, 1256-1264.
[2] Harman, D. (1982): Free Radicals in Biology (Pryor, W.A. ed.) vol. 5, pp. 255-275, Academic Press, New York.
[3] Halliwell, B. and Gutteridge, J.M.C. (1984) Biochem. J. 219, 1-14.
[4] Halliwell, B. (1981) in: Age Pigments, (Sohal, R.S. ed.) pp. 1-62. Elsevier/North-Holland, Amsterdam, New York.
[5] Tappel, A.L. (1968) Geriatrics, 23, 97-105.
[6] Burton, G.W., Joyce, A. and Ingold, K.U. (1983) Arch. Biochem. Biophys. 221, 281-290.
[7] Tappel, A.L. (1980) in: Free Radicals in Biology (Pryor, W.A. ed.) vol. 4, pp. 1-47, Academic Press, New York.
[8] Witting, L.A. (1980) in: Free Radicals in Biology (Pryor, W.A. ed.) vol. 4, pp. 295-319, Academic Press, New York.
[9] Ames, B.N., Cathcart, R., Schwiers E. and Hochstein, P. (1981) Proc. Natl. Acad. Sci. USA 78, 6958-6862.
[10] Fridovich, I. (1976) in: Free Radicals in Biology (Pryor, W.A. ed.) vol. 1, pp. 239-277, Academic Press, New York.
[11] Burton, G.W. and Ingold, K.U. (1984) Science 224, 569-573.
[12] Barber, A.A. (1961) Arch. Biochem. Biophys. 92, 38-43.
[13] Vidlakova, M., Erazimova, J., Norki, J. and Placer, Z. (1972) Clin. Chim. Acta 36, 61-66.
[14] Stocks, J., Gutteridge, J.M.C., Sharp, R.J. and Dormandy, T.L. (1974) Clin. Sci. Mol. Med. 47, 215-222.
[15] Stocks, J., Gutteridge, J.M.C., Sharp, R.J. and Dormandy, T.L. (1974) Clin Sci. Mol. Med. 47, 223-233.
[16] Barclay, L.R.C., Locke, S.J., MacNeil, J.M., VanKessel, J., Burton, G.W. and Ingold, K.U. (1984) J. Am. Chem. Soc. 106, 2479-2481.
[17] Yamamoto, Y., Haga, S., Niki, E. and Kamiya, Y. (1984) Bull. Chem. Soc. Jap. 57, 1260-1264.
[18] Burton, G.W., Hughes, L. and Ingold, K.U. (1983) J. Am. Chem. Soc. 105, 5950-5951.
[19] Burton, G.W. and Ingold, K.U. (1981) J. Am. Chem. Soc. 103, 6472-6477.
[20] Matsushita, S., Ibuki, F. and Aoki, A. (1963) Arch. Biochem. Biophys. 102, 446-451. Proctor, P. (1970) Nature, 228, 868-869.
[21] Oberley, L.W. (1982) in: Superoxide Dismutase (Oberley, L.W. ed.) vol. 2, pp. 127-165, CRC Press, Boca Raton, FL.
[22] Packer, J.E., Slater, T.F. and Willson, R.L. (1979) Nature 278, 737-738.
[23] Barclay, L.R.C., Locke, S.J. and MacNeil, J.M. (1983) Can. J. Chem. 61, 1288-1290.
[24] Doba, T., Burton, G.W. and Ingold, K.U., unpublished.
[25] Killander, J. (1964) Biochim. Biophys. Acta 93, 1-14.