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The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma

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The Total (Peroxyl) Radical-trapping Antioxidant Parameter (TRAP) of six freshly prepared human plasma samples and 45 frozen plasma samples has been determined. It is shown that contributions from urate (35–65%), plasma proteins (10–50%), ascorbate (0–24%) and vitamin E (5–10%) to TRAP account for all of the peroxyl radical-trapping antioxidant activity in the majority of the samples. The changes in concentrations of the plasma antioxidants during peroxyl radical attack show that the first line of defense is provided by the plasma sulfhydryl groups, even urate being spared during the initial stages of the reaction. The modes of action of all of these plasma antioxidants and possible interactions between them are discussed, with particular emphasis on the abilities of the water-soluble antioxidants to regenerate or spare the only lipid-soluble antioxidant, vitamin E.

Introduction

Cellular damage by oxy-radicals, including those associated with lipid peroxidation, is generally believed to be a significant factor in heart disease, rheumatoid arthritis, cancer, certain inflammatory disorders and even the aging process itself [1–9]. The modes of action of many of the natural antioxidants that are found in biological fluids and tissues have been subjects of intense investigation. The most important biological antioxidants would appear to be vitamin C (ascorbic acid) [10–12], vitamin E [7–9,13,14], uric acid [15], glutathione peroxidase [16], catalase, superoxide dismutase [17], transferrin and ceruloplasmin [18–20]. The relative importance of each of these antioxidants in vivo, i.e., the relative contributions of each antioxidant to the total antioxidant capacity of a biological fluid or tissue, is still not well understood. This can be attributed, at least in part, to the fact that most experiments that have been designed to explore biological antioxidants have, by their very nature, focused on one or more of the known antioxidants. Furthermore, these antioxidants will act cooperatively in vivo so as to provide greater protection to the organism against radical damage than could be provided by any single antioxidant acting alone.

Lipid peroxidation is a chain reaction in which the chain is carried by peroxyl radicals. Lipid hydroperoxides are the initial molecular products. Antioxidants which reduce the rate at which new chains are started are classified as preventive antioxidants [14,21]. Most preventive antioxidants act

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either by reducing hydroperoxides to molecular products without the production of radicals (e.g., glutathione peroxidase, catalase) or by sequestering and/or otherwise inactivating transition metal catalysts (e.g., transferrin, ceruloplasmin). Antioxidants which can trap radicals directly, thereby shortening the chain length, are classified as chain-breaking antioxidants [14,21]. This class includes vitamin E (a peroxyl trap) and superoxide dismutase (an $O_2^{\cdot-}$ trap).

One of the major problems encountered in the study of lipid peroxidation in systems of biological relevance is the difficulty associated with the unambiguous separation of preventive from chain-breaking antioxidant activity. Previous workers have shown that blood serum incubated with tissue homogenates (usually brain) possesses strong antioxidant properties [18,19,22–24]. Most of the observed antioxidant activity has been attributed to the presence in the serum of the iron-binding protein, transferrin, and the ferroxidase, ceruloplasmin, and hence these studies have shown that serum contains preventive antioxidants of the metal ion-inactivating type. The importance of this type of antioxidant in experiments of this type (i.e., not necessarily in vivo) has been confirmed by showing that, in the absence of serum, the spontaneous peroxidation of the tissue homogenates can also be suppressed by the addition of metal-chelating agents, such as EDTA [18]. While it is clear that serum contains powerful preventive antioxidants, experiments of this type cannot easily distinguish between preventive and chain-breaking antioxidants. Both the overall importance and individual importance of the chain-breaking antioxidants in biological tissues and fluids have remained largely unexplored in any quantitative sense.

We have undertaken a detailed study of the peroxyl radical, chain-breaking antioxidant activity of human blood plasma in order to provide quantitative information regarding the individual contributions of each chain-breaking antioxidant other than superoxide dismutase. In a preliminary communication [25], we reported that the contribution of vitamin E, vitamin C and uric acid could not account for all of the peroxyl radical, chain-breaking antioxidant activity of plasma, i.e., the known chain-breaking antioxidants did not account for the Total (Peroxyl) Radical-trapping Antioxidant Parameter (TRAP) of the plasma. The previously unrecognized chain-breaking antioxidant present in plasma was discovered to be the plasma proteins. This conclusion was based, in part, on the results of fractionation of the plasma proteins by gel filtration. We have now succeeded in identifying the chemical nature of the major source of the chain-breaking antioxidant activity of plasma proteins. The percentage contribution of the plasma proteins to TRAP has also been revised from a previously reported 57–73% [25] to 10–50%.

Materials and Methods

Human serum albumin and phosphate-buffered saline (10 mM, pH 7.4) were obtained from Sigma and used as received. L-Ascorbic acid (BDH Chemicals), sodium urate (Sigma), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma), 3,4-dihydroxybenzylamine hydrobromide (DHBA, Aldrich), 2,2′-azo-bis(2-amidinopropane hydrochloride) (ABAP, Polysciences) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) have been used in previously described work from this laboratory [25]. Desferal® was a gift from Ciba-Geigy Canada, Ltd.

Blood was obtained by venipuncture after an overnight fast (10–12 h). The blood was collected over Na_2EDTA (1–2 mg/ml blood) and immediately centrifuged (10000 × g for 10 min at 4° C). The supernatant (plasma) was used immediately for analysis and measurement of TRAP (see below). Other plasma samples, obtained from the blood of unfasted subjects, were stored at −80° C and analyzed at a later date.

The concentrations of urate and (reduced) ascorbate (i.e., not including ascorbate in its oxidized, dehydroascorbate form) in each sample were measured by HPLC using a varian Micro Pak NH_2-10 column eluted with a mixture of acetonitrile (69%) and aqueous NaH_2PO_4 (0.04 M, pH 4.8) at a flow rate of 1.5 ml/min with ultraviolet detection at 254 nm [25,26]. Samples were prepared for analysis by first adding methanol (2.0 ml) to the plasma (0.5 ml) to precipitate the plasma proteins, followed by the internal standard (aqueous 1.0 mM DHBA, 50 μl) and

n-heptane (1.0 ml). The aqueous and organic layers were separated by brief centrifugation after vortex-stirring. Urate and ascorbate were determined by injecting the aqueous extract (100 μl) onto the HPLC column [25]. Vitamin E was determined by injecting the heptane extract (100 μl) onto a silica HPLC column as described previously [13].

Plasma sulfhydryl concentrations were measured by the method described by Ellman [27]. Into a 1 × 0.5 cm cuvette was placed 900 μl of 0.2 M Na_2HPO_4 (pH 9.0, containing 2 mM EDTA), 100 μl of plasma and 20 μl of DTNB stock solution (10.0 mM in 0.05 M phosphate buffer at pH 7). From the absorbance at 412 nm ($\varepsilon = 13\,600$ $M^{-1} \cdot cm^{-1}$) the sulfhydryl group concentration was determined (after correction using an appropriate blank).

The experimental approach used to measure the TRAP of plasma, i.e., $TRAP_{exp}$, has taken advantage of the discovery that peroxidation of aqueous dispersions of oxidizable organic compounds can be reproducibly initiated by thermal decomposition of the water-soluble azo compound, ABAP [28,29]. At 37 °C, the temperature of the experiments described herein, ABAP decomposes thermally to yield useful quantities of peroxyl radicals at a known and constant rate R_i. The $TRAP_{exp}$ value, defined as the moles of peroxyls trapped by one liter of plasma, is determined from the length of time a plasma sample resists lipid peroxidation (τ_{plasma}, the induction period), as measured by the rate of uptake of oxygen, when subjected to attack by peroxyl radicals (Eqn. 1). The factor f in Eqn. 1 allows for the fact that in the experiment the plasma is diluted by the addition of buffer.

$$TRAP_{exp} = R_i \tau_{plasma} / f \quad (1)$$

We have now discovered that the original experimental procedure using a Clark-type oxygen electrode and very dilute plasma samples [25] gives $TRAP_{exp}$ values that are approx. 30% larger than those measured on essentially neat plasma (1.0 ml plasma diluted by addition of 0.2 ml phosphate-buffered saline containing 0.4 M ABAP) by the pressure transducer technique [13,28,30–32]. It was also found that the greater the dilution of the plasma by phosphate-buffered saline, the greater was the apparent value of $TRAP_{exp}$ (Fig. 1). We attribute these discrepancies to the fact that the oxygen electrode method, as originally described, used plasma which had been diluted by a factor of 100. Such high degrees of dilution are necessary in order that the supply of dissolved oxygen not be exhausted before the end of the induction period, i.e., not exhausted before τ_{plasma} can be measured. However, the very low lipid concentrations which resulted from this dilution reduced the chain length to such an extent that self-termination of two chains by reaction between two peroxyl radicals became competitive with termination by reaction of a peroxyl radical with a molecule of the antioxidant. Under these circumstances, the antioxidant lasted longer because it no longer terminated every chain. It is believed that this self-termination is responsible for the overlong induction periods. We have resolved this problem, while retaining the necessary high plasma dilution, by adding a small quantity of linoleic acid, a readily oxidizable lipid, to the highly diluted plasma samples (1.6% plasma)

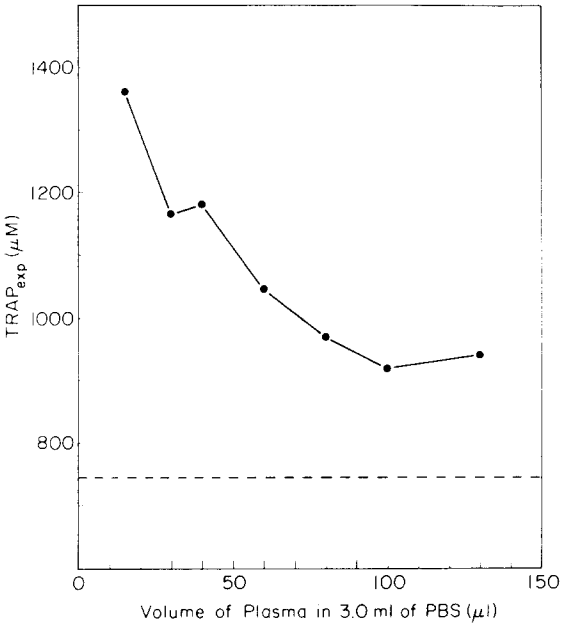

Fig. 1. The dependence of $TRAP_{exp}$ (μM) on the volume of plasma added to 3.0 ml of phosphate buffered saline (PBS; pH 7.4) using the oxygen electrode method (no added linoleic acid; Refs. 11 and 25). The $TRAP_{exp}$ value obtained for the same sample (50 μl) using the modified oxygen electrode method (i.e., with added linoleic acid) was 745 μM (dotted line).

[11]. The values of $TRAP_{exp}$ measured by this modified method are in excellent agreement with those obtained for slightly diluted plasma (83% plasma) by the pressure transducer method.

The modified oxygen electrode method for measuring $TRAP_{exp}$ has been described in detail [11]. The induction periods (τ_{plasma}) were measured with a YSI model 18053/18172 Dissolved Oxygen Monitor System. The plasma samples were prepared by vortex-mixing (in a 2 ml vial) 100 μl of plasma and 4 μl of linoleic acid for 30 s. A portion of this mixture (50 μl) was added to 3.0 ml of phosphate-buffered saline containing 4.0 mM ABAP at 37°C in the oxygen electrode cell. (Light must be excluded from the cell because ABAP is also subject to photodecomposition, which can cause variations in R_i.) At the end of the induction period, when the lipid peroxidation was proceeding rapidly, 25 μl of 0.4 mM TROLOX (a water-soluble analogue of vitamin E) was added (see Fig. 2). The second induction period, τ_{TROLOX}, can be used to calculate R_i (Eqn. 2). The factor of 2.0 appears in this equation because TROLOX traps two peroxyl radicals per molecule [13,28,31,32], i.e., $n = 2$ (vide infra). The $TRAP_{exp}$ value for the plasma sample is then calculated from Eqn. 3.

$$R_i = 2.0[\text{TROLOX}]/\tau_{\text{TROLOX}} \quad (2)$$

$$\text{TRAP}_{exp} = 2.0[\text{TROLOX}]\tau_{plasma}/f\tau_{\text{TROLOX}} \quad (3)$$

The contributions of the individual antioxidants to τ_{plasma} were determined in a series of separate experiments by adding known amounts of each antioxidant to standard plasma samples and measuring the extensions in τ_{plasma}.

The contribution of plasma proteins to $TRAP_{exp}$ was determined by performing gel filtration of plasma using a 0.9×20 cm column containing Sephadex G-25 eluted with phosphate-buffered saline, pH 7.4. Samples were collected in 0.5 ml fractions. The dilution of the plasma proteins in the combined eluent was estimated from its A_{280} (compared to the A_{280} in the undiluted plasma). This dilution factor was essentially identical to that obtained from the total volume dilution (6–8-times) of the fractions containing protein. Samples of the fractions were added to a sample

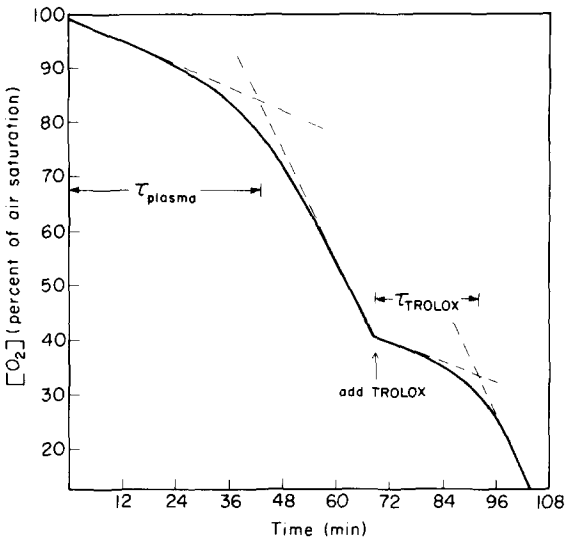

Fig. 2. Oxygen electrode trace obtained for plasma from subject AM (see Table II) using the modified oxygen electrode method: 50 μl of AM plasma containing 2 μl of linoleic acid in 3.0 ml of phosphate-buffered saline at 37°C. The second induction period is produced by the addition of 25 μl of 0.400 mM TROLOX.

of intact plasma in an oxygen electrode cell and the increase in τ_{plasma} was measured.

The changes in concentrations of the individual chain-breaking antioxidants in plasma (except ascorbate, see below) during the induction period were also monitored. ABAP (0.4 M, 3.0 ml) was added to neat plasma (20.0 ml) at 37°C and oxygen uptake was monitored using a pressure transducer. Samples (1.0 ml) were removed periodically for analysis. Analysis of plasma sulfhydryl content by the Ellman method [27] was not affected by the presence of ABAP. Sample preparation for HPLC analysis of urate was modified in order to remove the ABAP which interfered with this analysis. To 0.5 ml of plasma was added methanol (2.0 ml) to precipitate the plasma proteins, and heptane (1.0 ml), the sample was vortex-mixed and the aqueous/methanolic and organic layers were separated as described above. The aqueous/methanolic extract (1.0 ml) was passed through a column of Chelex-100 (which had been washed with methanol, bed volume approx. 1 ml), followed by an additional 1.0 ml of methanol. DHBA (20 μl of 1.0 mM) was added to the combined fractions and this solution (100 μl)

was injected onto the HPLC column for analysis of urate. Unfortunately, ascorbate did not survive this procedure and so it proved possible to measure only the initial ascorbate concentration in the plasma, not its loss during these experiments. The organic layer was used to determine the vitamin E concentration in the plasma.

Results

The $TRAP_{exp}$ values and concentrations of vitamin E, urate, ascorbate and sulfhydryl groups for six fresh plasma samples obtained from fasted human subjects are shown in Table I. The vitamin E and urate concentrations are within the usually reported physiological ranges [15], but some of the ascorbate levels are lower than is normal for fasted subjects [33,34]. Since the addition of ascorbate to a fresh plasma sample and its subsequent analysis by HPLC gave a recovery of more than 90% of the added ascorbate, we presume that the plasma ascorbate levels in Table I are accurate. Also the majority of the ascorbate levels measured in 45 samples from non-fasted subjects were within the normal range (20–120 μM) [35].

The $TRAP_{exp}$ values for fresh plasma samples were not measurably different from those found for the same samples after storage at $-80\,^{\circ}C$ for more than 2 weeks. The $TRAP_{exp}$ values for 45 previously frozen plasma samples fell within the range 571 μM to 1284 μM; average value ($\pm$S.D.) = 820 ± 148 μM; median value 800 μM (Fig. 3).

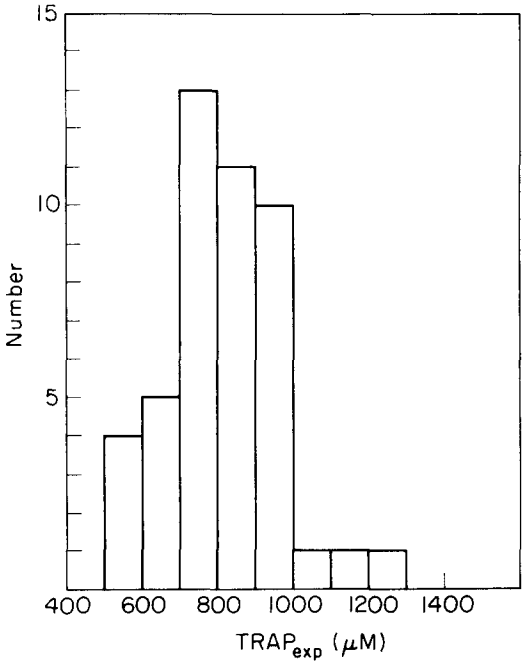

Fig. 3. Histogram of $TRAP_{exp}$ values of 45 previously frozen plasma samples from non-laboratory personnel.

The average concentrations of vitamin E, urate, ascorbate and sulfhydryl groups in these 45 plasma samples were 28 ± 9 μM, 364 ± 87 μM, 69 ± 40 μM and 424 ± 57 μM, respectively.

If the concentration of a chain-breaking antioxidant, j, is known, and if the number of peroxyl radicals trapped by each molecule of this antioxidant, n_j (the stoichiometric factor), is known,

TABLE I

CONCENTRATIONS (μM) OF CHAIN-BREAKING ANTIOXIDANTS IN AND $TRAP_{exp}$ VALUES (μM) FOR HUMAN PLASMA PREPARED FROM FRESHLY DRAWN BLOOD

Subject	[Vit. E]	[Urate]	[Ascorbate]	[SH]	$TRAP_{exp}$ (O_2 electrode) [a]	$TRAP_{exp}$ (transducer) [b]
AM	38	319	12	434	767	nm [c]
AW	21	311	0	311	726	nm [c]
SC	25	340	0	444	954	nm [c]
JV	17	340	21	379	671	666
GB	25	368	17	556	745	673
KI	45	468	90	408	1034	1031

[a] $TRAP_{exp}$ value determined with fresh plasma (diluted to 1.6% in phosphate-buffered saline) using the modified oxygen electrode method [11].

[b] $TRAP_{exp}$ value determined with fresh plasma (diluted to 83% in phosphate-buffered saline) using the pressure transducer method [13,28,30–32].

[c] nm = not measured.

then we can define a Contributing Antioxidant Parameter, CAP(j), which describes the contribution made by this antioxidant to $TRAP_{exp}$:

$$CAP(j) = n_j[j] \quad (4)$$

Stoichiometric factors of the plasma antioxidants

The stoichiometric factors is most reliably determined by measuring the increase in $TRAP_{exp}$, $\Delta TRAP_{exp}$, produced by the addition of a known concentration of antioxidant, i.e., by plotting $\Delta TRAP_{exp}$ vs. Δ [j]. Such plots should be linear and have a slope equal to n. That is, the n value of a 'well-behaved' antioxidant should not depend on the initial concentration of the antioxidant. Values of n are expected to be integral.

The n value for vitamin E is known to be 2.0 [13,28,30–32]. However, the n values for the other plasma antioxidants either have not been measured or have not been generally agreed upon [11,25,36].

The n value for ascorbate has been found to be dependent on the initial concentration of ascorbate, decreasing from a value of close to 2.0 at low initial ascorbate concentrations (approx. 1 μM) to a value which approaches zero at high initial concentrations ($\gg$ approx. 1 mM) [11]. This has been attributed to the facility with which vitamin C itself undergoes autoxidation [11]. In view of the 63-fold dilution of the plasma, which produces actual initial ascorbate concentrations in the oxygen electrode cell of between 0 and approx. 2 μM, the effective n value for ascorbate can be predicted to lie between 1.5 and 2.0. We have used an n value of 1.7 in our calculations. The value of 0.7 used in our earlier work [25] was derived without benefit of the knowledge that n varied with the concentration of ascorbate. Of course, the effective n value for ascorbate must be lower under the conditions used for determining $TRAP_{exp}$ by the pressure transducer technique, since this measurement is made at higher concentrations of plasma. However, since ascorbate is present at relatively low concentrations, the $TRAP_{exp}$ values measured with the pressure transducer will be only slightly less than values determined with the oxygen electrode.

We have determined an n value for urate in plasma using both the oxygen electrode method

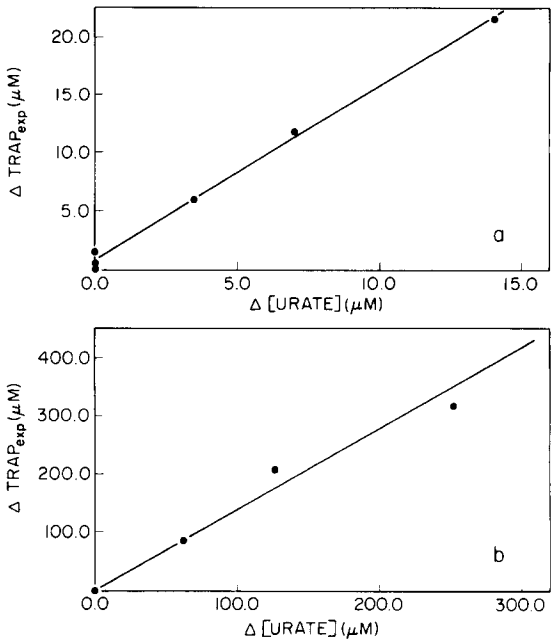

Fig. 4. Plot of increase in $TRAP_{exp}$, $\Delta TRAP_{exp}$ (μM), produced by the addition of urate, Δ[urate] (μM), to plasma using (a) the oxygen electrode technique and (b) the pressure transducer technique. The lines drawn have slopes of 1.3.

(initial urate concentrations betwen 2 and 15 μM with 1.6% plasma in phosphate-buffered saline), which gives $n = 1.3$ (Fig. 4a), and the pressure transducer method (initial urate concentrations between 60 and 250 μM with 50% plasma in phosphate-buffered saline), which also gives $n =$ 1.3 (Fig. 4b). It is clear that over this range of urate concentrations and in this system the n value for urate does not depend on the initial urate concentration. These n values were determined by adding urate to the plasma before the oxidation was initiated and measuring the increase in the induction period compared to the induction period without added urate. This n value of 1.3 for urate is therefore obtained in the presence of all the plasma's natural antioxidants and unmodified proteins. However, when urate was added after the end of the normal plasma induction period (thus producing a second induction period) an n value of 1.65 was obtained. Thus, a higher n value is found for urate in the absence of other plasma antioxidants and in the presence of proteins modified by free radical attack. (At the end of the induction period the normally yellow plasma is colorless and a white precipitate is present. This

precipitate has not been analyzed. However, it is probably due to proteins which have become insoluble through their modification by free radical attack.)

Niki et al. [36] have reported an n value of 2.0 for urate in liposome and micelle systems containing no other antioxidants at 37°C. This n value was assigned on the basis that sequential addition of equal concentrations of urate and TROLOX (for which n is known to be 2.0) gave equal induction periods when lipid peroxidation was initiated with ABAP. We find, however, that this is not the case for the ABAP-initiated peroxidation of egg lecithin unilamellar liposomes at 37°C. That is, we find that for equal concentrations of TROLOX and urate the corresponding ratio of τ_{TROLOX} to τ_{urate} is 1.2, which leads to an n value of $2.0/1.2 = 1.7$ for urate in this system. It is difficult to rationalize all of these different n values for urate, but it is clear that its effective n value depends on the specific system under study. We have chosen to use an n value of 1.3 for urate in our calculations, since this is the value we obtain with plasma under 'initial' conditions.

We now define a calculated TRAP value, $TRAP_{calc}$, which should be equal to $TRAP_{exp}$ if the concentrations and stoichiometric factors are known for every chain-breaking antioxidant present in the sample:

$$TRAP_{calc} = \sum CAP(j) = \sum n_j[j] \quad (5)$$

Using the measured concentrations of vitamin E, ascorbate and urate with n values of 2.0, 1.7 and 1.3, respectively, we can calculate CAP(E), CAP(U) and CAP(C), and hence the total contribution of these three antioxidants to $TRAP_{exp}$. The difference between $TRAP_{exp}$ and the sum of the calculated contributions from these three antioxidants (column 3 in Table II) represents a very significant fraction (20–40%) of $TRAP_{exp}$. We have attributed this 'missing' secondary antioxidant activity to the previously unrecognized chain-breaking antioxidant effect of the plasma proteins [25]. Gel filtration (Sephadex G-25) of the plasma leads to recovery of the protein portion of the plasma. This fraction contains all the vitamin E (present in the plasma lipoproteins) and the protein chain-breaking antioxidants. Titration of this eluent from gel filtration with peroxyl radicals in the usual way yields the chain-breaking antioxidant concentration due to vitamin E plus protein (column 4 in Table II). Subtraction of the individual CAP(E) values from each of these concentrations yields the chain-breaking antioxidant contribution of the plasma proteins (given in parentheses in column 4 in Table II). Given the uncertainties in these calculations, there is rather satisfactory agreement with the individual $TRAP_{exp} - \{CAP(E) + CAP(U) + CAP(C)\}$ values (column 3 in Table II). The previously unrecognized chain-breaking antioxidant in plasma is therefore confirmed to lie in the plasma proteins, and it appears

TABLE II

CONTRIBUTIONS OF PLASMA PROTEINS TO $TRAP_{exp}$ [a]

Subject	$TRAP_{exp}$ [b]	$TRAP_{exp} - \{CAP(E) + CAP(U) + CAP(C)\}$ [c]	Proteinaceous chain-breaking antioxidant [b,d]	$TRAP_{calc}$ [e]
AM	767	255	410 (334)	654
AW	726	279	187 (145)	549
SC	954	350	359 (309)	750
JV	671	198	255 (221)	598
GB	745	195	299 (249)	741
KI	1034	183	210 (120)	986

[a] All numbers refer to concentrations in μM units.

[b] Measured using the modified oxygen electrode technique.

[c] $\{CAP(E) + CAP(U) + CAP(C)\}$ calculated as $\{2.0[\text{vit. E}] + 1.3[\text{urate}] + 1.7[\text{ascorbate}]\}$.

[d] Measured on the eluent from gel filtration. Concentrations in parenthesis have been adjusted by subtracting the vitamin E contribution, CAP(E).

[e] $\{CAP(E) + CAP(U) + CAP(C) + CAP(SH)\} = \{2.0[\text{vit. E}] + 1.3[\text{urate}] + 1.7[\text{ascorbate}] + 0.33[SH]\}$.

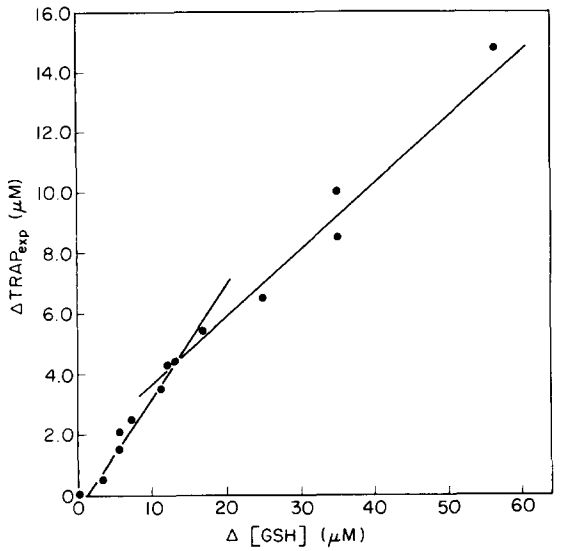

Fig. 5. Plot of the increase in $TRAP_{exp}$, $\Delta TRAP_{exp}$ (μM), produced by the addition of GSH, Δ[GSH] (μM), to plasma. The two lines drawn have slopes of 0.33 and 0.22, respectively.

to be due mainly to the presence of sulfhydryl (SH) groups (see below).

The *n* value for sulfhydryl groups was determined using both glutathione (GSH) and the plasma proteins themselves. First, using GSH, a plot of $\Delta TRAP_{exp}$ versus Δ[GSH] showed a slight curvature, with an initial slope of 0.33 (0–12 μM) and a final slope of 0.22 (12–60 μM) (Fig. 5). We suggest that this curvature and the non-integral *n* value are due to autoxidative destruction of GSH [37,38]. It should be added that GSH by itself will inhibit the autoxidation of the plasma lipids, especially under the conditions used with the pressure transducer. That is, a brief induction period was produced when GSH was added after all the natural plasma antioxidants had been consumed.

Second, individual plasma samples were divided into two portions. One portion was incubated for 12 h at 4°C with 20 mM *N*-ethylmaleimide (which is known to alkylate sulfhydryl groups) and was then subjected to gel filtration, yielding a plasma protein fraction containing no sulfhydryl groups detectable with Ellman's reagent. The other portion was subjected to gel filtration and the SH group concentration in the plasma proteins was determined with Ellman's reagent. Comparison of the chain-breaking antioxidant activities of the chemically blocked and fresh (control) plasma protein samples together with the known concentration of SH groups yielded *n* values for the protein sulfhydryls of 0.33 (plasma from subject JV), 0.28 (KI plasma), 0.17 (SC plasma) and 0.20 (DA plasma). One of the most interesting aspects of this experiment was that only 50–60% of the CAP of plasma protein fraction was destroyed by treatment with *N*-ethylmaleimide in the JV, SC and DA plasma samples, while essentially all of the CAP of the protein fraction in the KI plasma sample was destroyed. This leaves in the JV, SC and DA plasma proteins approx. 10% of $TRAP_{exp}$ which cannot be attributed to accessible sulfhydryl groups. The anomalous behavior of the KI plasma sample is not understood.

We have chosen an *n* value for protein sulfhydryl groups equal to 0.33, since this yields $TRAP_{calc}$ values based on the individual concentrations of E, U, C and SH (see column 5 in Table II) that are in very satisfactory agreement with the $TRAP_{exp}$ values (column 2 in Table II) for each individual. The agreement between $TRAP_{calc}$ and

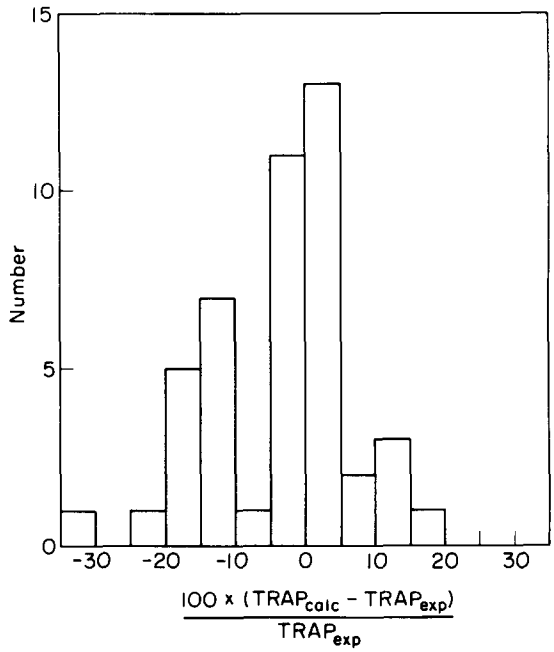

Fig. 6. Histogram of the percent difference between $TRAP_{calc}$ (= 2.0[vit. E] + 1.3[urate] + 1.7[ascorbate] + 0.33[SH]), and $TRAP_{exp}$ for 45 samples from non-laboratory personnel. Negative deviations indicate that $TRAP_{calc}$ is less than $TRAP_{exp}$.

$TRAP_{exp}$ is further emphasized in Fig. 6, which shows a histogram of the difference, $TRAP_{calc} - TRAP_{exp}$ (expressed as the percentage of the individual $TRAP_{exp}$ value) for 45 plasma samples from non-laboratory personnel. From Table II and this histogram we estimate that the uncertainty in the determination $TRAP_{calc} - TRAP_{exp}$ is no greater than approx. 15%.

Although plasma contains millimolar quantities of glucose, it was found that added glucose (5 mM) had no effect on τ_{plasma}.

The concentrations of vitamin E, urate and plasma sulfhydryl groups for two different plasma samples, during the period of inhibition of lipid peroxidation (i.e., τ_{plasma}), are shown in Fig. 7. The induction period comes to an end when all chain-breaking antioxidants have been consumed. The plasma sulfhydryl groups are the first to decline in concentration, then the urate and finally the vitamin E. The last-named antioxidant remains detectable until the very end of the induction period.

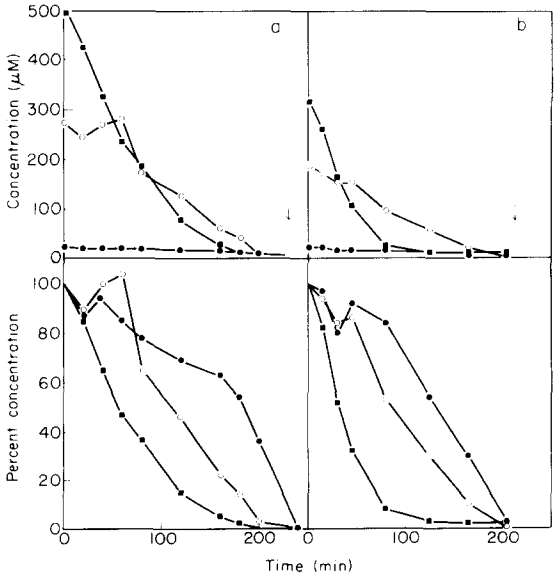

Fig. 7. Plot of the absolute (top) and relative (bottom) concentrations of vitamin E (●), urate (○) and sulphydryl groups (■) during the ABAP-initiated peroxidation of (a) DG plasma and (b) GB plasma (second sample). The arrows correspond to the end of the induction periods, i.e., to the start of rapid oxygen uptake as measured with the pressure transducer.

Discussion

Interactions between the plasma chain-breaking antioxidants

The interactions between the various chain-breaking antioxidants in the plasma may be important in maintaining efficient inhibition of lipid peroxidation. The interactions between vitamins C and E are well established [10,12,39–41]. It is generally recognized that vitamin E can only trap peroxyl radicals that are present in (and/or at the interface of) the lipid phase, whereas vitamin C, urate [36] and glutathione [42] can trap peroxyl radicals in the aqueous phase but not in the lipid phase. However, if vitamin E is present in the lipid phase, vitamin C can regenerate vitamin E from the α-tocopheroxyl radical [40,41]. Work currently in progress in our laboratory suggests that this is not the case for urate and glutathione.

It is interesting to note that urate protects ascorbate against oxidation by cupric ion [43] and against iron-induced oxidation [44]. In the latter study [44], it was also found that urate in human serum 'stabilized' ascorbate without any apparent consumption of the urate. These results reflect the fact that urate possesses preventive antioxidant activity (through metal ion chelation [45]) in addition to this chain-breaking antioxidant activity.

It is noteworthy that experiments carried out in the presence of the iron-chelating agents, Desferal® and EDTA, indicated that there was no effect on the concentration dependence of the *n* values of ascorbate and of glutathione.

The contribution of each antioxidant to $TRAP_{exp}$

Human plasma is highly resistant to peroxidation. Work by others [18–20,22,23] has shown that plasma contains preventive antioxidants. As we have shown herein, plasma has a chain-breaking antioxidant activity which allows it to trap approx. 700–1000 μM of radicals (see Table I and Fig. 3).

The individual chain-breaking antioxidants and their Contributing Antioxidant Parameters (CAP), expressed as their percentage contribution to $TRAP_{exp}$, are given in Table III. It should be noted that although vitamin E is the major lipid-soluble, chain-breaking antioxidant in plasma [13] it is responsible for only 5–10% of the plasma's

TABLE III

CONTRIBUTIONS OF INDIVIDUAL CHAIN-BREAKING ANTIOXIDANTS TO $TRAP_{exp}$ [a]

Antioxidant	Contribution to $TRAP_{exp}$ (%) [b]	Range (%)
Urate	58 ± 18	35–65
Plasma proteins	21 ± 10	10–50
Ascorbate	14 ± 8	0–24
Vitamin E	7 ± 2	5–10

[a] Average values for 45 samples from non-laboratory personnel.

[b] Mean ± S.D.

$TRAP_{exp}$ (see Tables I and III). We have previously shown that the chain-breaking antioxidant activity of the plasma proteins is a non-specific property of these proteins [25]. Induction periods were measured for various protein fractions obtained by gel filtration and were shown to parallel, approximately, the 280 nm absorbance of the individual fractions (which provides a simple measure of the total quantity of protein in each individual fraction). The longest induction periods were obtained with those fractions that contained human serum albumin. Furthermore, addition of 90 mg/ml of human serum albumin to plasma increased $TRAP_{exp}$ by 200 μM. This increase is comparable to the measured chain-breaking antioxidant concentration of the plasma proteins and is consistent with the fact that plasma protein concentrations normally range from 60 to 80 mg/ml [46]. The addition of several other proteins containing sulfhydryl groups, such as transferrin, ceruloplasmin, and even papain (which is not a plasma protein), to plasma samples also produced increases in the $TRAP_{exp}$ values. The amounts of transferrin and ceruloplasmin that were required to produce a measurable increase in TRAP were, however, 20- and 100-times their normal physiological concentrations, respectively! Clearly, the known [18] antioxidant activity of these two proteins is due almost entirely to their preventive antioxidant effect.

Positive values in the histogram shown in Fig. 6 indicate that $TRAP_{calc}$ is larger than $TRAP_{exp}$, while negative values indicate that $TRAP_{calc}$ is smaller than $TRAP_{exp}$. In principle, it is impossible for $TRAP_{calc}$ to be larger than $TRAP_{exp}$. However, uncertainties in the determination of both quantities would explain the positive deviations and would be expected to lead to some symmetric distribution around zero if no other antioxidants were present. The histogram shows evidence of being skewed towards negative values, which suggests that there may be chain-breaking antioxidants which have not been accounted for in at least some plasma samples. If so, they probably reside in the plasma proteins. This suggestion is supported by the inability (in three out of four cases) to eliminate completely the plasma protein contribution to $TRAP_{exp}$ by reaction with *N*-ethylmaleimide (see above). Since the calculated n values for the plasma sulfhydryl groups in these four samples varied from 0.17 to 0.34, the reasonably good correlation between $TRAP_{calc}$ and $TRAP_{exp}$ may be somewhat fortuitous. That is, the measured sulfhydryl group concentrations probably are proportional to the protein concentrations. The n value for the protein sulfhydryl groups of 0.33 would then be a composite value which encompasses all of the radical-trapping reactions of the proteins, i.e., it would correspond to the product of the weighted average of n values for the individual radical-trapping reactions and a factor which would describe the relationship between the concentration of these protein radical traps and the protein sulfhydryl group concentration. The histogram in Fig. 6 shows that these relationships are constant for the majority of the plasma samples, with the negative deviations coming from those samples for which the sulfhydryl group concentrations were low either because of improper sample handling or because the relationships between the protein radical-traps and sulfhydryl groups described above had changed.

The amino acid most likely to exhibit antioxidant activity is tyrosine, which (like vitamin E) contains a phenolic hydroxyl group. However, we find that monomeric tyrosine has no observable antioxidant behavior in aqueous solution. Thus, when it is added to the plasma at the start of an oxidation experiment it produces no increase in the induction period; when it is added after the normal induction period it does not produce a second induction period. Even when an equimolar amount of TROLOX was added together with the tyrosine at the end of the plasma induction period,

the second induction period was no greater than that produced by the TROLOX alone. Since *para*-cresol (which is structurally related to tyrosine insofar as chain-breaking antioxidant activity is concerned) is an antioxidant in hydrocarbon solvents [47,48], we presume that in aqueous solution hydrogen-bonding greatly reduces the efficiency of phenolic antioxidants. Similarly, monomeric tryptophan and histidine showed no chain-breaking antioxidant activity. Of course, in the proteins some of these residues may be present in much less polar, non-aqueous, environments, and such residues should be capable of trapping peroxyl radicals. Whether they actually would do so would depend on whether the peroxyl radicals could penetrate the protein to reach these potential radical-trapping sites.

The relative magnitude of the contributions of each antioxidant (see Table III) does not necessarily represent the relative importance of the particular antioxidant to the biological system. The changes in the concentrations of the chain-breaking antioxidants during the induction period (Fig. 7) show that vitamin E is present until the end of the induction period despite the fact that its initial concentration is always very low relative to urate and sulfhydryl groups. This result is consistent with the suggestion that vitamin E is regenerated by water-soluble antioxidants [10,39,40,49, 50]. However, in our experiments, we generate radicals in the aqueous phase of plasma. Our results can, therefore, equally well be interpreted as indicating that water-soluble peroxyl radicals react with water-soluble chain-breaking antioxidants in preference to penetrating into the oxidizable lipid fraction of the plasma. In such a case it would be more appropriate to refer to the sparing action on vitamin E of water-soluble chain-breaking antioxidants.

An interesting feature of Fig. 7 is the apparent inability of the low molecular weight chain-breaking antioxidants in plasma to protect the plasma proteins' sulfhydryl groups from peroxidative damage. It appears, in fact, that at the beginning of the oxidation even the urate is spared at the expense of these groups. The oxidative destruction of essential sulfhydryl groups of proteins and enzymes in the presence of lipid hydroperoxides has been reported [38,51,52]. However, the preferential consumption of sulfhydryl groups in plasma subjected to peroxyl radical attack implies that there is no compound actually present in the plasma that is able to 'repair' these groups. They therefore represent the most expendable source of antioxidants in plasma.

The large amount and variety of aqueous antioxidants (both preventive and chain-breaking) indicates that plasma is very well equipped to meet the threat posed by substances originating in the aqueous phase that are potentially capable of initiating lipid peroxidation. Probably, the most common aqueous peroxyl radical is hydroperoxyl, $HOO^{\cdot}$, which is generated in a pH-governed equilibrium with the superoxide anion radical. The latter is, of course, scavenged rapidly and efficiently by superoxide dismutase [17]. By contrast, vitamin E is the only significant lipid-based chain-breaking antioxidant, and it is present in only small quantities. (Note that the concentration of β-carotene [15] in plasma is too low for it to make an important contribution to $TRAP_{exp}$.) We suggest that relatively little radical chain generation actually originates in the lipid phase. However, when radicals do enter the lipid from the aqueous phase there are synergistic mechanisms available to minimize the loss of vitamin E.

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