

Ascorbate is an outstanding antioxidant in human blood plasma

(oxidant stress/lipid peroxidation/protein thiols/ α -tocopherol)

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Contributed by Bruce N. Ames, June 5, 1989

ABSTRACT We have shown recently that the temporal order of antioxidant consumption in human blood plasma exposed to a constant flux of aqueous peroxy radicals is ascorbate = protein thiols > bilirubin > urate > α -tocopherol and that detectable lipid peroxidation starts only after ascorbate has been consumed completely. In this paper, we show that it is indeed ascorbate that completely protects plasma lipids against detectable peroxidative damage induced by aqueous peroxy radicals and that ascorbate is the only plasma antioxidant that can do so. Plasma devoid of ascorbate, but no other endogenous antioxidant, is extremely vulnerable to oxidant stress and susceptible to peroxidative damage to lipids. The plasma proteins' thiols, although they become oxidized immediately upon exposure to aqueous peroxy radicals, are inefficient radical scavengers and appear to be consumed mainly by autoxidation. Our data demonstrate that ascorbate is the most effective aqueous-phase antioxidant in human blood plasma and suggest that in humans ascorbate is a physiological antioxidant of major importance for protection against diseases and degenerative processes caused by oxidant stress.

It is becoming increasingly evident that oxidants contribute to the development or exacerbation of many of the most common human diseases associated with aging, including cancer, heart attacks, stroke, arthritis, and cataract (1-4). Over our lifetimes, we are continuously exposed to oxidants of both endogenous and exogenous origin. Endogenous sources of oxidants include mitochondrial respiration, enzymes such as lipoxygenase and xanthine oxidase, and the NADPH oxidase/myeloperoxidase system of phagocytes (2, 5). Examples of external sources of oxidants are natural dietary constituents, UV radiation, natural radioactive gases (e.g., radon leaching from soils), and environmental pollutants such as car exhaust and cigarette smoke (1-4).

Our defenses against oxidant stress are the antioxidants synthesized in our body and the antioxidant vitamins taken up in the diet (1, 2). The antioxidants synthesized in our body include all antioxidant proteins (e.g., catalase and transition metal-binding proteins) and various small molecules, some of which are end products of metabolic pathways (e.g., bilirubin and urate). Their levels in our body cannot be manipulated by simple means. On the other hand, the levels of the antioxidant vitamins, such as ascorbate, α -tocopherol, and β -carotene, can be increased easily by dietary means or supplementation. In light of the well-known antioxidant properties of ascorbate (6), it is noteworthy that the U.S. recommended daily allowance (RDA) for ascorbate is based exclusively on its function in collagen synthesis (its anti-scorbutic effect) (7) and not on its antioxidant activity. In contrast, the RDA for α -tocopherol *a priori* has to be based on its antioxidant activity since this is its only well-established physiological function (8).

The reluctance to raise the RDA for ascorbate partly stems from *in vitro* findings, often obtained by using tissue homoge-

nates (9, 10), that ascorbate can have prooxidant activity in the presence of free transition metal catalysts (11). However, these *in vitro* findings are unlikely to be relevant to the *in vivo* situation in healthy organisms (6, 7), where most transition metal ions are not free, but attached to binding proteins, and thus are prevented from participating in free radical reactions outside the protein (2, 12, 13). Only under pathological conditions that cause release of heme or metal ions from their binding proteins—e.g., from hemoglobin (14) or ferritin (15)—could ascorbate act as a prooxidant. Under such conditions, however, not only ascorbate, but also α -tocopherol can be expected to be deleterious, since α -tocopherol, too, displays prooxidant effects in the presence of free iron ions (16).

In this study, we have used human blood plasma as a physiological model system to compare the antioxidant efficacy of ascorbate with that of α -tocopherol and other biological antioxidants. We have shown recently (17) that in plasma exposed to a water-soluble radical initiator or the oxidants generated by polymorphonuclear leukocytes no lipid peroxidation can be detected as long as ascorbate is present. This strongly suggested, yet did not prove, that in plasma ascorbate is capable of completely protecting the lipids against detectable peroxidative damage. During the phase of no detectable lipid peroxidation, not only ascorbate but also part of the plasma proteins' thiols were oxidized (17), suggesting that they also provide very effective antioxidant protection. Here we show that in plasma ascorbate is indeed an outstandingly effective scavenger of aqueous peroxy radicals, much more effective than any of the other endogenous antioxidants, and that the protein thiols have only very limited radical-trapping capabilities. Ascorbate proved to be solely protective rather than acting as a prooxidant, providing strictly increased benefit with increased concentrations.

MATERIALS AND METHODS

Materials. Isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), microperoxidase (MP-11), 5,5'-dithiobis(2-nitrobenzoic acid), *N*-ethylmaleimide, antioxidants, and Sephadex G-25-50 were purchased from Sigma. Diocetylamine and metaphosphoric acid were obtained from Aldrich and 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH) was from Polysciences. Ion pair mixture Q12 (0.5 M solution of dodecyltriethylammonium phosphate) was purchased from Regis (Morton Grove, IL), and sodium heparin Vacutainers (143 units for 10 ml of blood) were from Becton Dickinson. 15(S)-Hydroperoxyicosatetraenoic acid was obtained from Calbiochem, and other lipid hydroperoxide standards were prepared as described (18). The equipment used was the same as described (18) except that a 10- μ l instead of a 5- μ l flow cell was used in the fluorometer.

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane)hydrochloride; *n* value, stoichiometric factor, or number of peroxy radicals trapped by each molecule of an antioxidant; PBS, 50 mM phosphate-buffered saline, pH 7.4; RDA, U.S. recommended daily allowance. *To whom reprint requests should be addressed.

Incubation of Plasma. Plasma prepared from fresh heparinized blood (18) from healthy donors was preincubated at 37°C for 5 min. An aliquot of 750 mM AAPH in 50 mM phosphate-buffered saline (pH 7.4) (PBS), was added, giving a final AAPH concentration of 50 mM. The sample was incubated in a shaking water bath at 37°C under air and in the dark. AAPH is a water-soluble azo compound that thermally decomposes and thereby produces peroxy radicals at a constant rate (19). Immediately after the addition of AAPH (time 0) and at the time points indicated, aliquots were withdrawn for the determination of lipid hydroperoxides and antioxidants (see below). Where appropriate, ascorbate was added from a freshly prepared 50× concentrated stock solution of ascorbate in PBS to give the final concentrations indicated.

Treatment of Plasma by Gel Filtration. For each Sephadex G-25-50 column to be prepared, 10 ml of a suspension containing 6.25 g of filtered moist resin in 5 ml of PBS was transferred into a polypropylene Econo-Column (Bio-Rad). The column was centrifuged for 5 min at 600 × g and 4°C in a swinging bucket rotor. To equilibrate the resin, 2.5 ml of PBS was added and the column was spun for 15 min. This step was repeated once. For desalting, 0.5 ml of plasma was applied slowly to the dry resin, and the column was centrifuged for 15 min, collecting the eluate. Then, 250 μl of PBS was added and the column was spun again, collecting the eluate into the same tube. This procedure yielded 750 μl of 33% diluted plasma deprived of >97% of small free molecules including ascorbate and urate (measured as described below). Plasma fractions treated by gel filtration were pooled and incubated with 50 mM AAPH as described above.

Determination of Lipid Hydroperoxides. The various classes of lipid hydroperoxides were determined with an ultrasensitive HPLC/isoluminol chemiluminescence assay as described (18, 20). This assay measures the hydroperoxy groups themselves and not indirect indices of lipid peroxidation such as diene conjugates or breakdown products of lipid hydroperoxides. The same assay was also used for the quantitation of α-tocopherol (18).

Determinations of Bilirubin and Protein Thiols. These were done as described (17).

Determinations of Ascorbate and Urate. These were done by paired-ion reversed-phase HPLC coupled with electrochemical detection using a modification (R. Stocker, personal communication) of the method described by Kutnink *et al.* (21). Fifty microliters of plasma was mixed with an equal volume of cold 5% (wt/vol) metaphosphoric acid and centrifuged to remove the precipitated proteins. Fifty microliters of the supernatant was mixed with 15 μl of cold 2.58 M K₂HPO₄ buffer (pH 9.8) followed by the addition of 185 μl of HPLC eluant (see below). Forty microliters of this mixture was immediately chromatographed on an LC18DB column [25 cm × 4.6 mm (i.d.)] (Supelco) preceded by a guard column [2 cm × 4.6 mm (i.d.)] containing the same material. The eluant, delivered at a flow rate of 1.0 ml/min, consisted of 40 mM sodium acetate, 0.54 mM Na₂EDTA, 1.5 mM dodecyltriethylammonium phosphate, and 7.5% (vol/vol) methanol, taken to pH 4.75 with glacial acetic acid. The eluate was analyzed with an LC 4B amperometric electrochemical detector equipped with a glassy-carbon working electrode and a Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN). The applied potential was +0.5 V, with a sensitivity setting of 500 nA for urate and 50 nA for ascorbate. Urate and ascorbate eluted as single peaks with retention times of 5.8 and 11.5 min, respectively. After each set of analyses, a calibration was performed with freshly prepared standard solutions of urate and ascorbate in PBS.

RESULTS

Incubation of human blood plasma at 37°C with the water-soluble radical initiator AAPH (50 mM), conditions under

which peroxy radicals are produced at a rate of 3.0 μM/min (19), led to sequential depletion of the plasma antioxidants in the following temporal order: ascorbate = protein thiols > bilirubin > urate > α-tocopherol (17). Using an HPLC/isoluminol chemiluminescence assay that detects the various classes of lipid hydroperoxides at plasma levels as low as 0.03 μM (20), no lipid hydroperoxides could be detected during the initial period (0–50 min) of incubation of plasma with AAPH. This period coincided with the complete consumption of ascorbate. Thereafter, micromolar concentrations of lipid hydroperoxides were formed, even though protein thiols and bilirubin were still present at ≈60% and ≈85%, respectively, of their initial concentrations, and urate and α-tocopherol were present at unchanged initial concentrations (17).

When we added increasing amounts of ascorbate to plasma before it was challenged with AAPH, there was an increase in the period during which no lipid hydroperoxides could be detected (Fig. 1). For example, in the presence of 5 mM ascorbate there was no detectable lipid peroxidation for >6 hr, a period during which >1 mM aqueous peroxy radicals were produced by decomposition of AAPH at a rate of 3.0 μM/min (19). The lag phase preceding detectable lipid peroxidation did not increase linearly with ascorbate concentration (Fig. 1), reflecting decreased radical-trapping ability of ascorbate as its concentration increases (22).

To investigate antioxidant protection and lipid peroxidation in plasma devoid of ascorbate, plasma was treated by gel filtration using a column centrifugation technique (see *Materials and Methods*). This treatment removed small free molecules including ascorbate and urate (<1 μM and <5 μM left, respectively) but did not remove protein thiols or lipoprotein-associated α-tocopherol and lipids, which were recovered to >98%. Albumin-bound bilirubin also was not removed by the gel-filtration step and was recovered to ≈90%. When plasma treated by gel filtration was exposed to AAPH, peroxidative damage to lipids was induced immediately with the formation of hydroperoxides of phospholipids (Fig. 2A), cholesterol esters, and triglycerides (data not shown). Despite the presence of protein thiols, α-tocopherol, and bilirubin at near-physiological plasma concentrations, there was no lag phase preceding detectable lipid peroxidation (Fig. 2A). This demonstrates that the plasma proteins' thiols, although they become oxidized immediately upon exposure of normal plasma (17) or gel-filtrated plasma (Fig. 2B) to AAPH, cannot prevent detectable peroxidative damage to lipids. In addition, these findings corroborate our earlier conclusion (17) that in plasma exposed to aqueous peroxy radicals α-tocopherol cannot prevent initiation of

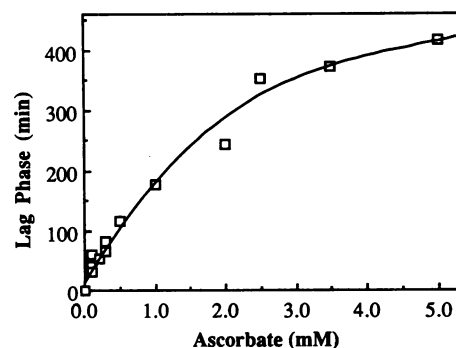


FIG. 1. Extension by ascorbate of the lag phase preceding detectable lipid peroxidation in plasma exposed to aqueous peroxy radicals. Plasma supplemented with ascorbate to the total (endogenous plus added) concentrations indicated was incubated with 50 mM AAPH, and the lag phase during which no lipid hydroperoxides could be detected (concentration, <0.03 μM) was determined.

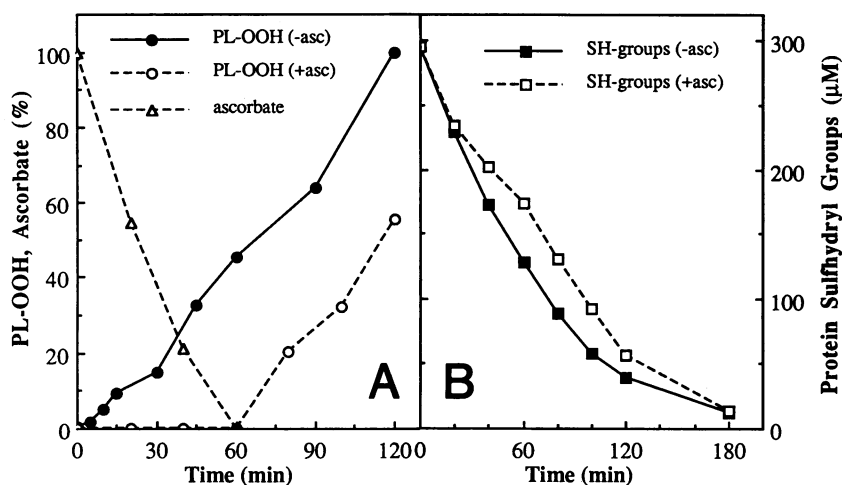


FIG. 2. Lipid peroxidation (A) and oxidation of the protein thiols (B) in plasma treated by gel filtration and exposed to aqueous peroxy radicals. Plasma was treated by gel filtration to remove endogenous ascorbate and then exposed to 50 mM AAPH in the absence (–asc, solid symbols) or presence (+asc, open symbols) of added ascorbate (100 μ M). The concentrations of the endogenous plasma antioxidants in the incubations were as follows: ascorbate, <1 μ M; urate, 4.5 μ M; protein thiols, 295 μ M; bilirubin, 4.2 μ M; α -tocopherol, 20.1 μ M. PL-OOH, phospholipid hydroperoxides. 100% = 66 μ M phospholipid hydroperoxides and 100 μ M ascorbate.

detectable lipid peroxidation and formation of micromolar concentrations of lipid hydroperoxides.

Adding back 100 μ M ascorbate to plasma treated by gel filtration before it was exposed to AAPH restored the period of complete protection against detectable lipid peroxidation (Fig. 2A). During this period, the added ascorbate was consumed completely. Only after completion of ascorbate oxidation did lipid peroxidation become detectable. Addition of ascorbate reduced the rate of oxidation of the protein thiols only slightly (Fig. 2B). Adding back 300 μ M urate, instead of ascorbate, to gel-filtered plasma did not prevent immediate initiation of lipid peroxidation by AAPH but merely lowered the rate at which lipid peroxidation occurred (data not shown).

The above results demonstrate that ascorbate can completely prevent initiation of detectable peroxidative damage to plasma lipids by aqueous peroxy radicals. We then investigated whether ascorbate also could interrupt ongoing lipid peroxidation in plasma. Plasma containing 81 μ M endogenous ascorbate was incubated with 50 mM AAPH, and after 150 min of incubation—90 min after endogenous ascorbate had been oxidized completely and lipid peroxidation had been initiated—100 μ M ascorbate was added. Addition of ascorbate brought about an immediate and complete cessation of peroxidation of phospholipids (Fig. 3A), cholesterol esters, and triglycerides (Fig. 3B). Once the added ascorbate had been consumed completely, peroxidation of all three lipid classes resumed. Depletion of the antioxidants bilirubin, urate (Fig. 3C), and α -tocopherol (Fig. 3D) was also transiently stopped after addition of ascorbate. In contrast, oxidation of the protein thiols was not affected (data not shown).

The latter finding and the results shown in Fig. 2B raised the interesting question of why ascorbate can prevent detectable lipid peroxidation but cannot effectively prevent oxidation of the protein thiols (which for their part cannot prevent detectable lipid peroxidation). One possible explanation is that the protein thiols are consumed by autoxidation processes that cannot be effectively inhibited by ascorbate. Indeed, evidence for autoxidation of the protein thiols was obtained when we determined the number of peroxy radicals trapped by each protein thiol (their stoichiometric factor or n value). To this end, plasma treated by gel filtration was incubated with 10 mM *N*-ethylmaleimide and then exposed to 50 mM AAPH at 37°C. *N*-Ethylmaleimide treatment blocked the protein thiols to 100% and caused an increase in the initial rate of lipid peroxidation from 2.10 to 2.82 μ M/min. Thus,

the protein thiols scavenged peroxy radicals at a rate of $2.82 - 2.10 \mu\text{M}/\text{min} = 0.72 \mu\text{M}/\text{min}$. The oxidation of the protein thiols occurred at an initial rate of 3.04 $\mu\text{M}/\text{min}$. The n value of the protein thiols, therefore, was calculated to be $0.72/3.04 = 0.24 \mu\text{M}/\text{min}$. Another two identical experiments yielded n values of 0.29 and 0.26, resulting in a mean n value of 0.26 ± 0.03 ($n = 3$). This n value of 0.26 implies that under our experimental conditions only $\approx 1/4$ of the plasma proteins' thiols scavenge peroxy radicals, while $\approx 3/4$ are "wasted," most probably by autoxidation. This process does not involve superoxide radicals since superoxide dismutase (10,000 units/ml) did not reduce the rate of AAPH-induced protein thiol oxidation (data not shown).

DISCUSSION

The results presented in this paper demonstrate that in human blood plasma ascorbate is the only endogenous antioxidant that can completely protect the lipids from detectable peroxidative damage induced by aqueous peroxy radicals. Under this type of oxidant stress, ascorbate is a much more effective antioxidant than the protein thiols, α -tocopherol, bilirubin, or urate. Ascorbate appears to trap virtually all peroxy radicals in the aqueous phase before they can diffuse into the plasma lipids. Once ascorbate has been consumed completely, the remaining water-soluble antioxidants, urate, bilirubin, and the protein thiols, can trap only part of the aqueous peroxy radicals. The peroxy radicals that escape these remaining antioxidants in the aqueous phase diffuse into the plasma lipids, where they initiate lipid peroxidation. After initiation, propagation of peroxidation in the lipids appears to be strongly inhibited, most probably by lipid-soluble α -tocopherol. This strong inhibition of propagation is indicated by the observation that lipid peroxidation comes to a complete standstill when ascorbate is added to plasma after lipid peroxidation has already been initiated (see Fig. 3A and B). It is important to note, however, that α -tocopherol, protein thiols, and urate can merely lower the rate at which lipid peroxidation occurs; they cannot prevent initiation of detectable lipid peroxidation by aqueous peroxy radicals. Only ascorbate can do so.

It is known that ascorbate can switch from anti- to prooxidant activity, depending on its concentration and the presence of free transition metal ions (6, 9–11). In plasma, however, as shown in this study, ascorbate preserves its antioxidant activity, even at very high concentrations. No

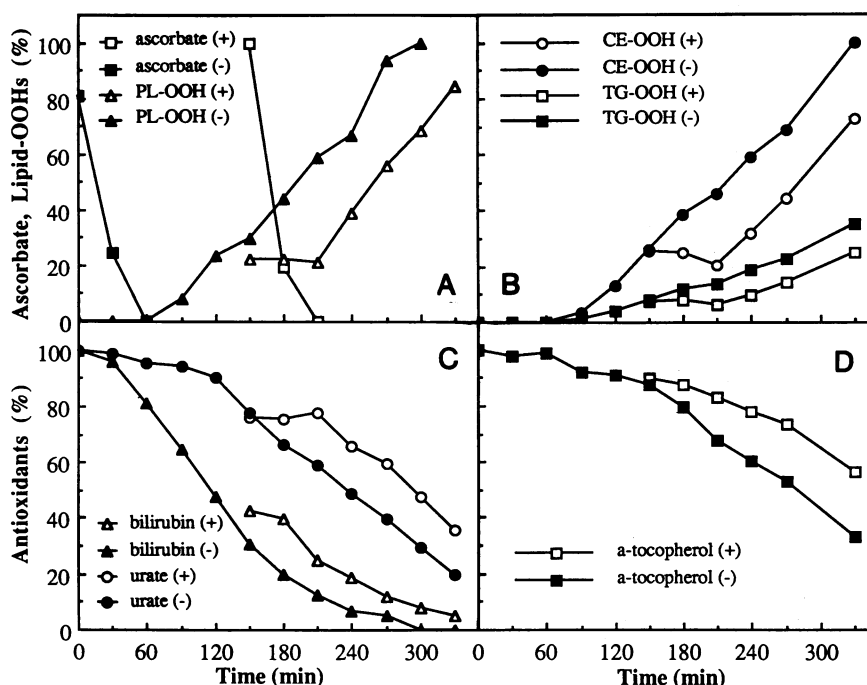


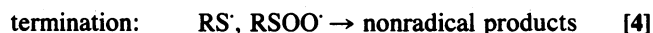
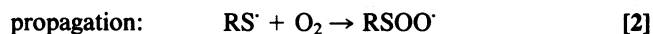
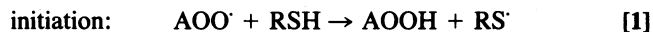
FIG. 3. Interruption by ascorbate of ongoing lipid peroxidation (A and B) and antioxidant depletion (C and D) in plasma exposed to aqueous peroxy radicals. Plasma containing $81 \mu\text{M}$ endogenous ascorbate was incubated with 50 mM AAPH, and after 150 min of incubation $100 \mu\text{M}$ ascorbate was added (+, open symbols). No ascorbate was added to the control incubation (-, solid symbols). Lipid-OOHs, lipid hydroperoxides; PL-OOH, phospholipid hydroperoxides; CE-OOH, cholesterol ester hydroperoxides; TG-OOH, triglyceride hydroperoxides. $100\% = 100 \mu\text{M}$ ascorbate, $75 \mu\text{M}$ phospholipid hydroperoxides, $146 \mu\text{M}$ cholesterol ester hydroperoxides or triglyceride hydroperoxides, $9.9 \mu\text{M}$ bilirubin, $334 \mu\text{M}$ urate, and $25.1 \mu\text{M}$ α -tocopherol.

prooxidant effect of ascorbate was observed up to a concentration of 5 mM (see Fig. 1). This confirms that in plasma transition metal ions are bound tightly and are not available for free radical reactions (2, 12, 13). The findings also imply that the higher the ascorbate concentration, the better, or longer lasting, the protection against aqueous oxidants (provided no free metal catalysts are present). Compared to average concentrations of ascorbate in human blood plasma ($27\text{--}51 \mu\text{M}$) (23), ascorbate levels in human tissues are generally far higher (24). They are particularly high in cornea, lens, and aqueous humor of the eye (up to 1.5 mM) and in adrenal and pituitary glands (up to 2.5 mM) (24, 25). Brain, heart, liver, spleen, kidneys, and pancreas also contain high concentrations of ascorbate (up to 0.8 mM) (24). These tissues, besides requiring ascorbate for enzymatic reactions, might need particularly good antioxidant protection because of relatively high fluxes of oxidants due to high metabolic rate coupled to high oxygen consumption (or, in the case of the eye, due to direct exposure to light). The brain, for example, makes up only $\approx 2\%$ of the total body weight, but consumes $\approx 18\%$ of the body's total oxygen consumption (26).

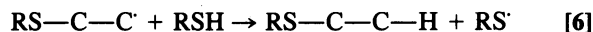
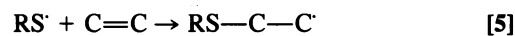
As evident from Fig. 1, the antioxidant protection by ascorbate does not correlate linearly with ascorbate concentration. With increasing concentrations of ascorbate its efficiency in scavenging free radicals decreases. This indicates that the number of peroxy radicals trapped by each molecule of ascorbate is concentration dependent. Indeed, it has been shown that the n value of ascorbate decreases from ≈ 1.7 at $1 \mu\text{M}$ to ≈ 0.5 at $50 \mu\text{M}$ (6, 22). This decrease in the n value most probably is due to "loss of ascorbate by peroxy-catalyzed autoxidation which is of increasing importance at higher initial concentrations of ascorbate" (6).

The plasma proteins' thiols, too, appear to undergo autoxidation under our experimental conditions. Their n value of 0.26 determined by us, which is in good agreement with the n value of 0.33 reported by Wayner *et al.* (27), indicates that only $\approx 1/4$ of the protein thiols engage in radical trapping,

while $3/4$ are "wasted" by autoxidation. As shown in this study, the autoxidation reactions are not inhibited by ascorbate and are superoxide independent. In analogy to a mechanism proposed by Ross *et al.* (28) for the consumption of glutathione by autoxidation, it is conceivable that in plasma aqueous peroxy radicals initiate self-perpetuating autoxidation of the protein thiols according to the following reaction sequence:



AOO $^{\cdot}$, AAPH-derived peroxy radical; RSH, protein thiol; RS $^{\cdot}$, protein thiol radical; RSOO $^{\cdot}$, protein thiol peroxy radical; RSOOH, protein thiol hydroperoxide. An alternative, or additional, possible mechanism for the peroxy radical-induced autoxidative consumption of the protein thiols is addition of RS $^{\cdot}$ to an olefinic double bond and formation of a carbon-centered radical, which subsequently abstracts hydrogen from another protein thiol:



Autoxidation reactions 2 and 3, and 5 and 6 are self-perpetuating radical chain reactions, since they are started by RS $^{\cdot}$ and produce RS $^{\cdot}$, which, therefore, plays a catalytic role.

In the presence of ascorbate, the peroxy radicals produced by AAPH are very effectively trapped by ascorbate (as indicated by oxidation of ascorbate in the absence of detect-

able lipid peroxidation). Therefore, in the presence of ascorbate, initiation reaction 1 can be expected to occur much less frequently. Yet, the rate of oxidation of the protein thiols is only slightly decreased by ascorbate. These observations may suggest that some of the dehydroascorbate molecules formed by oxidation of ascorbate with AOO[•] subsequently abstract hydrogen from RSH, thereby replacing AOO[•] in reaction 1. Thus, it is conceivable that ascorbate is regenerated from dehydroascorbate by protein thiols, analogous to the nonenzymatic reduction of dehydroascorbate by glutathione (29).

The question of whether oxidation of protein thiols should be considered antioxidant protection or oxidative damage is not settled and may depend on the protein affected. For example, it has been suggested that albumin's concentration and turnover rate in plasma ($t_{1/2}$, 20 days) are high enough to render oxidative damage to it biologically insignificant and that albumin, therefore, should be considered a sacrificial antioxidant (30). On the other hand, oxidation of thiols of plasma proteins that are not present at high concentrations and/or that are not readily replaceable might critically affect the biological activity of these proteins and then must be considered oxidative damage. As shown in this study, such possible oxidative damage to proteins, in contrast to peroxidative damage to lipids, is not effectively prevented by any of the plasma antioxidants.

In summary, our results demonstrate that in plasma, protein thiols, α -tocopherol, or urate cannot prevent initiation of detectable lipid peroxidation and formation of micromolar concentrations of lipid hydroperoxides induced by aqueous peroxy radicals, but can merely lower the rate at which lipid peroxidation occurs. In contrast, under this type of oxidant stress ascorbate is an outstandingly effective antioxidant: it not only completely protects the lipids from detectable peroxidative damage, but also spares α -tocopherol, urate, or bilirubin. We have shown previously that plasma ascorbate also appears to be outstandingly effective against the oxidants released from activated polymorphonuclear leukocytes (17) and belongs to the first line of antioxidant defense against lipid-soluble peroxy radicals (31). These findings imply that ascorbate should prove at least as helpful as α -tocopherol in the prevention and treatment of diseases and degenerative processes caused by oxidant stress. Our data suggest that an increase of the RDA for ascorbate from the current 60 mg to \approx 150 mg to maximize ascorbate's total body pool (32) would be beneficial to human health.

We thank R. Moos-Holling for drawing blood, and A. N. Glazer, K. U. Ingold, M. Profet, R. Stocker, and A. L. Tappel for critically reviewing the manuscript. This work was supported by National Cancer Institute Outstanding Investigator Grant CA39910 to B.N.A. and by National Institute of Environmental Health Sciences Center Grant ES01896.

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