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ASCORBATE FUNCTION AND METABOLISM IN THE HUMAN ERYTHROCYTE

James M. May

Departments of Medicine and Molecular Physiology and Biophysics, 736 Medical Research Building II, Vanderbilt University School of Medicine, Nashville, TN 37232-6303 USA

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1. ABSTRACT

Ascorbic acid, or vitamin C, is an important antioxidant in plasma, where it consumes oxygen free radicals and helps to preserve alpha-tocopherol (vitamin E) in lipoproteins. Erythrocytes, as the most plentiful cell in blood, help to preserve ascorbate in the blood plasma. In contrast to nucleated cells, which avidly concentrate ascorbate, the erythrocyte ascorbate concentration is the same as that in plasma. Erythrocytes nonetheless have a high capacity to regenerate the vitamin from its two electron-oxidized form, dehydroascorbic acid (DHA). DHA is rapidly taken up by these cells on the abundant glucose transport protein, GLUT1. Intracellular DHA is rapidly reduced to ascorbate by GSH in a direct chemical reaction, although enzyme-dependent mechanisms involving both glutaredoxin and thioredoxin reductase have also been demonstrated. Ascorbate, which carries a negative charge at physiologic pH, enters and leaves the cells slowly. Nonetheless, this slow release of ascorbate from erythrocytes can preserve both the plasma concentration of the vitamin, and prevent oxidation of alpha-tocopherol in low-density lipoprotein. In addition, intracellular ascorbate can spare and possibly recycle alpha-tocopherol in the erythrocyte membrane. In turn, alpha-tocopherol protects the cell membrane from lipid peroxidation. The ability of erythrocytes to recycle ascorbate, coupled with the ability of ascorbate to protect alpha-tocopherol in the cell membrane and in lipoproteins, provides a potentially important mechanism for preventing lipid peroxidative damage in areas of inflammation in the vascular bed, such as those involved with atherosclerosis.

2. INTRODUCTION

Vitamin C, or L-ascorbic acid, is synthesized in the liver of most mammals from glucose (1). However, humans, higher primates, and guinea pigs lack the ability to synthesize the vitamin, and so must rely on efficient mechanisms for its absorption, transport, and maintenance within tissues (2). Newly absorbed ascorbate is distributed to tissues in the blood plasma, in which it is one of the most important antioxidants. Not only does ascorbate directly consume oxidant free radicals in plasma, but it also protects and recycles vitamin E or alpha-tocopherol in lipoproteins and probably in erythrocytes. The two-electron oxidized form of ascorbate, dehydroascorbic acid (DHA) is in turn rapidly taken up by erythrocytes and recycled to ascorbate. The uptake, recycling, and function of ascorbate in the erythrocyte is the subject of this review.

3. REDOX CHEMISTRY OF ASCORBIC ACID

Ascorbic acid, or ascorbate, is an antioxidant because of the high reducing potential of its carbon-carbon double bond (figure 1), which readily donates one or two hydrogens and electrons to a variety of oxidants, including oxygen free radicals, peroxides, and superoxide (3). Each step of ascorbate oxidation is reversible, and this permits recycling back to ascorbate. The partially oxidized form of ascorbate, termed the (mono)ascorbyl free radical or AFR (figure 1), may serve as an electron acceptor or donor. The AFR is surprisingly stable, and can easily be detected by EPR at concentrations as low as 10 nM in biological fluids (4-6). Loss of the second electron results in dehydroascorbic acid, which is not an acid. This

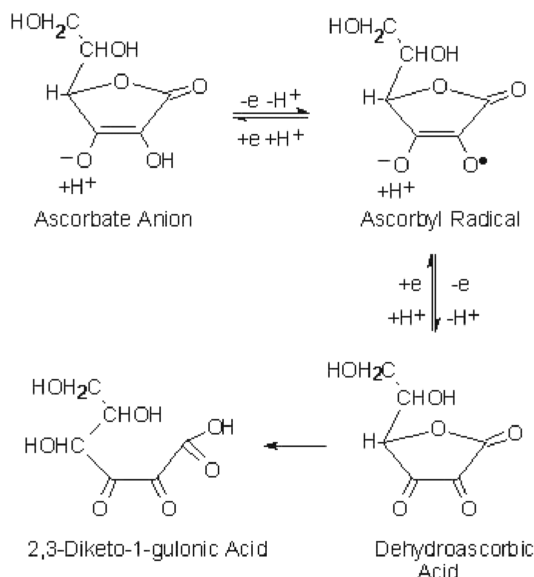

Figure 1. Oxidative metabolism of ascorbic acid.

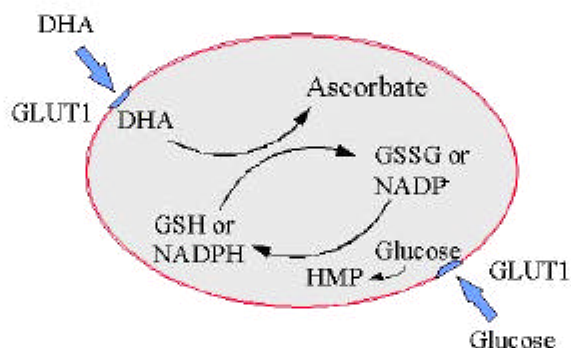

Figure 2. Erythrocyte recycling of DHA to ascorbate. HMP = hexose monophosphate pathway.

contrasts with ascorbate, which carries a negative charge at physiologic pH (the pK_a of the carbon-3 hydroxyl is 4.2). DHA is quite unstable at physiologic pH and temperature, with a half-life of about 6 min (7, 8). With hydrolysis of the lactone ring, DHA is converted to 2,3-diketo-1-gulonic acid (figure 1) (1, 9). This last step is probably irreversible in cells, although it can be reversed by mercaptoethanol *in vitro* (10). Loss of ascorbate through decomposition of DHA is obviously wasteful, and cells such as the erythrocyte have redundant mechanisms to recycle DHA back to ascorbate.

4. ERYTHROCYTE UPTAKE OF ASCORBATE AND DHA

Uptake of ascorbic acid by erythrocytes is very slow, with a half-time of hours, and occurs by simple diffusion (11-14). In contrast, most nucleated cells possess a sodium- and energy-dependent transporter with a high affinity for ascorbate that maintains low millimolar intracellular concentrations of the vitamin (2). Since erythrocytes lack an active transporter for ascorbate, they have intracellular concentrations of the vitamin that are similar to those in plasma, ranging from 20-60 micromolar in humans not taking ascorbate supplements (15, 16).

DHA is taken up by erythrocytes and other cells by facilitated diffusion on the glucose transporter. This was originally shown in human erythrocytes (2, 17), and has been elegantly demonstrated in *Xenopus laevis* oocytes. *Xenopus* oocytes naturally lack appreciable transport of glucose, ascorbate, and DHA. By expressing glucose transporters in oocytes, Vera, *et al*. (18) showed that DHA, but not ascorbate, enters cells via facilitated diffusion on the "GLUT" transporter type. Once it has entered erythrocytes or other cells, DHA is rapidly converted to ascorbate and trapped within the cells (figure 2) (13, 14, 17, 19). Uptake by facilitated diffusion of DHA, followed by reduction to ascorbate does generate at least a transient ascorbate gradient across the erythrocyte cell membrane (20), and probably accounts for plasma DHA concentrations of < 2 micromolar (15, 21, 22).

5. ASCORBATE RECYCLING IN ERYTHROCYTES: MECHANISMS AND CAPACITY

5.1 Mechanisms of erythrocyte ascorbate recycling

The mechanism by which erythrocytes reduce intracellular DHA to ascorbate was initially considered to be NADH-dependent (23). However, more recent studies have suggested that such recycling is primarily GSH- and NADPH-dependent (figure 2) (24). Excess GSH can chemically reduce DHA to ascorbate (7, 25). This reaction has been documented in dialyzed erythrocyte hemolysates following addition of GSH, and with a GSH-regenerating system (26). We found additional evidence for direct GSH-dependent recycling of DHA to ascorbate in resealed erythrocyte ghosts, in which the cytoplasm is diluted 40-fold. In ghosts that had been resealed to contain 4 mM GSH, recycling of DHA to ascorbate was several-fold more efficient than was recycling in ghosts resealed without additives, or than was recycling in ghosts resealed in the presence of 400 micromolar NADH (20).

Erythrocytes also have enzymes that can facilitate GSH-dependent reduction of DHA to ascorbate. The thioltransferase glutaredoxin has been shown to reduce DHA using GSH as a cofactor (27), and an enzyme with similar properties has been purified from human erythrocytes (28). The most likely mechanism for either direct (29) or enzyme-mediated (27) GSH-dependent DHA reduction involves nucleophilic addition of the thiyl anion of GSH to carbon-3 of DHA, followed by reduction by another molecule of GSH to form the ascorbate double bond and GSSG (figure 3). It was first shown in lens epithelial cells that added DHA depletes GSH and causes a rise in GSSG and in the activity of the hexose monophosphate shunt (29, 30). In erythrocytes incubated without glucose, we also found that addition of DHA in low millimolar concentrations decreases GSH and increases GSSG proportionately (20). In the presence of glucose, DHA did not affect the erythrocyte content of GSH. This

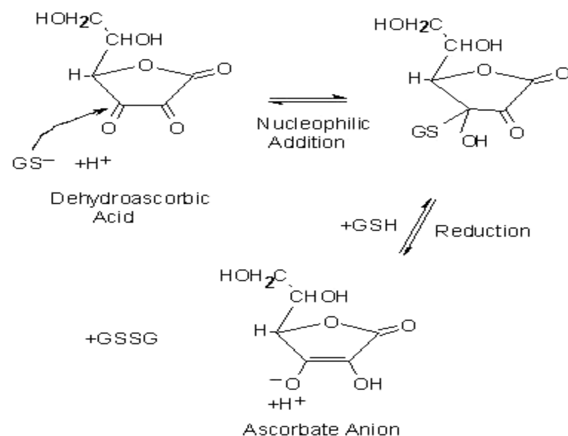

Figure 3. Proposed mechanism for direct reduction of DHA by GSH.

failure of GSH to fall in the presence of glucose probably reflects recycling of GSSG to GSH by glutathione reductase when adequate NADPH is available from the hexose monophosphate shunt (26). Two additional results also support a GSH-dependent mechanism of erythrocyte DHA reduction. First, depletion of cellular GSH by 50-70% with diamide decreased the ability of glucose-depleted cells to recycle DHA to ascorbate (20). Diamide selectively oxidizes GSH to GSSG (31), although the agent can react with pyridine nucleotides (32). Second, in electron paramagnetic resonance studies, we found no increase in the AFR signal during the first few minutes of uptake and reduction of 1 mM DHA by intact erythrocytes (20). If the AFR is an intermediate in the recycling of DHA to ascorbate, it should have been apparent in this experiment.

DHA can also be reduced to ascorbate by the NADPH-dependent enzyme thioredoxin reductase (33). This reaction is facilitated by the presence of thioredoxin. Thioredoxin reductase has been shown to be present in erythrocytes by immunologic methods (34), and has been purified from this cell type (35). Additional support for enzyme-dependent DHA reduction, whether mediated by GSH or NADPH, is our observation that DHA loading of erythrocytes is rapid and saturable, with an apparent K_m of 200 micromolar (16). We have also found that erythrocyte hemolysates support NADPH-dependent reduction of DHA to ascorbate, which is enhanced by 5 micromolar thioredoxin from *E. coli*, and inhibited by 10 micromolar aurothioglucose (Mendiratta, S. and May, J.M., unpublished observations). At a concentration of 10 micromolar, aurothioglucose is selective for thioredoxin reductase (36). Whereas thioredoxin can recycle DHA to ascorbate in the erythrocyte, GSH-dependent mechanisms are likely to predominate. This may not be the case for other cell types, since HL-60 cells show no decrease in their ability to recycle DHA to ascorbate when their GSH is severely depleted (37).

5.2 Capacity of erythrocytes for ascorbate recycling

The ability of erythrocytes to recycle DHA to ascorbate is substantial. We have used DHA-stimulated reduction of ferricyanide by erythrocytes to estimate this capacity. Ferricyanide is a mild oxidant that does not penetrate the erythrocyte cell membrane (38, 39). Its extracellular reduction to ferrocyanide can be easily measured in a sensitive spectrophotometric assay (40). Assay of ferricyanide reduction is non-destructive to the cells, and DHA-induced increases in ferricyanide reduction provide an integrated measure of ascorbate recycling. Orringer and Roer (23) proposed that DHA-induced ferricyanide reduction by erythrocytes involves uptake of DHA by the cells, intracellular reduction of DHA to ascorbate by an NADH-dependent process, exit of ascorbate from the cells, and direct reaction with ferricyanide. Subsequent studies (41, 42) have shown that extracellular ferricyanide reduces intracellular, rather than extracellular ascorbate. The mechanism of this trans-membrane electron transfer from ascorbate to ferricyanide has not been established. It may be mediated by a trans-plasma membrane oxidoreductase, which has long been known to use NADH as an electron donor (43, 44). We found that ascorbate can also serve as an electron donor to this process. In ascorbate-loaded cells, the vitamin may be the major electron donor (45). Basal rates of ferricyanide reduction by erythrocytes are increased many-fold by providing the cells with DHA for conversion to ascorbate. Further, DHA-induced ferricyanide reduction is saturable with regard to the extracellular DHA concentration, and parallels the intracellular ascorbate content of the cells (42, 45). Using the initial rate of ferricyanide reduction at non-saturating DHA concentrations, which is about 40 nmol (ml erythrocytes)$^{-1}$ min^{-1}, we have calculated that erythrocytes in a milliliter of blood can regenerate a 40 micromolar concentration of ascorbate in this blood about once every three minutes (42). Similar estimates of ascorbate recycling capacity were determined from more recent studies in which initial rates of DHA reduction to ascorbate were measured directly in erythrocytes (16).

The ascorbate recycling capacity of erythrocytes provides ascorbate to supplement the already substantial antioxidant defenses of the cells (42), and to serve as a source of ascorbate for export into plasma, as discussed in the next section.

6. ANTIOXIDANT FUNCTION OF ASCORBATE IN PLASMA AND IN ERYTHROCYTES

6.1 Ascorbate as an antioxidant in plasma

Ascorbate has been shown to be the primary antioxidant in plasma, since its presence is required to prevent lipid hydroperoxide formation in plasma lipoproteins (46-48). More specifically, ascorbate directly protects against peroxide-mediated oxidation of plasma low- density lipoprotein (LDL) (49, 50). LDL is the primary atherogenic lipoprotein in human plasma, and is cleared by LDL receptors and by a scavenger pathway (50). If the surface lipids and protein sulfhydryls of LDL become oxidized, its receptor-mediated clearance is markedly impaired, and its uptake occurs largely by the scavenger pathway in monocyte-derived macrophages (50, 51). The latter in turn become the lipid-laden foam cells found in atherosclerotic plaques (52). Ascorbate probably

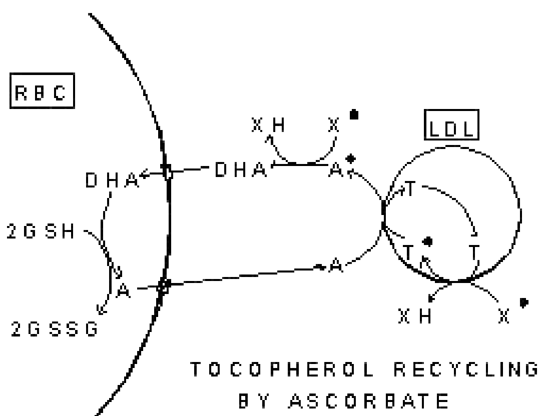

Figure 4. Tocopherol recycling by ascorbate. Abbreviations: RBC, human erythrocyte; ASC, ascorbate; T, alpha-tocopherol; X, free radical species.

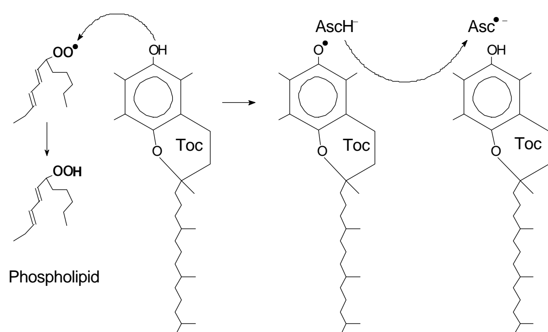

Figure 5. Recycling of lipid peroxyl radicals by alpha-tocopherol (Toc), and of alpha-tocopherol by ascorbate (Asc).

acts synergistically with the tocopherols in protecting against LDL oxidation (49, 53). Ascorbate can both consume oxygen free radicals before they can oxidize alpha-tocopherol, and can reduce alpha-tocopherol in LDL in the face of an oxidant stress (49, 54). These antioxidant effects of ascorbate in plasma may explain the early observations of Willis that acute and chronic scurvy in guinea pigs produced lesions in the arterial intima that are indistinguishable from those of human atherosclerosis (55).

Erythrocytes are likely to be an important source of ascorbate in plasma, if nothing else because of their abundance. As noted earlier, erythrocytes take up ascorbate (as opposed to DHA) very slowly, and ascorbate efflux is also on the order of hours (16). Nonetheless, it appears that ascorbate efflux from erythrocytes can maintain the steady-state plasma ascorbate concentration during prolonged incubations in oxygenated serum (16, 19, 56), and even in the presence of an oxidant stress generated by AAPH (42). The latter is a free radical initiator that releases water-soluble radicals at a constant rate as a function of the temperature. These radicals are efficiently scavenged by ascorbate in plasma (47).

We also found that erythrocytes and ascorbate generated by the cells protect alpha-tocopherol in LDL against oxidation by AAPH, as diagramed in figure 4 (16). This protection was due in part to consumption by erythrocytes of oxygen free radical species generated from AAPH. Nonetheless, loading erythrocytes with ascorbate by incubating them with DHA provided even greater protection against loss of alpha-tocopherol in LDL than did erythrocytes alone. The protective effect of DHA-loading on LDL alpha-tocopherol was reversed when ascorbate oxidase was present outside the cells, which suggests that it was due to ascorbate that had left the cells.

In addition to release of ascorbate from erythrocytes, the presence of an AFR reductase on the outer surface of the cells could also contribute to ascorbate recycling. One-electron oxidation of ascorbate outside erythrocytes would provide AFR to this enzyme for recycling back to ascorbate using intracellular reducing equivalents. Evidence for this activity has been presented for leukemic cells in culture (57, 58) and AFR reductase activity has been demonstrated in isolated erythrocyte membranes (59). The source of reducing equivalents for this process is presumably intracellular, but has not been identified.

By helping to maintain plasma ascorbate concentrations, erythrocyte ascorbate recycling may preserve alpha-tocopherol in LDL, prevent LDL oxidation, and scavenge oxidants released by monocytes and leukocytes. The latter effect will in turn prevent damage to the vascular endothelium.

6.2 Role of ascorbate in protecting erythrocytes from oxidative stress

A question related to whether ascorbate recycled by erythrocytes protects plasma lipoproteins from oxidative damage is whether ascorbate recycling protects the erythrocyte itself from oxidant stress, especially since this cell is equipped with so many different types of defenses against oxygen free radicals (60). In the cytoplasm, enzymatic defenses against both endogenous and exogenous oxidants include catalase (61), glutathione peroxidase/reductase, and superoxide dismutase (62). The primary non-enzymatic antioxidant defenses in the erythrocyte cytoplasm are GSH and ascorbate (63), although less well studied thiols such as ergothioneine (64, 65) may also contribute. In unpublished observations, we have found that ascorbate is more sensitive to oxidation by exogenous H_2O_2 generated by the glucose oxidase system than are GSH and alpha-tocopherol, but that it contributes little to defense of the cells against overwhelming oxidant stress.

Defenses against oxidant damage in the erythrocyte plasma membrane involve prevention and reversal of peroxidation of unsaturated fatty acids in the lipid bilayer (66). In the face of a profound external oxidant stress to the erythrocyte, the plasma membrane is

often the initial site of damage; the resulting peroxidation of membrane lipids then causes hemolysis (67). Quantitatively, alpha-tocopherol is the most important antioxidant in the cell membrane (66). This vitamin scavenges peroxide free radicals and converts them to less toxic lipid hydroperoxides (figure 5) (3). In so doing, it protects the cell membrane and decreases hemolysis (66, 68). Several studies have shown that the erythrocyte content of alpha-tocopherol correlates directly with the resistance of the cell to oxidant-induced hemolysis (69-71). Indeed, prior to the availability of more specific assays, H_2O_2-induced hemolysis was used as an indirect measure of the membrane content of this vitamin (72, 73). Further, addition of alpha-tocopherol to erythrocytes either *in vivo* or *in vitro* protects the cells against hemolysis induced by gamma irradiation (70) and by aqueous free radicals (74, 75). To a lesser extent, ubiquinol-10 (76, 77) and membrane protein sulfhydryls (78) may also contribute to protection of the membrane. The GSH-dependent phospholipid hydroperoxidase can reduce membrane lipid hydroperoxides (79, 80), and can also spare alpha-tocopherol in cellular membranes (81). This enzyme has not been shown to directly reduce alpha-tocopherol, however.

Given the primacy of alpha-tocopherol in protecting the erythrocyte membrane, it is crucial that it either be replaced or recycled when oxidized. Transfer of the vitamin from plasma lipoproteins likely provides the major source of new alpha-tocopherol to the cells, but such transfer is relatively slow, with a half-time of several hours (82, 83). Thus it is likely that alpha-tocopherol is recycled in the cell membrane from the alpha-tocopheroxyl free radical (figure 5). This process appears to be efficient, given the observation that erythrocyte membranes contain low concentrations of alpha-tocopherolquinone, the major two-electron oxidation product of the vitamin (69). The most plausible mechanism for alpha-tocopherol recycling involves ascorbate.

Ascorbate does not directly affect membrane lipid peroxidation (84), but it may perform this function indirectly by reducing the tocopheroxyl free radical in the lipid bilayer (85, 86). The mechanism of such recycling presumably involves reduction of the alpha-tocopheroxyl free radical at the aqueous-lipid interface of the membrane bilayer (figure 5) (3). Ascorbate has been shown to recycle the alpha-tocopheroxyl radical in variety of *in vitro* systems: in solution (85, 87), in lipid or detergent micelles (85, 87-89), in liposomes (84, 90, 91), in microsomes and mitochondria (92, 93), and in isolated erythrocyte membranes (94). However, there is controversy regarding whether ascorbate can recycle alpha-tocopherol in cells (95, 96).

If ascorbate recycles alpha-tocopherol in intact cells, the resistance to oxidation of alpha-tocopherol in the cell membrane should parallel changes in the intracellular ascorbate content. This was first demonstrated by Stocker, *et al*. (97), who reported that increased intracellular ascorbate protects membrane alpha-tocopherol in *Plasmodium vinckei*-infected erythrocytes. We recently found that increasing the erythrocyte ascorbate content by loading with DHA protects against ferricyanide- and AAPH-induced loss of erythrocyte alpha-tocopherol (98). Conversely, selective ascorbate depletion with the nitroxide Tempol (99) increases loss of alpha-tocopherol in response to both oxidants. The rationale for these experiments is that both oxidants are restricted to the extracellular space because of their size and charge, and thus should attack alpha-tocopherol in the membrane before interacting with intracellular ascorbate. The observed preservation of alpha-tocopherol in proportion to the cellular ascorbate content is in line with a recycling mechanism. A role for ascorbate recycling of alpha-tocopherol would also be supported by demonstrating that the vitamin is depleted with or before alpha-tocopherol and GSH in the face of an oxidant stress (100). This is complicated in intact cells by the recycling of DHA to ascorbate, since such recycling will tend to maintain the ascorbate concentration. In intact erythrocytes, we were able to show that loss of alpha-tocopherol lags behind that of ascorbate (100). On the other hand, Glascott, *et al*. (95, 96) found in cultured rat liver cells that ascorbate and alpha-tocopherol behaved independently during an exogenous oxidant stress. However, both erythrocytes and hepatocytes can recycle ascorbate, and rat hepatocytes can synthesize it. These capacities are likely to confound detection of any relationship between ascorbate and alpha-tocopherol. To overcome this problem, we used resealed erythrocyte ghosts, in which the capacity to recycle ascorbate is greatly diminished by a 40-fold dilution of the cytoplasmic contents. These ghosts can be tightly sealed in the presence of ascorbate, and followed for their ability to preserve alpha-tocopherol in the membrane in response to an oxidant stress. Intravesicular ascorbate protected against loss of alpha-tocopherol in the ghost membranes, whether induced by ferricyanide (101) or by AAPH (98). In the latter studies, alpha-tocopherol began to decrease only after intravesicular ascorbate was almost depleted. These results in both cells and ghosts show that ascorbate can spare, and perhaps recycle alpha-tocopherol in the cell membrane.

Despite the above *in vitro* evidence in support of a role for ascorbate in recycling alpha-tocopherol, there remain caveats in the reported *in vivo* experiments in the guinea pig model. Guinea pigs, like humans, cannot synthesize ascorbate, and so can be used to obtain different stages of ascorbate deficiency. Burton, *et al*. (102) studied the kinetics of alpha-tocopherol turnover at various levels of ascorbate depletion or repletion in these animals, and found that the turnover kinetics were not affected by the ascorbate status of the animal. On the other hand, Bendich *et al*. (103) found that ascorbate supplementation in ascorbate-deficient guinea pigs increased the alpha-tocopherol content in plasma at one level of alpha-tocopherol intake, and increased the alpha-tocopherol content in lung at all levels of alpha-tocopherol intake. As noted by Liebler (100), use of oxidatively stressed animals in such studies might provide a better test of whether ascorbate can "spare" alpha-tocopherol *in vivo*. Despite the conflicting *in vivo* results, the findings outlined above at the cell level do favor a sparing or recycling role for ascorbate in the preservation of alpha-tocopherol, both in plasma lipoproteins, and in the cell membrane.

7. PERSPECTIVE

Ascorbate in the blood plasma provides a crucial link between ascorbate uptake by the intestine and its delivery to the tissues. The vitamin also contributes to the antioxidant reserve of blood, especially in areas of oxidant stress in the vascular bed, such as those involved with inflammation or atherosclerosis. The ability of the erythrocyte to recycle ascorbate may be very important to the maintenance of this antioxidant reserve. However, ascorbate can be a two-edged sword, and function as a powerful prooxidant in the presence of transition metals such as the ferric ion (24). Given that the erythrocyte contains the largest store of iron in any cell, avoidance of hemolysis and release and degradation of hemoglobin is crucial. Sparing or recycling of alpha-tocopherol by ascorbate in the erythrocyte will tend to stabilize the erythrocyte membrane and thus prevent hemolysis. Thus the net effect is an antioxidant rather than prooxidant effect of the vitamin.

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Send correspondence to: Dr. James May , 736 Medical Research Building II, Vanderbilt University School of Medicine, Nashville, TN 37232-6303 USA. Tel: (615)-936-1653, Fax:(615)-936-1667, E-mail:james.may@ mcmail.vanderbilt.edu