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COMMENTARY

ON THE ANTIOXIDANT EFFECTS OF ASCORBIC ACID AND GLUTATHIONE

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Ascorbic acid (vitamin C) is required in the diets of humans, certain other primates, guinea pigs and several other species [1–5]. These animals, in contrast to most others, do not have the ability to synthesize ascorbic acid from glucose because they lack the enzyme L-gulonolactone oxidase, which is required for the formation of 2-keto-L-gulonolactone; this compound is spontaneously converted to ascorbic acid. Ascorbic acid deficiency leads to scurvy, which can be prevented in humans by administration of as little as 10 mg of ascorbic acid per day [6]. Many have thought that larger doses of ascorbic acid might be beneficial for health [3, 7]. Szent-Györgyi, soon after his pioneering research on ascorbic acid, considered the idea that it might have a useful role in the therapy of infections. Ascorbic acid has been claimed to be effective in the prevention and treatment of cancer [7–10], the common cold [11, 12], atherosclerosis [3, 13, 14], cataracts [15] and AIDS [16]. It has been claimed that increased intake of ascorbic acid has favorable effects on the immune system, healing of wounds, various types of stress including those due to physical exertion, cigarette smoking, and extremes of temperature, allergic responses, and mental health problems. These subjects have been reviewed [3, 7, 15, 17, 18]. In general, each of these claims has been contradicted, at least to some degree, by subsequent studies and the literature continues to reflect controversy apparently because it has not yet been possible to obtain reproducible objective evidence. A claim that a particular drug or treatment can cure a wide variety of conditions, perhaps everything from housemaid's knee to tuberculosis, is typically viewed with skepticism. So too might one be justifiably dubious about a theory according to which a wide variety of diseases are produced by oxidative phenomena and free radicals. Nevertheless, if such a theory is even partially valid, beneficial effects might be expected after administration of compounds such as ascorbic acid which has potent reducing properties and which is highly reactive.

In contrast to the claims made for large doses of ascorbic acid, there can be no doubt but that very small doses of ascorbic acid are sufficient to prevent scurvy; this has been observed frequently. Perhaps ascorbic acid performs a specific function that prevents scurvy, but this has not yet been discovered. It is more likely that ascorbic acid prevents scurvy through multiple effects, and indeed there is good evidence that ascorbic acid may affect the activity of a number of enzyme-catalyzed hydroxylation reactions.

It was suspected many years ago that there is a connection between ascorbic acid and glutathione. In 1928, Szent-Györgyi [1] reported evidence for dehydroascorbic acid reductase activity in animal tissues. The reduction of dehydroascorbic acid by animal tissues was also observed by Borsook *et al.* [19] who concluded that glutathione is involved in this reaction. Hopkins and Morgan [20] examined this reaction in plants. The reaction was considered for many years to take place non-enzymatically in animals, but eventually evidence for the participation of thiol transferase activity was obtained, and studies with purified enzyme preparations showed that the thiol transferases glutaredoxin and protein disulfide isomerase exhibit substantial glutathione-dependent dehydroascorbic acid reductase activity [21] [reaction (1)]. Many of the published findings are based on *in vitro* studies. However, convincing *in vivo* evidence linking glutathione to reduction of dehydroascorbic acid has been reported recently in studies on newborn rats and other animals [22–26].

Dehydroascorbic acid

$$+ 2\ \text{GSH} \rightarrow \text{ascorbic acid} + \text{GSSG} \qquad (1)$$

Glutathione, known prior to the discovery of ascorbic acid [27, 28], is widely distributed. This tripeptide (γ-glutamylcysteinylglycine), which functions in metabolism, catalysis, transport, and cellular protection, provides cells with their reducing milieu [29–34]. Glutathione deficiency in newborn rats and in guinea pigs, animals that are unable to synthesize ascorbic acid, is lethal, but death can be prevented by administration of high doses of ascorbic acid. The onset of scurvy in guinea pigs that are fed a diet deficient in ascorbic acid is delayed substantially by the administration of glutathione monoethyl ester, a glutathione delivery agent. These and related studies reviewed here, which have illuminated some of the physiological connections between ascorbic acid and glutathione, indicate that cellular glutathione is essential for the function of ascorbic acid.

Conversion of ascorbic acid to dehydroascorbic acid and its reversal

The functions of ascorbic acid are associated with

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its oxidation to dehydroascorbic acid. This is to be expected if ascorbic acid is required, for example, for reduction of enzyme-bound Fe^{3+}, or for neutralization of a reactive oxygen molecule. The relatively small amounts that are required in the diets of humans and guinea pigs for prevention of scurvy must be recycled. Administration of dehydroascorbic acid can prevent scurvy, clearly indicating that reduction of dehydroascorbic acid to ascorbic acid takes place *in vivo*. This process must be efficient since there is good evidence that dehydroascorbic acid is degraded rapidly and irreversibly [19]. When large amounts of ascorbic acid are administered, such a recovery process might be less important, and the effects observed, if any, could probably be ascribed to the high levels and reducing properties of the administered ascorbic acid.

Mitochondria do not appear to contain thiol transfer activity, and there is evidence that semidehydroascorbate may be reduced by an NADH-dependent system in mitochondria [35–39]. Such a reaction, which would tend to maintain mitochondrial levels of ascorbic acid, would function to conserve ascorbic acid in animals that depend upon its dietary intake. However, the marked decrease of tissue ascorbic acid levels found in glutathione deficiency (see below) indicates that the glutathione-dependent reductase reaction is of major physiological significance.

Effect of ascorbic acid on enzyme-catalyzed reactions

Scurvy is characterized by increased capillary fragility and associated hemorrhage, decreased and abnormal bone growth, and decreased synthesis of collagen. In early studies ascorbic acid-deficient guinea pigs were found to exhibit arterial changes that closely resemble those seen in human atherosclerosis [13]. The signs of scurvy in humans include poor wound healing, weakness, hemorrhagic phenomena, and hyperkeratosis [6]. These effects can be explained in part in terms of decreased collagen and bone synthesis. There is evidence that ascorbic acid influences a number of hydroxylation reactions. The several hydroxylases known to be involved in collagen formation, prolyl 4-hydroxylase, prolyl 3-hydroxylase, and lysyl hydroxylase, require a reducing agent for maximal activity *in vitro* and ascorbic acid appears to be the most effective of those studied [5].

Subcutaneous injection of carrageenan into guinea pigs produces massive collagen-containing granulomas, whose formation significantly increases the nutritional requirement for ascorbic acid; deprivation of dietary ascorbic acid decreases the formation of collagen [40, 41]. When minces of granulomas from scorbutic animals were incubated with labeled proline, there was decreased formation of labeled collagen hydroxyproline [40, 42–45]. Hydroxylation was greatly increased by adding ascorbic acid to the minces, indicating an apparent direct effect on conversion of peptide bound proline to peptide bound hydroxyproline [43, 44]. When hydroxylation was studied with 3,4-ditritiated L-proline, the reaction led to formation of tritiated hydroxyproline and equivalent amounts of tritiated water [44, 45]; this procedure was later used by several investigators. It was deduced that hydroxyproline is formed from peptide-linked proline (as predicted by the early studies of Stetten [46]) with this post-translational step occurring on ribosome-bound tRNA linked peptides having chain lengths in the range of 38–84 amino acid residues [47]. It is now known that peptide-bound hydroxyproline is essential for the stability of the triple helix of collagen, and this may explain some of the difficulties in interpreting certain early findings. Englard and Seifter [5] have written a scholarly review on the functions of ascorbic acid, and have considered the occasionally conflicting literature on the hydroxylation of proline in relation to collagen synthesis. They have also reviewed a number of the enzyme-catalyzed reactions that are affected by ascorbic acid. These include the α-ketoglutarate-dependent dioxygenases, which catalyze reaction (2).

$$\alpha\text{-Ketoglutarate} + O_2 + \text{substrate} \underset{\text{Ascorbate}}{\overset{Fe^{2+}}{\rightleftharpoons}} \text{succinate} + CO_2 + \text{hydroxylated substrate} \quad (2)$$

Dioxygenases that catalyze reactions of this type include prolyl 4-hydroxylase, prolyl 3-hydroxylase, lysyl hydroxylase, 6-*N*-trimethyl-L-lysine hydroxylase, and γ-butyrobetaine hydroxylase. The last two of these are involved in the biosynthesis of carnitine. The activities of these enzymes are decreased in the tissues of ascorbic acid-deficient animals as are those of 4-hydroxyphenylpyruvate hydroxylase, dopamine-β-hydroxylase, and peptidylglycine-α-amidating monooxygenase [5]. However, there appears to be little evidence for direct participation of ascorbic acid in these catalytic reactions. A reductant is required to maintain the activity of the enzymes, for example by reducing Fe^{3+} to Fe^{2+}; ascorbic acid is a highly effective reducing agent that functions to reactivate these enzymes, which tend to undergo oxidative inactivation. Studies on the mechanism of prolyl 4-hydroxylase indicate that the utilization of ascorbic acid is much less than stoichiometric and that the enzyme can go through a number of catalytic cycles in the absence of ascorbic acid before losing activity [48]. Nevertheless, inactivation is apparently a limiting factor *in vivo*. The observation that the β-subunit of prolyl 4-hydroxylase (a tetramer ($\alpha_2\beta_2$) consisting of two types of monomers) has protein disulfide isomerase activity [49], and the demonstration that the latter enzyme catalyzes glutathione-dependent dehydroascorbic acid reduction [reaction (1)] [21] strongly suggests that both glutathione and ascorbic acid contribute importantly to the proline hydroxylase reaction.

Dopamine-β-hydroxylase and peptidylglycine-α-amidase are found in tissues that contain high levels of ascorbic acid which may be required for constant reactivation of these copper-containing catalysts. Recently, evidence has been obtained that ascorbic acid plays a role in vitamin D metabolism in guinea pigs [50]. The findings suggest that still another hydroxylation reaction, one that is involved in the formation of 1,25-dihydroxycholecalciferol, may involve ascorbic acid. Further studies may lead to

biochemical understanding of the bone changes in scurvy.

Glutathione in cellular function

Glutathione provides cells with their reducing environment [31–33]. Reducing power arises *via* reduced pyridine nucleotides through the action of glutathione disulfide reductase, and is distributed to various molecules by the glutathione thiol-transferases. The cellular reducing environment may be augmented by other reactions, for example, by the activity of thioredoxin. Administration of large amounts of ascorbic acid may also contribute to the reducing properties of cells. The functions of glutathione include maintenance of the thiols of proteins and the reduced forms of other compounds such as ascorbic acid and α-tocopherol, reduction of ribonucleotides to the deoxyribonucleotide precursors of DNA, and protection of cells against oxidative damage, free radical damage, and other types of toxicity. Glutathione is synthesized in many types of cells from glutamate, cysteine, and glycine. These amino acids may be formed in cellular metabolism and may also be obtained from the diet. Glutathione is not required in the diets of animals nor is it taken up intact to a significant extent into portal blood from the gastrointestinal tract [34, 51]. Virtually all cells that contain glutathione are able to synthesize it by a two-step pathway catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase [reactions (3) and (4)].

L-Glutamate + L-cysteine

$$+ \text{ATP} \rightleftarrows \text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{ADP} + P_i \quad (3)$$

L-γ-Glutamyl-L-cysteine

$$+ \text{glycine} + \text{ATP} \rightleftarrows \text{glutathione} + \text{ADP} + P_i \quad (4)$$

Cellular utilization of glutathione is accounted for largely by export. Except for the erythrocyte, which appears to export glutathione disulfide, the major export form is glutathione [51]. Export of glutathione appears to function as part of a system for the protection of cell membranes, including a defense against lipid peroxidation which may be connected with the maintenance of α-tocopherol in the reduced state. Export of glutathione may provide a mechanism for reducing compounds in the immediate environment of the cell membrane. Although the level of glutathione in the blood plasma is very low (in the micromolar range as compared to intracellular levels in the millimolar range), it is probable that the levels of glutathione in the interstitial fluid are relatively high, reflecting cellular export of glutathione, and this may be of importance in cellular protection and possibly in the transport of certain molecules.

Glutathione is not transported efficiently into cells. Thus, cellular glutathione levels are not increased in animals treated with buthionine sulfoximine (an inhibitor of glutathione synthesis) when the plasma levels of glutathione are greatly increased. High plasma levels of glutathione produced by intraperitoneal injection of glutathione into buthionine sulfoximine-treated animals do not protect against damage due to inhibition of glutathione synthesis. The apparent "uptake" of extracellular glutathione, which has been reported occasionally, occurs largely, if not entirely, by pathways involving prior breakdown of glutathione to dipeptides and amino acids, transport of these, and intracellular synthesis of glutathione [34, 51].

The functions of glutathione have been probed *in vivo* by studies in which cellular glutathione levels are decreased markedly. Glutathione deficiency may be produced by administration of thiol-reactive compounds (e.g. diethylmaleate) and of oxidizing agents (e.g. diamide). However, these approaches have major limitations for experimental work because the available reagents are non-specific and thus produce other cellular effects [34]. Furthermore, an early effect of treatment with thiol reactive compounds or oxidizing agents is increased synthesis of glutathione because the rate-limiting step of glutathione synthesis catalyzed by γ-glutamylcysteine synthetase [reaction (3)] is feedback inhibited by glutathione [52]. Thus, administration of such a "glutathione depletor" may lead to a paradoxical increase in cellular glutathione levels [53, 54].

Inhibition of glutathione synthetase is not useful for production of uncomplicated experimental glutathione deficiency. Thus, patients with severe glutathione synthetase deficiency develop marked acidosis because they overproduce 5-oxoproline [55]. Since glutathione normally feedback inhibits the first step of its synthesis [reaction (3)], when there is very low glutathione synthetase activity glutathione levels decrease markedly, and this releases γ-glutamylcysteine synthetase from feedback inhibition. Formation of γ-glutamylcysteine thus increases greatly and this leads to increased formation of 5-oxoproline by the action of γ-glutamyl cyclotransferase. The formation of 5-oxoproline exceeds the capacity of 5-oxoprolinase, and 5-oxoproline therefore accumulates leading to life-threatening acidosis.

Inhibition of glutathione synthesis by inhibition of γ-glutamylcysteine synthetase is the preferred approach to a sustained decrease in cellular glutathione [34]. Buthionine sulfoximine and related compounds are highly selective inactivators of γ-glutamylcysteine synthetase and their administration to animals turns off cellular glutathione synthesis effectively [56–58]. Buthionine sulfoximine interacts with ATP at the active site of the synthetase where it is phosphorylated to form buthionine sulfoximine phosphate; this binds tightly, but not covalently, to the synthetase, thus inhibiting it irreversibly [34, 58, 59].

Glutathione deficiency and ascorbic acid

Severe glutathione deficiency produced by administration of L-buthionine-*SR*-sulfoximine to adult mice (e.g. 6 mmol/day for 9 days) leads to substantial tissue damage in skeletal muscle (myofiber degeneration [60]), lung (type 2 cell lamellar body disruption [61]), and marked mucosal degeneration in the jejunum and colon [62]. These effects, observed by electron microscopy, are prevented by administration of glutathione monoesters. Such esters are readily transported into cells and cleaved intracellularly to glutathione [63–66]; as noted above, glutathione

itself is not effectively transported and thus does not protect. In newborn rats, severe glutathione deficiency leads to much greater tissue damage and is lethal within a few days. The tissue damage in newborn rats includes effects on the lung (lamellar body destruction, loss of surfactant [23]), kidney (proximal tubular necrosis) [22, 23], liver (focal degeneration) [22, 23], brain (cerebral cortex mitochondrial swelling) [67], and lens (cataracts) [68]. These effects are not found after giving L-buthionine-*R*-sulfoximine, the diastereoisomer of L-buthionine-*SR*-sulfoximine that does not inhibit glutathione synthesis, and they are decreased or prevented by administration of glutathione monoester, thus supporting the conclusion that tissue damage and the lethality observed after giving L-buthionine-*SR*-sulfoximine are due to a deficiency of glutathione.

Tissue damage associated with glutathione deficiency is invariably accompanied by mitochondrial degeneration. Mitochondria, which do not synthesize glutathione but which obtain it by transport from the cytosol [69, 70], normally produce significant amounts of hydrogen peroxide [71]; the accumulation of peroxide in glutathione deficiency appears to be responsible for most of the mitochondrial and other types of cell degeneration. Such cellular damage is produced in glutathione deficiency by oxidation products that are normally formed (and destroyed) in cellular metabolism. Glutathione thus functions as an important antioxidant by virtue of its role in the destruction of hydrogen peroxide and lipid peroxides by providing substrate for the glutathione peroxidases. However, glutathione also functions as an antioxidant by promoting formation of the reduced forms of other antioxidants such as ascorbic acid.

The function of glutathione in maintaining tissue ascorbic acid levels has been clearly shown in experiments on newborn rats. When these animals were made glutathione deficient by administration of buthionine sulfoximine, they were found to have marked depletion of tissue (liver, kidney, lung, brain, eye) ascorbic acid. Both the ascorbic acid levels and the total ascorbic acid levels (ascorbic acid + dehydroascorbic acid) were decreased [22]. For example after treatment with L-buthionine-*SR*-sulfoximine (6 mmol/kg/day for 3.5 days), the liver ascorbic acid decreased from a control level of 2.51 ± 0.30 μmol/g to 0.42 ± 0.15 μmol/g; the total ascorbic acid decreased from a control level of 2.60 ± 0.28 μmol/g to 1.14 ± 0.23 μmol/g; these data indicate the presence of increased amounts of dehydroascorbic acid. Similar effects were observed in other tissues. When glutathione deficiency is produced in newborn rats by giving buthionine sulfoximine, about 90% of the animals die within 4–6 days. Mortality is decreased significantly by simultaneous administration of glutathione monoesters or of large doses of ascorbic acid (~2 mmol/kg/day) [22, 23]. Lower doses of ascorbic acid are less protective or are ineffective. Dehydroascorbic acid does not protect.

Not unexpectedly, administration of large doses of ascorbic acid to buthionine sulfoximine-treated newborn rats leads to a substantial increase of the tissue levels of ascorbic acid, indeed to a return to the control levels. However, interestingly, the tissue levels of glutathione of these rats are also increased significantly [22]. The levels of mitochondrial glutathione in the tissues of newborn rats treated with buthionine sulfoximine and ascorbic acid are substantially greater than the levels found after giving buthionine sulfoximine alone. Thus, the level of glutathione in liver mitochondria of newborn rats treated with buthionine sulfoximine was 0.31 ± 0.02 μmol/mg protein; after treatment with buthionine sulfoximine and ascorbic acid the level was 0.83 ± 0.20 μmol/mg protein [22]. The fold increases of mitochondrial glutathione observed when glutathione-deficient rats were given ascorbic acid were 2.7, 4.2, 6.0, and 2.7 in the liver, kidney, lung, and brain, respectively [22].

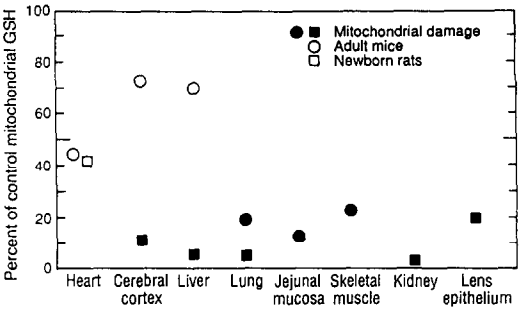

Fig. 1. Mitochondrial damage and glutathione levels of tissues of newborn rats (squares) and adult mice (circles). Closed symbols = mitochondrial damage (as observed by electron microscopy). From Ref. 23.

When newborn rats are made glutathione deficient by use of an experimental protocol in which only two doses of buthionine sulfoximine are given (on days 2 and 3 of life), they are found to have cataracts when they open their eyes on days 14–16 [22, 68]. Cataract formation can be almost completely prevented by administration of glutathione monoesters, but not by giving glutathione. Ascorbic acid was studied in this experimental model; although virtually all (97%) of the rats treated only with buthionine sulfoximine developed cataracts, when ascorbic acid (2 mmol/kg/day) was given together with buthionine sulfoximine, the incidence of cataracts was decreased markedly (9%). Administration of dehydroascorbic acid was also protective, suggesting that dehydroascorbic acid is reduced to ascorbic acid under these conditions. Animals given only two doses of buthionine sulfoximine in this protocol retained substantial capacity to synthesize glutathione in other tissues such as the liver. That dehydroascorbic acid protects against cataracts may be ascribed to its reduction by glutathione in the liver (or other organs) and transport of ascorbic acid via the plasma to the lens.

There is a good correlation between the decrease of mitochondrial glutathione levels and the occurrence of mitochondrial damage in the studies on newborn rats and adult mice (Fig. 1). For reasons not yet fully explored, the newborn animals are more sensitive to the effects of glutathione deficiency

than are older animals. Certain tissues are apparently more resistant to glutathione deficiency; these include the heart (Fig. 1) and the stomach [62]. Glutathione deficiency has little or no effect on the liver or kidney in adult mice, probably because these tissues continue to synthesize, or to be supplied with, ascorbic acid. That the brain of adult mice is essentially unaffected (as judged by electron microscopy) in buthionine sulfoximine-treated adult mice may be ascribed to the very poor transport of this inhibitor across the blood–brain barrier [72].

Glutathione deficiency in adult mice [61] leads to lamellar body disruption in lung type 2 cells, and in newborn rats [22, 23] to somewhat greater degeneration of these structures with loss of surfactant. Thus, electron microscopy showed decreased amounts of intraalveolar tubular myelin, which is secreted by the lamellar bodies. Lamellar body degeneration seems to be associated with oxidative damage to the perilamellar membrane, which contains the enzymes (phosphatidic acid phosphatase and choline phosphotransferase) which are key catalysts for the synthesis of phosphatidylcholine. Phosphatidylcholine, the main constituent of lung surfactant, is synthesized within the lamellar bodies of type 2 alveolar epithelial cells and is secreted as a protein complex into the alveolar subphase, where it is transformed into tubular myelin [73–76]. Glutathione deficiency induced by administration of buthionine sulfoximine to adult mice leads to markedly decreased levels of phosphatidylcholine in the lung and in the bronchoalveolar lining fluid [26]. Treatment with ascorbic acid (1–2 mmol/kg/day), which leads to greatly increased (about 2-fold) levels of lung mitochondrial glutathione, prevents damage to lung mitochondria and lamellar bodies as well as the decline of phosphatidylcholine levels in lung and alveolar lining fluid. This effect was dependent upon a dose of ascorbic acid of at least 1 mmol/kg/day; a dose of 0.5 mmol/kg/day was less effective and lower doses (0.05 and 0.005 mmol/kg/day) were not effective.

Although severe glutathione deficiency is lethal to newborn rats [and to guinea pigs (see below)], adult mice are able to survive such glutathione deficiency because they can synthesize ascorbic acid. When adult mice are treated with buthionine sulfoximine, the ascorbic acid level of the liver increases about 2-fold within 4 hr; the level of ascorbic acid then decreases and dehydroascorbic acid accumulates. In other tissues the ascorbic acid levels decrease and the levels of dehydroascorbic acid increase [23, 77]. Thus, an early effect of glutathione deficiency in adult mice is apparent induction of ascorbic acid synthesis in the liver. No such induction occurs in newborn rats, consistent with the view that significant amounts of ascorbic acid are not synthesized in these animals.

Ascorbic acid deficiency and glutathione

In view of the extensive body of knowledge indicating that glutathione functions in the reduction of dehydroascorbic acid, it was not entirely unexpected to find that glutathione deficiency decreases tissue ascorbic acid levels *in vivo*. However, it was not initially anticipated that administration of ascorbic acid would substantially decrease the mortality of glutathione-deficient newborn rats, nor was it expected that such treatment would increase tissue glutathione levels. These findings, which suggest that ascorbic acid and glutathione can perform similar functions *in vivo*, seem to elucidate a new aspect of the biochemistry of ascorbic acid. In a complementary approach, the effects of administration of glutathione monoethyl ester on ascorbic acid-deficient guinea pigs were examined.

When adult guinea pigs are treated with L-buthionine-*SR*-sulfoximine, they develop severe glutathione deficiency with tissue damage in liver (focal necrosis), kidney (proximal tubular damage), and lung (lamellar body degradation) in association with mitochondrial damage; they die within a few days [24]. The findings are closely similar to those made on newborn rats. Tissue damage and mortality are decreased or prevented by administration of ascorbic acid in doses of 1 mmol/kg three times per day; lower doses are less effective. Treatment with ascorbic acid spares mitochondrial glutathione as also found in newborn rats.

When guinea pigs were made deficient in ascorbic acid by feeding a scorbutic diet, they developed signs of scurvy, as expected, and died within 21–24 days. In ascorbic acid-deficient guinea pigs that were treated with glutathione monoethyl ester, the appearance of signs of scurvy (weight loss, bone changes, hematomas) were delayed significantly [25]. Mitochondrial glutathione levels were decreased markedly in scurvy. Interestingly, tissue ascorbic acid levels (as well as the glutathione levels) of the glutathione ester-treated animals were higher than in saline-treated controls. This may be explained by increased recovery of dehydroascorbic acid in the presence of higher levels of glutathione, and by effects of glutathione that directly replace those of ascorbic acid. Notably, guinea pigs given a scorbutic diet for 9 days exhibited higher than normal levels of glutathione in the liver and kidney. (Administration of glutathione did not lead to the effects observed after giving glutathione monoester.)

It is significant that administration of ascorbic acid can rescue glutathione-deficient newborn rats and guinea pigs and that administration of glutathione monoester delays the onset of scurvy in guinea pigs. A decreased level of ascorbic acid appears to provide a metabolic signal that leads to increased glutathione synthesis, whereas glutathione deficiency in adult mice seems to turn on ascorbic acid synthesis. The findings suggest that there may be symmetrical control mechanisms, and that the glutathione and ascorbic acid systems function together in antioxidant protection against endogenously formed reactive oxygen compounds. The observed metabolic redundancy emphasizes the physiological importance of this antioxidant function. Although glutathione synthesis takes place in virtually all cells, glutathione synthesized in the liver is the chief source of plasma glutathione. Thus, the liver exports glutathione, which is transported to and utilized by many tissues [51]. In the adult rat and mouse, ascorbic acid is

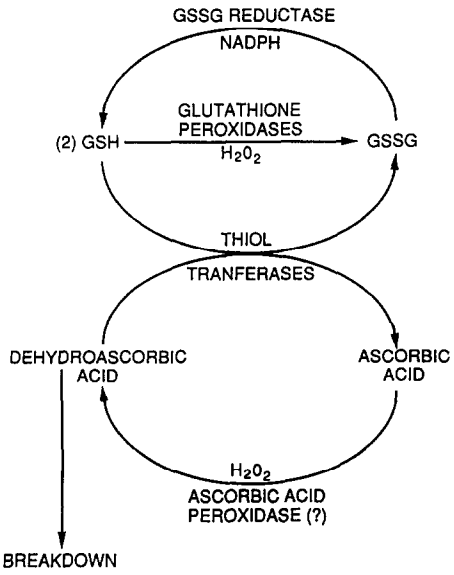

Fig. 2. Destruction of peroxides and related active oxygen forms by ascorbic acid and glutathione (GSH). Glutaredoxin and protein disulfide isomerase catalyze GSH-dependent reduction of dehydroascorbate. GSSG = glutathione disulfide. Adapted from Fig. 2 of Ref. 22.

synthesized in the liver [4] and is transported to other organs via the plasma.

Discussion

Both glutathione and ascorbic acid can function in the destruction of reactive oxygen compounds (Fig. 2 [22]). Glutathione functions in the reduction of hydrogen peroxide and other peroxides in reactions catalyzed by the glutathione peroxidases (selenium-containing and other). Ascorbic acid can also interact with hydrogen peroxide and other forms of reactive oxygen; such reactions can occur nonenzymatically, but the reaction of hydrogen peroxide with ascorbic acid is enzyme-catalyzed in chloroplasts, cyanobacteria and soybean nodules [78]. Possibly animal tissues also contain ascorbic acid peroxidase activity.

The *in vivo* findings are in certain respects directly analogous to some of the *in vitro* studies. Thus, glutathione (and other thiols) can replace ascorbic acid in several hydroxylation reactions [5]. Administration of ascorbic acid can increase the availability of glutathione for various functions by sparing the glutathione requirement for the reduction of dehydroascorbic acid. Similarly, the increased availability of glutathione in glutathione monoester-treated ascorbic acid-deficient guinea pigs may increase efficiency of recovery of dehydroascorbic acid, and thus the availability of ascorbic acid.

Patients with inborn errors associated with deficiency of glutathione synthesis (e.g. inherited deficiencies of glutathione synthetase or of γ-glutamylcysteine synthetase [55]), are analogous to glutathione-deficient experimental animals. Treatment of these patients with ascorbic acid should therefore be given serious consideration. Since relatively high doses of ascorbic acid (1–2 mmol/kg/day) were needed to prevent mortality in the experimental studies, one would be inclined to recommend trials of high doses of ascorbic acid for such patients. Ascorbic acid therapy may also be useful in the treatment of premature infants who have a deficiency of glutathione associated with cysteine deficiency due to lack of cystathionase [79]. Recent studies, which showed that pre-term infants (not respiratory distressed) have significantly lower levels of peripheral blood plasma total glutathione than do full-term infants [26], suggest that glutathione deficiency may be more common in certain newborn infants than previously thought. Unfortunately only a few data are now available about the glutathione status in various human diseases. Glutathione deficiency may occur in liver disease, adult respiratory syndrome, and AIDS; further studies would be desirable.

Certain drug- and radiation-resistant tumors have high cellular levels of glutathione and increased capacity for glutathione synthesis [for recent reviews, see Refs. 34, 80 and 81]. For example, treatment of human ovarian tumors grown in nude mice with melphelan or cisplatin often leads to resistance to these and other anticancer agents, and also to radiation. The resistant tumor cells have increased levels of glutathione; in experimental studies, resistance was reversed by treatment with buthionine sulfoximine. Although such treatment would be expected to decrease the synthesis of glutathione in normal cells as well as in tumors, it would be anticipated that the tumor cells, which have a higher requirement for glutathione, would be more sensitive to anticancer therapy after depletion of glutathione than the normal cells, which generally have a large excess of glutathione [34]. The success of this approach in experimental models has led to initiation of clinical trials of buthionine sulfoximine therapy [82].

In the light of the experimental work on the relationships between ascorbic acid and glutathione, it is relevant to consider the ascorbic acid status of patients receiving such therapy. Treatment with large doses of ascorbic acid would be expected to reverse effects produced by buthionine sulfoximine. Whether ascorbic acid might have the same effect on normal and tumor cells is not yet known; conceivably ascorbic acid might in some instances protect normal cells selectively but this possibility remains to be tested. In any event, ascorbic acid treatment would be a logical approach for treatment of patients exhibiting signs of glutathione deficiency produced by treatment with buthionine sulfoximine, and could therefore serve as a potential rescue therapy.

In addition to conditions in which there is a clear indication or likelihood of glutathione deficiency, therapy with reducing agents should also be considered for a host of conditions that are thought to be associated with the effects of "oxidative stress" or "free radical" damage. The diseases or conditions that fall within this category include ageing, cancer, atherosclerosis, viral infections including AIDS, stroke, myocardial infarction, arthritis and still

others. It is difficult to accept the view that the fundamental *cause* of each of these conditions is oxidative processes and associated free radical damage. It seems more likely that such oxidative stress is the result of specific pathological or degenerative processes that ultimately make cells susceptible to oxidative damage. Such oxidative stress thus seems to be a result rather than a primary cause. When experimental animals are deprived of glutathione by inhibition of its synthesis, they suffer the effects of reactive species that are normally formed in metabolism, but which cannot be destroyed when there is a lack of glutathione. Pathological processes that affect mitochondria, cytosolic components, or membranes can probably affect many cellular functions including protective and repair mechanisms. The occurrence of oxidative damage to various cell structures might well be associated with defects in turnover, disposal of damaged molecules, and repair processes which may be limiting in ageing or in particular clinical syndromes. (Cellular oxidative phenomena may be protective in some instances, for example, in the destruction of viruses and bacteria.)

It is nevertheless reasonable to explore therapeutic possibilities that may have some expectations for benefit because it is likely that many diseases are associated with at least some degree of oxidative stress, and this in itself can lead to serious consequences. Therapy directed at neutralization of oxidative phenomena may be successful to the extent that oxidation contributes to the overall disease process. Such treatment may not be completely curative because the basic pathological process may not be affected. The *in vivo* demonstration of the functioning of the ascorbic acid–glutathione system suggests that compounds that increase cellular glutathione levels might be useful in therapy. Administration of cysteine delivery agents such as *N*-acetyl-L-cysteine and L-2-oxothiazolidine-4-carboxylate leads to increased cellular glutathione levels; the latter compound is probably more effective than the former. L-2-Oxothiazolidine-4-carboxylate is, in contrast to L-cysteine, well transported into cells where it is converted to L-cysteine by 5-oxoprolinase [34, 83–85]. Other compounds whose administration leads to increased tissue glutathione levels include glutathione monoesters [63–66] and γ-glutamylcysteine [86]. [For additional consideration of the modulation of glutathione metabolism, see [Refs. 30–34, 51, 66, 87–89]. Relatively little toxicity has been found when compounds that increase glutathione levels or ascorbic acid are given; nevertheless, very high doses of these agents can produce toxic effects especially in the presence of transition metal ions.

Although several clinical approaches are of interest, the present discussion is limited to aspects of the atherosclerosis problem. Recent experimental work has led to the idea that oxidation of low density lipoprotein (LDL)* is a key step in the development of atherosclerosis [90–93]. Circulating monocytes, which attach to and penetrate the arterial wall, differentiate into macrophages, which become loaded with cholesterol to become foam cells. It is postulated that a modified form of LDL is involved since the rate at which native LDL is taken up is insufficient to produce foam cells. Study of several types of modified LDL led to the conclusion that an oxidized form of LDL is involved. Oxidized LDL is cytotoxic and apparently promotes uptake of monocytes into the arterial intima. Although many of the studies have involved use of LDL that was oxidized by potent chemical systems, there are data consistent with *in vivo* formation of oxidized LDL [92].

Evidence that is relevant to the hypothesis that oxidized LDL is involved in atherosclerosis has come from studies on the effects of probucol (4,4′-(isopropylidenethio)bis [2,6-di-*t*-butylphenol]), a drug that decreases plasma cholesterol levels. This drug also exhibits antioxidant activity and it decreases oxidized LDL formation *in vitro* [93]. It is possible that the antiatherosclerotic effect of probucol is a function of its antioxidant activity [94]. An analog of probucol that has no effect on cholesterol levels but that exhibits antioxidant activity had less effect in preventing atherosclerosis [95]. Sparrow *et al.* [96] found that administration of the reducing compound *N*,*N*′-diphenyl-1,4-phenylenediamine (DPPD) to cholesterol fed rabbits decreased the progression of atherosclerosis without affecting total plasma cholesterol levels. DPPD feeding also increased levels of high density lipoprotein and of plasma triglycerides, suggesting that DPPD may have some effects on lipid metabolism. It is likely that probucol and other compounds that have reducing properties affect both the formation of oxidized LDL as well as the metabolism of cholesterol (which includes pathways involving hydroxylation steps). DPPD, which is a mutagen, is unsuitable for therapy [96], and probucol also has undesirable side-effects [97]. Probucol and ascorbic acid were compared in an *in vitro* cell-free system containing 2.5 μM Cu^{2+} and in human monocyte macrophages [97]. Both agents decreased oxidation of LDL to about the same extent, but ascorbic acid was much more effective in protection of the tocopherol and carotene present in LDL.

Since glutathione provides reducing power for maintenance of the reduced forms of ascorbic acid and α-tocopherol, it is relevant to examine the effects of glutathione deficiency on oxidation of LDL. In one study, cultured bovine endothelial cells that were incubated with oxidized LDL were found to suffer a significant loss of glutathione and underwent lysis [98]. These cells became much more sensitive to oxidized LDL when treated with buthionine sulfoximine. It was found that treatment of the cells with L-2-oxothiazolidine-4-carboxylate decreased the toxicity of oxidized LDL. These interesting studies suggest that glutathione may have a significant function in the protection of cells from the effects of oxidized LDL. *In vivo* studies (e.g. on buthionine sulfoximine-treated animals) would be of great interest.

The *in vitro* studies do not necessarily reflect effects that occur *in vivo*. The nature of the oxidation process is not yet clear, nor is it known where LDL oxidation takes place; possibilities include the

* Abbreviations: LDL, low density lipoprotein; and DPPD, *N*,*N*′-diphenyl-1,4-phenylenediamine.

plasma, various cells, and the arterial intima. Studies in which human blood plasma was treated with 2,2′-azobis(2-amidinopropane)hydrochloride (which produces peroxyl radicals) not unexpectedly showed a disappearance of ascorbic acid. It was concluded that ascorbic acid is an outstanding antioxidant in human blood plasma, and that ascorbic acid "is a physiological antioxidant of major importance for protection against disease and degenerative processes caused by oxidant stress" [99]. Others concluded that oxidative "events within the lesion itself are more important than events in the plasma" [100, 101], and that "there is no good evidence for substantial amounts of circulating lipid hydroperoxides in human plasma in atherosclerosis or in any other disease state." Various questions remain about the origin of plasma ascorbic acid, its fate after oxidation and its possible renewal by reduction, relationship to tissue glutathione levels, and turnover. Administration of ascorbic acid or other antioxidants might be effective in the plasma or elsewhere, but this would probably depend upon the levels achieved and on various permeability factors. It is of interest that administration of a high dose of ascorbic acid to guinea pigs led to increased plasma and tissue levels of α-tocopherol [102].

The current interest in oxidation of LDL, and the possibility of conducting therapeutic trials with antioxidants such as ascorbic acid, seem to reflect a reincarnation of ideas expressed about 40 years ago. Thus, Willis [13] reported in 1953 that "acute and chronic scurvy (in guinea pigs) were effective in producing lesions of the arterial intima indistinguishable from the lesions which have been described in human atherosclerosis." Atherosclerosis was observed in scorbutic guinea pigs that had normal cholesterol levels. Feeding of cholesterol to scorbutic guinea pigs decreased ascorbic acid levels and administration of ascorbic acid inhibited atherosclerosis produced by cholesterol feeding. Willis suggested that "massive doses of parenteral ascorbic acid may be of therapeutic value in the treatment of atherosclerosis and the prevention of intimal hemorrhage and of thrombosis" [13]. He emphasized that ascorbic acid deficiency affected the arterial ground substance, which is rich in collagen. Ginter, who also studied atherosclerosis in scorbutic guinea pigs [14, 103], concluded that chronic ascorbic acid deficiency decreases transformation of cholesterol to bile acids, a process that involves hydroxylation reactions. Other aspects of the relationships between ascorbic acid and cardiovascular disease, mainly epidemiological studies, are encouraging [104]. It is of interest, and somewhat surprising, in the light of the relatively recent general acceptance of the cholesterol hypothesis of atherosclerosis, to realize that this idea had also been expressed in the early literature [see, for example, Ref. 105].

The studies reviewed above emphasize the physiologically significant connection between glutathione and ascorbic acid. The lethal effects of glutathione deficiency on animals that do not synthesize ascorbic acid—and their prevention by treatment with ascorbic acid—provide dramatic evidence of this interrelationship, as does the observation that treatment of ascorbic acid-deficient guinea pigs with glutathione monoester delays the onset of scurvy. The recent work on the subunits of prolyl-4-hydroxylase [49] suggests that glutathione has an important role in the function of a key enzyme involved in collagen synthesis. This enzyme, which tends to undergo inactivation readily, is reactivated by ascorbic acid. Glutathione-dependent reduction of the dehydroascorbic acid formed in the reactivation reaction can apparently be catalyzed by the β-subunit of prolyl 4-hydroxylase [21, 49]. The proximity of this glutathione-dependent reductase activity to the α-ketoglutarate-dependent dioxygenase activity is noteworthy. Studies on other enzymes of this type will be of interest.

In addition to chronic administration of large doses of ascorbic acid and therapies based on other antioxidants and compounds that increase tissue levels of glutathione, other types of experimentation would be of interest. The effects of restoration of the gene for L-gulonolactone oxidase should be examined in guinea pigs. Other genetic approaches that could be studied in experimental animals include development of model transgenic animals with high levels of the synthetases required for glutathione synthesis.

General acceptance of the idea that relatively high doses of ascorbate (i.e. much more than 60 mg/day) may promote optimal health has possibly been delayed, at least in part, by the appearance of exaggerated claims [see Ref. 106], but also by a general attitude of conservatism. In this respect, however, one should not expect antioxidants to be a panacea. Although oxidative stress probably plays a role in ageing and various diseases, it is unlikely to be the underlying cause of all of these conditions. Many intriguing experimental approaches involving glutathione, ascorbic acid and related compounds remain to be examined, and, hopefully future clinical trials will be designed to give definite and objective answers to questions about the effectiveness of specific agents.

REFERENCES

1. Szent-Györgyi A, CLXXII. Observations on the function of peroxidase systems and the chemistry of the adrenal cortex. Description of a new carbohydrate derivative. *Biochem J* **22**: 1387–1409, 1928.
2. Waugh WA and King CG, Isolation and identification of vitamin C. *J Biol Chem* **XCVII**: 325–331, 1932.
3. Levine M, New concepts in the biology and biochemistry of ascorbic acid. *N Engl J Med* **314**: 892–902, 1986.
4. Chatterjee IB, Majumder AK, Nandi BK and Subramanian N, Synthesis and some major functions of vitamin C in animals. *Ann NY Acad Sci* **258**: 24–47, 1975.
5. Englard S and Seifter S, The biochemical functions of ascorbic acid. *Annu Rev Nutr* **6**: 365–406, 1986.
6. Hodges RE, Baker EM, Hood J, Sauberlich HE and March SC, Experimental scurvy in man. *Am J Clin Nutr* **22**: 535–548, 1969.
7. Irwin MI and Hutchins BK, A conspectus of research on vitamin C requirements of man. *J Nutr* **106**: 823–879, 1976.
8. Block G, Vitamin C and cancer prevention: The

epidemiological evidence. *Am J Clin Nutr* **53**: 270S–282S, 1991.
9. Cameron E and Pauling L, Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. *Proc Natl Acad Sci USA* **73**: 3685–3689, 1976.
10. Moertel CG, Fleming TR, Creagan ET, Rubin J, O'Connell MJ and Ames MM, High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy. A randomized double-blind comparison. *N Engl J Med* **312**: 137–141, 1985.
11. Cook JD and Monsen ER, Vitamin C, the common cold, and iron absorption. *Am J Clin Nutr* **30**: 235–241, 1977.
12. Coulehan JL, Eberhard S, Kapner L, Taylor F, Rogers K and Garry P, Vitamin C and acute illness in Navajo schoolchildren. *N Engl J Med* **295**: 973–977, 1976.
13. Willis GC, An experimental study of the intimal ground substance in atherosclerosis. *Can Med Assoc J* **69**: 17–22, 1953.
14. Ginter E, Marginal vitamin C deficiency, lipid metabolism, and atherogenesis. *Adv Lipid Res* **16**: 167–220, 1978.
15. Bendich A, Machlin LJ, Scandurra O, Burton GW and Wayner DDM, The antioxidant role of vitamin C. *Adv Free Radic Biol Med* **2**: 419–444, 1986.
16. Harakeh S, Jariwalla RJ and Pauling L, Suppression of human immunodeficiency virus replication by ascorbate in chronically and acutely infected cells. *Proc Natl Acad Sci USA* **87**: 7245–7249, 1990.
17. Baker EM, Vitamin C requirements in stress. *Am J Clin Nutr* **20**: 583–590, 1967.
18. Prinz W, Bortz R, Bregin B and Hersch M, The effect of ascorbic acid supplementation on some parameters of the human immunological defence system. *Int J Vitam Nutr Res* **47**: 248–257, 1977.
19. Borsook H, Davenport HW, Jeffreys CEP and Warner RC, The oxidation of ascorbic acid and its reduction *in vitro* and *in vivo*. *J Biol Chem* **117**: 237–279, 1937.
20. Hopkins FG and Morgan EJ, Some relations between ascorbic acid and glutathione. *Biochem J* **30**: 1446–1462, 1936.
21. Wells WW, Xu DP, Yang Y and Rocque PA, Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. *J Biol Chem* **265**: 15361–15364, 1990.
22. Mårtensson J and Meister A, Glutathione deficiency decreases tissue ascorbate levels in newborn rats: Ascorbate spares glutathione and protects. *Proc Natl Acad Sci USA* **88**: 4656–4660, 1991.
23. Mårtensson J, Jain A, Stole E, Frayer W, Auld PAM and Meister A, Inhibition of glutathione synthesis in the newborn rat: A model for endogenously produced oxidative stress. *Proc Natl Acad Sci USA* **88**: 9360–9364, 1991.
24. Griffith OW, Han J and Mårtensson J, Vitamin C protects adult guinea pigs against tissue damage and lethality caused by buthionine sulfoximine-mediated glutathione depletion. *FASEB J* **5**: 4708, 1991.
25. Han J, Mårtensson J, Meister A and Griffith OW, Glutathione ester but not glutathione delays onset of scurvy in guinea pigs fed a vitamin C-deficient diet. *FASEB J* **6**: 5631, 1992.
26. Jain A, Mårtensson J, Mehta T, Krauss AN, Auld PAM and Meister A, Ascorbic acid prevents oxidative stress in glutathione-deficient mice; Effects on lung type 2 cell lamellar bodies, lung surfactant, and skeletal muscle. *Proc Natl Acad Sci USA* **89**: 5093–5097, 1992.
27. De Rey-Pailhade J, Sur un corps d'origine organique hydrogenant le soufre a froid. *C R Hebd Seances Acad Sci* **106**: 1683–1684, 1888.
28. Meister A, On the discovery of glutathione. *Trends Biochem Sci* **13**: 185–188, 1988.
29. Larsson A, Orrenius S, Holmgren A and Mannervik B (Eds.), *Functions of Glutathione. Biochemical, Physiological, Toxicological, and Clinical Aspects*. Raven Press, New York, 1983.
30. Meister A and Anderson ME, Glutathione. *Annu Rev Biochem* **52**: 711–760, 1983.
31. Dolphin D, Poulson R and Avramovic O (Eds.), *Glutathione: Chemical, Biochemical, and Medical Aspects*, Parts A and B, *Coenzyme and Cofactors* Series, Vol. III. John Wiley, New York, 1989.
32. Taniguchi N, Higashi T, Sakamoto Y and Meister A (Eds.), *Glutathione Centennial. Molecular Perspectives and Clinical Implications*. Academic Press, New York, 1989.
33. Meister A, Glutathione metabolism and its selective modification. *J Biol Chem* **263**: 17205–17208, 1988.
34. Meister A, Glutathione deficiency produced by inhibition of its synthesis and its reversal; Applications in research and therapy. *Pharmacol Ther* **51**: 155–194, 1991.
35. Stahl RL, Liebes LF and Silber R, Glutathione dehydrogenase (ascorbate). *Methods Enzymol* **122**: 10–12, 1986.
36. Alcain FJ, Buron MI, Villalba JM and Navas P, Ascorbate is regenerated by HL-60 cells through the transplasmalemma redox system. *Biochim Biophys Acta* **1073**: 380–385, 1991.
37. Coassin M, Tomasi A, Vannini V and Ursini F, Enzymatic recycling of oxidized ascorbate in pig heart: One-electron vs two-electron pathway. *Arch Biochem Biophys* **290**: 458–462, 1991.
38. Iyanagi T, Yamazaki I and Anan KF, One-electron oxidation–reduction properties of ascorbic acid. *Biochim Biophys Acta* **806**: 255–361, 1985.
39. Diliberto EJ Jr, Dean G, Carter C and Allen PL, Tissue, subcellular, and submitochondrial distributions of semidehydroascorbate reductase: Possible role of semidehydroascorbate reductase in cofactor regeneration. *J Neurochem* **39**: 563–568, 1982.
40. Barnes MJ and Kodicek E, Biological hydroxylations and ascorbic acid with special regard to collagen metabolism. *Vitam Horm* **30**: 1–43, 1972.
41. van B. Robertson W and Schwartz B, Ascorbic acid and the formation of collagen. *J Biol Chem* **201**: 689–696, 1953.
42. Gould BS and Woessner JF, Biosynthesis of collagen. The influence of ascorbic acid on the proline, hydroxyproline, glycine, and collagen content of regenerating guinea pig skin. *J Biol Chem* **226**: 289–300, 1957.
43. van B. Robertson W and Hewitt J, Augmentation of collagen synthesis by ascorbic acid *in vitro*. *Biochim Biophys Acta* **49**: 404–406, 1961.
44. Stone N and Meister A, Function of ascorbic acid in the conversion of proline to collagen hydroxyproline. *Nature* **194**: 555–557, 1962.
45. Meister A, Stone N and Manning JM, Conversion of proline to hydroxyproline in collagen synthesis. *Adv Chem Ser* **44**: 67–81, 1964.
46. Stetten MR, Some aspects of the metabolism of hydroxyproline, studied with the aid of isotopic nitrogen. *J Biol Chem* **181**: 31–37, 1949.
47. Manning JM and Meister A, Conversion of proline to collagen hydroxyproline. *Biochemistry* **5**: 1154–1165, 1966.
48. Myllylä R, Kuutti-Savolainen E-R and Kivirikko KI, The role of ascorbate in the prolyl hydroxylase reaction. *Biochem Biophys Res Commun* **83**: 441–448, 1978.

49. Koivu J, Myllylä R, Helaakoski T, Pihlajaniemi T, Tasenen K and Kivirikko KI, A single polypeptide acts both as the β subunit of prolyl 4-hydroxylase and as a protein disulfide-isomerase. *J Biol Chem* **262**: 6447–6449, 1987.
50. Sergeev IN, Arkhapchev YP and Spiricheve VB, Ascorbic acid effects on vitamin D hormone metabolism and binding in guinea pigs. *J Nutr* **120**: 1185–1190, 1990.
51. Meister A, Metabolism and function of glutathione. In: *Glutathione: Chemical, Biochemical and Medical Aspects* (Eds. Dolphin D, Poulson R and Avramovic O), Chap. 11, pp. 367–474. John Wiley, New York, 1989.
52. Richman PG and Meister A, Regulation of γ-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. *J Biol Chem* **250**: 1422–1426, 1975.
53. Deneke SM and Fanburg GL, Regulation of cellular glutathione. *Am J Physiol* **257**: L163–L173, 1989.
54. Plummer JL, Smith BR, Sies H and Bend JR, Chemical depletion of glutathione *in vivo*. *Methods Enzymol* **77**: 50–59, 1981.
55. Meister A and Larsson A, Glutathione synthetase deficiency and other disorders of the γ-glutamyl cycle. In: *The Metabolic Basis of Inherited Disease* (Eds. Scriver CR, Beaudet AL, Sly WS and Valle D), 6th Edn, Chap. 31, pp. 855–868. McGraw-Hill, New York, 1989.
56. Griffith OW, Anderson ME and Meister A, Inhibition of glutathione biosynthesis by prothionine sulfoximine (*S-n*-propyl homocysteine sulfoximine), a selective inhibitor of γ-glutamylcysteine synthetase. *J Biol Chem* **254**: 1205–1210, 1979.
57. Griffith OW and Meister A, Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S-n*-butyl homocysteine sulfoximine). *J Biol Chem* **254**: 7558–7560, 1979.
58. Griffith OW, Mechanism of action, metabolism, and toxicity of buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J Biol Chem* **257**: 13704–13712, 1982.
59. Meister A, Inhibition of glutamine synthetase and γ-glutamylcysteine synthetase by methionine sulfoximine and related compounds. In: *Enzyme-Activated Irreversible Inhibitors* (Eds. Seiler N, Jung MJ and Koch-Weser J), pp. 187–211. Elsevier-North Holland Biomedical Press, Amsterdam, The Netherlands, 1978.
60. Mårtensson J and Meister A, Mitochondrial damage in muscle occurs after marked depletion of glutathione and is prevented by giving glutathione monoester. *Proc Natl Acad Sci USA* **86**: 471–475, 1989.
61. Mårtensson J, Jain A, Frayer W and Meister A, Glutathione metabolism in the lung: Inhibition of its synthesis leads to lamellar body and mitochondrial defects. *Proc Natl Acad Sci USA* **86**: 5296–5300, 1989.
62. Mårtensson J, Jain A and Meister A, Glutathione is required for intestinal function. *Proc Natl Acad Sci USA* **87**: 1715–1719, 1990.
63. Puri RN and Meister A, Transport of glutathione as γ-glutamylcysteinylglycyl ester, into liver and kidney. *Proc Natl Acad Sci USA* **80**: 5258–5260, 1983.
64. Wellner VP, Anderson ME, Puri RN, Jensen GL and Meister A, Radioprotection by glutathione ester: Transport of glutathione ester in human lymphoid cells and fibroblasts. *Proc Natl Acad Sci USA* **81**: 4732–4735, 1984.
65. Anderson ME, Powrie F, Puri RN and Meister A, Glutathione monoethyl ester: Preparation, uptake by tissues, and conversion to glutathione. *Arch Biochem Biophys* **239**: 538–548, 1985.
66. Anderson ME and Meister A, Glutathione monoesters. *Anal Biochem* **183**: 16–20, 1989.
67. Jain A, Mårtensson J, Stole E, Auld PAM and Meister A, Glutathione deficiency leads to mitochondrial damage in brain. *Proc Natl Acad Sci USA* **88**: 1913–1917, 1991.
68. Mårtensson J, Steinherz R, Jain A and Meister A, Glutathione ester prevents buthionine sulfoximine-induced cataracts and lens epithelial cell damage. *Proc Natl Acad Sci USA* **86**: 8727–8731, 1989.
69. Griffith OW and Meister A, Origin and turnover of mitochondrial glutathione. *Proc Natl Acad Sci USA* **82**: 4668–4672, 1985.
70. Mårtensson J, Lai JCK and Meister A, High affinity transport of glutathione is part of a multicomponent system essential for mitochondrial function. *Proc Natl Acad Sci USA* **87**: 7185–7189, 1990.
71. Boveris A and Chance B, The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* **134**: 707–716, 1973.
72. Steinherz R, Mårtensson J, Wellner D and Meister A, Transport into brain of buthionine sulfoximine, an inhibitor of glutathione synthesis, is facilitated by esterification and administration of dimethylsulfoxide. *Brain Res* **518**: 115–119, 1990.
73. Buckingham S, Heinemann HO, Sommers SC and McNary WF, Phospholipid synthesis in the large pulmonary alveolar cell. *Am J Pathol* **48**: 1027–1041, 1966.
74. Spitzer HL, Rice JM, MacDonald PC and Johnston JM, Phospholipid biosynthesis in lung lamellar bodies. *Biochem Biophys Res Commun* **66**: 17–23, 1975.
75. Williams MC, Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. *J Cell Biol* **72**: 260–276, 1977.
76. King RJ, Composition and metabolism of the apolipoproteins of pulmonary surfectant. *Annu Rev Physiol* **47**: 775–788, 1985.
77. Mårtensson J and Meister A, Glutathione deficiency increases hepatic ascorbic acid synthesis in adult mice. *Proc Natl Acad Sci USA*, in press.
78. Dalton DA, Russell SA, Hanus FJ, Pascoe GA and Evans HJ, Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proc Natl Acad Sci USA* **83**: 3811–3815, 1986.
79. Sturman JA, Gaul G and Raiha NCR, Absence of cystathionase in human fetal liver: Is cystine essential? *Science* **169**: 74–76, 1970.
80. Vistica DT and Ahmad S, Acquired resistance of tumor cells to L-phenylalanine mustard: Implications for the design of a clinical trial involving glutathione depletion. In: *Glutathione Centennial: Molecular Perspectives and Clinical Implications* (Eds. Taniguchi N, Higashi T, Sakamoto Y and Meister A), Chap. 21, pp. 301–315. Academic Press, New York, 1989.
81. Ozols RF, Hamilton TC, Masuda H and Young RC, Manipulation of cellular thiols to influence drug resistance. In: *Mechanisms of Drug Resistance in Neoplastic Cells* (Eds. Tew KD and Woolley PV), Chap 19, pp. 289–305. Bristol Myers Symposium No. 9, Academic Press, New York, 1988.
82. Hamilton T, O'Dwyer P, Young R, Tew K, Padavic K, Comis R and Ozols R, Phase I trial of buthionine sulfoximine (BSO) plus melphalan (L-PAM) in patients with advanced cancer. *Proc Annu Meet Am Soc Clin Oncol* **9**: A281, 1990.
83. Williamson JM and Meister A, Stimulation of hepatic glutathione formation by administration of L-2-oxothiazolidine-4-carboxylate, a 5-oxo-L-prolinase substrate. *Proc Natl Acad Sci USA* **78**: 936–939, 1981.
84. Williamson JM, Boettcher B and Meister A, An

intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis. *Proc Natl Acad Sci USA* **79**: 6246–6249, 1982.
85. Anderson ME and Meister A, Marked increase of cysteine levels in many regions of brain after administration of 2-oxothiazolidine-4-carboxylate. *FASEB J* **3**: 1632–1636, 1989.
86. Anderson ME and Meister A, Transport and direct utilization of γ-glutamylcyst(e)ine for glutathione synthesis. *Proc Natl Acad Sci USA* **80**: 707–711, 1983.
87. Meister A, Selective modification of glutathione metabolism. *Science* **220**: 471–477, 1983.
88. Meister A, Novel drugs that affect glutathione metabolism. In: *Mechanisms of Drug Resistance in Neoplastic Cells* (Eds. Tew KD and Woolley PV), Chap. 7, pp. 99–126. Bristol Myers Symposium No. 9, Academic Press, New York, 1988.
89. Anderson ME and Meister A, Intracellular delivery of cysteine. *Methods Enzymol* **143**: 313–325, 1987.
90. Parthasarathy S, Khoo JC, Miller E, Barnett J, Witztum JL and Steinberg D, Low density lipoprotein rich in oleic acid is protected against oxidative modification: Implications for dietary prevention of atherosclerosis. *Proc Natl Acad Sci USA* **87**: 3894–3898, 1990.
91. Quinn MT, Parthasarathy S, Fong LG and Steinberg D, Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci USA* **84**: 2995–2998, 1987.
92. Ylä-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL and Steinberg D, Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* **84**: 1086–1095, 1989.
93. Parthasarathy S, Young SG, Witztum JL, Pittman RC and Steinberg D, Probucol inhibits oxidative modification of low density lipoprotein. *J Clin Invest* **77**: 641–644, 1986.
94. Chisolm GM, Antioxidants and atherosclerosis: A current assessment. *Clin Cardiol* **14**: 25–30, 1991.
95. Mao SJT, Yates MT, Parker RA, Chi EM and Jackson RL, Attenuation of atherosclerosis in a modified strain of hypercholesterolemic Watanabe rabbits with use of a probucol analogue (MDL 29,311) that does not lower serum cholesterol. *Arterioscler Thromb* **11**: 1266–1275, 1991.
96. Sparrow CP, Doebber TW, Olszewski J, Wu MS, Ventre J, Stevens KA and Chao Y-s, Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant *N,N'*-diphenylphenylenediamine. *J Clin Invest* **89**: 1885–1891, 1992.
97. Jialal I and Grundy SM, Preservation of the endogenous antioxidants in low density lipoprotein by ascorbate but not probucol during oxidative modification. *J Clin Invest* **87**: 597–601, 1991.
98. Kuzuya M, Naito M, Funaki C, Hayashi T, Asai K and Kuzuya F, Protective role of intracellular glutathione against oxidized low density lipoprotein in cultured endothelial cells. *Biochem Biophys Res Commun* **163**: 1466–1472, 1989.
99. Frei B, England L and Ames BN, Ascorbate is an outstanding antioxidant in human blood plasma. *Proc Natl Acad Sci USA* **86**: 6377–6381, 1989.
100. Halliwell B, Free radicals, reactive oxygen species and human disease: A critical evaluation with special reference to atherosclerosis. *Br J Exp Pathol* **70**: 737–757, 1989.
101. Halliwell B and Gutteridge JMC, The antioxidants of human extracellular fluids. *Arch Biochem Biophys* **280**: 1–8, 1990.
102. Bendich A, D'Apolito P, Gabriel E and Machlin LJ, Interaction of dietary vitamin C and vitamin E on guinea pig immune response to mitogens. *J Nutr* **114**: 1588–1593, 1984.
103. Ginter E, Cholesterol: Vitamin C controls its transformation to bile acids. *Science* **179**: 702–704, 1973.
104. Simon JA, Vitamin C and cardiovascular disease: A review. *J Am Coll Nutr* **11**: 107–125, 1992.
105. Duff GL, Experimental cholesterol arteriosclerosis and its relationship to human arteriosclerosis. *Arch Pathol* **20**: 259–304, 1935.
106. Barinaga M, Vitamin C gets a little respect. *Science* **254**: 374–376, 1991.