Winkler BS, Orselli SM, Rex TSThe redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. Free Radic Biol Med 17:333–349

ARTICLE *in* FREE RADICAL BIOLOGY AND MEDICINE · NOVEMBER 1994

Impact Factor: 5.71 · DOI: 10.1016/0891-5849(94)90019-1 · Source: PubMed

3 AUTHORS, INCLUDING:

Tonia S Rex
Vanderbilt University
41 PUBLICATIONS **1,220** CITATIONS

SEE PROFILE

Pergamon

Free Radical Biology & Medicine, Vol. 17, No. 4, pp. 333–349, 1994

0891-5849/94 $6.00 + .00

0891-5849(94)E0082-T

Review Article

THE REDOX COUPLE BETWEEN GLUTATHIONE AND ASCORBIC ACID: A CHEMICAL AND PHYSIOLOGICAL PERSPECTIVE

BARRY S. WINKLER, STEPHEN M. ORSELLI, and TONIA S. REX
Eye Research Institute, Oakland University, Rochester, MI, USA

(*Received* 6 *January* 1994; *Revised* 1 *March* 1994; *Accepted* 23 *March* 1994)

Abstract—This article provides a comprehensive analysis of the redox reaction between glutathione/glutathione disulfide and ascorbic acid/dehydroascorbic acid. It includes an historical perspective of the progression of the experiments, first begun more than 60 years ago and continuing today with heightened importance. Indeed, the antioxidant capacity of glutathione and ascorbic acid, whether singly or in combination, linked via the redox couple, is a subject of intense interest for studies by bench scientists and clinicians, particularly because a growing body of evidence suggests that free radicals may be involved in a variety of diseases. The authors begin with a detailed summary of "test tube" experiments (the chemical perspective) that have revealed the conditions that regulate the rate of the redox coupling between glutathione and dehydroascorbic acid and that promote or inhibit the decomposition of dehydroascorbic acid in ordinary, buffered aqueous media; results obtained in the authors' laboratory are used for illustration purposes and uniformity of presentation. The authors then proceed to a critical examination of the extent to which the redox couple between glutathione and ascorbic acid operates in a cell, using the often published antioxidant cascade (See Fig. 1) as the model for the analysis (the physiological perspective). The evidence for and the evidence against the presence of the enzyme dehydroascorbate reductase in animal cells is outlined in a balanced way in an attempt to make sense of this continuing controversy. Next, the authors carefully document the many studies showing that exogenous dehydroascorbic acid is transported into cells where it is reduced to ascorbic acid by glutathione. Finally, they probe the functional significance and efficiency of the redox couple in monolayer cultures of human retinal pigment epithelial (RPE) cells, as a prototypical cellular model. The authors include the results of new experiments showing that incubation of RPE cells with a nitroxide, TEMPOL, leads to the selective oxidation of intracellular ascorbic acid. This approach is desirable because it dissects the cascade at a specific site and permits measurements of the levels of ascorbic acid and glutathione in the cells before, during, and after oxidation. The results show that only partial regeneration of ascorbic acid is obtained when control conditions are restored. However, if either ascorbic acid or dehydroascorbic acid is added to the media during the recovery period following treatment of cells with TEMPOL, then full recovery of ascorbic acid is observed. These results raise certain concerns whether the activity of the redox couple between glutathione and dehydroascorbic acid is sufficient to restore the level of ascorbic acid in oxidatively challenged cells, when exogenous dehydroascorbic acid is unavailable. This leads to the suggestion that the transmembrane uptake of ascorbic acid and dehydroascorbic acid (with subsequent redox reduction to ascorbic acid) is an important component in the overall cellular machinery that regulates the intracellular concentration of ascorbic acid.

Keywords—Glutathione, Ascorbic acid, Redox couples, Free radicals, Oxidative stress, Antioxidants

INTRODUCTION

We begin with several beautifully written statements, which appeared in a 1936 article by Hopkins and Morgan.[1] "Ascorbic acid and glutathione are the most conspicuous and, so far as is at present known, the most active reducing substances in living tissues. In spite

Address correspondence to: Barry S. Winkler, Eye Research Institute, Oakland University, Rochester, MI 48309-4401, USA.

Barry S. Winkler received his BA in biology from Harpur College (now SUNY-Binghamton) in 1965 and conducted his MA and PhD work at the University of Buffalo Medical School in physiology under Professor Werner K. Noell. After receiving his PhD in 1971, Dr. Winkler joined the Eye Research Institute, Oakland University, Rochester, Michigan, where he is currently professor of Biomedical Sciences. His research principally involves studies on energy metabolism, oxidative processes, light damage, and electrophysiological responsiveness of the mammalian retina. Dr. Winkler is a regular member (1991–1995) of the Visual Sciences C Study Section, Division of Research Grants, National Institutes of Health and currently serves as chairperson of this review panel.

Stephen M. Orselli received his BS in biology from Oakland University in 1990. Following graduation, Mr. Orselli worked in the quality assurance department at Perrigo Co., Allegan, Michigan. Since 1991, Mr. Orselli has been a research assistant in Dr. Winkler's laboratory. He is currently pursuing his MS in hazardous waste management.

Tonia S. Rex is an undergraduate in the Honors College of Oakland University. She combines major studies in biochemistry, while continuing to perform in the modern dance and ballet programs at the University. She plans to go to medical school.

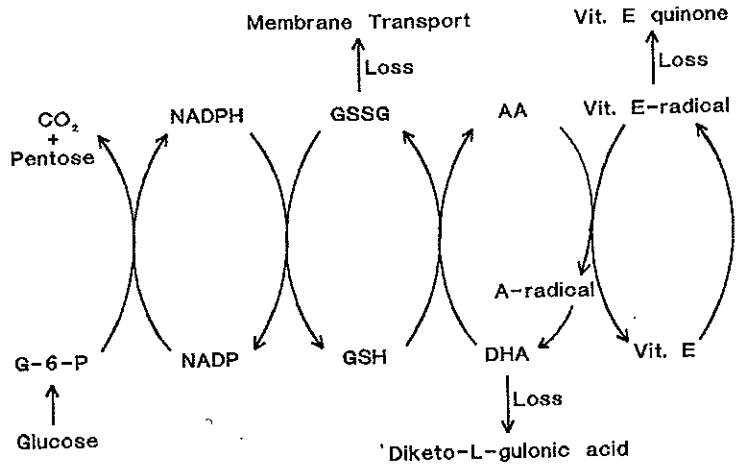


Fig. 1. The main features of the cellular antioxidant cascade are shown, with emphasis on the cyclical redox reactions, which link glutathione (GSH), ascorbic acid (AA), and vitamin E (Vit.E). The oxidized forms of these compounds, respectively, are glutathione disulfide (GSSG), semidehydroascorbate radical (A-radical) and dehydroascorbic acid (DHA), and vitamin E-radical (Vit. E radical). The reduction of GSSG requires NADPH as a cofactor (which is produced by cellular glucose metabolism) and is catalyzed by glutathione reductase (not shown). The reduction of DHA to AA occurs nonenzymatically; as discussed in the text, it is by no means certain whether in animal tissues this reaction is catalyzed by an enzyme. There is also uncertainty whether the reduction of Vit. E-radical by AA is enzyme-catalyzed. Also shown are several nodal sites where the oxidized forms of the compounds may be lost from the cycles: GSSG may exit from cells; DHA may be irreversibly degraded to diketo-L-gulonic acid; and Vit. E-radical may be converted to the relatively nonreactive Vit. E quinone.

of the fact that their fundamental constitutions and physiological functions are so different they have certain qualities in common. Both agree for instance in the circumstance that though their reduced and oxidized forms may co-exist in a tissue, they form redox systems which are not thermodynamically but only chemically reversible. Meanwhile, the question arises whether, as reducing substances with different redox potentials, they can exert combined activities, or display interrelations of importance'' (p. 1446). The partial redox reactions that underlie the energetically feasible coupling between glutathione and ascorbic acid, and that are the focus of this article, are:

$$2GSH \rightarrow GSSG + 2H^+ + 2e^- \ (+0.23V) \quad (1)$$

$$DHA + 2H^+ + 2e^- \rightarrow AA \ (+0.08V) \quad (2)$$

$$2GSH + DHA \rightarrow GSSG + AA \ (+0.31V) \quad (3)$$

Today, nearly 60 years later, the question posed by Hopkins and Morgan is largely unanswered. This article attempts to provide some answers to this question. Our approach includes a retrospective analysis of published accounts, together with prospective speculations that have emerged as a result of recent and novel experiments in our laboratory.

Szent-Gyorgyi[2] was the first to show that the oxidized form of hexuronic acid (the earlier name for ascorbic acid) is reduced by glutathione. Hopkins and Morgan extended this result and provided extensive quantitative data on the rate of oxidation of ascorbic acid in aqueous medium under different conditions, for example, different pHs, on the catalytic role of copper, and on the reduction of the oxidized form of ascorbic acid (now called dehydroascorbic acid) by glutathione. Borsook et al.[3] also published a comprehensive series of experiments on the redox coupling between glutathione and dehydroascorbic acid (interestingly, this article was submitted on June 8, 1936, whereas Hopkins and Morgan submitted theirs on June 30, 1936!). Borsook et al.[3] emphasized that dehydroascorbic acid is unstable and that its half-life at physiological pH and 37°C is only a few minutes. Moreover, they conducted the first major set of physiological experiments by following the fate of dehydroascorbic acid and ascorbic acid when these were added to blood, plasma, and intact isolated tissues, and also after ingestion. In 1941, Crook[4] wrote that ''the ability of tissue extracts from plants and animals, either to inhibit the oxidation of ascorbic acid or to reduce added dehydroascorbic acid ('DHA') is well known'' (p. 226). Work over the past 50 years in many laboratories,[5–30] including our own,[31,32] using a variety of plant and animal tissues, has confirmed and extended these early observations. The first part of this article (the chemical perspective) examines the dependence of the reaction between glutathione and dehydroascorbic acid in aqueous media on the physicochemical conditions, such as reactant

concentrations, pH, and temperature. This description sets the stage for the second part of the article (the physiological perspective), which deals with the interaction between glutathione and ascorbic acid under very specific physiologically relevant conditions in cultured cells and intact tissues.

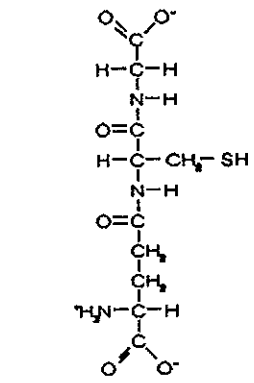
Interest in the interaction between glutathione and ascorbic acid relates directly to their roles in biological oxidations, a point that was well recognized by Szent-Gyorgyi.[2] It is undisputed that a trilogy of naturally occurring compounds is central to the intracellular antioxidant defense systems of mammalian cells. These compounds are glutathione, ascorbic acid, and vitamin E. Glutathione and ascorbic acid are water soluble and carry out their roles principally in the cytoplasm and mitochondria, whereas vitamin E is the principal chain-breaking, lipid soluble antioxidant in membranes. Each compound is a major free radical reductant in cells; these actions are distinct from the scavenging of oxidants and free radicals by enzymes like catalase, glutathione peroxidase, and superoxide dismutase. For reviews on the antioxidant roles of glutathione, ascorbic acid, and vitamin E, the reader is urged to consult the references.[33–48]

What makes glutathione, ascorbic acid, and vitamin E special is that they interact in a series of coupled oxidation–reduction reactions. These cyclical reactions are illustrated in Figure 1 (see legend for details), with apologies to the many authors who have previously published this scheme in one form or another over many years. It nevertheless is worth emphasizing that the regeneration of the reduced forms of the compounds by the set of coupled reactions provides for amplification of the antioxidant capacities of each compound. In the absence of redox coupling, the quenching of oxidants would be limited by the intracellular concentration of each compound. Although there is considerable experimental evidence linking the oxidation of glutathione to glutathione disulfide (GSSG) with stimulation of the glucose-dependent hexose monophosphate pathway, which yields the NADPH used by glutathione reductase in the regeneration of glutathione,[41,49–52] there is virtually no information on the extent to which the other cyclical reactions in Figure 1 (glutathione and ascorbic acid, and ascorbic acid and vitamin E) are linked in cells exposed to an oxidative insult. Indeed, Melhorn[25] recently stated that "the efficiency of vitamin C (ascorbic acid) regeneration has not been resolved" (p. 2724), although he concluded that the coupling is adequate, because following an oxidative challenge, red blood cells fed dehydroascorbic acid were able to again form ascorbic acid. To evaluate the physiological significance of the redox couple between glutathione and ascorbic acid (the interaction between ascorbic acid/vitamin E will be left to others[53–63]), we have followed the approach of Melhorn[25] and have employed a nitroxide, 4-OH Tempo (TEMPOL),[64–72] to specifically oxidize ascorbic acid in cells and tissues with the goal to answer the following question: To what extent can endogenous ascorbic acid be regenerated following an oxidative challenge? Our results with cells and tissues support our earlier experiments[31,32] in simple buffered solutions: following an oxidative challenge, the reduction of dehydroascorbic acid is not fast enough to offset its irreversible degradation under physiological conditions of temperature (37°C) and pH (7.4). Thus, the regeneration of endogenous ascorbic acid in cells is incomplete. The consequences of the inadequacy of this regeneration are addressed more fully in the physiological perspective section. Our intent is not to convince the reader that we have solved the question regarding the efficiency of redox coupling between glutathione and ascorbic acid, but rather to emphasize that new approaches are needed to provide unambiguous answers to the question posed by Hopkins and Morgan[1] many years ago.

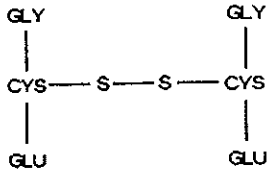
THE CHEMICAL PERSPECTIVE

For the sake of clarity, all the results were obtained in experiments performed in our laboratory, and they are displayed in the figures in a standardized fashion. This is intended simply to facilitate their presentation. As noted in the introduction, there is already an extensive literature that deals with the direct chemical reaction between dehydroascorbic acid and glutathione, and our findings should not be viewed as novel. Rather, they serve as an excellent summary composite of the many different investigations over the years. For convenience, the structures of the compounds are shown in Figure 2, along with their abbreviations, which will be used in the text that follows: glutathione, GSH; glutathione disulfide, GSSG; ascorbic acid, AA; dehydroascorbic acid, DHA; semidehydroascorbic acid, A-radical.

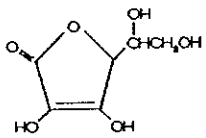
There are probably few experiments that are easier to perform than mixing GSH and DHA together and monitoring the formation of AA in an aqueous medium. In part, this is because a simple spectrophotometric method is available for monitoring AA formation, which is based on the change in absorbance at 265 nm; absorbance units are converted to concentration units using a molar extinction coefficient of 12.5. The results of a typical set of experiments of the sort just noted, as a function of the pH of a 0.1 M sodium phosphate solution, are demonstrated in Figure 3. At



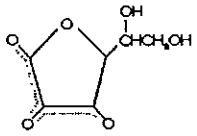
REDUCED GLUTATHIONE (GSH)



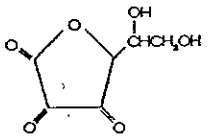
GLUTATHIONE DISULFIDE (GSSG)



ASCORBIC ACID (AA)



SEMIDEHYDROASCORBIC ACID (A-radical)



DEHYDROASCORBIC ACID (DHA)

Fig. 2. The structures of the pertinent compounds discussed throughout the text.

the start of the experiment, each 1-ml cuvette contained 5 mM GSH. The DHA was always freshly prepared as a concentrated solution in distilled water and kept on ice, both the low pH and low temperature serving to stabilize the compound.[1,3] After 2 min (at the arrow), 0.1 mM DHA was added. It is clear that the rate and amount of AA formed is dependent on pH. At pH = 8.1, the reaction was fastest, and more than 90% of the DHA was converted to AA. Reaction rates were slower, and the steady-state amount of AA formed was lower as the pH was decreased. Indeed, at pH = 6.15, there was virtually no reaction between GSH and DHA, although at this pH the DHA is quite stable. The dependence of the reaction on pH suggests that the GS^- moiety is the form of GSH that reacts with DHA. Consistent with this suggestion is the finding that the rate of AA formation at pH = 8 is about 10-fold greater than the measured rate at pH = 7.0. Figure 4 is a summary graph showing averaged data from many experiments in which the initial rate of AA formation is plotted against the concentrations of GSH and DHA. The family of curves shows that the reaction rates are directly related to the concentrations of reactants. These rates were low, for example, less than 2 nmol/min, when the starting DHA concentration was < 0.02 mM for all GSH concentrations (1–5 mM) tested. On the other hand, the initial velocity was about 17 nmol/min with 0.2 mM DHA and 5 mM GSH.

Figure 5 compares the effectiveness of several compounds that, from redox potential considerations alone, might be expected to reduce DHA to AA. Of the compounds shown, neither α-ketoglutarate nor lactate reduce DHA, whereas lipoic acid reduces DHA at a considerably faster rate than does GSH. Although we show here that lipoic acid at < 1 mM rapidly reduces DHA, this concentration is orders of magnitude greater than its cellular content, which is primarily found bound to protein complexes. Therefore, it is reasonable to assume that lipoic acid is not likely to participate to any significant extent in the reduction of DHA in cells. Other compounds that were tested and that failed to directly reduce DHA include NADH, NADPH, and ethanol.

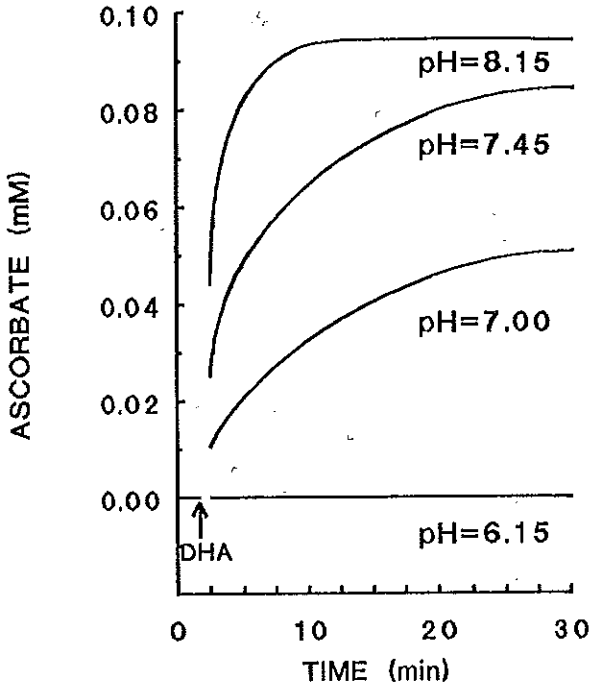


Fig. 3. Spectrophotometric recordings (265 nm) of the production of ascorbic acid that result from the direct, nonenzymatic reaction between dehydroascorbic acid (DHA) and glutathione. At the start, cuvettes contain 5 mM GSH in 0.1 M sodium phosphate buffered media at the different pHs illustrated. At arrow, 0.1 mM DHA is added (from concentrated solution). Upward-going tracings show increase in absorbance at 265 nm and, thus, formation of ascorbic acid, expressed in mM. Each curve represents the average of five individual experiments.

It has long been known that DHA is very unstable at physiological pH and irreversibly decomposes.[1] Its decomposition product, diketogulonic acid, does not react with GSH[3] and, as we have found, neither do the subsequent breakdown products, oxalic acid and threonic acid. Thus, it is the irreversible hydrolysis of DHA that is responsible for the failure to convert 100% of the starting amounts of DHA to AA at any of the pHs tested in Figure 3. The greater the rate of hydrolysis relative to the rate of reduction, the lower will be the final percentage recovery of AA. Conversely, the lower the rate of hydrolysis, the greater will be the extent of recovery. Indeed, it was Borsook et al.[3] who so carefully documented that "the irreversible change in dehydroascorbic acid begins at about pH = 4 and becomes progressively faster with increasing pH above this point" (p. 245). We have addressed this issue with the following sets of experiments whose results are shown in Figures 6–8. In the protocols 0.1 mM AA was added to 1 ml of phosphate buffered media at different pHs (Fig. 6, pH = 7.4; Fig. 7, pH = 8.1; Fig. 8, pH = 6.15). As others have done, we added 0.001 mM cupric ions to promote the autoxidation of AA in these room temperature experiments.[31] The decrease in AA concentration due to cupric ion catalyzed autoxidation was followed over time. In Figures 6–8, this decrease is represented as the continuous downward tracing. A comparison of these tracings reveals that the rate of autoxidation is increased with increasing pH. The rate of reappearance of AA following the addition of 5 mM GSH (at arrows) at various times during the ongoing process of autoxidation is also shown in Figures 6–8. At pHs 7.4 and 8.1, the addition of GSH led to recovery of AA, but the extent of recovery was decreased the longer the elapsed time between the inclusion of cupric ions and the addition of GSH. The magnitude of recovery was also less when the experiment was carried out at pH = 8.1 in comparison to pH = 7.4. Thus, when GSH was added back after 15 min, about 30% of AA was regenerated at pH = 8.1, but more than 60% was recovered at pH = 7.4. These are but two of many examples in the literature showing that DHA hydrolysis is a significant variable in the ability of GSH to regenerate AA. Interestingly, at pH = 6.15, the addition of GSH slowed the ongoing rate of autoxidation, due to

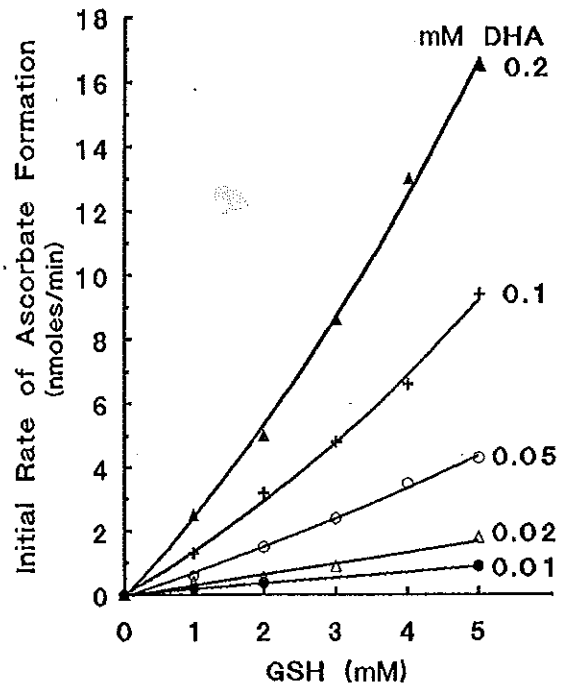


Fig. 4. A summary graph of the initial rates of ascorbic acid that is formed, expressed as nmol/min, as a function of the concentrations of glutathione and dehydroascorbic acid. Initial rates were obtained typically during the first minute following addition of dehydroascorbate to the glutathione-containing media. During this period, the rates were linear. Phosphate-buffered medium at pH = 7.4. Each point is the average of at least 5 experiments.

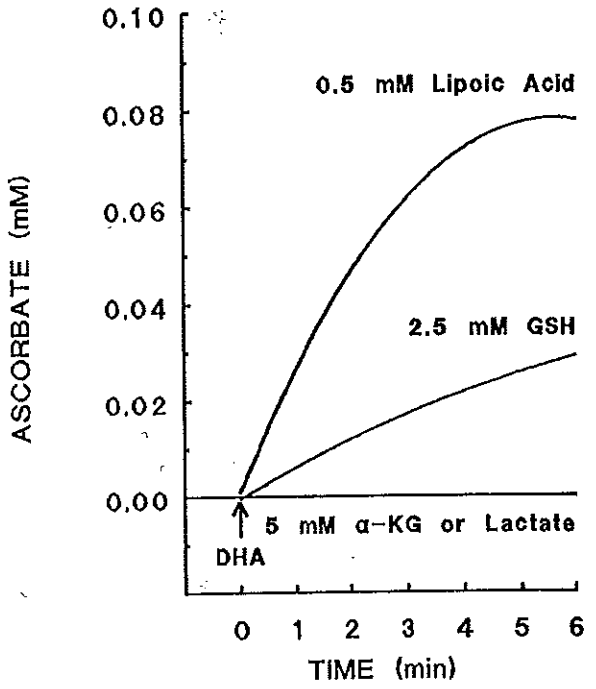


Fig. 5. A graph of the effectiveness of various compounds in reacting with 0.1 mM dehydroascorbic acid to form ascorbic acid, expressed in mM. Clearly, lipoic acid is more effective than glutathione, but neither α-ketoglutarate nor lactate reduce dehydroascorbic acid (n = 4 for each compound, except GSH where n = 10).

chelation of the cupric ions,[1,3,31] but failed to increase the level of AA. This is consistent with the results in Figure 3 showing that GSH and DHA do not react at any significant rate at this low pH. So, although it is well known that low pH protects DHA from hydrolysis, it also inhibits the direct reaction between GSH and DHA; perhaps other reducing agents are more effective in an acidic environment and could be used to reduce DHA, although the physiological relevance of such a result would be uncertain, at least for mammalian tissues.

In addition to a dependence on pH, the stability of DHA in aqueous medium is influenced by temperature. The results presented in Figures 3–8 were carried out at room temperature (20–22°C), but we were also interested to evaluate these effects at 37°C, the temperature most relevant to the physiology of mammalian tissues. Figure 9 illustrates the results of an experiment in which the autoxidation of AA, catalyzed by 0.5μM cupric ions, was followed at 37°C, and 5 mM GSH was added to the media exactly in the same manner as in the room temperature experiments.[31] Because the pH of this medium was 7.4, these results are directly comparable to the curves in Figure 6. Clearly, at 37°C the regeneration of AA by GSH is considerably less than at 23°C, consistent with a greater rate of DHA hydrolysis, and thus, greater irreversible loss at the higher temperature.

The oxidation of ascorbic acid proceeds in one-electron steps via an intermediary A-radical, which subsequently disproportionates yielding DHA and AA. Indeed, A-radical is enzymatically reduced to AA in plants and animal tissues by an NADH-dependent enzyme, that is, semidehydroascorbate reductase.[73–83] As indicated earlier, NADH does not directly reduce DHA. From thermodynamic considerations, GS^- would not be expected to react to any significant extent with A-radical (46). We thought, nevertheless, that it would be worthwhile to directly visualize whether any reaction occurs to diminish A-radical. Our approach involved adding ascorbate oxidase to phosphate buffered medium (pH = 7.4) containing 1 mM AA. This combination of enzyme and substrate led to the production of a steady level of A-radical, which was detected by ESR (Fig. 10). After 10 min, 5 mM GSH was added to the medium, and changes in the A-radical were subsequently monitored at 1, 5, and 15 min. It is clear that despite a nearly 1000-fold excess of GSH relative to A-radical, that is, mM vs. μM, the A-radical persisted. In fact, although not shown, the ESR signal was

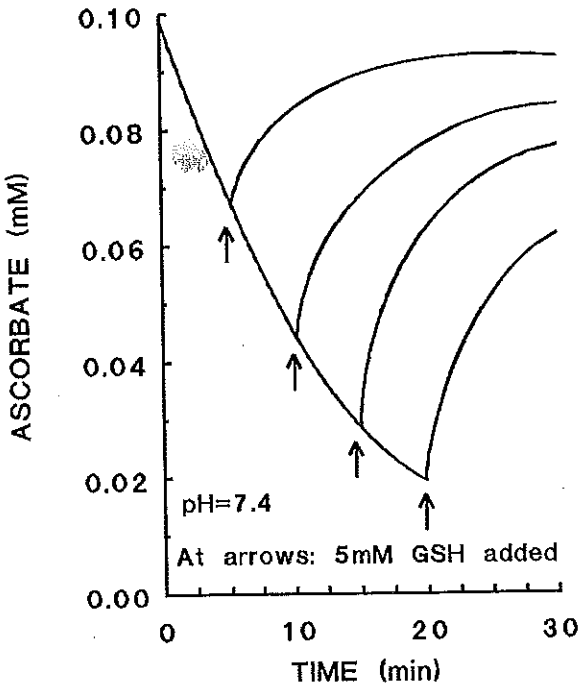


Fig. 6. The effects of adding 5 mM glutathione (arrows) at different times to media containing 0.1 mM ascorbic acid and 0.001 mM cupric ions. Media conditions: Phosphate-buffered, room temperature, pH = 7.4. Note rate and extent of recovery of ascorbic acid following the addition of glutathione. See text for details.

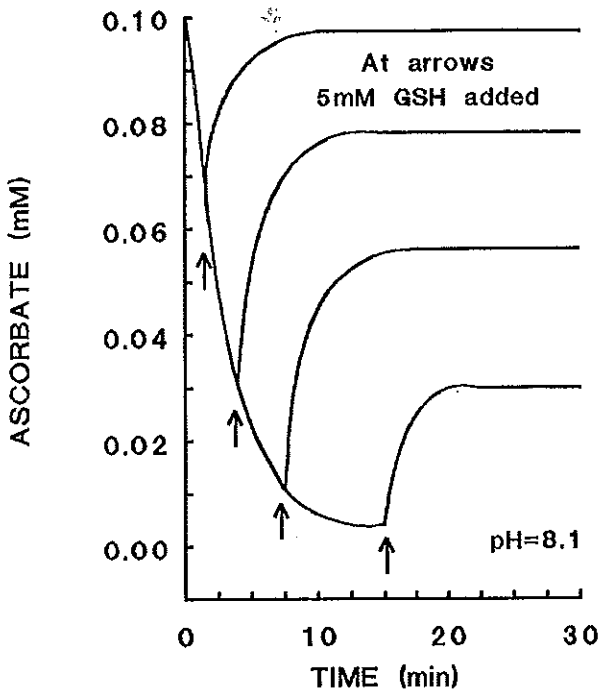


Fig. 7. Identical procedures as described in Figure 6, except experiment was conducted at pH = 8.1.

virtually unchanged 60 min later. Thus, no significant reaction was observed between GSH and A-radical, in accord with the approximate $k_{eq} = 2 \times 10^{-5}$. The two bottom tracings in Figure 10 show that the A-radical is not produced when only DHA is present or when DHA and GSH are mixed together. This latter finding may seem surprising, because AA is produced in this condition and it is believed that A-radical is generated in the presence of AA and DHA.[73] Experiments in which either 5 mM AA alone or a mixture of 5 mM AA and 2 mM DHA was tested (room temperature, air, pH = 7.4) showed that there was no significant difference in the A-radical that was formed (data not shown). Thus, under these conditions, there is no reaction between AA and DHA to form A-radical, its production depending only on the rate of oxidation of AA. Because GSH prevents the oxidation of the newly formed AA by chelating cupric ions,[1,3,31] this would seem to be the most likely reason why the A-radical signal is not observed.

Mechanistic considerations: One-electron vs. two-electron transfer

One might consider the mechanism of the reaction between GS^- and DHA. The overall reaction was given in the introduction. It is interesting to evaluate if this reaction could occur in two one-electron steps, as shown later.

$$GS^- + DHA \rightarrow GS^{\bullet} + A\text{-}rad^{\bullet -} \quad K_{eq} = 3 \times 10^{-19} \quad (4)$$

$$GS^{\bullet} + GS^- \rightarrow GSSG^{\bullet -} \quad (5)$$

$$GSSG^{\bullet -} + DHA \rightarrow GSSG + A\text{-}rad^{\bullet -} \quad (6)$$

$$2A\text{-}rad^{\bullet -} + 2H^+ \rightarrow AA + DHA \quad (7)$$

$$2GS^- + DHA + 2H^+ \rightarrow GSSG + AA \quad K_{eq} = 3.2 \times 10^{10} \quad (8)$$

It is clear that the first step is thermodynamically improbable based on the electrochemical potentials (46) of the partial reactions that lead to a calculated $K_{eq} = 3 \times 10^{-19}$. Although the overall reaction rate is favored because $K_{eq} = 3.2 \times 10^{10}$, if the mechanism involves these sequential steps, then the net rate would be very slow. It is possible to estimate the maximum rate of reaction (4) above for the condition where GS^- = 5 mM and DHA = 0.1 mM. With these concentrations and an estimated maximum forward rate constant (k_f) of 3×10^{-10} $M^{-1}s^{-1}$ (this rate constant was obtained using a maximum value for the reverse reaction (k_r), that is, the maximum diffusion controlled rate of 10^9 $M^{-1}s^{-1}$, and K_{eq}), we calculate the rate of the for-

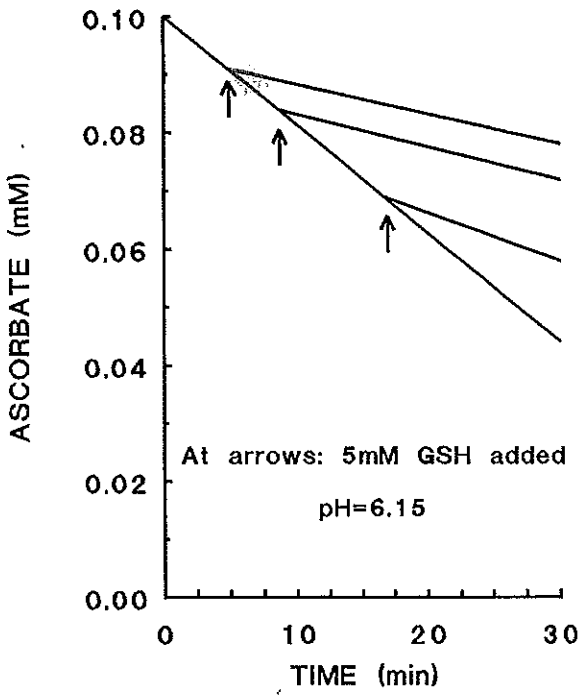
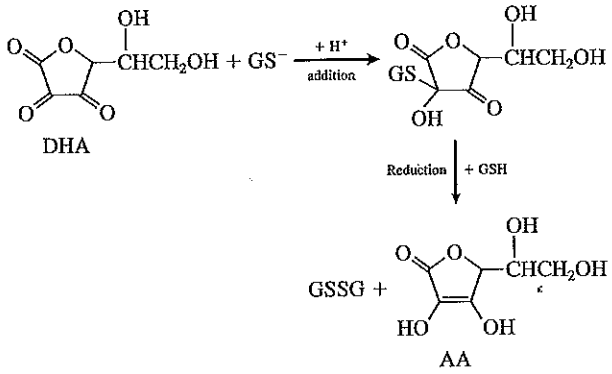


Fig. 8. Identical procedures as described in Figure 6, except pH = 6.15.

ward reaction (4) to be 1.5×10^{-16} Ms^{-1}. The data in Figure 3 (for pH = 7.0) show, however, that the initial rate of the formation of AA when the starting concentrations of GSH and DHA were 5 and 0.1 mM, respectively, amounted to approximately 8 nmol/min. Because the reaction was run in a total volume of 1 ml, this value converts to 1.3×10^{-7} Ms^{-1}. The fact that the measured rate of the reaction is much faster than can be accounted for by one-electron chemistry suggests that a two-electron transfer is a more likely possibility for the reaction between GS^- and DHA. A reasonable and comparatively simple possibility for the two-electron reduction of DHA by GSH is as follows:



The initial step is nucleophilic addition of the conjugate base of GSH to the central carbonyl of DHA followed by a reduction step by GSH that results in the formation of AA and GSSG.

Because lipoic acid has a lower pKa than GSH, it is perhaps not surprising that it is more reactive than GSH in reducing DHA (see Fig. 5). Given the arguments presented earlier, it also seems reasonable to suggest that reduction of DHA by the intramolecular dithiol lipoic acid is favored by entropy.

THE PHYSIOLOGICAL PERSPECTIVE

The major question under consideration concerns the efficiency of the redox couple between GSH and DHA in the regeneration of AA in cells exposed to an oxidative challenge. Burton et al.[84] recently cautioned "that even the most carefully modeled in vitro system may fail to reproduce the in vivo reality" (p. 205). In their particular case, they had failed to find evidence for an interaction between vitamin C and vitamin E in vivo, a conclusion that is completely at odds with the substantial in vitro data[53–63] showing that vitamin C reacts directly with the free radical form of vitamin E. In contrast, an interaction between GSH and AA is supported by both in vitro and in vivo studies. Indeed,

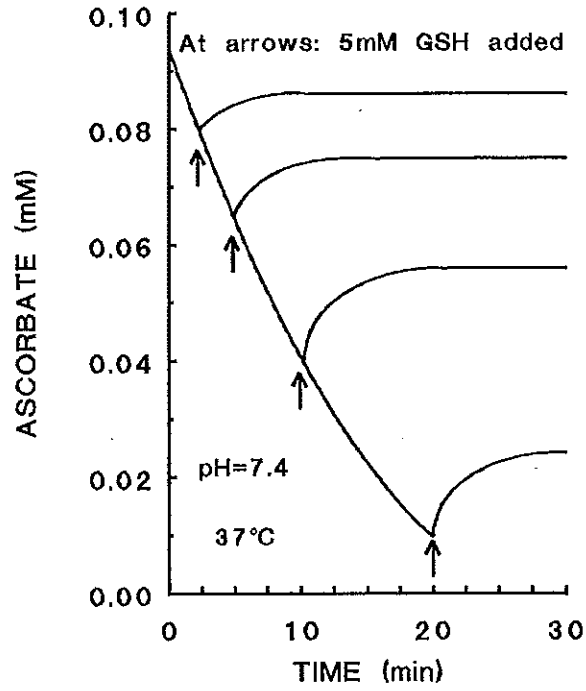


Fig. 9. Identical procedures as described in Figure 6, except temperature is 37°C.

Meister and colleagues,[45,85–92] in a continuing series of in vivo experiments that manipulated independently the cellular levels of GSH and AA, provide strong evidence that GSH and AA function together as antioxidants. The particular term used by Meister to describe the interaction between GSH and AA is "sparing" (the same term is used to describe the interaction between vitamins C and E). Meister defines "sparing" in two ways: (1) the reaction of GSH and AA with common cellular oxidants, for example, H_2O_2, thus the displaying of overlapping functions; and (2) promotion of the reduction of DHA to AA by GSH. Although not meant as a criticism of Meister's in vivo studies, there is, in fact, little direct evidence in his studies to support either of these possibilities. This is because there are no measurements of the levels of putative cellular oxidants under the different manipulative regimens, although it is recognized that this may not be feasible. With respect to the second sparing action, namely, the reaction between GSH and DHA, Mårtensson and Meister[87] report that following GSH depletion there is a marked decrease in AA and increase of DHA. However, there is some question regarding the validity of the DHA measurements. A spectrophotometric method was used that relies on measurements of AA and of total ascorbate (AA + DHA), the difference representing the level of DHA; HPLC methodology

would be a better choice for measurements of DHA.[24] In particular, they report that DHA accounts for 40% of the total ascorbate in a normal, clear lens, for example, 0.31 μmol/g DHA vs. 0.78 μmol/g total ascorbate in Figure 6 of reference 87. They raise the possibility that this oxidation may have occurred during surgical isolation of the lens. Yet, measurements of total lens glutathione over many years using surgical procedures probably very similar to those employed by Mårtensson and Meister reveal a high concentration of GSH and virtually no GSSG.[33,41] Despite these few concerns, it is clear that the studies of Meister and colleagues have provided significant, new information that in vivo GSH can spare ascorbate and that ascorbate can spare GSH.

One factor that would certainly influence the validity of applying conclusions from measurements of the rate of the nonenzymatic reaction between GSH and DHA in test tube experiments to events in cells is whether this reaction is catalyzed by an enzyme. Insofar as animal tissues are concerned, there is lingering uncertainty over this issue. For example, nearly 30 years ago, Schneider and Standinger[73] wrote that "whereas in plant cells there exists an enzyme system (dehydroascorbate reductase) catalyzing the reduction of dehydroascorbic acid to ascorbic acid, thus preserving the supply of ascorbic acid in the cell, an analogous enzyme does not occur in the animal cell" (p. 158). More recently, Melhorn[25] states that "although several mammalian tissues appear to contain semidehydroascorbate reductase, I am not aware of any convincing evidence for mammalian DHA reductase" (p. 2724). On the other side of the ledger, Rose and Bode,[103] in discussing an essentially identical version of the scheme shown in Figure 1 of this article, state that "one aspect that could be considered speculative is the indication that regeneration of ascorbic acid from dehydro-L-ascorbic acid by GSH is nonenzymatic" (p. 282). Although at face value, in view of the overwhelming evidence showing the direct, chemical reaction between GSH and DHA, the statement of Rose and Bode makes little sense, we interpret their statement to mean that they believe that the enzymatic reaction is much faster than the directly coupled reaction. A faster enzymatic reaction would thus reduce the contribution of the direct reaction, but it would most certainly not render it "speculative." Rose and colleagues have evaluated the reduction of DHA by GSH in homogenates of a variety of animal tissues and reported the process "had several characteristics of being enzyme-mediated" (p. 277).[103]

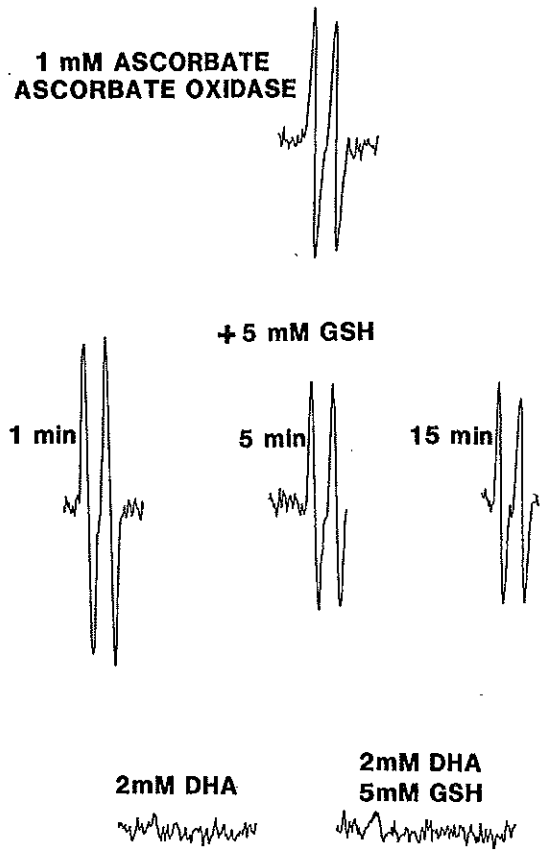


Fig. 10. First derivative ESR spectra of semidehydroascorbate radical (A-radical) under various conditions. Top trace: A-radical signal that is produced in the presence of 1 mM ascorbate and ascorbate oxidase. Middle three tracings: represent signals obtained 1, 5, and 15 min after 5 mM GSH was added to the sample. Bottom left tracing: 2 mM DHA alone does not produce A-radical. Bottom right tracing: A mixture of 2 mM DHA and 5 mM GSH does not produce A-radical. Two hyperfine components separated by 1.8G at g = 2.005 are characteristic of the A-radical.

To make sense of this apparent controversy over the direct vs. enzymatic reaction between GSH and DHA, it seems prudent to take a closer look at the literature. DHA reductase activity was detected in haemolysates of human erythrocytes by Hughes.[6] This is a widely quoted article by investigators who favor the existence of the enzyme. Yet, it has been completely overlooked that Hughes noted that "activity measurements above pH = 7.0 were difficult because of the increasing rapidity of the chemical (that is noncatalyzed) reaction with increase in pH above 7.0" (p. 1069). Essentially the same problem was encountered by Bigley et al.,[14] who wrote that "the pH optimum of the enzymatic reaction is close to 7.4. Since the rapidity of the nonenzymatic reaction increases measurement error in the higher pH range the standard assay was performed at pH = 6.85" (p. 18). In fact, a careful inspection of Figure 1 (p. 18) of the Bigley et al. article shows that at pH = 7.4 there was no

significant difference between the direct and catalyzed reaction rates. Stahl et al.[17] reported that detection of DHA reductase activity depended on the method used to measure the activity. When a coupled assay monitoring the oxidation of NADPH by glutathione reductase (340 nm) was used, evidence for an enzyme-catalyzed reaction was obtained. In contrast, when Stahl et al. used a direct spectrophotometric assay (265 nm), no evidence was found for an enzyme. In their article, they write that "although the discrepancy between the direct and coupled assays remains unresolved, the possibility exists that with the coupled assay GSSG generation by another pathway in homogenates accounts for an apparent reaction rate above that of the nonenzymatic blank" (p. 121). It has recently been proposed that GSH-dependent reduction of DHA is catalyzed by glutaredoxin and protein disulfide isomerase.[22] There are two aspects of the article by Wells et al.[22] that deserve comment. First, the maximum velocity of the reaction catalyzed by porcine recombinant liver thioltransferase was higher when the coupled assay rather than the direct assay was employed; this supports the comment of Stahl et al.[17] Second, and more important, is the pH data of Wells et al. They report that the optimum pH was 6.8 "in agreement with the studies of Bigley et al." (p. 15362; see reference 14). But, in fact, Wells et al. misquote Bigley et al. (see previous text for correct pH optimum). Thus, the extent to which glutaredoxin catalyzes the reduction of DHA at pH 7.4 remains unclear. This leaves the recent reports of Choi and Rose,[20] Rose,[21] Bode et al.,[30] and Rose and Bode[103] as the only other studies presenting evidence in support of DHA reductase in animal tissues. It should be emphasized that, on a quantitative basis, the optimum enzyme-catalyzed reaction rates reported in these several articles are typically less than 1 nmol/min/mg protein, a very low rate of activity, indeed, and considerably below the nonenzymatic rate under comparable conditions. In contrast, the work of Coassin et al.[27] (pig tissues) and Melhorn[25] (human erythrocytes) find no evidence for DHA reductase activity. Furthermore, work in our laboratory,[32] with cytosolic supernatants of a variety of ocular tissues from rat and rabbit, has also failed to detect the presence of DHA reductase. An example of our experiments, using rat retina, is shown in Figure 11. It can be seen that the nonenzymatic rate of AA formation from the reaction between GSH and DHA continues (pH = 7.4) unchanged following the addition of the retinal supernatant. The same result, that is, no difference in reaction rate before and after retinal supernatant, was obtained when the experiments were conducted at pH = 6.8 and when either 0.2 mM NADPH or 0.2 mM NADH was included in the assay medium. Taken together, the available evidence leads us to conclude that the nonenzymatic, direct reaction between GSH and DHA is the major physiologically relevant reaction underlying the reduction of DHA to AA in mammalian tissues.

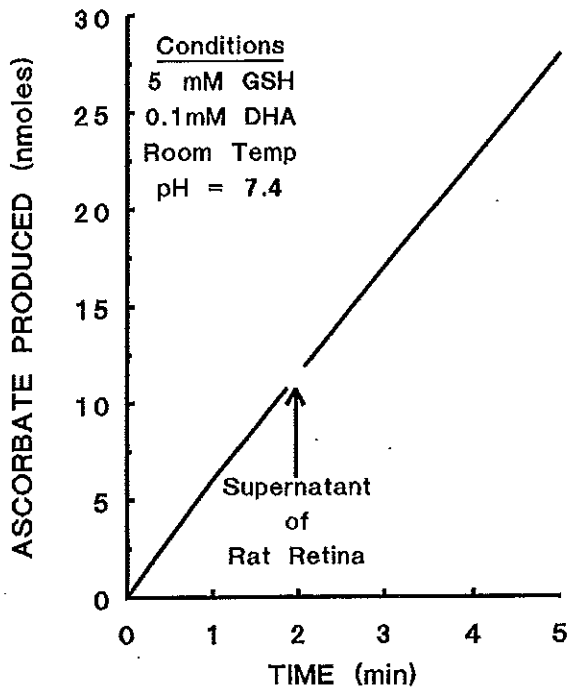


Fig. 11. A comparison of the averaged rate of production of ascorbic acid due to the reaction between 5 mM glutathione and 0.1 mM dehydroascorbic acid before and after the addition of a cytosolic supernatant of rat retina (n = 8). The graph reveals that there is no significant difference in the rate of reaction. The assay conditions are as shown in the graph; the buffer was 0.1 M sodium phosphate. Preparation of retina was as follows: a rat retina was homogenized in 0.5 ml of buffer, centrifuged at 10,000 g for 15 min, and the clear supernatant was used in the assay. Aliquot size ranged from 50 to 200μl. Total volume in cuvette was 1 ml. One rat retina contains approximately 1.2 mg protein.

In the long history of studies involving incubation of cells and tissues with DHA, a clear-cut and reproducible result has been reported, almost without exception: cells rapidly take up DHA and reduce it to AA.[1–4,6–17,20,21,25,27,30,93–106] Many of these studies, as well as others,[107,109] have also shown that AA is taken up by cells, but generally at a slower rate in comparison to the rate of uptake of DHA under similar conditions. The one exception cited earlier is found in the study of Hornig et al.[93] in which it was reported that guinea pig erythrocytes took up DHA but did not convert it to AA in any appreciable amount, that is, mainly DHA was found in the cells. We note that Hornig et al. carried out their incubations at pH = 6.3–6.4. As shown in Figure 3, at this low pH there is virtually no

reaction between GSH and DHA. We believe this is the reason why Hornig et al. found little AA in the cells fed DHA. Indeed, we suggest that incubations at this low pH may be an excellent way to "trap" DHA in cells! With respect to the mechanism of DHA uptake, Vera et al.[105] provide direct evidence, which confirms and extends earlier suggestions,[10,97,110] that DHA is transported into cells by the glucose transporters. In the case of AA uptake, its transport has been reported, depending on the tissue, to be Na-dependent,[94,100,101,107] Na-independent,[94] or sugar-dependent.[108]

That cells take up DHA and readily convert it to AA is a significant experimental advantage for several reasons. First, the experimentalist has the opportunity to manipulate the intracellular levels of these compounds using straightforward procedures: cells are simply incubated in the presence of DHA for variable periods of time, and then the levels of DHA and/or AA are measured. Second, when the incubation medium contains only DHA, the subsequent measurement of AA in the cells reflects the unambiguous intracellular reduction of DHA to AA, essentially uncontaminated by the external environment. This set of circumstances can be contrasted with the situation involving GSH/GSSG, because, as Meister[45] has carefully documented, neither of these compounds is efficiently transported into cells. It is for this reason that Meister chose an inhibitor of GSH synthesis, L-buthionine-SR-sulfoximine, and a GSH analogue, GSH monoethyl ester, respectively, to decrease or increase the intracellular level of GSH.

We are now in a position to close this discussion by focusing on an evaluation of the physiological efficiency of the redox coupling between GSH and DHA. Let us consider an hypothetical cell that contains known amounts of GSH and AA. The function of the redox couple is best probed using an agent that specifically oxidizes AA to DHA and that has no direct interaction with GSH. Following the oxidizing episode, the level of AA and GSH is measured over time. A comparison of the concentrations of GSH and AA before and after the oxidative insult yields an estimate of the efficiency of the couple, full recovery of AA reflecting 100% efficiency. The first question is whether there are available agents with the required specificity. The more commonly employed cellular oxidants, such as H_2O_2 and t-butylhydroperoxide (tBHP), react nonenzymatically with both GSH and AA and, therefore, are poor choices in this experimental paradigm. A better choice, as reported by Melhorn,[25] involves the use of nitroxides that are reduced intracellularly by AA. Indeed, Melhorn used nitroxide reduction kinetics and spin trapping to evaluate the free radical

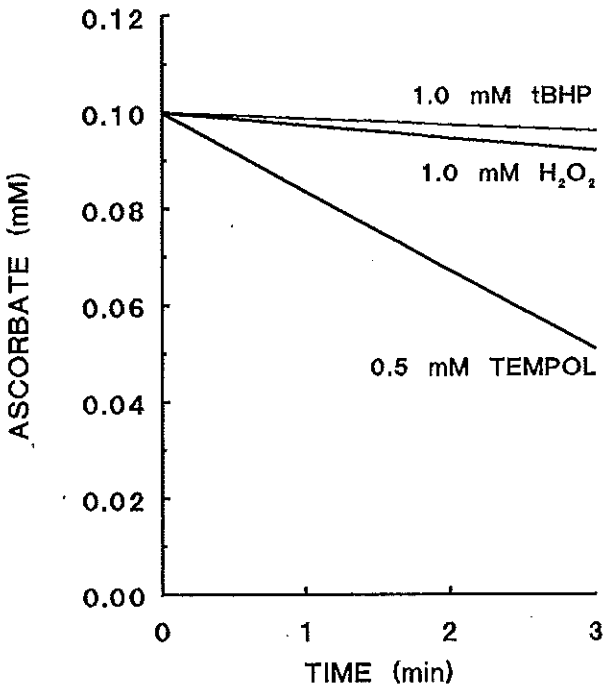


Fig. 12. A comparison of the rate of loss of ascorbic acid in a 0.1 M sodium phosphate (pH = 7.4) solution (room temperature) that contains either 1.0 mM tertiary butylhydroperoxide (tBHP), 1.0 mM hydrogen peroxide (H_2O_2) or 0.5 mM TEMPOL.

scavenger role of AA in human red blood cells. We can confirm that the nitroxide, 4-hydroxy tempo (TEMPOL), effectively oxidizes AA but spares GSH. In Figure 12, we show that TEMPOL oxidizes AA at a rate that is considerably faster than the oxidation of AA by either H_2O_2 or tBHP. That TEMPOL does not oxidize GSH in a phosphate buffered medium (pH = 7.0–7.4) was shown in other experiments in which 1mM GSH and 5mM TEMPOL were incubated together for 5–60 min. Subsequent measurements of GSH[31] (essentially a set of standard curves) revealed that the concentration of GSH was unchanged over the duration of the experiment (data not shown).

Melhorn[25] carried out clever and highly original experiments that showed that human erythrocytes could be depleted of AA using the nitroxide DMTA (N,N,-dimethylamino-2,2,6,6-tetramethyl piperidine-N-oxyl). The level of AA was not directly measured in his study but, rather, was inferred from the rate of reduction of DMTA. For example, Melhorn stated that "repeated washing of the cells in the presence of 1 mM DMTA substantially decreased intracellular ascorbate, as judged by the greatly diminished initial nitroxide reduction rate" (p. 2726). Furthermore, and most relevant to this discussion, Melhorn reported that "treatment of ascorbate-depleted cells with DHA re-

stored nitroxide reduction activity and yielded a markedly nonlinear relationship between DMTA reduction rates and DHA concentrations'' (p. 2726). This result, however, does not address the issue of the efficiency of the redox couple between GSH and DHA, because the cells were resupplied with DHA after incubation with DMTA. Rather, the correct implication of the finding as stated by Melhorn (and with which we agree), is that ''the restoration of nitroxide reducing activity when ascorbate-deplete cells were incubated with DHA indicates that thiols are not depleted and the pentose phosphate pathway is not substantially impaired by the procedure'' (p. 2729). We wish to be very careful in our interpretation of the experiments of Melhorn, because his article is the seminal and, to date, only publication to our knowledge that uses a nitroxide to specifically oxidize AA inside a living cell.

We have both followed and extended in several important ways the example of Melhorn[25] to further dissect and probe the physiological interaction between GSH and AA in control and oxidatively challenged monolayer cultures of human retinal pigment epithelial (RPE) cells. Measurements were made of the cellular levels of AA[111] and GSH[52] before, during, and after a 15-min exposure of the cells to 1 mM TEMPOL, a nitroxide analog of DMTA that rapidly penetrates and exits from cells.[70] The results of these experiments, conducted at 37°C and pH = 7.4, are displayed in Figure 13A–F (see legend to Fig. 13 for methodological details). At the start of the experiments, these 2nd–4th-passaged, confluent cell cultures contained no detectable AA, whereas the GSH content at t = 0 was 68 ± 3 nmol/mg protein (n = 15 ± SD). We therefore incubated the cells for 30 min with either 2 mM DHA (panels A–C) or 2 mM AA (panels D–F) to promote the uptake and, in the case of DHA, its reduction to AA. It can be seen that incubating the cells in serum-free MEM containing 2 mM DHA led to the rapid appearance of AA, 200 nmol AA/mg protein being formed, and that much of this AA was retained in these cells when they were subsequently incubated in DHA-free MEM medium (control; open bar above each graph). In panel B, we show that a 15-min exposure to 1 mM TEMPOL caused nearly a 75% loss in the cellular content of AA. Based on the known chemistry and thermodynamic properties of the reaction between TEMPOL and AA (46), it is reasonable to assume that DHA is a product of this reaction in the cells, although direct measurements of DHA have not yet been made. When these cells were returned to DHA-free MEM medium, there was only a small, incomplete recovery of AA over the next 60 min. To our knowledge, this represents the first attempt in the literature to measure the extent of the endogenous recovery of AA in oxidatively challenged cells. The poor recovery of AA under this condition suggests a low efficiency in the redox regeneration of AA in human RPE cells. In contrast to the incomplete recovery shown in panel B, resupplying DHA (panel C) to AA-depleted cells (the Melhorn experiment) led to full recovery of AA, that is, the levels of AA were the same before and after exposure of the cells to TEMPOL. This shows that TEMPOL, like DMTA,[25] does not disrupt GSH-dependent reduction of exogenously supplied DHA. Although not shown, the GSH content of the cells was not significantly different from the averaged t = 0 value at all time points during the experimental paradigms. Essentially the same observations were made when AA was supplied to RPE cells (panels D–F). Thus, RPE cells took up AA, TEMPOL caused a decline in its concentration, recovery of AA was incomplete when cells were returned to MEM, but it was complete when cells were resupplied with AA.

That GSH is required in RPE cells for the reduction of exogenous DHA was shown in separate experiments in which cells were pretreated with 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU),[52] which irreversibly inactivates glutathione reductase, prior to incubating the cells with 2 mM DHA. In this case, we found that less than 20 nmol AA/mg protein were formed, and GSH content declined by approximately 75% after 30 min in DHA-containing medium. A substantial loss of GSH has also been found in lens epithelial cells treated in a similar way, that is, pretreatment with BCNU followed by exposure to DHA.[112] As expected, treatment of RPE cells with BCNU did not prevent the cells from accumulating AA, when the cells were incubated in the presence of 2 mM AA.

An assumption in the foregoing discussion is that DHA is produced intracellularly as a result of treatment of RPE cells with TEMPOL. Thus, the failure to regenerate AA (panels B and E) when cells are returned to MEM suggests that the coupling between GSH and the endogenously produced DHA is inefficient, probably because the rate of reduction is slower than the rate of hydrolysis of DHA. However, we have not yet measured the DHA content in TEMPOL-treated cells. We have tried to obtain some information on this point in test tube experiments similar to those shown in Figure 6. We have found that when TEMPOL, instead of cupric ions, is used to promote the oxidation of AA, the addition of GSH leads to partial recovery of AA. Because GSH does not react with A-radical (Fig. 10), the observed recovery of AA must be due to reaction with the DHA that was formed during the TEMPOL-induced oxidation of AA. Still, measurements of the

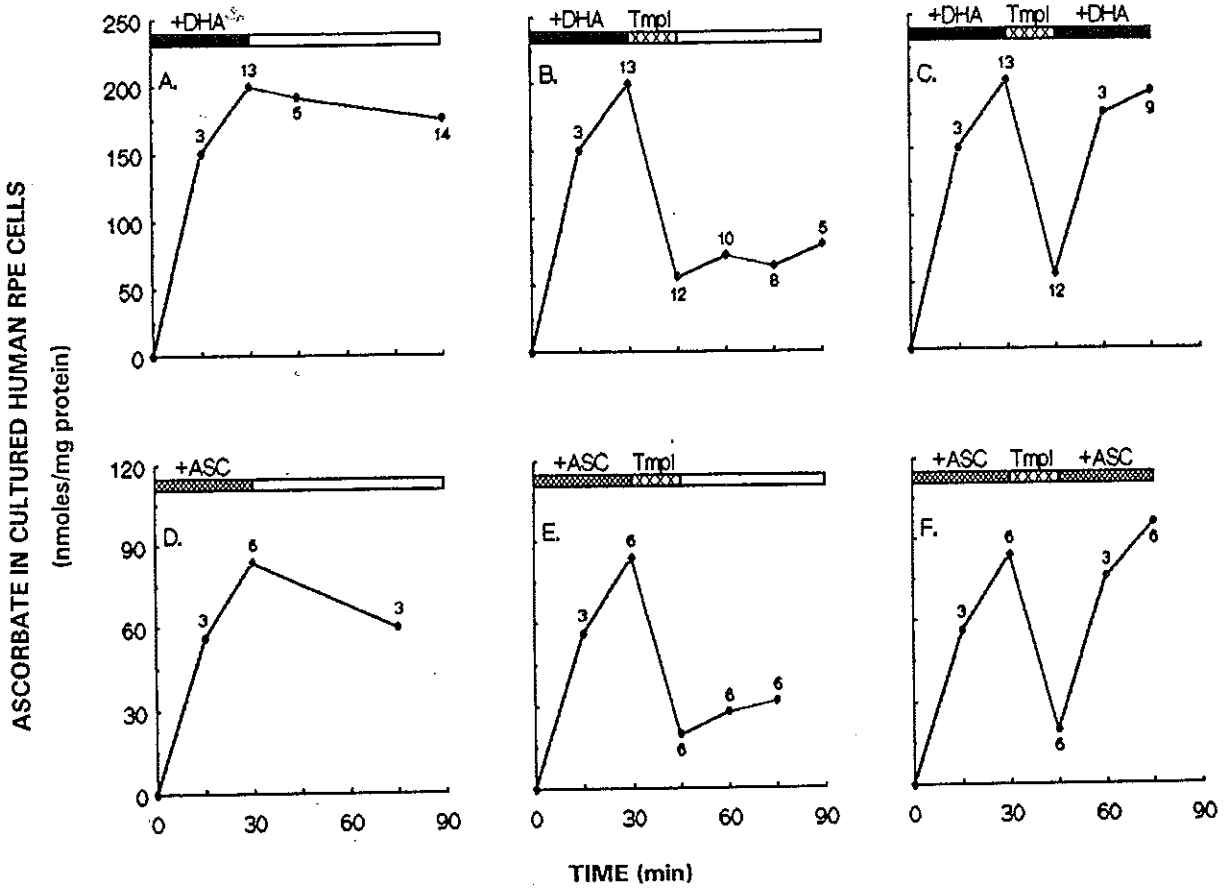


Fig. 13. Levels of ascorbic acid, expressed as nmol/mg protein, in monolayer cultures of human retinal pigment epithelial (RPE) cells. In panels A–C, cells are incubated in MEM medium containing 2 mM DHA (solid bar) for 30 min. Following this load-up period, the cells are incubated as follows: Panel A, cells are returned to control, MEM media (open bar); panel B, cells are incubated with 1 mM TEMPOL (Tmpl) for 15 min (cross-hatched bar) and then returned to control, MEM media; Panel C, cells are incubated with 1 mM Tmpl for 15 min and then incubated with MEM media containing 2 mM DHA. Panels D–F: sequence is similar to that described for panels A–C, except ascorbic acid is present in MEM media where noted. Methods are as follows: RPE cells are grown to confluency in MEM media containing 10% fetal calf serum. When confluent (60 mm culture dishes, 3–4 ml media per dish), the growth media is replaced with serum-free MEM media, which is then used throughout the incubations. Dehydroascorbic acid (DHA) is freshly weighed out 5 min before needed, while ascorbic acid (AA) is made as a 100-fold concentrated solution and diluted to appropriate concentration just before use. All incubations are performed at 37°C in the presence of 20% O_2/75% N_2/5% CO_2, and pH of all media is 7.4. At any given time in the experiment, the culture dishes are rinsed 3 times with ice cold 0.9% NaCl, and the cells (typically 2×10^6 cells/dish) are scraped into a total volume of 0.5 ml of 5 mM EDTA. Then 0.1 ml of 20% TCA is added. The suspension is sonicated for 1–2 min, centrifuged at 10,000 g for 10 min. Ascorbic acid in the clear supernatant is determined according to the method of Omaye, Turnbull, and Sauberlich.[111] The most important part of the assay is the inclusion of PCMB to tie up SH groups, because otherwise the assay reads SH as well as ascorbic acid, and this may lead to a serious overestimate of the concentration of ascorbic acid. Ascorbic acid standards are run each and every experiment. Glutathione is measured using the standard DTNB reaction.[52] The numbers next to the data points represent the number of individual culture dishes that are analyzed. Although not shown, standard deviations are all less than 10% of the averaged value at any given time point. Microscopic evaluation of the confluent RPE cells revealed that the cells were normal at the end of each experimental regimen.

oxidation products of AA in TEMPOL treated cells are needed. For example, it was originally assumed that DHA was produced as a consequence of the transformation of dopamine into norepinephrine by the action of dopamine-B-hydroxylase in chromaffin granules, but it is now very well accepted that the product is, in fact, A-radical.[77,78,113–125] It was previously reported that semidehydroascorbate reductase activity was detected in a variety of bovine ocular tissues, including RPE-choroid.[79] In this mixed tissue preparation, the specific activity was 0.75 nmol/min/mg protein. In our experience using homogenates (not supernatants) of human RPE cells ($n = 6$) incubated under standard assay conditions for measuring this enzyme,

for example, AA, ascorbate oxidase, H_2O_2 and NADH, we are unable to consistently detect its presence. More work on this enzyme may be needed, especially in conjunction with quantitative ESR measurements of the A-radical in cells.

CONCLUDING REMARKS

We hope the reader understands and better appreciates the long history of experimental investigations that have focused on the question of the importance of the redox couple between GSH and AA. This body of work has unequivocally established that GSH directly and nonenzymatically reduces DHA to AA in simple solutions and in animal cells and tissues. Indeed, this result is the basis for the inclusion of the GSH/GSSG and AA/DHA redox cycles in the antioxidant cascade shown in Figure 1 in this article, and in countless other articles. So, in the grand tradition of "I'm okay, you're okay," the redox couple is alive and well, because cells take up exogenous DHA and in the presence of GSH convert it to AA in the cytoplasm. But, herein lies a problem. The scheme shown in Figure 1 assumes that the oxidized and reduced pools of both GSH/GSSG and AA/DHA are in the cytoplasm. It follows that the only source of DHA for reduction by GSH is from the oxidation of the intracellular pool of AA. When we used TEMPOL to specifically oxidize the intracellular AA in cultured human RPE cells, only partial regeneration of the AA was observed when the cells were returned to TEMPOL-free media. These results raise concerns regarding the efficiency of the GSH/DHA redox couple, when DHA comes exclusively from oxidation of intracellular AA. These concerns, expressed previously[31] on the basis of results obtained in test tube experiments and expressed again here on the basis of experiments with living cells, lead us to suggest that the cellular reduction of DHA under this condition is insufficient to offset its decomposition. The problem arises because of the marked instability of DHA at physiological temperature (37°C) and pH (7.4) of mammalian cells, as noted in the earliest publications in this field.[1–4] On the other hand, the fact that AA could be recovered fully when TEMPOL-treated RPE cells were subsequently bathed in TEMPOL-free media containing DHA or AA supports our earlier suggestion[31] that transmembrane transport processes play an important role in regulating the level of AA in normal and oxidatively challenged cells. We therefore suggest that the antioxidant cascade (Fig. 1) be amended to include two additional arms to illustrate entry of exogenous AA and DHA into the cytoplasm. With this amendment, AA can be formed by reaction between GSH and intracellularly derived DHA whether the DHA comes from oxidation of AA or from uptake of exogenous DHA. Clearly, the intracellular AA pool can also be replenished by uptake of exogenous AA, without involvement of redox cycling. Sorting out the relative contributions of these pathways, in the context of the typical extracellular concentrations of AA (mM) and DHA (μM), is a challenge for the future.

Acknowledgements—This work was supported in part by Grant EY 05100 and by Core Grant for Vision Research EY05230 from the National Eye Institute, National Institutes of Health. The authors thank Dr. Michael Sevilla for performing the ESR measurements of the ascorbyl radical and for providing advice on the chemistry of free radicals. They also thank Dr. Arthur Bull and Dr. Robert Stern for insightful discussion on reaction schemes. The authors are particularly indebted to Dr. Michael Hartzer and Ms. Mei Cheng, who grew, fed, maintained and supplied us with countless culture dishes of human retinal pigment epithelial cells. The authors thank Ms. Alice Carleton for typing the manuscript.

REFERENCES

1. Hopkins, F. G.; Morgan, J. M. C. Some relations between ascorbic acid and glutathione. *Biochem. J.* **30**:1446–1462; 1936.
2. Szent-Gyorgyi, A. Observations on the function of peroxidase systems and the chemistry of the adrenal cortex. *Biochem. J.* **22**:1387–1409; 1928.
3. Borsook, H.; Davenport, H. W.; Jeffreys, C. E. P.; Warner, R. C. The oxidation of ascorbic acid and its reduction in vitro and in vivo. *J. Biol. Chem.* **117**:237–279; 1937.
4. Crook, E. M. The system dehydroascorbic acid-glutathione. *Biochem. J.* **35**:226–236; 1941.
5. Hughes, R. E. The use of homocysteine in the estimation of dehydroascorbic acid. *Biochem. J.* **64**:203–208; 1956.
6. Hughes, R. E. Reduction of dehydroascorbic acid by animal tissues. *Nature* **203**:1068–1069; 1964.
7. Hughes, R. E.; Kilpatrick, G. S. The reduction of dehydroascorbic acid by haemolysates of pernicious anemia erythrocytes. *Clin. Chim. Acta* **9**:241–244, 1964.
8. Hendry, J. M.; Easson, L. H.; Owen, J. A. The uptake and reduction of dehydroascorbic acid by human leucocytes. *Clin Chim. Acta* **9**:498–499; 1964.
9. Anderson, E. I.; Spector, A. Oxidation-reduction reactions involving ascorbic acid and the hexosemonophosphate shunt in corneal epithelium. *Invest. Ophthalmol. & Vis. Sci.* **10**:41–53, 1971.
10. Bigley, R. H.; Stankova, L. Uptake and reduction of oxidized and reduced ascorbate by human leukocytes. *J. Exp. Med.* **139**:1084–1092; 1974.
11. Stankova, L.; Bigley, R.; Wyss, S. R.; Aebi, H. Catalase and dehydroascorbate reductase in human polymorphonuclear leukocytes (PMN): Possible functional relationship. *Experientia* **35**:852–853; 1979.
12. Basu, S.; Som, S.; Deb, S.; Mukherjee, D.; Chatterjee, I. B. Dehydroascorbate acid reduction in human erythrocytes. *Biochem. Biophys. Res. Comm.* **90**:1335–1340; 1979.
13. Bigley, R.; Stankova, L.; Roos, D.; Loos, J. Glutathione-dependent dehydroascorbate reduction: A determinant of dehydroascorbate uptake by human polymorphonuclear leukocytes. *Enzyme* **25**:200–204; 1980.
14. Bigley, R.; Riddle, M.; Layman, D.; Stankova, L. Human cell

dehydroascorbate reductase. *Biochim. Biophys. Acta* **659:**15–22; 1981.
15. Stahl, R. L.; Liebes, L. F.; Farber, C. M.; Silber, R. A spectrophotometric assay for dehydroascorbate reductase. *Anal. Biochem.* **131:**341–344; 1983.
16. Hossain, M. A.; Asada, K. Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. *Plant & Cell Physiol.* **25:**85–92; 1984.
17. Stahl, R. L.; Liebes, L. F.; Silber, R. A reappraisal of leukocyte dehydroascorbate reductase. *Biochim. Biophys. Acta* **839:**119–121; 1985.
18. Tel-Or, E.; Huflejt, M.; Packer, L. The role of glutathione and ascorbate in hydroperoxide removal in cyanobacteria. *Biochem. Biophys. Res. Comm.* **132:**533–539; 1985.
19. Stahl, R. L.; Liebes, L. F.; Silber, R. Glutathione dehydrogenase (ascorbate). *Meth. Enzymol.* **122:**10–12; 1986.
20. Choi, J.-L.; Rose, R. C. Regeneration of ascorbic acid by rat colon. *Proc. Soc. Exp. Med.* **190:**369–374; 1989.
21. Rose, R. C. Renal metabolism of the oxidized form of ascorbic acid (dehydro-L-ascorbic acid). *Amer. J. Physiol.* **256:**F52–F56; 1989.
22. Wells, W. W.; Xu, D. P.; Yang, Y.; Rocque, P. A. Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. *J. Biol. Chem.* **265:**15361–15364; 1990.
23. Morgolis, S. A.; Paule, R. C.; Ziegler, R. G. Ascorbic and dehydroascorbic acids measured in plasma preserved with dithiothreitol or metaphosphoric acid. *Clin. Chem.* **36:**1750–1755; 1990.
24. Dhariwal, K. R.; Washko, P. W.; Levine, M. Determination of dehydroascorbic acid using high-performance liquid chromatography with coulometric electrochemical detection. *Anal. Biochem.* **189:**18–23; 1990.
25. Melhorn, R. J. Ascorbate- and dehydroascorbic acid-mediated reduction of free radicals in the human erythrocyte. *J. Biol. Chem.* **266:**2724–2731; 1991.
26. DiPerro, S.; Borraccino, G. Dehydroascorbate reductase from potato tubers. *Phytochem.* **30:**427–429; 1991.
27. Coassin, M.; Tomasi, A.; Vannini, V.; Ursino, F. Enzymatic recycling of oxidized ascorbate in pig heart: One electron vs. two-electron pathway. *Arch. Biochem. Biophys.* **290:**458–462; 1991.
28. Bates, C. J. Use of homocysteine to stabilize ascorbic acid, or to reduce dehydroascorbic acid, during HPLC separation of large volumes of tissue extracts. *Clin. Chim. Acta* **205:**249–252; 1992.
29. Washko, P. W.; Welch, R. W.; Dhariwal, K. R.; Wang, Y.; Levine, M. Ascorbic acid and dehydroascorbic acid analyses in biological samples. *Anal. Biochem.* **204:**1–14; 1992.
30. Bode, A. M.; Yavarow, C. R.; Fry, D. A.; Vargas, T. Enzymatic basis for altered ascorbic acid and dehydroascorbic acid levels in diabetes. *Biochem. Biophys. Res. Comm.* **191:**1347–1353; 1993.
31. Winkler, B. S. In vitro oxidation of ascorbic acid and its prevention by GSH. *Biochim. Biophys. Acta* **925:**258–264; 1987.
32. Winkler, B. S. Unequivocal evidence in support of the nonenzymatic redox coupling between glutathione/glutathione disulfide and ascorbic acid/dehydroascorbic acid. *Biochim. Biophys. Acta* **1117:**287–290; 1992.
33. Kinoshita, J. Selected topics in ophthalmic biochemistry. *Arch. Ophthalmol.* **72:**554–572; 1964.
34. Fridovich, I. The biology of oxygen radicals. *Science* **201:**875–880; 1978.
35. McCay, P. B. Vitamin E: Interactions with free radicals and ascorbate. *Ann Rev. Nutr.* **5:**323–340; 1985.
36. Englard, S.; Seifter, S. The biochemical functions of ascorbic acid. *Ann. Rev. Nutr.* **6:**365–406; 1986.
37. Bendich, A.; Machlin, L. J.; Scandurra, O. The antioxidant role of vitamin C. *Adv. in Free Rad. Biol. & Med.* **2:**419–444; 1986.
38. Halliwell, B. Oxidants and human disease: Some new concepts. *FASEB J.* **1:**358–364; 1987.
39. Frei, B.; Stocker, R.; Ames, B. N. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Natl. Acad. Sci.* **85:**9748–9752; 1988.
40. Pascoe, G. A.; Reed, D. J. Cell calcium, vitamin E and the thiol redox system in cytotoxicity. *Free Radic. Biol. Med.* **6:**209–224; 1989.
41. Reddy, V. N. Glutathione and its function in the lens: An overview. *Exp. Eye Res.* **50:**771–778; 1990.
42. Packer, L. Protective role of vitamin E in biological systems. *Am. J. Clin. Nutr.* **53:**1050S–1055S; 1991.
43. Chow, C. K. Vitamin E and oxidative stress. *Free Radic. Biol. Med.* **11:**215–232; 1991.
44. Sies, H.; Stahl, W.; Sundquist, A. R. Antioxidant functions of vitamins. *Ann N.Y. Acad. Sci.* **669:**7–20; 1992.
45. Meister, A. On the antioxidant effects of ascorbic acid and glutathione. *Biochem. Pharm.* **44:**1905–1915; 1992.
46. Buettner, G. R. The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* **300:**535–543; 1993.
47. Rose, R. C.; Bode, A. M. Biology of free radical scavengers: An evaluation of ascorbate. *FASEB J.* **7:**1135–1142; 1993.
48. Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci.* **90:**7915–7922; 1993.
49. Winkler, B. S.; Giblin, F. J. Glutathione oxidation in retina: Effects on biochemical and electrical activities. *Exp. Eye Res.* **36:**287–297; 1983.
50. Larsson, A.; Orrenius, S.; Holmgren, A.; Mannervik, B., eds. *Functions of glutathione.* New York: Raven Press; 1983.
51. Reed, D. J. Regulation of reductive processes by glutathione. *Biochem. Pharm.* **35:**7–13; 1986.
52. Winkler, B. S.; DeSantis, N.; Solomon, F. Multiple NADPH-producing pathways control glutathione (GSH) content in retina. *Exp. Eye Res.* **43:**829–847; 1986.
53. Packer, J. E.; Slater, T. F.; Willson, R. L. Direct observations of a free radical intervention between vitamin E and vitamin C. *Nature* **278:**737–738; 1979.
54. Niki, E.; Saito, T.; Kawakami, A.; Kamiya, Y. Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. *J. Biol. Chem.* **259:**4177–4182; 1984.
55. Scarpa, M.; Rigo, A.; Maiorino, M.; Ursini, F.; Gregolin, C. Formation of α-tocopherol radical and recycling of α-tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes. *Biochim. Biophys. Acta* **801:**215–219; 1984.
56. Lambelet, P.; Saucy, F.; Loliger, J. Chemical evidence for interactions between vitamins E and C. *Experientia* **41:**1384–1388; 1985.
57. Niki, E. Interaction of ascorbate and α-tocopherol. *Ann. N.Y. Acad. Sci.* **498:**186–199; 1986.
58. Stocker, R.; Hunt, N. H.; Weidemann, M. J.; Clark, I. A. Protection of vitamin E from oxidation by increased ascorbic acid content within plasmodium vinckei-infected erythrocytes. *Biochim. Biophys. Acta* **876:**294–299; 1986.
59. Packer, L.; Maguire, J. J.; Melhorn, R. J.; Servinova, E.; Kagan, V. E. Mitochondria and microsomal membranes have a free radical reductase activity that prevents chromanoxyl radical accumulation. *Biochem. Biophys. Res. Comm.* **159:**229–235; 1989.
60. Vatassery, G. T.; Smith W. E.; and Quach, H. T. Ascorbic acid, glutathione and synthetic antioxidants prevent the oxidation of vitamin E in platelets. *Lipids* **24:**1043–1049; 1989.
61. Melhorn, R. J.; Sumida, S.; Packer, L. Tocopheroxyl radical persistence and tocopherol consumption in liposomes and in vitamin E-enriched rat liver mtochondria and microsomes. *J. Biol. Chem.* **264:**13448–13452; 1989.
62. Mukai, K.; Nishimura, M.; Kikuchi, S. Stopped-flow investigation of the reaction of vitamin C with tocopheroxyl radical in

aqueous triton X-100 micellar solutions. *J. Biol. Chem.* **266**:274–278; 1991.
63. Sharma, M. K.; Buettner, G. R. Interaction of vitamin C and vitamin E during free radical stress in plasma: An ESR study. *Free Radic. Biol. Med.* **14**:649–653; 1993.
64. Quintanilha, A. T.; Packer, L. Surface localization of sites of reduction of nitroxide spin-labeled molecules in mitochondria. *Proc. Natl. Acad. Sci.* **74**:570–574; 1977.
65. Swartz, H. M.; Sentjurc, M.; Morse, P. D. Cellular metabolism of water-soluble nitroxides: Effect on rate of reduction of cell/nitroxide ratio, oxygen concentrations and permeability of nitroxides. *Biochim. Biophys. Acta* **888**:82–90; 1986.
66. Belkin, S.; Mehlhorn, R. J.; Hideg, K.; Hankovsky, O.; Packer, L. Reduction and destruction rates of nitroxide spin probes. *Arch. Biochem. Biophys.* **256**:232–243; 1987.
67. Branca, M.; Denurra, T.; Turrini, F. Reduction of nitroxide free radical by normal and G6PD deficient red blood cells. *Free Radic. Biol. Med.* **5**:7–11; 1988.
68. Mitchell, J. B.; Samuni, A.; Krishna, M. C.; DeGraff, W. G.; Ahn, M. S.; Samuni, U.; Russo, A. Biologically active metal-independent superoxide dismutase mimics. *Biochemistry* **29**:2802–2807; 1990.
69. Samuni, A.; Winkelsberg, D.; Pinson, A.; Hahn, S. M.; Mitchell, J. B.; Russo, A. Nitroxide stable radicals protect beating cardiomyocytes against oxidative damage. *J. Clin. Invest.* **87**:1526–1530; 1991.
70. Mitchell, J. B.; DeGraff, W.; Kaufman, D.; Krishna, M. C.; Samuni, A.; Finkelstein, E.; Ahn, M. S.; Hahn, S. M.; Gamson, J., Russo, A. Inhibition of oxygen-dependent radiation-induced damage by the nitroxide superoxide dismutase mimic. *Tempol. Arch. Biochem. Biophys.* **289**:62–70; 1991.
71. Gelvan, D.; Saltman, P.; Powell, S. R. Cardiac reperfusion damage prevented by a nitroxide free radical. *Proc. Natl. Acad. Sci.* **88**:4680–4684; 1991.
72. Reddan, J. R.; Sevilla, M. D.; Giblin, F. J.; Padgaonkar, V.; Dziedzic, D. C.; Leverenz, V.; Misra, I. C.; Peters, J. L. The superoxide dismutase mimic TEMPOL protects cultured rabbit lens epithelial cells from hydrogen peroxide insult. *Exp. Eye Res.* **56**:543–554; 1993.
73. Schneider, W.; Staudinger, H. Reduced nicotinamide-adenine dinucleotide-dependent reduction of semidehydroascorbic acid. *Biochim. Biophys. Acta* **96**:157–159; 1965.
74. Green, R. C.; O'Brien, P. J. The involvement of semidehydroascorbate reductase in the oxidation of NADH by lipid peroxide in mitochondria and microsomes. *Biochim. Biophys. Acta* **293**:334–342; 1973.
75. Orringer, E. P.; Roer, M. E. S. An ascorbate-mediated transmembrane-reducing system of the human erythrocyte. *J. Clin. Invest.* **63**:53–58; 1979.
76. Goldenberg, H. Insulin inhibits NADH-semidehydroascorbate reductase in rat liver plasma membrane. *Biochem. Biophys. Res. Comm.* **94**:721–726; 1980.
77. Diliberto, E. J.; Allen, P. L. Semidehydroascorbate as a product of the enzymic conversion of dopamine to norepinephrine. *Mol. Pharm.* **17**:421–426; 1980.
78. Diliberto, E. J.; Dean, G.; Carter, C.; Allen, P. L. Tissue, subcellular, and submitochondrial distributions of semidehydroascorbate reductase: Possible role of semidehydroascorbate reductase in cofactor regeneration. *J. Neurochem.* **39**:563–568; 1982.
79. Khatami, M.; Roel, L. E.; Li, W.; Rockey, J. H. Ascorbate regeneration in bovine ocular tissues by NADH-dependent semidehydroascorbate reductase. *Exp. Eye Res.* **43**:167–175; 1986.
80. Shigeoka, S.; Yasumoto, R., Onishi, T.; Nakano, Y.; Kitaoka, S. Properties of monodehydroascorbate reductase and dehydroascorbate reductase and their participation in the regeneration of ascorbate in Euglena gracilis. *J. Gen. Microbiol.* **133**:227–232; 1987.
81. Bando, M.; Obazawa, H. Activities of ascorbate free radical reductase and $H2O_2$-dependent NADH oxidation in senile cataractous human lenses. *Exp. Eye Res.* **50**:779–784; 1990.
82. Hausladen, A.; Kunert, K. J. Effects of artificially enhanced levels of ascorbate and glutathione on the enzymes monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase in spinach (Spinacia oleracea). *Physiologia Plantarum* **79**:384–388; 1990.
83. Alcain, F. J.; Buron, M. I.; Villalba, J. M.; Navas, P. Ascorbate is regenerated by HL-60 cells through the plasmalemma redox system. *Biochim. Biophys. Acta* **1073**:380–385; 1991.
84. Burton, G. W.; Wronska, U.; Stone, L.; Foster, D. O.; Ingold, K. U. Biokinetics of dietary RRR-α-tocopherol in the male guinea pig at three dietary levels of vitamin C and two levels of vitamin E: Evidence that vitamin C does not "spare" vitamin E in vivo. *Lipids* **25**:199–210; 1990.
85. Mårtensson, J.; Steinherz, R.; Jain, A.; Meister, A. Glutathione ester prevents buthionine sulfoximine-induced cataracts and lens epithelial cell damage. *Proc. Natl. Acad. Sci.* **86**:8727–8731; 1989.
86. Jain, A.; Mårtensson, J.; Stole, E.; Auld, P. A. M.; Meister, A. Glutathione deficiency leads to mitochondrial damage in brain. *Proc. Natl. Acad. Sci.* **88**:1913–1917; 1991.
87. Mårtensson, J.; Meister, A. Glutathione deficiency decreases tissue ascorbate levels in newborn rats: Ascorbate spares glutathione and protects. *Proc. Natl. Acad. Sci.* **88**:4656–4660; 1991.
88. Mårtensson, J.; Jain, A.; Stole, E., Frayer, W; Auld, P. A. M.; Meister, A. Inhibition of glutathione synthesis in the newborn rat: A model for endogenously produced oxidative stress. *Proc. Natl. Acad. Sci.* **88**:9360–9364; 1991.
89. Meister, A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal: Applications in research and therapy. *Pharmacol. Therapeutics* **51**:155–194; 1991.
90. Mårtensson, J.; Meister, A. Glutathione deficiency increases hepatic ascorbic acid synthesis in adult mice. *Proc. Natl. Acad. Sci.* **89**:11566–11568; 1992.
91. Jain, A.; Mårtensson, J.; Mehta, T.; Krauss, A. N.; Auld, P. A. M.; 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.* **89**:5093–5097; 1992.
92. Mårtensson, J.; Han, J.; Griffith, O. W.; Meister, A. Glutathione ester delays the onset of scurvy in ascorbate-deficient guinea pigs. *Proc. Natl. Acad. Sci.* **90**:317–321; 1993.
93. Hornig, D.; Weber, F.; Wiss, O. Uptake and release of [I-14C] ascorbic acid and [I-14C] dehydroascorbic acid by erythrocytes of guinea pigs. *Clin. Chim. Acta* **31**:25–35; 1971.
94. Bianchi, J.; Wilson, F. A.; Rose, R. C. Dehydroascorbic acid and ascorbic acid transport systems in the guinea pig ileum. *Amer. J. Physiol.* **250**:G461–G468; 1986.
95. Wagner, E. S.; White, W.; Jennings, M.; Bennett, K. The entrapment of [14C] ascorbic acid in human erythrocytes. *Biochim. Biophys. Acta* **902**:133–136; 1987.
96. Kern, H. L; Zolot, S. L. Transport of vitamin C in the lens. *Curr. Eye Res.* **6**:885–896; 1987.
97. Mooradian, A. D. Effect of ascorbate and dehydroascorbate on tissue uptake of glucose. *Diabetes* **36**:1001–1004; 1987.
98. Rose, R. C. Transport of ascorbic acid and other water-soluble vitamins. *Biochim. Biophys. Acta* **947**:335–366; 1988.
99. DiMattio, J. A comparative study of ascorbic acid entry into aqueous and vitreous humors of the rat and guinea pig. *Invest. Ophthalmol. & Vis. Sci.* **30**:2320–2330; 1989.
100. Rose, R. C.; Choi, J.-L. Intestinal absorption and metabolism of ascorbic acid in rainbow trout. *Am. J. Physiol.* **258**:R1238–R1241; 1990.
101. Zhou, A.; Nielsen, J. H.; Farver, O.; Thorn, N. A. Transport of ascorbic acid and dehydroascorbic acid by pancreatic islet cells from neonatal rats. *Biochem. J.* **274**:739–744; 1991.
102. Bode, A. M.; Vanderpool, S. S.; Carlson, E. C.; Meyer, D. A.; Rose, R. C. Ascorbic acid uptake and metabolism by corneal

endothelium. *Invest. Ophthalmol. & Vis. Sci.* **32:**2266–2271; 1991.
103. Rose, R. C.; Bode, A. M. Ocular ascorbate transport and metabolism. *Comp. Biochem. Physiol.* **100A:**273–285; 1991.
104. Vera, J. C.; Rivas, C. I.; Fischbarg, J.; Golde, D. W. Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. *Nature* **364:**79–82; 1993.
105. Lam, K.-W.; Yu, H.-S.; Glickman, R. D.; Lin, T. Sodium-dependent ascorbic and dehydroascorbic acid uptake by SV-40-transformed retinal pigment epithelial cells. *Ophthalmic. Res.* **25:**100–107; 1993.
106. Tirrell, J. G.; Westhead, E. W. The uptake of ascorbic acid and dehydroascorbic acid by chromaffin granules of the adrenal medulla. *Neuroscience* **4:**181–186; 1979.
107. Khatami, M.; Stramm, L. E.; Rockey, J. H. Ascorbate transport in cultured cat retinal pigment epithelial cells. *Exp. Eye Res.* **43:**607–615; 1986.
108. Khatami, M.; Li, W.; Rockey, J. H. Kinetics of ascorbate transport by cultured retinal capillary pericytes. *Invest. Ophthalmol. & Vis. Sci.* **27:**1665–1671; 1986.
109. Delamere, N. A.; Williams, R. N. A comparative study on the uptake of ascorbic acid by the iris-ciliary body of the rabbit, guinea pig and rat. *Comp. Biochem. Physiol.* **88B:**847–849; 1987.
110. Bigley, R.; Wirth, M.; Layman, D.; Riddle, M.; Stankova, L. Interaction between glucose and dehydroascorbate transport in human neutrophils and fibroblasts. *Diabetes* **32:**545–548; 1983.
111. Omaye, S. T.; Turnbull, J. D.; Sauberlich, H. E. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Meth. Enzymol.* **62:**3–8; 1979.
112. Giblin, F. J.; Winkler, B. S.; Sasaki, H.; Chakrapani, B.; Leverenz, V. Reduction of dehydroascorbic acid in lens epithelium by the glutathione redox cycle. *Invest. Ophthalmol. & Vis. Sci.* **34(Suppl):**1298; 1993.
113. Diliberto, E. J.; Allen, P. L. Mechanism of dopamine-β-hydroxylation. *J. Biol. Chem.* **256:**3385–3393; 1981.
114. Njus, D.; Knoth, J.; Cook, C.; Kelley, P. M. Electron transfer across the chromaffin granule membrane. *J. Biol. Chem.* **258:**27–30; 1983.
115. Srivastava, M.; Duong, L. T.; Fleming, P. J. Cytochrome b561 catalyzes transmembrane electron transfer. *J. Biol. Chem.* **259:**8072–8075; 1984.
116. Levine, M.; Morita, K.; Heldman, E.; Pollard, H. B. Ascorbic acid regulation of norepinephrine biosynthesis in isolated chromaffin granules from bovine adrenal medulla. *J. Biol. Chem.* **260:**15598–15603; 1985.
117. Wakefield, L. M.; Cass, A. E. G.; Radda, G. K. Functional coupling between enzymes of the chromaffin granule membrane. *J. Biol. Chem.* **261:**9739–9745; 1986.
118. Wakefield, L. M.; Cass, A. E. G.; Redda, G. K. Electron transfer across the chromaffin granule membrane. *J. Biol. Chem.* **261:**9746–9752; 1986.
119. Huyghe, B.; Pollock Klinman, J. Activity of membranous dopamine β-monooxygenase within chromaffin granule ghosts. *J. Biol. Chem.* **266:**11544–11550; 1991.
120. Dhariwal, K. R.; Black, C. D. V.; Levine, M. Semidehydroascorbic acid as an intermediate in norepinephrine biosynthesis in chromaffin granules. *J. Biol. Chem.* **266:**12908–12914; 1991.
121. Dhariwal, K. R.; Shirvan, M.; Levine, M. Ascorbic acid regeneration in chromaffin granules. *J. Biol. Chem.* **266:**5384–5387; 1991.
122. Njus, D.; Kelley, P. M. The secretory-vesicle ascorbate-regenerating system: A chain of concerted H^+/e^--transfer reactions. *Biochim. Biophys. Acta* **1144:**235–248; 1993.
123. Jalukar, V.; Kelley, P. M.; Njus, D. Reaction of ascorbic acid with cytochrome b561. *J. Biol. Chem.* **266:**6878–6882; 1991.
124. Kelley, P. M.; Jalukar, V.; Njus, D. Rate of electron transfer between cytochrome b561 and extravesicular ascorbic acid. *J. Biol. Chem.* **265:**19409–19413; 1990.
125. Kelley, P. M.; Njus, D. A kinetic analysis of electron transport across chromaffin vesicle membranes. *J. Biol. Chem.* **263:**3799–3804; 1988.