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## Vitamin C physiology: the known and the unknown and Goldilocks

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### Abstract

Vitamin C (Ascorbic Acid), the antiscorbutic vitamin, cannot be synthesized by humans and other primates, and has to be obtained from diet. Ascorbic acid is an electron donor and acts as a cofactor for fifteen mammalian enzymes. Two sodium-dependent transporters are specific for ascorbic acid, and its oxidation product dehydroascorbic acid is transported by glucose transporters. Ascorbic acid is differentially accumulated by most tissues and body fluids. Plasma and tissue vitamin C concentrations are dependent on amount consumed, bioavailability, renal excretion, and utilization. To be biologically meaningful or to be clinically relevant, *in vitro* and *in vivo* studies of vitamin C actions have to take into account physiologic concentrations of the vitamin. In this paper, we review vitamin C physiology; the many phenomena involving vitamin C where new knowledge has accrued or where understanding remains limited; raise questions about the vitamin that remain to be answered; and explore lines of investigations that are likely to be fruitful.

### Keywords

Vitamin C; Dehydroascorbic Acid; Vitamin C Transport; Scurvy; Dose Concentration Relationship; Recommended Dietary Allowance

### Introduction

Vitamin C (Ascorbic acid, abbreviated as AA; the terms vitamin C and ascorbic acid are used interchangeably) is synthesized by all plants and most animals (Smirnoff et al., 2001). It is a vitamin for humans because the gene for gulonolactone oxidase, the terminal enzyme in the AA synthesis pathway has undergone mutations that make it non-functional (Linster & Van Schaftingen, 2007). Animals that have lost the ability to synthesize ascorbic acid do not have a phylogenetic relationship with each other. These animals include non-human

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primates, guinea pigs, capybara and some birds and fish (Chaudhuri & Chatterjee, 1969, Chatterjee, 1973, Cueto et al., 2000). Deficiency of ascorbic acid produces the fatal disease scurvy, which can be cured only by the administration of vitamin C.

In this paper, we review general aspects of vitamin C biology as it pertains to human health. Those aspects of vitamin C that have been extensively reviewed elsewhere are only briefly discussed here. These include the importance of proper sample processing and reliability of assays to ensure that vitamin C measurements are accurate and precise (Levine et al., 1999b); the role of vitamin C as an antioxidant (Padayatty et al., 2003); and the chemistry and biology of pharmacologic (high dose) intravenously administered vitamin C (Levine et al., 2011) (Parrow et al., 2013). A large number of epidemiological and intervention studies have examined the effects of vitamin C consumption and/or supplementation on physiological parameters, biomarkers and clinical end points. In general these studies found either no effect attributable to vitamin C intake or reported ambiguous results. Although these results are not reviewed here in great detail, we emphasize the importance of dose concentration relationships in designing and interpreting studies. Getting concentrations and amounts “just right”, as Goldilocks has described in clear fashion, is discussed throughout. The attached figures and figure legends are largely self-contained, and repetition of figure material in the manuscript is minimized.

## History of scurvy and the discovery of vitamin C

Although scurvy has been known since ancient times (Clemeston, 1989), it became a notable cause of large scale deaths in the last five hundred years. Scurvy afflicted large land armies and cities in Northern Europe, especially in winter and during siege warfare. In the age of exploration, scurvy became the principle factor limiting sea voyage, often killing many sailors after two to three months at sea. In perhaps the first controlled clinical trial, James Lind showed that scurvy could be cured by citrus fruits (Lind, 1953b). However, this simple remedy was not used widely for several decades. The finding did not fit in with the extant scientific knowledge, and existing theories of the causation of disease had no concept of nutritional deficiency. Ascorbic acid was first isolated by Albert Szent-Gyorgyi in 1928 and shown to be the antiscorbutic factor by Szent-Gyorgyi and King in 1932 (Svirbely & Szent-Gyorgyi, 1932, King & Waugh, 1932).

## Scurvy

The earliest symptom of scurvy is subtle, and was described by James Lind in his treatise on the scurvy (1753) as lassitude (Lind, 1953b). It was a predictable affliction of sailors who developed the disease after a month or two at sea. In its early stage, sailors lost initiative and the will to work, but could work normally when compelled to do so. Frank scurvy is now rare but in full form presents with striking signs and symptoms. These include hypochondriasis and depression; perifollicular hyperkeratosis with coiled hairs; swollen and friable gingivae; anemia, petechial hemorrhage, erythema, and purpura; arthralgia and/or joint effusions; breakdown of old wounds; bleeding into the skin, subcutaneous tissues, muscles, joints and subperiosteal hemorrhages; fever; shortness of breath; infections; and confusion (Hodges et al., 1971, Hood et al., 1970). Subjects can present with only some

symptoms or signs, and in these cases diagnosis is often missed initially (Bernardino et al., 2012). The clinical picture is confusing in the presence of multiple vitamin deficiencies (Blanchard et al., 2014) or when atypical symptoms such as dyspnea are predominant (Kupari & Rapola, 2012). Untreated, the condition is fatal.

There is not a definitive low vitamin C plasma concentration at which scurvy develops. Studies using radiolabeled vitamin C predict that body stores in healthy humans are about 1500 mg. Scurvy is thought to occur when this falls below 300 mg (Hodges et al., 1971), with plasma vitamin C concentrations  $<10 \mu\text{M}$ . However, these experiments were done at a time when vitamin C measurements used colorimetric assays that detected not only vitamin C but other unknown interfering substances. Overestimation of vitamin C was more marked when the concentrations of vitamin C measured were low (Baker et al., 1969, Baker et al., 1971, Hodges et al., 1971). Modern vitamin C assays are much more accurate and precise. These generally use high performance liquid chromatography (HPLC) to separate vitamin C from other substances and flow-by electrochemistry (amperometry), flow-through electrochemistry (coulometry), ultraviolet (UV), or fluorescence to detect and measure vitamin C. Recently, mass spectrometry is another tool to detect vitamin C, coupled to liquid chromatography (called LC-MS)(Leveque et al., 2000, Szultka et al., 2014, Gentili et al., 2008). At present, the most thoroughly characterized method is HPLC with electrochemical detection (Levine et al., 1999b). Thus, because of inaccurate assays, the measurements of plasma vitamin C concentrations in scurvy may have shown values that were two to three fold higher than the true value. In depletion-repletion studies which utilized a modern HPLC electrochemical assay, healthy young men and women attained plasma vitamin C concentrations of  $8 \mu\text{M}$  without developing scurvy (Levine et al., 1996b, Levine et al., 2001b). However, many subjects experienced lassitude, and it was considered unsafe to further deplete them of vitamin C. Therefore, scurvy remains a clinical diagnosis, confirmed by low plasma vitamin C concentrations, but as yet without a definite diagnostic concentration. Values below  $10 \mu\text{M}$  are perhaps not far from incipient scurvy, but physical signs may appear only at much lower values, perhaps as low as  $3\text{-}5 \mu\text{M}$ . In epidemiological studies, plasma vitamin C concentrations less than  $11.4 \mu\text{M}$  ( $0.2 \text{ mg/dl}$ ) are deemed to indicate deficiency (Jacob et al., 1987, Schleicher et al., 2009).

## Chemistry and metabolism of vitamin C

Chemically, vitamin C is an electron donor, or reducing agent, and electrons from ascorbate account for all of its known physiological effects. Vitamin C chemistry is detailed in figure 1. Because electrons from vitamin C can reduce oxidized species, or oxidants, vitamin C is often termed an antioxidant, but this terminology is misleading. Electrons from ascorbate can reduce metals such as copper and iron, leading to formation of superoxide and hydrogen peroxide, and subsequent generation of reactive oxidant species. Thus, under some circumstances ascorbate, through its action as a reducing agent, will generate oxidants. This chemistry occurs *in vivo* when pharmacologic ascorbate concentrations, in the millimolar range, are achieved in the plasma and in the extra cellular fluids, and can also occur with physiologic concentration of ascorbate in cell culture media when metals are present (Parrow et al., 2013).

Ascorbate loses electrons sequentially. When one electron is lost, the first product is ascorbate radical. Most radical species have short lives less than one millisecond. Ascorbate radical is different, in that the half life can be in many seconds, or even minutes, depending on absence of oxygen and electron acceptors, especially iron (Buettner, 1993). For example, under some conditions ascorbate radical can be measured in blood and extracellular fluid samples (Chen et al., 2007). When a second electron is lost, a more stable species formed, in comparison to ascorbate free radical. The formed species is dehydroascorbic acid, which exists in hydrated and anhydrous forms. As discussed below, dehydroascorbic acid has affinity for facilitated glucose transporters and is transported by a number of them (Corpe et al., 2013) (Rumsey et al., 1997) (Rumsey et al., 2000a). Both dehydroascorbic acid and ascorbate radical are reversibly reduced to ascorbate. Dehydroascorbic acid half-life is only minutes, due to hydrolytic ring rupture. Once the ring structure is lost, the product 2,3 diketogulonic acid cannot reform its precursors dehydroascorbic acid, ascorbate radical, and ascorbate.

## Known and postulated actions of vitamin C

### Enzymology and vitamin C

As just described, all known and postulated actions of vitamin C Table 1 are accounted for by a single chemical property: that AA is an electron donor and thus a reducing agent. The most well characterized actions are those as an enzyme cofactor, including those in which it is an actual cosubstrate. Ascorbate acts as an electron donor for fifteen mammalian and three fungal enzymes (Englard & Seifter, 1986, Levine, 1986). These include two monooxygenases (Dopamine  $\beta$ -Hydroxylase, Peptidylglycine  $\alpha$ -Amidating Monooxygenase), twelve dioxygenases (6 Prolyl 4-Hydroxylases; Prolyl 3-Hydroxylase; Lysyl Hydroxylase; Asparaginyl hydroxylase; Trimethyllysine Hydroxylase;  $\gamma$ -Butyrobetaine Hydroxylase; and 4-Hydroxyphenylpyruvate Dioxygenase) and one amine oxidase. Although details of these enzyme reactions and the role of ascorbate are described elsewhere (Levine et al., 2006) a brief summary follows.

Dopamine  $\beta$ -Hydroxylase, found in neurosecretory vesicles and in adrenal chromaffin granules (Levine et al., 1941b) is necessary for the synthesis of norepinephrine in the nervous system and in the adrenal glands. It requires molecular oxygen and ascorbate (Levine et al., 1941b), and the reaction consumes ascorbate (Fleming & Kent, 1991, Stewart & Klinman, 1988). Peptidylglycine  $\alpha$ -Amidating Monooxygenase, found in secretory vesicles is required to amidate many peptide hormones (Eipper & Mains, 1991, Glembotski, 1986) (Prigge et al., 2000) (Kumar et al., 2015) to make them biologically active. These include many hypothalamic and gastrointestinal hormones. The enzyme requires molecular oxygen, copper and ascorbate, and consumes ascorbate (Prigge et al., 2000). However, for amidation, other electron donors can replace ascorbate *in vitro* (Prigge et al., 2000). Trimethyllysine Hydroxylase and  $\gamma$ -Butyrobetaine Hydroxylases are required for carnitine synthesis from the essential amino acids lysine and methionine. The enzyme requires iron,  $\alpha$ -ketoglutarate and a reductant, of which ascorbate is the most optimal, at least *in vitro* (Dunn et al., 1984). Because carnitine is obtained from diet and also synthesized in the body, the relative importance of these two sources, and the role of ascorbate in its synthesis are

difficult to characterize *in vivo* (Thoma & Henderson, 1984, England & Seifter, 1986) (Rebouche, 1991a, Rebouche, 1991b). 4-Hydroxyphenylpyruvate Dioxygenase is required for the catabolism of tyrosine (Lindblad et al., 1970). Ascorbate deficiency leads to impaired tyrosine catabolism and increased plasma concentrations of tyrosine (England & Seifter, 1986, Levine et al., 1941a).

For enzymes in which ascorbate is involved in prolyl or lysyl hydroxylation, ascorbate acts as a cofactor rather than a cosubstrate. As a cofactor, the number of product molecules generated is in great excess in comparison to number of molecules of ascorbate utilized. Note that prolyl 4 hydroxylase has three isoenzymes involved in collagen hydroxylation and another three in the hydroxylation of HIF -1 (Pekkala et al., 2003) (Myllyharju, 2008).

### Collagen Hydroxylation

Common symptoms of scurvy include wound dehiscence, poor wound healing and loosening of teeth, all pointing to defects in connective tissue (Lind, 1953a) (Crandon et al., 1940, Hirschmann & Raugi, 1999). Collagen provides connective tissue with structural strength. Vitamin C catalyzes enzymatic (Peterkofsky, 1991) post-translational modification of procollagen to produce and secrete adequate amounts of structurally normal collagen by collagen producing cells (Kivirikko & Myllyla, 1985) (Prockop & Kivirikko, 1995). Precollagen, synthesized in the endoplasmic reticulum, consists of amino acid repeats rich in proline. Specific prolyl and lysyl residues are hydroxylated, proline is converted to either 3 hydroxyproline or 4 hydroxyproline, and lysine is converted to hydroxylysine. The reactions catalyzed by prolyl 3-hydroxylase, prolyl 4-hydroxylase, and lysyl hydroxylase (Peterkofsky, 1991) (Prockop & Kivirikko, 1995) (Pekkala et al., 2003) require vitamin C as a cofactor. Hydroxylation aids in the formation of the stable triple helical structure of collagen, which is transported to the Golgi apparatus and eventually secreted by secretory granules. In the absence of hydroxylation, secretion of procollagen decreases (Peterkofsky, 1991) and it probably undergoes faster degradation. However, some hydroxylation can occur even in the absence of vitamin C (Parsons et al., 2006). Secreted procollagen is enzymatically cleaved to form tropocollagen that spontaneously forms collagen fibrils in the extracellular space. These fibrils form intermolecular collagen cross links, giving collagen its structural strength. Independent of its effects on hydroxylation, ascorbate may stimulate collagen synthesis (Geesin et al., 1988) (Sullivan et al., 1994). Collagen synthesis may be decreased in scorbutic animals (Peterkofsky, 1991, Kipp et al., 1996, Tsuchiya & Bates, 2003). Reduced collagen cross links may be a marker of vitamin C deficiency in the guinea pig (Tsuchiya & Bates, 2003) but this may not be specific to vitamin C deficiency. Though many features of human scurvy appear to be due to weakening of connective tissue, it has not been shown that these lesions are due to defective collagen synthesis.

### HIF-1 Hydroxylation

In addition to its role in the hydroxylation of collagen, vitamin C may also play a role in the hydroxylation of specific proline residues in Hypoxia Inducible Factor -1 alpha (HIF-1 $\alpha$ ) by a separate set of prolyl 4 hydroxylases. These enzymes contain non haeme iron, and require ascorbate, molecular oxygen, and 2-oxoglutarate (Bruick & McKnight, 2001) (Myllyharju, 2003) (Pekkala et al., 2003) (Knowles et al., 2003) (Dengler et al., 2014). HIF-1 is a

transcription factor that is a key to oxygen sensing in multicellular animals (Taabazuing et al., 2014) (Semenza, 2014). It consists of an oxygen regulated  $\alpha$  subunit and a constitutively expressed  $\beta$  subunit (Dengler et al., 2014). Under normal conditions, including normal oxygen tension, HIF-1 $\alpha$  is hydroxylated at specific proline and asparagine residues. Hydroxylated HIF -1  $\alpha$  is targeted for degradation by proteasomes (Dengler et al., 2014). Under hypoxic conditions, whether due to reduced atmospheric oxygen or due to local ischemia, hydroxylation is inhibited and HIF-1  $\alpha$  is stabilized. Metal induced ascorbate depletion in cultured cells also inhibits hydroxylation and stabilizes HIF-1  $\alpha$  (Kaczmarek et al., 2007) though metals may also have other effects on this system. Because unhydroxylated HIF-1 $\alpha$  is stable, it accumulates long enough to translocate into the nucleus. In the nucleus, HIF-1 $\alpha$  forms dimers with HIF-1  $\beta$ , and the dimer binds to DNA to transcribe target genes (Dengler et al., 2014). In perfused lungs, HIF-1  $\alpha$  was induced by hypoxia in one hour but was degraded under normoxic conditions in minutes (Yu et al., 1998). HIF-1 activates gene transcription by binding to parts of the DNA within the Hypoxia Response Element, a *cis* acting regulatory element. HIF-1 may regulate several hundred genes, but regulation is limited to a smaller number of specific genes for each cell type (Semenza, 2011) (Taabazuing et al., 2014). HIF-1 plays an important role in normal physiology (Semenza, 2011). As reviewed elsewhere, HIF-1 has a role in the control of erythropoiesis (Franke et al., 2013); in lung disease (Shimoda & Semenza, 2011); in heart disease (Semenza, 2014); in diabetes (Catrina, 2014, Ichiki & Sunagawa, 2014); and in cancer (Semenza, 2013) (Borsi et al., 2015) . All of these conditions have varying degrees of oxygen deprivation at the tissue level. Ascorbate has demonstrable effects on HIF-1 *in vitro*. Ascorbate inhibited HIF -1 activity in cell culture and prevented gene transcription specific to HIF-1 stimulation (Vissers et al., 2007, Kuiper et al., 2014). Increased systolic pulmonary artery pressure induced by hypoxia in (ascorbate and iron replete) healthy subjects was unaffected by intravenously administered vitamin C but was reduced by iron infusion (Talbot et al., 2014). Whether these effects were mediated by HIF-1 is not known though HIFs are thought to be mediators of hypoxia induced pulmonary hypertension (Shimoda & Laurie, 2014).

HIF-1 in turn is negatively regulated by Factor Inhibiting HIF (FIH). FIH is a hydroxylase that also requires ascorbate (Flashman et al., 2010). FIH appears necessary for hydroxylation of asparagine and/or aspartate residues on HIF 1, and also on the structural protein ankyrin. Hydroxylation *in vitro* was dependent on ascorbate or ascorbate analogs that are electron donors (Yang et al., 2011b). It is not known if other intracellular electron donors will suffice, ie. glutathione. Manipulation of HIF-1 and the factors involved in its enzymatic pathways may present attractive therapeutic targets (Myllyharju, 2008). The concentration -dependent role of ascorbate *in vivo* in human physiology and disease states that may potentially act via HIF-1 are not yet known.

### Enzymology in vivo and in situ

The role of vitamin C as a cofactor or cosubstrate for many enzymes has not been well studied *in vivo*. Neither is it certain that only vitamin C can fulfill these roles *in vivo*. It is possible that other reducing agents can replace ascorbate, to varying extents. Some signs and symptoms of scurvy appear to be due to specific defects in enzyme actions where vitamin C is known to play a role. Lassitude and signs of autonomic dysfunction might be due to



impaired norepinephrine biosynthesis. Breakdown of wounds, bleeding gingivae, loosening of teeth and other apparent defects in connective tissue might be due to impaired hydroxylation reactions in collagen, producing either smaller amounts of functional collagen, or weaker collagen or both. Impaired amidation of peptide hormones (Eipper et al., 1992) may produce widespread hormone defects, but such specific defects have not been experimentally shown to be the cause of human or animal scurvy.

Most enzyme reactions where vitamin C acts as a cofactor require relatively low concentrations of the vitamin in comparison to the normal *in vivo* concentration. It is possible that as consequence of vitamin C transporter activity, many tissues contain significant amounts of vitamin C even in the face of deficiency, though such measurements in humans are not available. Further, only limited data exist regarding intracellular distribution of vitamin C. In scurvy, the cell as a whole may contain significant amounts of the vitamin but it may not be available at the requisite concentrations at specific enzyme reaction sites within the cell, ie. within specific subcellular organelles. One example of how ascorbate concentrations can affect enzymatic reactions is for norepinephrine biosynthesis. The concentration of ascorbate is approximately 12-15 mM in adrenal medullary chromaffin granules, the site of norepinephrine biosynthesis. The enzyme that produces norepinephrine from dopamine is dopamine beta-monoxygenase. The  $K_m$  of this enzyme for ascorbate is < 1 mM, meaning that this enzyme works at close to maximal rate unless severe ascorbate lack occurs (Dhariwal et al., 1989) (Dhariwal et al., 1991a). It is unknown whether adrenal vitamin C is so depleted in scurvy that concentrations in adrenal medullary secretory granules are less than 1 mM.

It is also possible that intracellular vitamin C may play additional roles that are different from that as cofactors for enzymes. For example, ascorbate may act as an intracellular chemical reducing agent (antioxidant). While some of these reactions have been demonstrated *in vitro*, it is unknown whether they have clinical relevance. Glutathione is another intracellular reducing substance, often present at millimolar concentrations (Montero et al., 2013, Wu et al., 2004). It is not known whether there are specific intracellular non-enzymatic chemical reactions that utilize ascorbate as an electron donor, but not glutathione.

## Ascorbic acid and dehydroascorbic acid transport

Vitamin C is transported by specific transporters termed Sodium-dependent Vitamin C Transporter (SVCT) 1 and 2 (Tsukaguchi et al., 1999) (Daruwala et al., 1999, Wang et al., 1999, Wang et al., 2000). The distribution of these transporters in animals and humans has been determined by studying mRNA expression of the two transport proteins. Although antibodies are available, their reliability has not been consistent. Functional studies have clarified the role of these transporters in certain tissues using cell and animal models. SVCT1 and 2 belong to a family of nucleobase transporters that are highly conserved through evolution.

SVCT1 is the absorptive vitamin C transporter and occurs in the intestine, in renal tubules and in the liver (Fig 2). SVCT1 knockout mice, in which the transporter is not expressed,

lose large amounts of vitamin C in the urine compared to wild type mice. Urinary loss occurs because vitamin C that is filtered at the glomerulus is not reabsorbed, because SVCT1 is absent (Corpe et al., 2010). However, SVCT1 knockout mice absorb both vitamin C itself as well as a structural analog, 6-bromo ascorbate, from the intestinal lumen. As discussed in detail below, oxidized 6-bromo ascorbate is not transported by glucose transporters (GLUTs), and there are no SVCT1 transporters in SVCT1 knockout mice. Therefore, these mice transport AA and 6-bromo ascorbate from the intestinal lumen either via SVCT2 or through an unknown transporter.

Another means to study vitamin C transport mechanisms was creation of SVCT2 knockout mice. SVCT2 is widely distributed and based on mRNA expression is the predominant tissue transporter for vitamin C. SVCT2 knockout mice die at birth, and fetal tissues show very low vitamin C concentrations in all tissues measured (Sotiriou et al., 2002).

As noted above, the ascorbate oxidation product dehydroascorbic acid (DHA) exists in several different forms and is transported by GLUTs (Corpe et al., 2013) (Rumsey et al., 1997) (Rumsey et al., 2000a, Vera et al., 1993). Several GLUTs have a higher affinity for DHA than for glucose. The hydrated form of DHA forms bicyclic hemiketal structures, some of which resemble glucose in three dimensions (Fig 2) (Corpe et al., 2005). As soon as DHA is transported, it undergoes immediate intracellular reduction to ascorbate. The process of extracellular oxidation to DHA, DHA transport, and immediate intracellular reduction is termed ascorbate recycling (Washko et al., 1993). Based on data from *in vitro* cell models, DHA has been proposed to serve either as an alternate path or dominant path for ascorbate accumulation (Vera et al., 1993) (Nualart et al., 2003) (Agus et al., 1997).

*In vivo*, our understanding of the roles of DHA and GLUTs in vitamin C economy in the intact organism is unclear, especially for humans. Available data indicate that it is unlikely that DHA transport is the major pathway for ascorbate transport for most tissues. This is because the SVCT2 knockout mouse dies at birth and has virtually no ascorbate in all tissues measured other than the liver, where ascorbate is synthesized (Sotiriou et al., 2002). If DHA transport via GLUTs could serve as a salvage pathway, ascorbate should be present in at least some tissues. Mice are different than humans, and it remains possible that humans have DHA pathways that mice lack. Also, not every tissue was measured for ascorbate in the SVCT2 knockout mouse, and it is possible that some tissues do utilize the ascorbate recycling pathway. A recent example comes from experiments in red blood cells from both mice and humans (Tu et al., 2015). Red blood cells are one cell type that exclusively utilizes dehydroascorbic acid as the transported substrate. Once transported inside red blood cells, dehydroascorbic acid is immediately reduced to vitamin C.

In the physiological state, the amount of DHA in plasma is estimated as <1-2% of that of ascorbate. Liquid chromatography mass spectrometry assays in the near future may be able to address our current inability to measure DHA directly in plasma. These assays will have to account for DHA instability and hydrolysis in plasma as a biological fluid, and will either have to correct for hydrolysis or provide sample stability. At present there are no direct means to measure DHA accurately. Current techniques are to measure ascorbate, and then to re-measure ascorbate after the sample is reduced. The value before reduction is subtracted



from the value after reduction. Accuracy is reduced because a large number is usually subtracted from a large number, and the resulting difference may not be distinguishable from zero. Under conditions where ascorbate oxidation occurs locally, it is possible that DHA concentrations are higher than the estimated 1-2% plasma values. Local DHA concentrations may be higher when cells produce reactive oxidant species, such as that by activated neutrophils and monocytes. In addition, when ascorbate is given pharmacologically, it is possible that DHA concentrations rise proportionately. Vitamin C analogs with halogen substitutions at the 6<sup>th</sup> carbon position may be useful in the future in dissecting vitamin C transport mechanisms, especially the contribution of DHA uptake to overall ascorbate content in cells. The halogen analogs are transported by SVCTs, but when oxidized, are not transported by GLUTs. Substitution of the hydroxyl group with halogen prevents the oxidized halogen DHA species from forming a three dimensional configuration, as a bicyclic hemiketal, that is similar to glucose (Corpe et al., 2005)(Fig 2).

Interest remains in characterizing the role of DHA transport in ascorbate economy because of DHA transport by GLUTs, and the possibility of dysregulated DHA transport with hyperglycemia, as in diabetes. Nearly 40 years ago, a general hypothesis was linked vitamin C to diabetes via DHA (Mann & Newton, 1975). Unfortunately, until recently specific explanations were lacking that tied diabetes pathophysiology to DHA transport or aberrant DHA transport (Will & Byers, 1996) (Chen et al., 2006). Promising new data from mouse and human red blood cells indicate that DHA is the only transported species into these cells, and hyperglycemia inhibited transport *in vitro* and *in vivo* (Tu et al., 2015). Red blood cells from diabetic subjects had lower vitamin C and were more rigid, with decreased structural protein beta-spectrin. Thus, red blood cells with low vitamin C concentrations in diabetes could contribute to or even cause microvascular hypoxia that is the hallmark of diabetic vascular disease (Tu et al., 2015, May, 2015). Because glucose transporters differ in mouse and human red blood cells, clinical research in humans without and with diabetes will be essential in future studies.

To summarize: SVCT 1 and 2 transport vitamin C into cells. In addition, vitamin C accumulation into cells under some circumstances may occur by other mechanisms: ascorbate recycling, or direct transport of DHA. As indicated above, ascorbate recycling means that extracellular ascorbate is oxidized to dehydroascorbic acid, which is transported by facilitative glucose transporters and then effectively trapped by intracellular reduction to ascorbate.

Regardless of how transport occurs, either via SVCTs or via ascorbate recycling, vitamin C transported into some cells must exit from them, as in intestine and kidney. Also, other tissues secrete vitamin C. These include liver, the site of vitamin C synthesis in animals able to synthesize the vitamin; adrenal; reproductive organs; and stomach. Mechanisms of exit, or efflux, are not known. Because vitamin C is an anion at physiologic pH, it cannot simply diffuse across the cell membrane to extracellular fluid. Efflux transporters for the vitamin have not been identified (Eck et al., 2013). For intestinal epithelium and proximal renal tubular cells, vitamin C is absorbed by luminal transporters such as SVCT1. Once inside intestinal epithelia and renal tubular cells, the vitamin must exit, presumably by efflux transporters on the basolateral surface, to enter plasma or extracellular fluid. Vitamin C is

secreted into the gastric juice, cerebrospinal fluid, and aqueous humor, all of which have higher concentrations than those in plasma (Fig 3). Vitamin C is also secreted by the adrenal gland in humans in response to ACTH. Vitamin C secretion from the adrenals in humans is rapid, within minutes. The amount secreted is sufficient to increase local plasma vitamin C concentrations several fold in the adrenal vein (see section: Human Adrenals secrete vitamin C in response to ACTH), although insufficient to increase systemic concentrations (Padayatty et al., 2007). Animal data indicate that testes and ovaries might also secrete vitamin C in response to hormone signaling (Koba et al., 1971) (Musicki et al., 1996). For rapid hormone dependent secretion of vitamin C to occur, a transport mechanism is likely to be responsible, but is as yet unidentified. Transport kinetics properties of SVCT1 and 2 are ideal for transporting the vitamin into cells, but not for its secretion into plasma or extra cellular fluids from cells (Eck et al., 2013).

The transporter SVCT1 is responsible for vitamin C re-absorption in the proximal convoluted tubule of the kidney. SVCT1 knockout mice are unable to reabsorb vitamin C in the urine and have high perinatal mortality. The high perinatal mortality can be reversed almost completely by supplementing the mother with vitamin C (Corpe et al., 2010). In these knockout mice, low vitamin C concentrations that do no apparent harm to adult mice are deleterious to the fetus in the peripartum period. Clinical studies to test whether vitamin C could improve outcomes in high risk pregnancies have been performed in patients with histories of pre-eclampsia. Effects were inconsistent, in large part because vitamin C intake was enough to produce tissue saturation even in the control groups, as discussed below. Women were not enrolled based on low or high vitamin C concentrations, in part because measurements were not performed. It remains unknown whether vitamin C could improve pregnancy outcomes in patients with low concentrations of the vitamin (Rumbold et al., 2015, Rossi & Mullin, 2011).

Several Single Nucleotide Polymorphisms (SNPs) have been described in SVCTs. SNPs can be in the protein coding (exonic SNP) region of the gene or in other regions including introns (intronic SNP), promoter or intergenic regions. Some exonic SNPs may change the amino acid sequence of the protein product (nonsynonymous SNP, missense SNP). For other exonic SNPs, the variation in nucleotide sequence does not result in a change in the amino acid sequence of the protein (synonymous SNP). Nonsynonymous SNPs are more likely to change SVCT protein function, which may then be reflected in altered vitamin C physiology. However even SNPs that do not change amino acid sequence of the protein product (intronic SNPs, synonymous SNPs) may produce a functional change *in vivo*, because the difference in the DNA sequence may have many effects, including influencing promoter activity or messenger RNA stability (Shastry, 2009) .

Several SNPs have been found in the human SVCT1 gene that, by reducing vitamin C renal reabsorption, could recapitulate low vitamin C concentrations (Corpe et al., 2010) (Timpson et al., 2010). The relevant polymorphisms are more common in populations from sub-Saharan Africa (Eck et al., 2004) (Erichsen et al., 2006) than those from other parts of the world (Timpson et al., 2010) and may simply reflect the greater genetic diversity found in Africa (Jorde et al., 2000, Witherspoon et al., 2007, Tishkoff & Williams, 2002, Tishkoff & Verrelli, 2003, Reed & Tishkoff, 2006). Alternately, the relatively easy availability of

vitamin C rich food may have reduced the danger from impaired vitamin C transport by SVCT1 and allowed such SNPs to persist in this population. Other, as yet unknown selective pressures may also account for these SNPs. Another possibility is that such SNPs are rarer in the non-African populations because these populations may have been subject to deprivations, especially that of vitamin C containing food, in their migration out of Africa. In the presence of low vitamin C intake, it is necessary to conserve vitamin C, and SNPs that result in vitamin C loss would be rapidly selected out.

In addition to the common SVCT1 SNPs whose effect on vitamin C transport have been studied *in vitro* (see below) (Corpe et al., 2010), many uncommon or rare SVCT1 SNPs have been identified. With increasing use of DNA sequencing in research, it has become apparent that these SNPs are numerous with several hundred identified in publically available databases (ENSEMBL, 2015a, NCBI, 2015a). Because there are so many different SVCT1 SNPs (though each individual SNP is uncommon), the uncommon or rare SVCT1 SNPs cumulatively may account for a greater proportion of SVCT1 SNPs in the general population than that accounted for by the well-studied common SVCT1 SNPs. The population distribution of these uncommon SNPs is not known and it is possible that the preponderance of SVCT1 SNPs described in African populations may reflect incomplete knowledge or ascertainment bias (Michels et al., 2013).

The effect of SVCT1 exon SNPs on vitamin C transport has been studied *in vitro* using the *Xenopus laevis* oocyte expression system. When either the common type of SVCT1 or SVCT1 containing SNPs frequently found in humans was expressed in oocytes, oocytes that expressed SVCT1 transporters harboring SNPs were found to have reduced ability to accumulate vitamin C from the surrounding media (Corpe et al., 2010). Data from these *in vitro* vitamin C transport studies were used to estimate plasma vitamin C concentrations in humans using a mathematical model based on data from normal subjects (Graumlich et al., 1997, Padayatty et al., 2004). The calculations showed that SVCT1 SNPs significantly reduced steady state fasting vitamin C concentration at intakes in the range of 30 to 2500 mg/day (Corpe et al., 2010). Population studies have shown reduced plasma vitamin C concentration in subjects with specific SVCT1 SNPs, though these reductions were less than that predicted by the pharmacokinetic modeling described above (Michels et al., 2013) (Timpson et al., 2010) (Cahill & El-Sohemy, 2009). In people with low vitamin C intake, such polymorphisms in SVCT1 and SVCT2 were associated with low vitamin C concentrations in the lens and aqueous humor (Senthilkumari et al., 2014). A negative association was seen between two SVCT2 SNPs (one intronic and the other exonic) and colorectal adenoma, but colorectal adenomas were not associated with the common SVCT1 SNPs (Erichsen et al., 2008). In another study, a synonymous SVCT1 SNP conferred an increased risk of Crohn's disease (Amir Shaghghi et al., 2014). In these association studies, there were no vitamin C measurements reported for plasma, tissue or urine. Therefore it is not possible to attribute the findings to alterations in vitamin C physiology (Michels et al., 2013). A metaanalysis of five studies with a total of more than 18000 patients found no relationship between SVCT1 SNPs and metabolic parameters that contribute to the risk of cardiovascular disease (Wade et al., 2015). Currently, the clinical and physiological significance of intronic and exonic SVCT polymorphisms are unknown. The role of genetic

polymorphisms on vitamin C physiology is reviewed elsewhere (Michels et al., 2013, Shaghghi et al., 2016).

SVCT2 is found throughout the body and transports vitamin C into tissues. As noted above, SVCT2 knockout mice die immediately after birth; and fetal organs have undetectable or very low vitamin C concentrations in comparison to heterozygous and wildtype mice (Sotiriou et al., 2002). In vitamin C synthesizing species such as mice, the developing embryo does not synthesize vitamin C in the early part of fetal development. Placental transport of vitamin C from the mother to the fetus is essential for fetal survival, and such transport is mediated by SVCT2 in placenta. Placental vitamin C transport maintains higher vitamin C concentration on the fetal side of the placenta than on the maternal side. Therefore, vitamin C concentrations are higher in the umbilical cord blood than in the maternal blood.

That SVCT2 knockout mice do not survive is the best available *in vivo* evidence that vitamin C acquisition via the uptake of DHA by glucose transporters is not physiologically dominant nor is it adequate to stave off fatal scurvy at the tissue level. However, because ascorbate was not measured in every tissue from SVCT2 knockout mice, it was still possible that the DHA pathway was utilized by one or more tissues or cell types *in vivo*. Thus, DHA is the exclusive pathway utilized by mouse and human red blood cells (Tu et al., 2015). Red blood cell ascorbate was not measured in SVCT2 knockout mice because an assay for RBC was not available at that time (Li et al., 2012).

The SVCT2 gene harbors many more SNPs (more than 2000) than the SVCT1 gene but they are either intronic or synonymous SNPs (a few nonsynonymous SNPs have been recently identified in public databases) (ENSEMBL, 2015b, NCBI, 2015b). It may be that SVCT2 is less tolerant of changes in its amino acid sequence reflecting its critical importance in transporting vitamin C into a wide variety of cells (Michels et al., 2013) (shown by lethality at birth in SVCT2 knockout mice) (Sotiriou et al., 2002).

## Absorption and tissue distribution of vitamin C

Vitamin C is absorbed from the small intestine in humans, achieving peak plasma vitamin C concentrations approximately 120-180 minutes after ingestion. Although SVCT 1 is believed to be the candidate transporter, other SVCTs may exist. Alternatively, some ascorbate may be oxidized in intestine, transported as DHA by GLUT2, and then immediately reduced (Corpe et al., 2013). Of note, intestinal transport of DHA by GLUTs, with subsequent reduction to ascorbate in the enterocyte or mesenteric system, does not account for the experimental findings that there is absorption of an ascorbic acid analog in mice that do not have SVCT1. When oxidized to its dehydro form, this analog cannot form a bicyclic hemiketal structure (Fig 2), and is not transported by GLUTs, even though the analog is absorbed in SVCT knockout mice.

After absorption, because vitamin C is water soluble, it is distributed from blood throughout the extracellular space. Tissues accumulate vitamin C (Fig 3) against a concentration gradient, most likely via SVCT2 as discussed above. Tissue concentrations are dependent

upon plasma and extracellular fluid vitamin C concentrations, which in turn are dependent on dietary intake of vitamin C. Tissue concentrations of vitamin C, which are frequently millimolars (fig 3), are far in excess of what is required for its actions as a coenzyme. Millimolar concentrations of intracellular vitamin C may simply serve as a reservoir of the vitamin, or may have other unknown functions.

It should be noted that for many human tissues, measurements of accurate vitamin C concentrations in health and in disease states are not known. The major portion of vitamin C in humans is in the liver, brain, and skeletal muscle. Although skeletal muscle vitamin C concentrations are not high when compared to other cell types (Fig 3), skeletal muscle constitutes 31-38% of body mass (Janssen et al., 2000). Skeletal muscle vitamin C concentrations appear to be in equilibrium with dietary intake of the vitamin (Carr et al., 2013). Estimates of tissue vitamin C in humans (Fig 3) are based on data compiled from multiple sources, often using post mortem samples and inaccurate AA essays. When human body fluids or tissues are not promptly and properly processed, vitamin C may be lost by oxidation and/or sample handling practices, and assay results may be artificially low. To calculate body stores of vitamin C that result from differing vitamin C intake, it is possible in theory to match tissue / cell concentrations to plasma concentrations. Unfortunately, only limited matched values are available, for monocytes, lymphocytes, neutrophils, mixed mononuclear cells, skeletal muscle, and red blood cells (Levine et al., 1996b) (Levine et al., 2001b) (Tu et al., 2015, Carr et al., 2013). These data are insufficient to calculate total body stores of vitamin C in relation to doses. Prior to use of modern HPLC assays, radiolabelled ascorbate administered to humans was used to estimate body stores (Baker et al., 1969, Kallner et al., 1979, Baker et al., 1971). Limitations of these data are that the radiolabel was assumed to remain as ascorbate in humans, but no mass measurements were performed to confirm the assumption.

For animal vitamin C concentrations, rodent data are most abundant. Reported tissue concentrations of vitamin C in the rat are much higher than those in humans (Fig 3). This may be because fresh tissue is easily obtained under controlled conditions from rodents, thus avoiding oxidation and irreversible degradation of vitamin C. Note that plasma vitamin C was reported to be 90  $\mu\text{M}$  in rat (Fig. 3), although more recent studies with modern assays have shown plasma concentrations of 40 – 70  $\mu\text{M}$  in rat (Corpe et al., 2013) and mouse (Corpe et al., 2010). Higher plasma concentrations described in older reports on rodents may have been due to inaccurate spectrophotometric/colorimetric assays. These assays can over-estimate the amount of vitamin C when measured concentrations are at the low end of an assay range, as is the case for plasma samples. It is also possible that the higher reported measurements may have indicated a true increase in plasma vitamin C concentrations precipitated by acute stress. Because of interfering substances, spectrophotometric/colorimetric assays tend to be inaccurate when vitamin C concentrations are low, as in the micromolar range. Spectrophotometric/colorimetric assays are less affected by interfering substances (presumably because they are present in fixed amounts) when ascorbate concentrations are higher, especially in the mM range, as is the case with many tissues. It is also possible that there are species differences in plasma and tissue vitamin C concentrations, and in particular differences between vitamin C synthesizing (rat, mouse) and non-synthesizing (primate) species. Reliable vitamin C concentrations (i.e. derived from

properly obtained and processed tissue samples and measured by HPLC assays) for many human tissues are as yet not available (especially in relation to simultaneously measured plasma vitamin concentrations), with exceptions noted above for circulating cells and skeletal muscle.

Red blood cells are the only body compartment other than saliva (Fig 3) that have vitamin C concentrations that are similar to or lower than that of plasma (Li et al., 2012) (Evans et al., 1982) (Jacob et al., 1987). Until recently RBC vitamin C was usually measured (Butler & Cushman, 1940, Barkhan & Howard, 1958, Kassan & Roe, 1940)}(Evans et al., 1982) (Jacob et al., 1987) using spectrophotometric/ colorimetric assays prone to artifact, such that vitamin C concentrations were either overestimated or indeterminate (Li et al., 2012, Mendiratta et al., 1998, Iheanacho et al., 1995, Okamura, 1980, Westerman et al., 2000, Lubschez, 1945, Iggo et al., 1956, Washko et al., 1992, Rumsey et al., 2000b). With advent of HPLC techniques to measure vitamin C in red blood cells, there is now promise of learning pathophysiology of vitamin C in disease, especially in diabetes (Tu et al., 2015).

In contrast to other tissues, RBCs obtain vitamin C via a dehydroascorbic acid pathway. Progenitor erythroid cells have SVCT2 transporters, but these are lost in the process of maturation, such that circulating RBCs do not have any known vitamin C transporter. Circulating RBCs have slightly lower vitamin C concentrations than plasma. RBCs obtain ascorbate via transport of trace concentrations of DHA, found in plasma, on glucose transporters followed by immediate intracellular reduction. RBCs can reduce DHA via the protein glutaredoxin, or by direct chemical reduction from the millimolar intracellular RBC concentrations of glutathione. RBCs are unlikely to be a simple storage reservoir for plasma vitamin C based on direct efflux from RBCs, although this hypothesis has been considered (Montel-Hagen et al., 2008), because ascorbate efflux from RBC is comparatively slow (May et al., 1996, Mendiratta et al., 1998, May et al., 2001). Because RBCs are more numerous than other blood cells, they do account for most of the vitamin C in circulating cells of whole blood (Barkhan & Howard, 1958, Li et al., 2012).

The function of ascorbate in RBCs is uncertain. Until recently (Li et al., 2012), function has been difficult to address because of inability to accurately measure ascorbate in RBCs. In addition to the postulated role of ascorbate efflux from RBCs, other evidence supports a function of ascorbate based transmembrane electron transfer across the RBC. The hypothesis is that ascorbate is an electron donor for transmembrane electron transfer from within the RBC to an acceptor externally, most likely ascorbate radical formed from ascorbate oxidation in plasma. Therefore, electrons from ascorbate within RBCs would help to maintain plasma ascorbate, but without direct exit of ascorbate itself from the RBC (May et al., 2000) (May et al., 2004). The hypothesis is consistent with observations about vitamin C stability in blood and plasma samples (Dhariwal et al., 1991b) (Levine et al., 1999b) (Kassan & Roe, 1940). Vitamin C in plasma samples prepared from human whole blood, stored for as long as 24 hours, is more stable than vitamin C in human plasma or serum samples that have been separated from RBCs and stored for the equivalent amount of time (Dhariwal et al., 1991b). Vitamin C may also have a separate role in maintaining the red blood cell structural protein  $\beta$ -spectrin. In mice that cannot make vitamin C and that have low vitamin C concentrations, red blood cell  $\beta$ -spectrin is decreased.  $\beta$ -Spectrin returns to



normal levels within days after deficient mice are re-supplemented (Tu et al., 2015). Other red blood cell structural proteins are not affected by variations in vitamin C concentrations. It is unknown why ascorbate is necessary for maintenance of  $\beta$ -spectrin. It is possible that there are specific amino acids on  $\beta$ -spectrin (i.e. arginine, asparagine) that are hydroxylated in an ascorbate-dependent fashion, analogous to ascorbate dependent hydroxylation of proline in collagen and HIF-1 $\alpha$ .

## Vitamin C physiology

Current knowledge of vitamin C physiology in humans is largely limited to vitamin C dose concentration relationships, bioavailability and renal excretion in healthy young subjects (Levine et al., 1996b, Levine et al., 2001b). Many investigations have documented depletion rates, absorption, plasma and cellular concentrations, and excretion of vitamin C in normal humans and in some disease states. (Vinson & Bose, 1988) (Mangels et al., 1993, Gregory, 1993) (Blanchard, 1991b, Blanchard et al., 1989, Blanchard, 1991a, Baker et al., 1971, Hodges et al., 1969). Results were variable, perhaps because of uncertain dietary control, imprecise vitamin C assays, or pharmacokinetics that was not performed at steady state (Piotrovskij et al., 1993).

Plasma vitamin C concentrations depend on dietary intake; vitamin C absorption by the gastrointestinal tract; distribution in body fluids and uptake by tissues; irreversible metabolism of vitamin C (utilization); and vitamin C excretion by the kidneys. All of these factors may be altered in disease, and may also vary depending on body composition, genetics, and perhaps other factors such as physical activity. However, the most important variable identified so far that determines plasma vitamin C concentration is dietary intake.

Many studies have detailed the relationship between dietary intake of vitamin C and the concentrations of vitamin C in plasma and in circulating cells. Low dietary intake and plasma concentrations of the vitamin are common, even in affluent countries. In a survey of 7277 non-institutionalized civilians in the US in 2003-2004 (Schleicher et al., 2009), mean plasma vitamin C concentrations in subjects more than 6 years of age were 48  $\mu$ M in males, and 54.8  $\mu$ M in females. However, 8.2% of males and 6% of females had plasma vitamin C concentrations < 11.4  $\mu$ M, a concentration where frank scurvy could occur. Among men, 18% of smokers but only 5.3% of non-smokers had such low values. Among women, 15.3% of smokers and 4.2% of non-smokers had similarly low values. These values are to a large extent dependent on dietary intake and perhaps smoking, but some disease states may increase loss by oxidative degradation of the vitamin or through increased renal loss.

Absolute bioavailability of an oral dose of vitamin C can be determined when the subject is at steady state for a specific dose, as in depletion-repletion studies performed under carefully controlled conditions (Young, 1996, Graumlich et al., 1997) (Levine et al., 2001b)(Figs 4 A and 4B). Absolute bioavailability describes what percentage of an orally administered dose is absorbed when compared to an intravenous dose. For example, bioavailability of a 30 mg oral dose can be determined when the subject has consumed this dose (15 mg taken twice daily, total of 30 mg/ day) long enough for plasma and tissue concentrations of the vitamin to be at equilibrium for the dose. This equilibrium condition is termed steady-state.

At the time of steady-state, plasma vitamin C concentrations after administration of a 30 mg oral and intravenous dose can be used to calculate absolute bioavailability of the 30 mg dose. Bioavailability of the intravenous dose is considered as complete, or 100%, because the intestine is by-passed with intravenous administration. When tissues are in equilibrium with plasma, the calculations are valid because the administered dose is not suddenly taken up by tissues. Conversely, if subjects were not in equilibrium for any dose, then absorption and bioavailability calculations could be unreliable. For example, if a subject at equilibrium for a 30 mg/day dose was then given a much larger dose instead, say 200 mg, tissues would suddenly be exposed to higher vitamin C concentrations, plasma concentrations could change unpredictably, and bioavailability calculations therefore become unreliable or of questionable value. Current data on absolute bioavailability pertain to doses of 15 to 1250 mg when the subject was at steady state for each specific dose (Graumlich et al., 1997).

While useful for understanding absorption, the steady- state condition rarely pertains to real life. When a patient takes oral vitamin C at 30 mg or 1000 mg, current data cannot accurately predict bioavailability for the dose unless that patient consumes the same dose chronically and is at steady- state for that dose (assuming no other vitamin C intake in food). Bioavailability is very high (meaning close to 100%) when small doses of (for example 30 mg/day) are given to subjects with low plasma vitamin C concentrations. In the carefully controlled depletion-repletion studies, pure vitamin C was given as a solution by mouth in the fasting state. In real life, vitamin C in food may not be fully bioavailable because of physical sequestration of the vitamin within foods, or because vitamin C is degraded or its absorption inhibited by other food components. It is also possible that some substances in food can enhance vitamin C absorption. Therefore, published bioavailability data has to be interpreted cautiously when applied to real life clinical practice. A summary of vitamin dose concentration relationships, bioavailability, amount absorbed and the amount excreted in the urine is shown in figure 5.

## Vitamin C in disease states

In addition to its deficiency state scurvy, plasma and tissue vitamin C may be altered in many disease states. Vitamin C is the most potent concentration-dependent water soluble antioxidant in the body (Frei et al., 1989) and the principal antioxidant that quenches aqueous peroxy radicals and lipid per oxidation products in plasma *ex vivo* (Frei et al., 1990). If in the process ascorbate is irreversibly oxidized, vitamin C will be consumed and dietary requirements for the vitamin increase. *In vitro*, ascorbate is preferentially oxidized in plasma before other antioxidants (uric acid, tocopherols, and bilirubin). Although antioxidant effects of vitamin C and other antioxidants have been shown *in vitro*, significance of the findings is unclear unless such effects can be shown *in vivo*. It has not been possible to definitively attribute pathological processes to antioxidant deficiency, nor to lack of specific antioxidants.

Many common clinical conditions are thought to result in pro-oxidant states that contribute to pathology, including that attributable to cigarette smoking and diabetes. Smokers (Schechtman et al., 1991, Lykkesfeldt et al., 2000, Smith & Hodges, 1987, Kallner et al., 1981, Schechtman et al., 1989) and diabetic subjects (Dorchy, 1999, Seghieri et al., 1994, Yue

et al., 1990, Som et al., 1981, Stankova et al., 1984, Will et al., 1999, Sinclair et al., 1994, Sargeant et al., 2000) (Chen et al 2006; Tu et al 2015) (Kaviarasan et al., 2005) have lower plasma vitamin C concentrations than controls. These findings may be due to low dietary intake and/or increased metabolism of vitamin C. *In vitro and in vivo*, hyperglycemia reduces red cell vitamin C concentrations (Tu et al., 2015). *In vivo*, it is unclear whether reductions in plasma vitamin C concentrations are due to direct glucose toxicity or indirectly from other metabolic consequences of diabetes. Vitamin C concentrations may also be low in acute illnesses, including myocardial infarction (Hume et al., 1972, Riemersma et al., 2000), acute pancreatitis (Bonham et al., 1999, Scott et al., 1993), sepsis (Fowler et al., 2014) and in patients with critical illness (Berger & Oudemans-van Straaten, 2015). It is possible that low plasma and tissue vitamin C concentrations contribute to the pathology or is a consequence of the disease process. On the other hand low concentrations may be merely associated with a disease condition but not causal. In these disease conditions, circulating oxidants may be present in the plasma. While such oxidants could have no effect on vitamin C *in vivo*, they still could oxidize vitamin C in blood once it has been withdrawn from the patient and is awaiting analysis. Other substances that interfere with vitamin C assays may also be present, and it is necessary to take the possibility of measurement artifacts into consideration (Padayatty & Levine, 2000). Disease states are associated with sub-optimal nutrition, and low vitamin C concentrations may simply reflect poor intake (Dallongeville et al., 1998). The role of vitamin C as an antioxidant has been reviewed in detail elsewhere (Padayatty et al., 2003). Because vitamin C is excreted by the kidneys, it can accumulate in renal failure, and also is lost in dialysis fluid during hemodialysis (Sullivan & Eisenstein, 1970, Balcke et al., 1984, Handelman, 2011, Clase et al., 2013). Vitamin C replacement in patients with renal failure has to take both these factors into consideration to avoid both vitamin C deficiency and vitamin C toxicity (Pru et al., 1985, Balcke et al., 1984, Ono, 1986).

## Vitamin C in relation to oral health

Vitamin C measurements have been performed in different oral tissues, but clinical interpretation is often problematic. The earliest studies of vitamin C using guinea pig as a model of a scurvy-prone animal showed that teeth take up substantial amounts of administered vitamin C (Burns et al., 1951). C13 labeled vitamin C is accumulated by the parotid and submandibular glands in the ascorbate replete (Hornig et al., 1974) and ascorbate depleted guinea pig (Hornig et al., 1972) (Hornig et al., 1972). Some uptake was noted in the periodontal tissue and pulp of teeth (Hornig et al., 1972) (Hornig et al., 1974). Secretory granules in the acinar cells of rat parotid glands contain millimolar concentrations of vitamin C and it is cosecreted with amylase (Vonzastrow et al., 1984). In hypophysectomized rats compared to normal rats, vitamin C accumulation is reduced in many tissues, including salivary glands (Hornig et al., 1972) by as yet unknown mechanisms. Vitamin C is secreted by the salivary glands and is found in low concentrations in saliva. Studies of salivary vitamin C concentrations in humans have shown widely varying results, from undetectable (Feller et al., 1975) to very low concentrations or near or higher than plasma concentrations (Anonymous, 1986, Diab-Ladki et al., 2003, Schock et al., 2004, Liskmann et al., 2007, Rai et al., 2011, Rai et al., 2007, Buduneli et al., 2006, Gumus et al.,

2009, Feller et al., 1975, Makila & Kirveskari, 1969, Moore et al., 1994, Bates et al., 1972, Vaananen et al., 1994, Leggott et al., 1986b, Leggott et al., 1986a, Makila, 1968, de Sousa et al., 2015, Saral et al., 2005). On balance, it seems likely that salivary vitamin C concentrations are lower than those of plasma. Salivary vitamin C concentrations increase within hours on consumption of vitamin C (Makila & Kirveskari, 1969). Vitamin C concentrations do not vary between submandibular, lingual and parotid salivary glands (Makila & Kirveskari, 1969), but decrease with increased salivary flow in submandibular but not in parotid glands (Makila & Kirveskari, 1969). In a later study, plasma ascorbic acid concentrations as expected were synchronous with vitamin C depletion and repletion, but salivary ascorbic acid concentrations were unchanged, perhaps because they were at the lower limit of detection of the assay (Leggott et al., 1986b). These widely discordant reports of vitamin C concentrations in saliva may be attributable to assays that were affected by interfering substances (Feller et al., 1975), or because of differences in sample collection and processing. Standardized methodology, meticulous sample processing and modern methods of analysis that are specific and precise may resolve this issue. The function of vitamin C in saliva remains unknown.

In scurvy, oral signs and symptoms are prominent, and include bleeding and spongy gingivae with eventual loosening and loss of teeth. The underlying pathology in scurvy may be an impaired ability to replace collagen or dental tissues lost due to turnover or bacterial action. In the guinea pig, which has continuously growing teeth, teeth growth stops when vitamin C is withdrawn (Dalldorf & Zall, 1930). In the US studies on experimental scurvy in men, signs of scurvy began to appear about 40 days after vitamin C withdrawal (Hodges et al., 1969, Hodges et al., 1971, Hood et al., 1970). Oral lesions consisted of gingival swelling, gingival hemorrhage, and sublingual petechial hemorrhages. The lesions began along the margins of the gingivae and extended to interdental papillae, but periodontal membrane was intact on X-ray. The lesions were more marked in those with gingivitis. There were only minor gingival lesions in a subject with healthy gingivae and none in an edentulous subject. Some subjects also had Sjogren's syndrome, including xerostomia and enlargement of the parotid and submandibular salivary glands (Hodges et al., 1969, Hodges et al., 1971, Hood et al., 1970). In these studies, signs and symptoms of scurvy occurred later and were less severe than that reported in historical records, probably because scurvy in sailors or other populations occurred under much more adverse conditions. Those affected by either land or sea scurvy most likely suffered from multiple nutritional deficiencies and infections, and were usually involved in strenuous physical work. In self-induced experimental scurvy in a physician investigator, signs of scurvy appeared only after 161 days of consuming a self-reported vitamin C free diet (Crandon et al., 1940). The subject had good oral hygiene and had no nutritional deficiency other than that of vitamin C. No gross changes were seen in the teeth or gingivae except for interruption of lamina seen on x-ray, which may be a reliable sign of scurvy. Gingival biopsy was normal. From these studies, it is clear that the gingival lesions of scurvy only occur in those with teeth, and with preexisting gingivitis and/or dental caries. In a vitamin C depletion-repletion (and a second depletion) study of healthy men, low plasma vitamin C was not associated with periodontal disease, but was associated with gingival inflammation and bleeding (Leggott et al., 1986a).

The role of vitamin C in maintenance of normal dental health, in the absence of scurvy, is unclear. It is possible that adequate tissue, plasma and salivary vitamin C concentrations are essential for the normal health of teeth and gingivae. Low plasma vitamin C is associated with poverty, and it is difficult to attribute poor dental health to any one factor, including plasma vitamin C (Vaananen et al., 1994). It has been hypothesized that low cellular concentration of vitamin C is a cause of dry mouth, (Enwonwu, 1992) or that vitamin C increases production of saliva by increasing prostaglandin production (Horrobin & Campbell, 1980). In an uncontrolled study, treatment of Sjogrens syndrome (not resulting from vitamin C deficiency) with a mixture of substances including vitamin C was reported to improve symptoms (Horrobin et al., 1981) but no improvement was found in another study (McKendry, 1982). An association has recently been found between a rare SNP in SVCT1 and aggressive periodontitis (de Jong et al., 2014). It is too early to draw conclusions from such genetic studies, but it is an area that has promise.

Vitamin C acting locally may have erosive effects on teeth. There are reports of dental erosion from vitamin C consumption (Giunta, 1983, Imfeld, 1996) but these are uncommon considering the wide use of the vitamin. *In vitro*, prolonged exposure to ascorbic acid causes dental erosions (Giunta, 1983) visible on scanning electron microscopy (Meurman & Murtomaa, 1986). Chewable vitamin tablets (or vitamin C 500mg wafers used experimentally) reduced salivary pH from 7 to about 4.5 in two and a half minutes (Hays et al., 1992). In healthy subjects, no changes in the teeth were seen after one week of vitamin C consumption even in the absence of mechanical cleaning (Meurman & Murtomaa, 1986). However, it is possible that chronic consumption of chewable (Giunta, 1983) or effervescent (Meurman & Murtomaa, 1986) vitamin C may erode dental enamel. It has been suggested that sucrose containing vitamin C syrup may also cause acid damage to teeth (Woods, 1981).

## Experimental findings relating to vitamin C

There are numerous experimental phenomena which involve vitamin C in humans or with human cells, but with uncertainty as to how the findings relate to normal physiology, pathophysiology, clinical use, or disease treatment Described below are three such findings and clinically relevant questions for each.

### Activated human neutrophils accumulate vitamin C

When exposed to pathogenic bacteria, human neutrophils are activated and produce oxidants that kill the pathogen. These oxidants may cause collateral damage to the neutrophils themselves and to other tissues. When freshly obtained normal human neutrophils are exposed to clinical isolates of *E. coli*, *Enterococcus faecalis*, *Moraxella catarrhalis*, *Klebsiella oxytoca*, *Acinetobacter baumannii* or *C. albicans*, the neutrophils are activated and accumulate vitamin C rapidly from the surrounding culture media until all available external vitamin C is exhausted (Wang et al., 1997) (Washko et al., 1993). Vitamin C concentrations inside the neutrophils increase from starting concentrations of about 1 mM to values as high as 10 to 12 mM (Wang et al., 1997)(Fig. 6). The likely mechanism is via ascorbate recycling (Stankova et al., 1975) (Bigley & Stankova, 1974) (Hemila et al., 1984) (Anderson & Lukey, 1987). As described above, this means that oxidants from neutrophils accelerate oxidation of

external ascorbate to dehydroascorbic acid, which is transported on glucose transporters, and immediately reduced internally to ascorbate. Why do vitamin C concentrations increase so much and so rapidly in activated neutrophils? One possibility is that vitamin C is used to protect neutrophils from the oxidants that they make. Conversely, vitamin C could increase oxidant generation, to ensure that pathogens are killed. Answers to these questions may have clinical relevance. Thus, is ascorbate clinically important to enhance neutrophil action in healthy patients? Since the mechanism of increased ascorbate in neutrophils involves glucose transporters, could extra vitamin C be useful for neutrophil action in diabetic subjects? Could extra vitamin C, by its actions in neutrophils, improve outcomes in sepsis? (Fowler et al., 2014) (Voigt et al., 2002) (Fowler et al., 2014, Ferron-Celma et al., 2009) . Would extra ascorbate improve bacterial killing in patients with Chronic Granulomatous Disease? Patients with this disease have recurrent bacterial infections that are uncommon in healthy people, the infections are difficult to treat, and neutrophils from patients with this disease do not increase their ascorbate concentrations in presence of bacteria (Curnutte, 1993, Thrasher et al., 1994, Roos et al., 1996, Holland, 2010, Wang et al., 1997).

## Red blood cells and vitamin C

As discussed above, circulating red blood cells also transport DHA and reduce it intracellularly to AA. There is evidence that plasma ascorbic acid is maintained by ascorbic acid within red blood cells. In clinical samples, vitamin C in plasma is maintained much longer when the plasma is not separated from red blood cells until the time of sample processing (Dhariwal et al., 1991b). Plasma, with red blood cells removed will lose vitamin C fairly rapidly unless special precautions are taken to prevent loss. How red blood cells stabilize plasma vitamin C may be due to transfer of electrons from ascorbic acid within the red cell across the red blood cell membrane by electron transfer proteins (Su et al., 2006) (May et al., 2000) (VanDuijn et al., 2000). These red blood cells proteins are found in species that cannot make vitamin C, such as humans, but are absent from species that synthesize vitamin C such as rodents. It is possible that electron transfer across red blood cells has importance in disease or in conditions where plasma vitamin C is decreased, such as diabetes. Indeed, despite recent intriguing advances discussed above (Tu et al., 2015), the function of vitamin C itself in red blood cells is unknown. If vitamin C has an important function in RBCs, does this have relevance to transfused red cells, either for functional consequences, or as a function of storage of blood for transfusion?

## Human Adrenals secrete vitamin C in response to ACTH

Adrenal glands are known to have very high concentrations of vitamin C and Albert Szent-Györgyi first isolated it from ox adrenals. Human adrenal glands *in vivo* secrete vitamin C in response to ACTH which stimulates adrenal cortex to produce the essential hormone cortisol. Vitamin C secretion is rapid, starting and ending within minutes of ACTH administration in humans (Fig 7), and precedes cortisol secretion (Padayatty et al., 2007). What is the function of vitamin C release from adrenal glands? Vitamin C secretion in response to prostaglandin or hormonal stimulation has also been shown in the testes and ovary in animals (Koba et al., 1971) (Petroff et al., 1998) (Guarnaccia et al., 2000). Does vitamin C release have a similar function in these steroid secreting glands? In humans,



vitamin C concentrations in the adrenal vein peak at approximately five times the plasma concentration. Peak plasma vitamin C concentrations approaching values found transiently in the adrenal vein may be attained in the general circulation in those who take very large doses (1- 3 grams/day) of vitamin C supplements (Padayatty et al., 2004). Is mimicking of, or interference with, adrenal function induced by such concentrations?

## Recommended Dietary Allowance for vitamin C

The Recommended Dietary Allowance (RDA) for vitamin C in the United States and Canada is 75 mg for women and 90 mg for men (Food and Nutrition Board, 2000b). Many countries have increased the RDA for vitamin C in the past 15 years so that they are now similar to those in the United States and Canada, or even slightly higher.

The early RDA for vitamin C was based on the amount of vitamin C needed to prevent scurvy with an added amount to ensure a margin of safety and to account for individual variability. Human experiments conducted in UK (Peters et al., 1953), Canada (Johnstone et al., 1946) and USA (Hodges et al., 1969, Baker et al., 1969, Baker et al., 1971, Hodges et al., 1971) concluded that 10 mg or less of vitamin C/day was sufficient to prevent scurvy. These studies formed the basis for an RDA for vitamin C for several decades. However these studies had many flaws, including imperfect dietary control, the possibility of multiple nutrient deficiencies, and use of imprecise vitamin C assays. Nevertheless, the effect of the intake of a known amount of vitamin C on observable clinical outcome was shown.

An ideal RDA should be based on an intake that leads to optimum health, and not merely prevent a deficiency state, in this case scurvy. While the goal is laudable, getting there has so far been elusive. This is because we do not quite know how to measure optimum health, for any vitamin. Thus, it is predictable that we do not have data to indicate what intake of vitamin C is optimal for health.

The Food and Nutrition Board of the Institute of Medicine (of The National Academy of Sciences, USA) revised the RDA for vitamin C in the year 2000 using different guidelines than vitamin C body stores or clinical deficiency criteria used previously. For vitamin C, these guidelines incorporated ability of vitamin C to confer antioxidant protection. *In vitro* findings from human neutrophils were combined with published clinical data on vitamin C dose concentration relationships in healthy young men to estimate intake values (Food and Nutrition Board, 2000b). Unlike the earlier recommendations that related the amount of vitamin C intake only to a clinical outcome, *in vitro* data were utilized that described how clinically relevant vitamin C concentrations resulted in inhibition of superoxide production. Unfortunately, we do not know whether the newer criteria apply to health of intact humans or are relevant to *in vivo* physiology.

How was the current RDA calculated? Neutrophil vitamin C data detailed above consistent with minimal urinary loss was used to calculate the Estimated Average Requirements (EAR) for the vitamin. EAR for vitamin C is that amount of average vitamin C intake per day that is estimated to meet the nutrient requirements of 50% of the healthy population in that age and sex group. EAR was then used to calculate the RDA, taking into consideration the

standard deviation of EAR. For vitamin C, a coefficient of variation of 10% is assumed as data on standard deviation of requirements is not available. RDA is the amount of average vitamin C intake per day that would meet the nutritional requirements of 97-98% of the healthy individuals in the particular age and sex group, (Hellwig et al., 2006, Food and Nutrition Board, 2000b). For example, the EAR for vitamin C is 75 mg/day for men aged 19-30 years old, and the RDA for vitamin C is 90 mg/day for this age and sex group (Hellwig et al., 2006, Food and Nutrition Board, 2000b). These criteria are based on intake of the vitamin from foods, and not from supplements.

Basing recommendations in part on *in vitro* data and extrapolating these data to humans has limitations (Harper, 1975). However, due to absence of key human data, such extrapolations were deemed necessary by the Food and Nutrition Board for vitamin C. A similar rationale was used for vitamin E recommendations (Food and Nutrition Board, 2000a).

The RDA for vitamin C was as low as 10mg/day but has gradually increased over the years so that now it is nearly 100 mg/day. Intake of 10mg/day of vitamin C would result in plasma vitamin C concentrations of about 10  $\mu$ M while consumption of 100 mg/day of vitamin C will result in plasma vitamin C concentrations of about 50  $\mu$ M, close to the plasma concentrations above which vitamin C is lost in the urine. Once vitamin C is lost in urine, plasma and tissue levels do not increase much more with oral dosing, except transiently with doses found in supplements. In the NIH depletion repletion studies, fatigue occurred when plasma concentrations were less than about 20  $\mu$ M (Levine et al., 1996b) (Padayatty & Levine, 2001a). It is likely that some people in the general population will experience fatigue when vitamin C concentrations are between 10  $\mu$ M and 20  $\mu$ M, without any other manifestation of vitamin C deficiency. These low concentrations may be avoided by the new higher RDA, which may be an unintended benefit of the higher RDA. Conversely, fatigue is a common symptom in clinical practice, and is rarely due to vitamin C deficiency. The current RDA is higher than that amount for which there is a proven clinical benefit to prevent scurvy, although there may be other benefits that we are currently unable to quantitate clinically.

In summary, how much vitamin C intake is ideal for optimum health is not known. It is likely that requirements for vitamin C will vary (Yew, 1975) with genetically determined variations in vitamin C transport or metabolism, and with disease states. For example, at a time when scurvy was rampant in sailors who were at sea for prolonged periods, not all men developed the disease. All men on board (except the few officers) consumed the same or very similar diets, and while many (as many as half) developed fatal scurvy, others were unaffected or barely affected. Thus there was substantial inter individual variation in susceptibility to scurvy. Since vitamin C is easily acquired from foods, in clinical practice it is perhaps better to consider what foods have general health benefits.

With all of this information, then, how much vitamin C should we consume, and how should we consume it? Consumption of five servings of a variety of fruits and vegetables a day will provide ample amounts of vitamin C (approximately 200 -250 mg of vitamin C). Many observational studies and limited intervention trials have shown the benefits of increased fruit and vegetable intake on reducing cardiovascular risk and all-cause mortality (Wang et

al., 2014, Hartley et al., 2013, Woodside et al., 2013) (Hung et al., 2004). A diet rich in fruit and vegetable intake is associated with many factors including high socioeconomic status (in Western countries), education, and a healthy lifestyle. Fruits and vegetables also displace higher calorie foods, provide bulk and fiber, and micronutrients and phytochemicals. Plasma concentrations of many substances, including carotenoids and vitamin C, are elevated in those with higher consumption of a variety of fruits and vegetables. However, association is not causation, and the mechanism of the beneficial effects of fruits and vegetables is not known. In view of available information, the best advice is to eat a wide variety of fruits and vegetables of many colors. Five servings per day will produce near saturation of plasma and tissues by providing 200 to 250 mg/day of vitamin C.

## Relevance of experimental studies to ascorbate physiology: remembering Goldilocks

While a large number of studies have shown effects of vitamin C on many physiological parameters or cellular function, the relevance of these findings to clinical physiology and pathophysiology remains unclear. Many such studies used un-physiological doses of vitamin C or attained plasma or tissue concentrations far from the physiological range, either too high or too low. Some of these concerns have been reviewed elsewhere (Levine et al., 2011) and we have limited our discussion to the necessity of careful interpretation of these studies. In essence there are problems that Goldilocks would understand fully, both in experiments in the laboratory and experiments in people. For interpretation to be sound, vitamin C conditions should be “just right”, to reflect physiology. When there is either too little vitamin C, or too much vitamin C, then it is hard to interpret the outcomes.

## Interpretation of *in vitro* studies using vitamin C

Most mammalian cells grow well in cell culture media that contain no ascorbate, and are scorbutic. In contrast, the intact animal cannot survive in the absence of vitamin C, either synthesized by the animal or obtained from food. Cultured cells used *in vitro* have either adapted to survive and proliferate in the absence of ascorbate, or have subtle abnormalities due to ascorbate deficiency that are not fatal. In either case, these cells may not be representative of normal human physiology in which cells are at all times exposed to ascorbate concentrations in the range of 10  $\mu\text{M}$  to 70  $\mu\text{M}$  (occasionally higher, in those who take vitamin C supplements). Further, vitamin C is the most abundant water soluble antioxidant in circulation. Cell culture studies exploring antioxidant mechanisms that are performed without ascorbate will at the very least be unrepresentative of *in vivo* physiology. When ascorbate supplementation is undertaken, ideally cells should be exposed to physiological concentrations of ascorbate but not to very high (>100  $\mu\text{M}$ ) or very low (<10  $\mu\text{M}$ ) vitamin C concentrations that are outside the physiological range. Ascorbate in cell culture conditions is unstable, and a constant concentration of ascorbate can be maintained only by repeated supplementation with appropriate amounts. Unfortunately, there often has been limited appreciation of how to apply ascorbate physiology to cell experiments. As a consequence, physiologic ascorbate concentrations are added to cells for short periods of time, and then effects are determined. Most often this time is insufficient for cells to achieve

physiologic intracellular concentrations. Thus, the meaning of findings becomes questionable.

The other side of the spectrum is that for some *in vitro* experiments, investigators have added very high, or pharmacologic, concentrations of ascorbate, in the mM range. Addition of mM concentrations may result in the generation of ascorbate radical and hydrogen peroxide. These compound, in turn, have many actions on cells that are independent of ascorbate physiology (Levine et al., 2011). Such actions on cells could provide new insights to use of ascorbate in disease treatment, especially cancer treatment. However, it must be remembered that pharmacologic ascorbate actions may not be relevant to ascorbate physiology.

### Interpretation of *in vivo* studies using vitamin C

Similarly, *in vivo* infusion of mM concentrations of ascorbate have been used to demonstrate many effects including vasodilatation (Levine et al., 1996a, Motoyama et al., 1997, Beckman et al., 2001, Vita et al., 1998, Jackson et al., 1998, Duffy et al., 2001, Kaufmann et al., 2000). The results, even when repeatable, do not represent vitamin C physiology but rather pharmacological use of vitamin C (Padayatty & Levine, 2001b). Such studies are useful to demonstrate specific effects such as anti-oxidant actions or as proof of concept (Kaufmann et al., 2001). Other studies using infused vitamin C do so with the understanding that pharmacologic properties are being studied: for example, in cancer treatment (Levine et al., 2011) (Yun et al., 2015). While these studies may be very useful for understanding new uses of ascorbic acid as a drug, such studies however are not useful in determining the optimum dietary intake of vitamin C or ascorbate oral supplementation to achieve a desired clinical affect. Therefore, the results of *in vitro* and *in vivo* experiments with vitamin C have to be interpreted taking all these of factors into consideration, particularly the dose concentration relationship of vitamin C.

### Goldilocks and intervention trials: importance of dose-concentration relationships

Many small and large scale intervention trials have studied the effect of vitamin C supplementation (alone or with other nutrients) on clinical outcome. These have shown either no effect or very small effects. These negative results may indicate that vitamin C had no clinical effect on the condition studied. Alternately, these studies may have had control and treatment groups that were not substantially different with regard to plasma and tissue vitamin C concentrations, (Levine et al 1999a) (Padayatty & Levine, 2009) making it impossible to detect any effect attributable to the vitamin. Such an outcome is likely unless the study is carefully designed, because vitamin C dose concentration relationship is not linear but sigmoidal. In patients who are in a vitamin C depleted state, plasma vitamin C concentrations increases only modestly after small doses of vitamin C (<30mg/day) but much more so after slightly larger doses (>100 mg/day) before reaching a plateau. For example, in men enrolled in the NIH depletion repletion study, fasting steady state plasma vitamin C concentrations were 8.7  $\mu$ M for a daily vitamin C intake of 30 mg. For a 100 mg/day intake, plasma values increased to 56  $\mu$ M, and for an intake of 400 mg/day, plasma

values increased to 70  $\mu\text{M}$ . Corresponding values for women were: for 30 mg/day-12.6  $\mu\text{M}$ ; for 100mg/day-62  $\mu\text{M}$ ; and for 400 mg/day-73  $\mu\text{M}$ . Therefore, intakes much higher than the RDA for vitamin C (90 mg/day for men, 75 mg/day for women in the US) resulted in only small increases in plasma vitamin C concentrations (see figures 4A and 4B) (Levine et al., 1996b, Levine et al., 2001b). These findings have several important implications. First, if an intervention study starts with well-nourished control and intervention groups (consuming about 100 mg/day of vitamin C in food), then supplemental vitamin C would only modestly increase plasma and tissue vitamin C concentrations. (Levine et al 1999a) (Padayatty & Levine, 2009). Such minor changes may not have any demonstrable benefit, and predictably so. Second, it is necessary to select populations that have low plasma vitamin C concentrations, so that supplementation (Levine et al 1999a) (Padayatty & Levine, 2009) will produce a clear difference between the control and treatment groups with regard to plasma and tissue vitamin C concentrations (Padayatty & Levine, 2009). Thus the potential for demonstrating benefit (or harm) is greatly increased (Lykkesfeldt & Poulsen, 2010). To ensure this, it is necessary to measure plasma vitamin C concentrations at baseline, and ideally, after supplementation (Padayatty & Levine, 2009) (Padayatty & Levine, 2014). Careful attention to dose concentration relationships is essential to detect any effect from vitamin C supplementation and to ensure that intervention studies have a reasonable chance of detecting benefit or harm (Morris & Tangney, 2011). Finally, these principles apply not just to vitamin C, but to other vitamins. When the goal is to learn whether increase in a vitamin changes outcome, it is essential to select study populations for whom supplementation could change vitamin concentrations more than marginally. These concentrations must be measured both before and after intervention with that vitamin, and in control groups as well.

The importance of the sigmoidal dose concentration response is shown by the following examples. In a study of the effect of antioxidants on eclampsia, 283 pregnant women at increased risk of pre-eclampsia were given either vitamin C 1000 mg/day (with vitamin E) or placebo. The study population had measured plasma vitamin C concentrations of about 30  $\mu\text{M}$ , which increased to  $\sim 50$   $\mu\text{M}$  in the treatment group (Chappell et al., 1999). PAI-1/PAI-2 ratio (PAI-1/2: Plasminogen Activator Inhibitor 1 or 2, a marker of pre-eclampsia) was reduced in the supplemented group, as was pre-eclampsia. To verify the findings, a subsequent study was performed in a larger cohort of 1877 pregnant women at risk of pre-eclampsia and perinatal complications. Vitamin C 1000 mg/day (plus vitamin E) was administered, but plasma vitamin C concentrations were not measured (Rumbold et al., 2006). However, calculated plasma vitamin C concentrations were approximately 70 to 80  $\mu\text{M}$  in both the control and treatment groups, despite supplementation in the treatment group (Padayatty & Levine, 2006). Not surprisingly, the study showed no benefit. In a follow up study of vitamin C 1000 mg/day (plus vitamin E) to prevent hypertension in 10154 pregnant women, plasma vitamin C was not measured and supplementation showed no benefit (Roberts et al., 2010). This outcome was also predictable, because the study population was well nourished at baseline. These, and similar studies cannot reliably distinguish between a true lack of effect of vitamin C and an apparent lack of effect, because the intervention groups were likely already replete in vitamin C and were not substantially different from the control groups with regard to the vitamin, even with supplementation (Rumbold et al., 2006,

Padayatty & Levine, 2006, Sesso et al., 2008, Padayatty & Levine, 2009, Fortmann et al., 2013, Padayatty & Levine, 2014, Isanaka et al., 2012, Padayatty & Levine, 2013). In vitamin C supplementation studies, as well as those for other nutrients, consideration of dose-concentration relationships is critical to good study design (Levine et al 1999a) (Padayatty & Levine, 2009) (Morris & Tangney, 2011) (Lykkesfeldt & Poulsen, 2010).

### Some stark unknowns

While recent studies have shed new light on many aspects of vitamin C physiology, conundrums remain. A few of these conundrums are listed below.

- 1) Most animals synthesize vitamin C but some have lost the ability to do so. Those animals that lack vitamin C synthetic ability do not bear any phylogenetic relationship to each other, implying many independent mutations all resulting in the same phenotype. No common environmental influence is apparent. To date, there is no satisfactory evolutionary explanation for the apparent random loss of vitamin C synthetic ability. It remains possible that other animals have not been recognized to have lost the ability to synthesize vitamin C. Identification of all non-synthesizers possibly could enhance recognition of a pattern, but so far none is evident.
- 2) Vitamin C is concentrated by most tissues in humans and in many mammals. However, only much lower concentrations, sometimes 1-2 orders of magnitude lower, are required for the known enzymatic actions of vitamin C. Why do tissues so avidly acquire vitamin C? A possible explanation is that maintaining concentrations in great excess above required function may be an evolutionary protective or “safety” function. In this case, it would take time for enzymatic consequences, and deficiency, to develop if intake suddenly stopped. Alternatively, intracellular accumulation at much higher concentrations than those needed for enzymatic action may indicate that vitamin C has other, as yet unknown, functions.
- 3) High concentrations (>5 mM as compared to  $\mu\text{M}$ ) of vitamin C are produced in extracellular fluid only with intravenous or parenteral administration of ascorbate. Ascorbate at pharmacological concentrations produces hydrogen peroxide (Fig 8). Hydrogen peroxide or oxidant species that result from peroxide damage cancer cells but not normal cells. In blood, these oxidant species are below the threshold of measurement, perhaps due to the large reducing capacity of red blood cells. In nature, pharmacologic concentrations of ascorbate do not occur in the blood or extracellular fluids. But such concentrations occur within cells of many tissues. With administration of ascorbic acid intravenously or parenterally, mM concentrations of ascorbic acid occur in the extracellular fluid as well as within cells. For normal cells, there are redundant intracellular mechanisms to detoxify the harmful oxidants from ascorbate administration as a drug. These mechanisms may also protect cells from oxidants that result from the normal mM intracellular concentrations of ascorbate that are part of physiology. For both examples, which detoxification pathways are the most important ones regarding ascorbate, or is there purposeful redundancy? Which pathways are deficient in the many types of cancer cells that are sensitive to hydrogen peroxide? As ascorbate generates oxidants, are the key damaging pathways initiated within cells or outside them, or both, depending on the cell type? Is intracellular damage initiated at



the transcription (DNA) level, translation (RNA) level, or post-translational (protein or organelle or membrane) level? (Parrow et al., 2013).

There are many known pathways to detoxify ROS in cells. Ascorbate is sequestered in some secretory vesicles. However, whenever it has been investigated, ascorbate was not found to be in any other intracellular compartment. Such compartmentalization remains possible for some cells types but has not been found.

4) In humans, ascorbate is rapidly released/secreted by the adrenal gland in response to its tropic hormone ACTH. In animals, the testes and ovary also secrete ascorbate. Ascorbate secretion also occurs in the stomach. What functions does ascorbate release serve? What are the mechanisms of ascorbate secretion?

### Known, with incomplete evidence

Our understanding of ascorbate physiology has many uncertainties. These extend from its molecular actions to its recommended dietary allowance. Lack of vitamin C inevitably results in scurvy. Many of the signs and symptoms of scurvy match known enzymatic actions of ascorbate. For example, loosening of teeth and tissue breakdown may reflect inability to cross link collagen, leading to structural weakness of connective tissue. Lassitude may reflect impaired nor epinephrine synthesis, defective amidation of peptide hormones or impaired carnitine synthesis (or all three). However, there is no experimental evidence to conclusively link specific enzyme defects to the signs and symptoms of scurvy. Additionally the role of ascorbate in many of these enzyme actions has not been clearly demonstrated *in vivo*. *In vitro*, specificity data are incomplete regarding ascorbate as the required electron donor (as opposed to other reducing agents). Nevertheless, scurvy cannot be cured without administering vitamin C, and therefore some biochemical pathways must have a specific requirement for vitamin C.

Vitamin C is by its chemical nature an electron donor, commonly called an antioxidant. However the widely held assumption that vitamin C has an important role as an antioxidant in humans is unproven. To date, supplementation with vitamin C has shown no benefit in disease conditions proposed to be caused by, or resulting in, oxidant stress (Padayatty et al., 2003). Besides, many supplement trials are plagued by the problem of inadequate differences between the treatment and controls groups with regard to vitamin C concentrations, addressed in detail above.

### Some common questions about vitamin C

1- Is vitamin C effective in the prevention or treatment of common cold? Cochrane Reviews (Douglas et al., 2000, Hemilä et al., 2007) (Hemila & Chalker, 2013) conducted meta-analyses of published studies in which vitamin C was used for the prophylaxis or the treatment of common cold. 29 placebo (but not necessarily randomized) controlled trials were analyzed, in which oral vitamin C was used at doses of 200 mg or more per day. In the general population, vitamin C supplementation taken prophylactically had no effect on the incidence of common cold, but had a modest effect in reducing the duration (by 8% in adults, 13% in children) and severity of symptoms of cold. In those who underwent severe

exercise (five trials), such as in Marathon runners, the risk of cold was halved. Treatment of common cold with vitamin C (seven trials) was ineffective. In summary, vitamin C probably will not prevent colds for most people. In some people, there may be mild or modest effect on duration, especially those who are heavy exercisers. Because of its low cost and safety, and consistent though modest benefit, vitamin C supplementation can be considered on an individual basis in patients with cold who have no contra indications to vitamin C supplementation (Hemila & Chalker, 2013).

2 - Is there evidence for or against the use of gram doses of vitamin C taken as supplements? Vitamin C bioavailability declines rapidly with dose. When 1250 mg of vitamin C is administered orally to healthy young men at steady state for that dose, bioavailability is 46%. Almost all the absorbed dose is excreted in urine within 24 hours (Levine et al., 1996b, Graumlich et al., 1997). No clinical benefits have been shown for consumption of vitamin C above the recommended dietary allowance. Current recommendations are that doses above 2 g/day should be avoided to prevent side effects, particularly bloating and osmotic diarrhea. Whether these doses lead to hyperoxaluria is unclear (Food and Nutrition Board, 2000b, Handelman, 2007, Auer et al., 1998). Supplemental vitamin C can cause hemolysis in those with glucose 6 phosphate dehydrogenase deficiency (Mehta et al., 1990) and in patients with Paroxysmal Nocturnal Hemoglobinuria (Iwamoto et al., 1994b) (Iwamoto et al., 1994a). Vitamin C consumption may also predispose to oxalate accumulation in those with impaired renal function (Canavese et al., 2005). In those without contraindications, supplemental vitamin C appears to be safe (Levine et al., 1999a, Food and Nutrition Board, 2000b). It is possible that vitamin supplements may have local effects such as erosion of teeth (Giunta, 1983). Other side effects may occur rarely. There have been case reports of oesophageal erosions, renal impairment (Nakamoto et al., 1998, Nasr et al., 2006, Rathi et al., 2007, Mashour et al., 2000) and unexpected interactions with other drugs or supplements (Bromley et al., 2005).

3 - What are the therapeutic uses of high dose intravenously administered vitamin C? Intravenously administered vitamin C produces pharmacologic concentrations not possible with oral intake. Vitamin C, given intravenously in doses of up to 100 g have been used for several decades in treating infections, cancer and other conditions (Padayatty et al., 2010). Rigorous trials to test benefit have not yet been undertaken. Intravenous ascorbate is widely used by integrative medicine (complementary and alternate medicine) practitioners. Intravenously administered vitamin C (Padayatty et al., 2004) has a favorable safety profile without any apparent ill effect (Hoffer et al., 2008) Padayatty et al., 2010). At mM plasma concentrations, or pharmacologic concentrations, vitamin C acts as a pro- oxidant. Animal studies show that only these pharmacologic concentrations of ascorbate produce ascorbate radical and hydrogen peroxide in the extracellular fluid (Fig 8) (Chen et al., 2005). Pharmacologic ascorbate concentrations have antitumor activity *in vitro* and in animal studies (Chen et al., 2008, Chen et al., 2007) (Parrow et al., 2013, Yun et al., 2015). In small phase I clinical studies, intravenously administered ascorbate was safe. Survival was doubled in patients with metastatic pancreatic cancer, but only retrospective controls were used. Phase II and Phase III studies have not yet been conducted to test benefit of intravenously administered vitamin C in patients with cancer. Conducting such trials rigorously, with

controls, and considering dose size and frequency are necessary. Such trials will address questions about ascorbate efficacy as an anticancer drug (Parrow et al., 2013).

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## List of abbreviations

<b>AA</b>	Ascorbic Acid
<b>ACTH</b>	Adrenocorticotrophic Hormone
<b>CGD</b>	Chronic Granulomatous Disease
<b>DHA</b>	Dehydroascorbic Acid
<b>EAR</b>	Estimated Average Requirements
<b>FIH</b>	Factor Inhibiting HIF
<b>GLUT</b>	Glucose Transporter
<b>GRD</b>	Glutathione Reductase
<b>GSH</b>	Glutathione
<b>GSSG</b>	Glutathione Disulfide
<b>HIF</b>	Hypoxia Inducible Factor
<b>HPLC</b>	High Performance Liquid Chromatography
<b>NADPH</b>	Nicotinamide Adenine Dinucleotide Phosphate
<b>RDA</b>	Recommended Dietary Allowance
<b>RNOS</b>	Reactive Nitrogen Oxide Species
<b>ROS</b>	Reactive Oxygen Species
<b>SNP</b>	Single Nucleotide Polymorphism
<b>SVCT</b>	Sodium dependent Vitamin C Transporter

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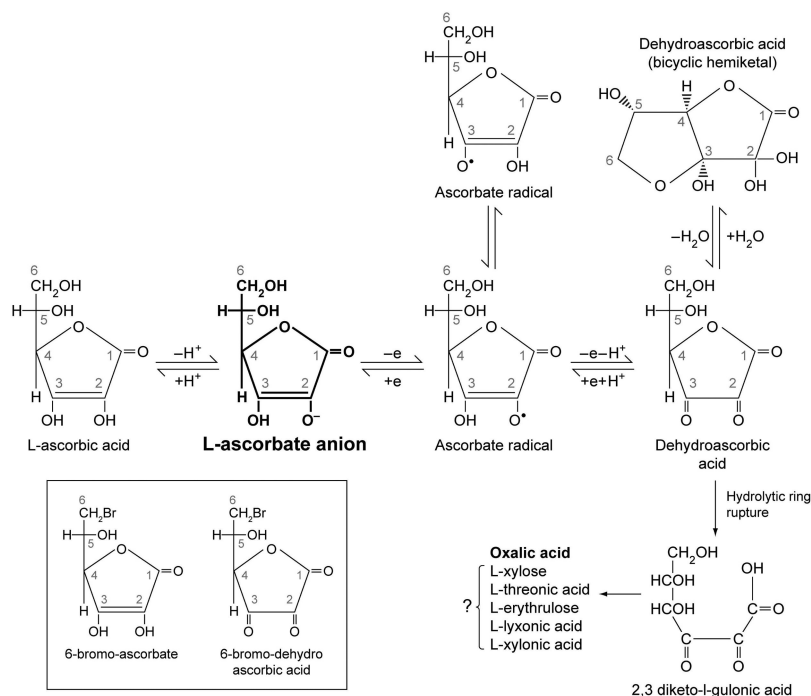
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**Figure 1. Ascorbic acid metabolism**

Ascorbic acid metabolism and halogenated analogues of ascorbic acid. Vitamin C (ascorbic acid) under physiological conditions is >99% in the form of ascorbate anion (shown in bold) (Beuttner & Schafer, 2004). It can sequentially donate two electrons from the double bond between carbons two and three. Loss of the first electron (oxidation) produces the free radical ascorbate radical (semidehydroascorbic acid). Some reactive free radicals produced by biological processes can be harmful because of their highly reactive nature. These can be reduced by ascorbic acid but in the process ascorbic acid is itself is converted (oxidized) into ascorbate radical. Ascorbate radical has a half-life of  $10^{-3}$  seconds to several minutes, depending on the presence of oxygen and metals. Under physiologic conditions, ascorbate radical is comparatively unreactive compared to other free radicals. Ascorbate radical can be reduced back to vitamin C. Alternately, it can lose a second electron (oxidized) to form dehydroascorbic acid. Thus, because vitamin C loses electrons, it acts as an antioxidant or free radical scavenger (Buettner, 1993).

Dehydroascorbic acid is also unstable with a half-life of several minutes (Lewin, 1976).

While dehydroascorbic acid exists in different forms, the dominant form *in vivo* is likely to be a hydrated hemiketal (Lewin, 1976, Corpe et al., 2005). Dehydroascorbic acid undergoes hydrolysis, with irreversible ring rupture to form 2,3-diketogulonic acid, whose metabolic products include oxalate, threonate, and possibly xylose, xylonic acid, and lynxonic acid (Lewin, 1976). Oxalic acid is the clinically significant metabolic product in humans (bold).

In some animals, products of vitamin C catabolism may enter the pentose phosphate pathway or other pathways of carbohydrate metabolism (Banhegyi & Loewus, 2004). Dehydroascorbic acid may be reduced back sequentially to ascorbate radical and ascorbic acid by glutathione or directly to ascorbic acid by enzyme dependent mechanisms (Linster & Van Schaftingen, 2007). Under suitable conditions (millimolar concentrations of ascorbic acid, presence of metal ions), hydrogen peroxide may be formed. Analogues of vitamin C

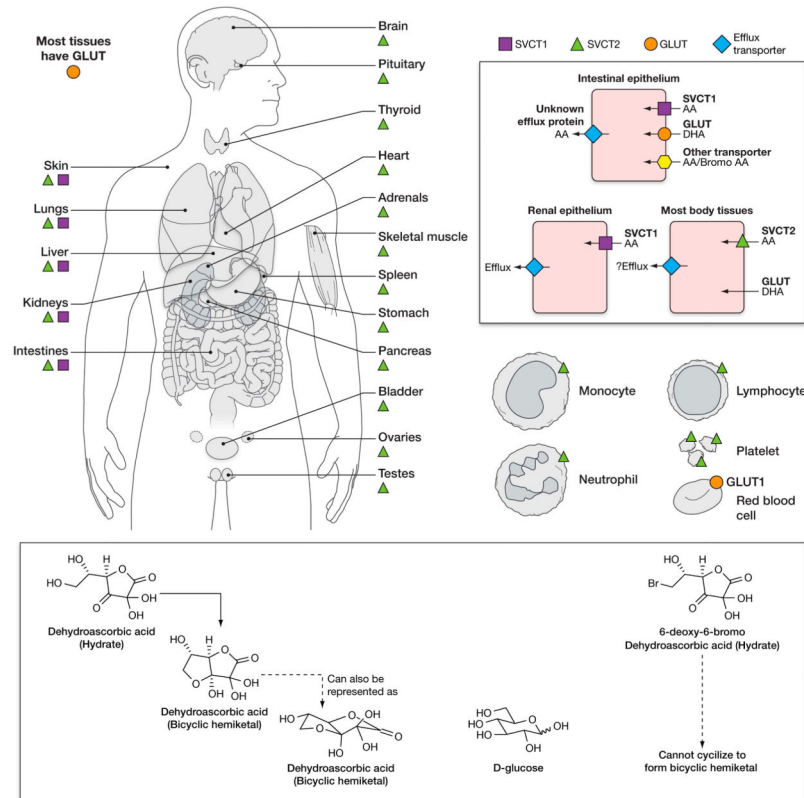
have been synthesized by replacing the OH group at carbon 6 with bromine or iodine. These halogenated analogues of vitamin C (shown in box) can be oxidized to bromo dehydroascorbic acid and iodo dehydroascorbic acid respectively. However, oxidized halogen ascorbate analogues cannot form a cyclized hydrated hemiketal (Fig. 2). Note that many of the reactions above have been shown only *in vitro* or in animals and not under physiological conditions *in vivo* or in humans. From (Washko et al., 1992). Modified and reproduced with permission of Analytical Biochemistry.

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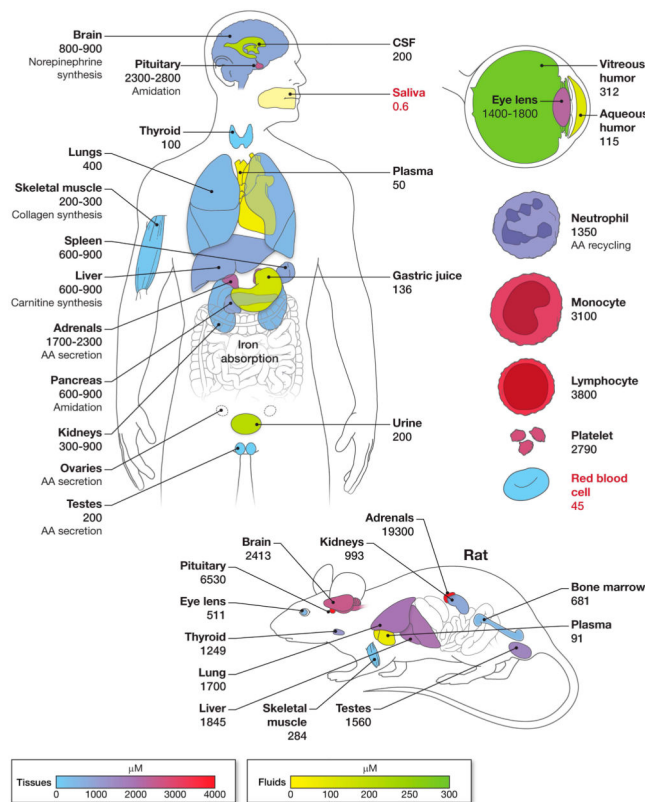
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### Figure 2. Vitamin C transporters

Distribution of vitamin C transporters in human tissues. Sodium Vitamin C Transporters (SVCTs) are mainly responsible for vitamin C transport into cells in humans and other mammals. SVCT1 is primarily expressed in absorptive tissues, including the intestinal epithelium and the proximal convoluted tubules and the descending part of the loop of Henle in the kidney. SVCT 1 is also expressed in liver. SVCT2 is expressed in most body tissues, as are Glucose Transporters (GLUTs). SVCT 1 and 2 transport ascorbic acid but not dehydroascorbic acid into cells. GLUTs 1, 2, 3, 4 and 8 (but not other GLUTs) (Corpe et al., 2013, Rumsey et al., 2000a, Rumsey et al., 1998, Burzle & Hediger, 2012), transport dehydroascorbic acid but not ascorbic acid into cells. Some GLUTs have a higher affinity for DHA than for glucose. The transporter responsible for exporting ascorbic acid from cells into the extracellular fluid or plasma has not been identified. The only ascorbate containing cell known to lack an SVCT is the mature red blood cell. The red blood cell obtains its ascorbate by transporting dehydroascorbic acid and immediately reducing it internally. Dehydroascorbic acid is transported into human red blood cells by GLUT1, and into mouse red blood cells by GLUT 3 and/or 4 (Tu et al., 2015). D-Glucose closely resembles the ring form (hydrated hemiketal form) of dehydroascorbic acid, which is likely to account for its transport by some GLUTs. When bromo ascorbic acid is oxidized to form bromo dehydroascorbic acid, this compound is not transported by GLUTs. SVCT distribution was inferred by the presence of specific mRNA for SVCT1 and 2, and in some cases by antibodies. Figure based on data from: (Tsukaguchi et al., 1999, Savini et al., 2008) (Daruwala et al., 1999, Wang et al., 1999, Wang et al., 2000).

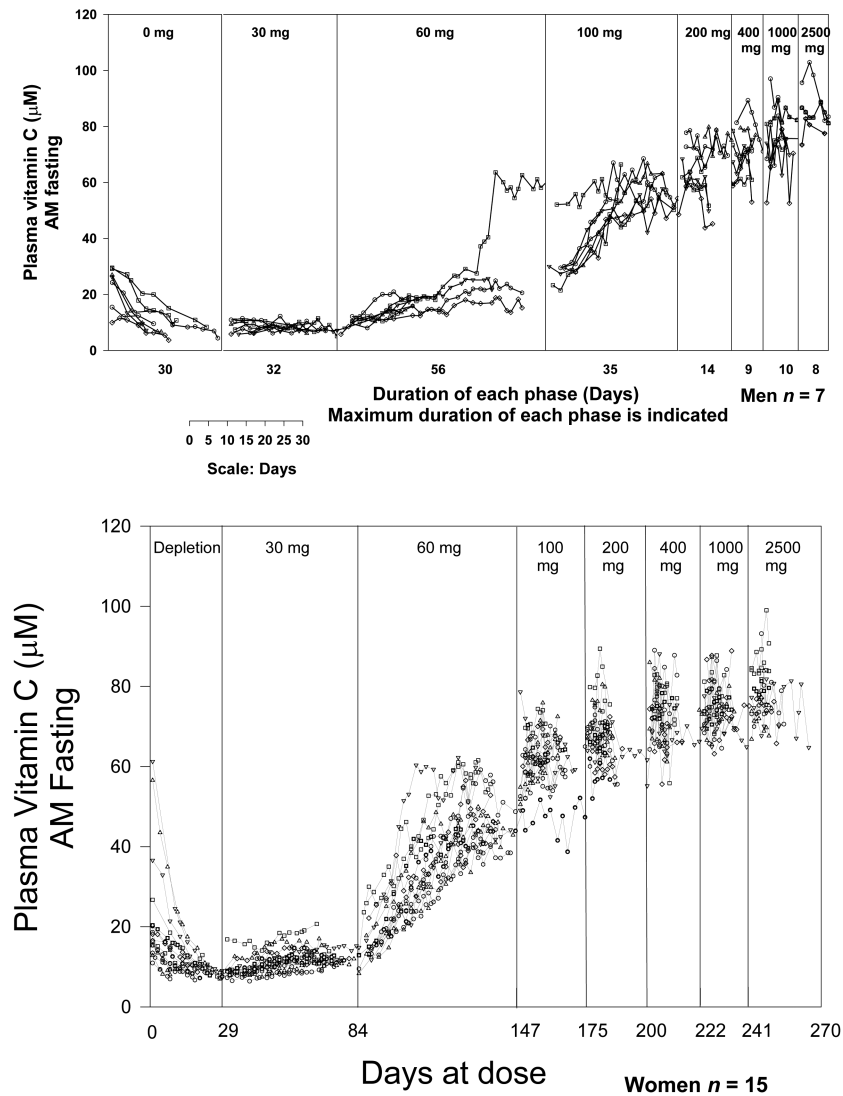


**Figure 3. Vitamin C concentrations in human and rat tissues and fluids**

Concentration of vitamin C in body tissues and body fluids are shown in  $\mu\text{M}$ . Ascorbic acid concentrations are much higher in tissues than in plasma. Tissues and body fluids are shown in separate concentration dependent color schemes. Organs that are uncolored (white) indicate that data on vitamin C concentrations are unavailable. For clarity, some organs shown are slightly displaced from their correct anatomical positions. Vitamin C concentrations shown are derived from published reports. Values for white blood cells and urinary vitamin C concentrations were obtained from depletion repletion studies in young healthy women, at the study phase when they were at steady state for vitamin C intake of 100 mg per day (Levine et al., 2001b), which is close to the recommended dietary allowance for vitamin C. Note that the red blood cell is the only cell with internal concentrations of vitamin C that are below those in plasma (values less than plasma are shown in red). Of body fluids, only saliva has a vitamin C concentration lower than that of plasma. Putative functions of vitamin C as a cofactor for enzymes are indicated, as are selected clinical or experimental observations of phenomenon involving vitamin C. Values shown should be considered approximate as they were obtained from tissues collected under non uniform conditions including clinical and post mortem samples. Some vitamin C concentrations shown are the mean values of results from two or more studies. Further, the dietary intake or plasma concentrations of vitamin C were not known in many cases and the methods used in sample collection and storage, and for vitamin C assays, varied widely. Values may also vary with age and possibly in disease states. Data obtained from (Hornig, 1975, Voigt et al., 2002) (Dostalova, 1982, Koliakos et al., 2002, Vobecky et al., 1982, Evans et al., 1982, Omaye et al., 1987, Berger et al., 1996, Taylor et al., 1997, Rathbone et al., 1989, Sobala et al., 1989,

Levine et al., 2001b, Jacob et al., 1987). Reported salivary vitamin C concentrations varied widely. Salivary vitamin C concentration shown (median 0.6  $\mu\text{M}$ , range 0.2-19  $\mu\text{M}$ ) in nonsmokers was measured by HPLC. Corresponding median plasma vitamin C concentration was 53  $\mu\text{M}$  (range 16  $\mu\text{M}$ -89  $\mu\text{M}$ )(Schock et al., 2004). Note that tissue vitamin C concentrations shown for the rat are much higher than for humans. Explanations are that fresh tissue samples can be obtained from animals (while many human tissue samples were obtained post mortem), or that there are true species differences. Compared to other tissues, AA concentrations in the rat pituitary and adrenal glands are higher, and therefore they are shown in arbitrary colors (and not according to the concentration dependent color scale) (Hornig, 1975). Vitamin C concentration data for mice are similar to that of the rat (Sotiriou et al., 2002, Corpe et al., 2010).





#### Figure 4. Vitamin C dose concentration relationship

Vitamin C concentrations in plasma and circulating cells were studied in young healthy men (Levine et al., 1996b) and women (Levine et al., 2001b) each of whom were given six to seven different doses of the vitamin in two depletion-repletion studies. Seven healthy men (4A) and fifteen healthy women (4B), all nonsmokers, age 19-27 years were studied as inpatients. To decrease hospitalization time, outpatient subjects prior to admission were instructed to consume a diet containing < 60 mg of vitamin C. When inpatients, throughout hospitalization they consumed a defined diet containing less than 5 mg of vitamin C daily (King et al., 1997). Deficiencies of other nutrients were prevented by supplementation. When plasma vitamin C concentrations achieved nadir of <10 µM, vitamin C in solution was administered at 15 mg orally in the fasted state twice daily (30 mg total per day) until steady state for the dose was achieved. Vitamin C dose was increased to 30 mg twice daily (60 mg total per day) until steady state was achieved for this dose. In this way subjects received the following doses in mg/day: 30, 60, 100, 200, 400, 1000, and 2500. Vitamin C was measured by HPLC with coulometric electrochemical detection. Doses are indicated at the top of the

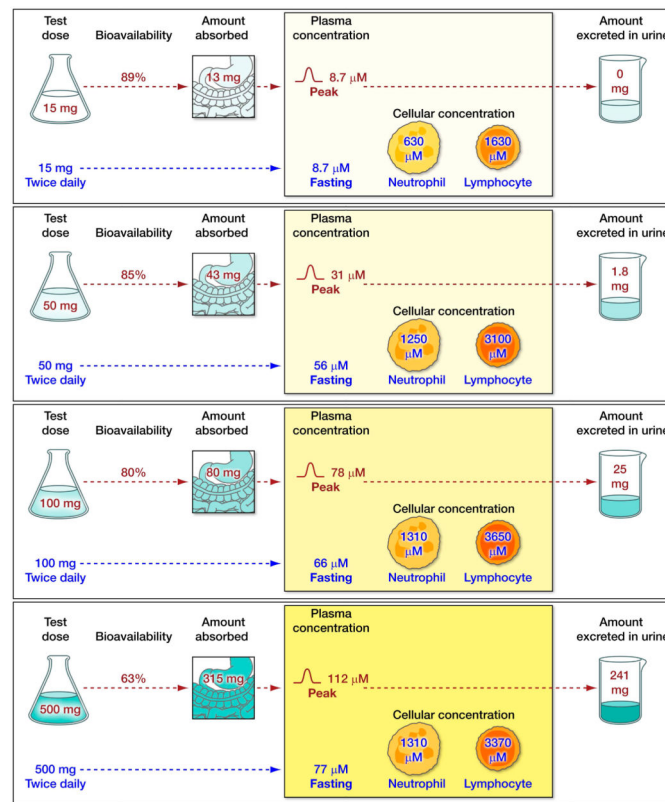
figure. Each symbol represents a different subject. There is a one-day gap between all doses for bioavailability sampling. Each vertical line represents the start of a new dose. Some subjects reached steady state concentrations earlier than others; the duration shown is longest time taken by a subject to reach steady state. Two subjects were studied at one time and the graphs show data collated at the end of all studies. Modified and reproduced with permission from Biofactors (Levine et al., 2001a) and The Proceedings of the National Academy of Sciences (Levine et al., 2001b).

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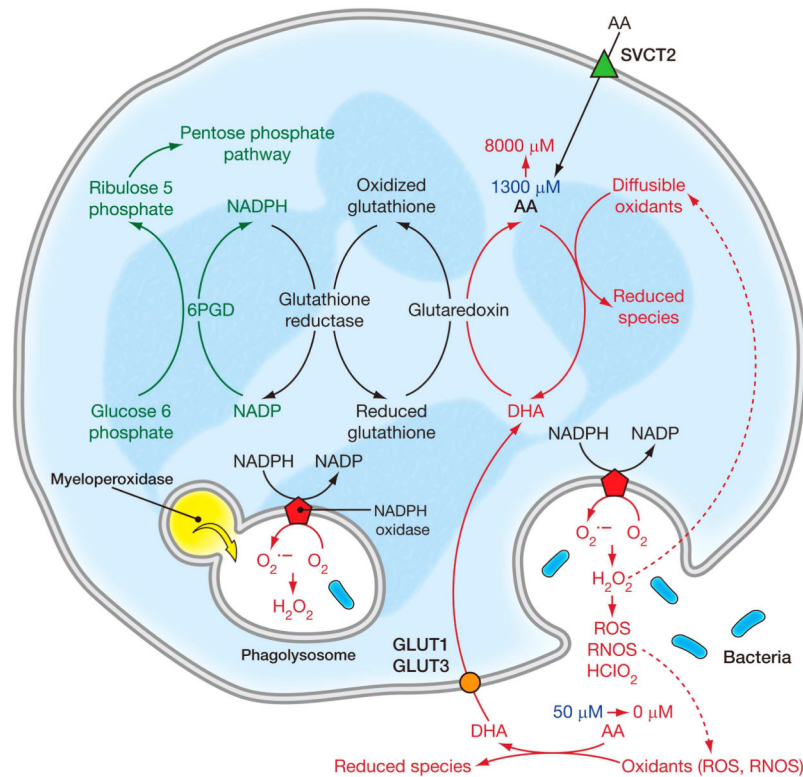


**Figure 5. Ascorbate flux in humans**

The relationship between the daily intake of known doses of vitamin C and its absorption, distribution and excretion are shown for four different daily intakes of vitamin C. Data were obtained from studies in seven healthy young men (Levine et al., 1996b) detailed in Fig 4. Ascorbate flux is shown in four panels. The upper part of each panel shows the relationship between an oral dose of vitamin C and: bioavailability; amount absorbed by the gastrointestinal tract; peak plasma concentrations reached; and the amount of the vitamin excreted in urine. Bioavailability studies were performed when patients were at steady-state for that dose (with some modifications, see below). Following each bioavailability study, subjects were given twice daily doses of the vitamin until they reached steady state for that dose. The lower part of each panel shows the steady-state condition for a specific dose. The panels for 50 and 100 mg doses can be taken as examples. When subjects were at steady-state for vitamin C intake of 50 mg twice daily (100mg total daily dose), fasting plasma vitamin C concentrations were 56 $\mu\text{M}$ , and neutrophil and lymphocyte vitamin C concentrations were 1250  $\mu\text{M}$  and 3100  $\mu\text{M}$  respectively. At this stage, bioavailability was studied in part by using a test dose of 100mg by mouth (labelled “test dose”). Urine excretion data (25 mg) are shown for this oral dose. Not shown are data for the same dose administered intravenously, to permit bioavailability to be calculated. Bioavailability for the 100 mg test dose was 80%: 80 mg of vitamin C was absorbed, and the resultant peak plasma vitamin C concentration attained was 78  $\mu\text{M}$ . 25 mg of vitamin C was excreted in the urine in the following 24 hours. The upper part of each of the four panels shows bioavailability doses of 15, 50, 100 or 500 mg. The lower part of the four panels show steady state for vitamin C intake of 30 mg/day, 100 mg/day (close to the recommended dietary allowance),

200 mg/day (the approximate amount provided by five servings of fruits and vegetables per day), or 1000mg/day (a dose that is used as a dietary supplement). Data shown are mean values for all patients studied. Amount absorbed was calculated from bioavailability data (Graumlich et al., 1997, Levine et al., 1996b). Separate color schemes, for which the intensity of color is related to the amount or concentration of vitamin C, are used for test doses; amounts absorbed and excreted in the urine; and for fasting steady state intracellular vitamin C concentrations.

Bioavailability studies were performed as follows: bioavailability for 15 mg dose at the end of depletion phase, for 50 mg when the subjects were at steady state for 60 mg, for 100 mg when the subjects were at steady state for 100 mg, and for 500 mg when the subjects were at steady state for 400 mg. Note that the end of the depletion phase was not a true steady-state. Mean fasting plasma vitamin C concentration at the end of depletion was  $7.62 \pm 1.64 \mu\text{M}$ . Fasting steady state plasma vitamin C concentrations at the time of oral bioavailability tests were: for 50 mg dose,  $24.8 \mu\text{M} \pm 14.1$ ; for 100 mg dose,  $56 \mu\text{M} \pm 4.5$ ; and for 500 mg dose,  $70 \mu\text{M} \pm 6.9$ . Each subject received a total of seven different doses of vitamin C but only data from four bioavailability studies and for four steady state conditions are shown. Details of methods used were previously published (King et al., 1997, Graumlich et al., 1997, Levine et al., 1996b, Levine et al., 2001b).



**Figure 6. Ascorbic acid recycling in Human Neutrophils**

Ascorbic acid (AA) and dehydroascorbic acid (DHA) transport and recycling in human neutrophils *in vitro* (Stankova et al., 1975) (Bigley & Stankova, 1974) (Hemila et al., 1984) (Anderson & Lukey, 1987, Wang et al., 1997). When normal human neutrophils *in vitro* were activated by pathogens (*E coli*, *Enterococcus faecalis*, *Moraxella catarrhalis*, *Klebsiella oxytoca*, *Acinetobacter baumannii* or *C. albicans*) (Wang et al., 1997), neutrophils accumulated ascorbic acid. Intracellular vitamin C concentrations increased from ~ 1.3mM to 8mM, and vitamin C in the culture media decreased to undetectable concentrations from 50μM. These findings did not occur when neutrophils were used from patients with Chronic Granulomatous Disease (Wang et al., 1997). Patients with this condition are unable to make superoxide, and have dysfunctional neutrophils that cannot kill certain pathogens. The observed *in vitro* phenomena can be accounted for by the proposed model described below. Normal human neutrophils maintain internal vitamin C concentrations of about ~ 1.3mM by uptake of AA by sodium-dependent vitamin C transporter 2 (SVCT2). Activated neutrophils secrete reactive oxygen species because membrane associated NADPH oxidase is able to transfer electrons across the cell membrane, using NADPH in the process. H<sub>2</sub>O<sub>2</sub> is formed, which is a precursor for production of reactive oxygen species (ROS and RNOS). These oxidize extracellular AA to DHA. Neutrophil activation with oxidant formation and its consequent results are shown in red. DHA is rapidly transported into the neutrophil by glucose transporters, probably GLUT1 and GLUT3, and immediately reduced to AA by glutaredoxin, producing a 4 to 10-fold increase in neutrophil internal AA concentration. When bacteria are engulfed by the neutrophil, a phagolysosome is formed. Myeloperoxidase containing vesicles fuse with the phagolysosome, such that the oxidant generating reactions

occur in a sequestered space. Although superoxide cannot cross the plasma membrane,  $H_2O_2$  can readily diffuse into the cell. Oxidants generated by  $H_2O_2$  can be reduced by internal AA. Glutathione (GSH), used by glutaredoxin during DHA reduction, is regenerated from glutathione disulfide (GSSG) by glutathione reductase (GRD) and NADPH. NADPH essential for these reactions is derived predominantly from the pentose phosphate pathway. Two molecules of NADPH are produced by the oxidative phase of pentose phosphate pathway (shown in green) when glucose 6 phosphate is converted to ribulose 6 phosphate (shown in green). Ribulose 6 phosphate undergoes further metabolism in the non-oxidative phase of the pentose phosphate pathway. As NADPH is oxidized to NADP, electrons are transferred to GRD so it can reduce GSSG to GSH. Modified and reproduced from (Rumsey & Levine, 1998), with permission of the Journal of Nutritional Biochemistry.

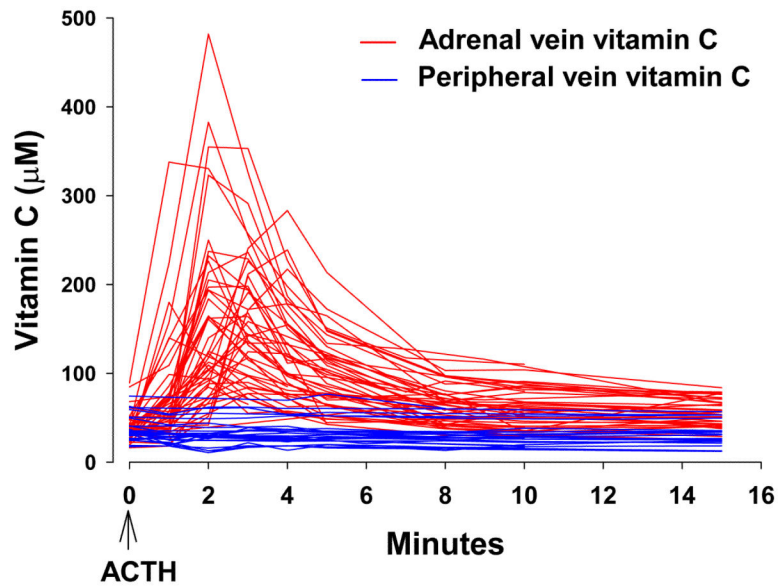
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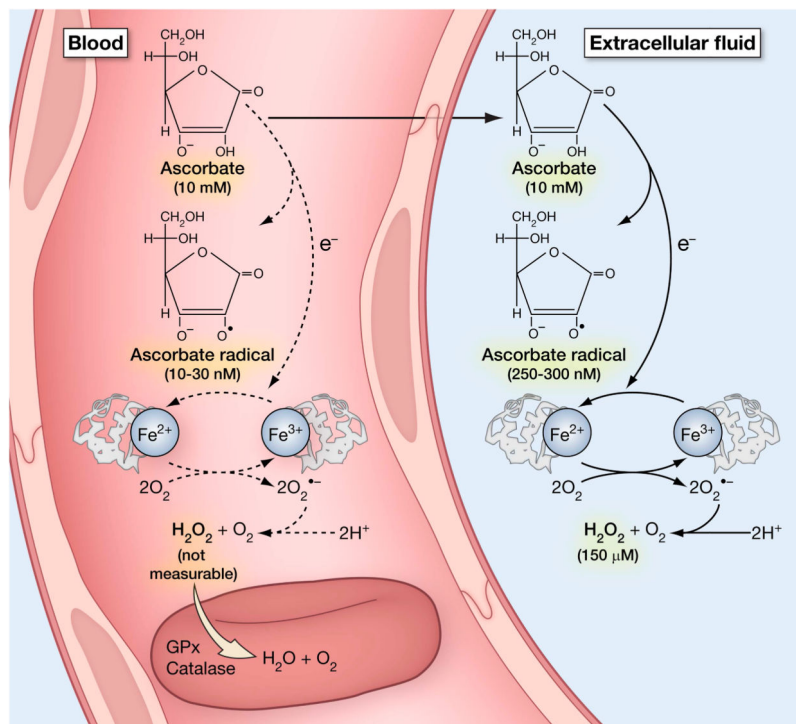
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**Figure 7. Vitamin C secretion by human adrenal glands**

Vitamin C concentrations were measured in the adrenal and peripheral veins of 26 patients with primary hyperaldosteronism. Under radiographic guidance, catheters were placed in both adrenal veins, and blood samples were taken after stimulation with adrenocorticotrophic hormone (ACTH). Vitamin C concentrations in each of the adrenal ( $n = 47$ ) and peripheral ( $n = 26$ ) veins sampled are shown. In 5 patients, blood samples were obtained from only one adrenal vein because of unusual venous anatomy or difficulties with adrenal vein catheterization. In the adrenal veins, peak vitamin C concentrations (Mean  $\pm$  SD:  $176 \pm 71$   $\mu\text{mol/L}$ ) were reached between 1 and 4 min, and were significantly ( $P < 0.0001$ , paired  $t$  test) higher than corresponding peripheral plasma vitamin C concentrations ( $35 \pm 15$   $\mu\text{mol/L}$ ). In patients in whom adrenal vein vitamin C concentration could be measured in only one adrenal gland, that single value was used in the calculation. In patients in whom both adrenals were successfully sampled, the mean of the two adrenal vein vitamin C concentrations was used for calculation, but all values are shown. Modified and reproduced from (Padayatty et al., 2007), with permission from American Journal of Clinical Nutrition.



**Figure 8. Ascorbate radical formation *in vivo***

Concentrations of ascorbate, ascorbate radical ( $\text{Asc}^{\bullet -}$ ) (radical is denoted by a superscript dot) and  $\text{H}_2\text{O}_2$  in blood and extracellular space, and proposed mechanisms that result in measurable  $\text{Asc}^{\bullet -}$  and  $\text{H}_2\text{O}_2$  in extracellular fluid but not in blood. Whether given by oral or parenteral routes, ascorbate rapidly equilibrates between blood and extracellular fluid, possibly through intercellular junctions. When pharmacological doses are administered parenterally, blood and extracellular fluid ascorbate concentrations reached 10 to 20 mM. Concentrations of  $\text{Asc}^{\bullet -}$  in blood reached 10-30 nM but  $\text{H}_2\text{O}_2$  was not detectable in blood.  $\text{Asc}^{\bullet -}$  and  $\text{H}_2\text{O}_2$  reached concentrations of 250-300 nM and 150  $\mu\text{M}$  respectively in extracellular fluid. A proposed scheme that accounts for these observations are as follows: In extracellular fluid, ascorbate loses one electron to form  $\text{Asc}^{\bullet -}$  (solid lines). The electron reduces a protein-centered metal, for example  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ . Candidate proteins may be either in extracellular fluid, or on cell membranes facing outward.  $\text{Fe}^{2+}$  donates an electron to oxygen, forming superoxide ( $\text{O}_2^{\bullet -}$ ) which undergoes dismutation to form  $\text{H}_2\text{O}_2$  (Qian & Buettner, 1999). In blood, these reactions are damped or inhibited (dashed lines), or the resultant products diffuse into red blood cells where they are rapidly extinguished/reduced. In blood, the formation of  $\text{Asc}^{\bullet -}$  may be inhibited by red blood cell membrane bound reducing proteins (May et al., 2001) and/or by large plasma proteins that do not distribute into the extracellular space.  $\text{H}_2\text{O}_2$  that is formed in blood will be immediately destroyed by plasma catalase and red blood cell GSH peroxidase, so that no  $\text{H}_2\text{O}_2$  is detectable (Chen et al., 2007, Gaetani et al., 1996, Johnson et al., 2005) (Chen et al., 2005). The identities of the metal-centered proteins are not known.  $\text{H}_2\text{O}_2$  that diffuses into the blood or tissues from extracellular fluid is inactivated by reducing substances in these compartments. Ascorbate was measured by HPLC,  $\text{Asc}^{\bullet -}$  by Electron Paramagnetic Resonance (EPR) and  $\text{H}_2\text{O}_2$  by fluorescence (Chen et al., 2007). The values shown are approximations from measurements

in animals given ascorbate by the intra peritoneal or intravenous routes (Chen et al., 2007) (Chen et al., 2005, Chen et al., 2008). Modified and reproduced from (Chen et al., 2007), with permission from the National Academy of Sciences.

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**Table 1**

Putative enzymatic and non-enzymatic effects of vitamin C in mammals and fungi are listed in the table. Three mammalian enzymes (prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase) take part in collagen biosynthesis. Prolyl 4-hydroxylase has six isoenzymes, three of which hydroxylate proline residues in procollagen, and another three hydroxylate proline residues in Hypoxia-Inducible Factor (HIF). HIF regulates several hundred genes and its activity is modulated by hydroxylation. Factor Inhibiting HIF (FIH) in turn modulates HIF by hydroxylation of asparagine residues in HIF (Dann et al., 2002, Lando et al., 2002). FIH also hydroxylates asparagine, aspartate and histidine residues in ankyrin repeat domains (Yang et al., 2011a, Yang et al., 2011b). Enzymatic functions of vitamin C have been demonstrated *in vitro* but generally not *in vivo*. In clinical experiments, iron absorption is increased by vitamin C but its effect on hemoglobin concentration is uncertain. Postulated antioxidant and pro oxidant roles of vitamin C have been experimentally demonstrated but are unproven in humans. Vitamin C is a reducing agent and may exert beneficial effects as a water soluble antioxidant. Some antioxidant functions have been demonstrated *in vitro* but have not been proven in humans. Under certain conditions vitamin C may be a pro-oxidant, especially when it is present in pharmacologic concentrations. Note that all actions of ascorbate are based on its ability to donate electrons. Where electrons go determines anti-oxidant or pro-oxidant consequences. Modified from Padayatty SJ, Daruwala R, Wang Y, et al. Vitamin C: Molecular Actions to Optimum Intake. In: Cadenas E, Packer L, eds. Handbook of Antioxidants. 2<sup>nd</sup> ed. New York: Marcel Dekker, Inc.; 2002:117-145. With permission of Marcel Dekker Inc., New York.

<b>Cofactor for enzymes</b>	
<i>Enzyme</i>	<i>Function of enzyme</i>
<b>Mammalian enzymes</b>	
Dopamine $\beta$ -monooxygenase Peptidyl-glycine $\alpha$ -amidating monooxygenase	Norepinephrine biosynthesis (Levine et al., 1991) Amidation of peptide hormones (Prigge et al., 1999)
Prolyl 4-hydroxylase Three Collagen isoenzymes Prolyl 3-hydroxylase Lysyl hydroxylase	Collagen hydroxylation (Prockop & Kivirikko, 1995)
Prolyl 4-hydroxylase Three Hypoxia-inducible Factor (HIF) isoenzymes	Hypoxia-inducible Factor (HIF) hydroxylation (Myllyharju, 2008)
Asparaginyl hydroxylase or FIH-1 (Factor Inhibiting HIF)	Regulation of HIF (Dann et al., 2002, Lando et al., 2002)
Trimethyllysine hydroxylase $\gamma$ -Butyrobetaine hydroxylase	Carnitine biosynthesis (Rebouche, 1991a)
4-hydroxyphenylpyruvate dioxygenase	Tyrosine metabolism (Lindblad et al., 1970)
Flavin adenine dinucleotide-dependent amine oxidase (lysine-specific demethylase 1)	Histone demethylation (Tsukada & Zhang, 2006)
<b>Fungal enzymes</b>	
Deoxyuridine 1'-hydroxylase Thymine 7-hydroxylase Pyridine deoxyribonucleoside 2' hydroxylase	Reutilization pathways for pyrimidines or the deoxyribose moiety of deoxynucleosides (Wondrack et al., 1978) (Stubbe, 1985)
<b>Reducing agent</b>	
<i>Site</i>	<i>Action</i>

Small intestine	Promote iron absorption (Hallberg et al., 1987)
<b>Antioxidant</b>	
<i>Site</i>	<i>Action</i>
Cells	Regulate gene expression and mRNA translation, prevent oxidant damage to DNA and intracellular proteins (Hitomi & Tsukagoshi, 1996, Toth et al., 1995, Padayatty et al., 2003, Qiao & May, 2011, Sram et al., 2012).
Plasma	Increase endothelium dependent vasodilatation, reduce extracellular oxidants from neutrophils, reduce low density lipoprotein oxidation, quench aqueous peroxy radicals and lipid peroxidation products (Polidori et al., 2004, Richards et al., 2015, Ceriello et al., 2013, Traber & Stevens, 2011).
Stomach	Prevent formation of N-nitroso compounds (Helser et al., 1992, Aditi & Graham, 2012).
<b>Pro oxidant</b>	
<i>Target</i>	<i>Effect</i>
DNA	DNA damage (Podmore et al., 1998)
Lipid hydroperoxidase	Decomposition of lipid peroxidase leading to DNA damage (Lee et al., 2001)
Downstream targets of hydrogen peroxide	Damage to cancer cells (Chen et al., 2007) (Chen et al., 2008) (Parrow et al., 2013)