Cellular functions of ascorbic acid: a means to determine vitamin C requirements

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Optimal ascorbic acid (vitamin C) requirements in humans are unknown. In situ kinetics is a biochemical approach to determine requirements for vitamin C and other vitamins. In situ kinetics requires that cellular functions of ascorbic acid are characterized. Vitamin-C-dependent cellular reactions are directly related to vitamin C concentrations inside and outside cells. By coupling intracellular and extracellular functions of ascorbic acid to vitamin concentration, in situ kinetics provides a novel approach to determining vitamin C requirements.

Introduction

Ascorbic acid (vitamin C) is essential for human health. Without ascorbic acid, the deficiency disease scurvy occurs and is fatal if untreated. The amount of ascorbic acid necessary for maintaining health is unknown. This review will focus on how this problem can be answered by understanding cellular functions of ascorbic acid. Knowledge of vitamin C and cellular function may play a central role in determining the dietary allowance.

The current recommended dietary allowance (RDA) for ascorbic acid is 60 mg in the United States and 30 mg in Australia (recommended dietary intake, RDI). The RDA is based on three parameters. The first is prevention of the deficiency disease scurvy, as described in the Iowa prison studies[1-4]. Five prisoners were given ascorbic-acid-free diets for months, with or without small doses of ascorbic acid. Signs and symptoms of scurvy were prevented when as little as 10–12 mg of ascorbic acid was ingested. To provide reserves to prevent scurvy, multiples of this ingestion quantity were selected as goals for dietary ingestion; for example, six-fold more than the minimum 10 mg was chosen in the US.[5] In countries where the quantity is lower, such as Greece and Australia, the recommendation can be considered a minimum amount to prevent scurvy in most people.

A second parameter in suggesting a dietary recommendation was based on urinary excretion of vitamin C. The hypothesis was that once urinary excretion occurred, body stores of the vitamin were saturated. From the Iowa prisoners, urinary excretion of vitamin C occurred when ingestion was approximately 60 mg daily[1,3,4]. This proposal was re-examined in outpatients believed to be ingesting no dietary ascorbic acid[6]. These volunteers were given an ascorbic acid dose plus radiactive ascorbic acid tracer to determine catabolism, compartmental-ization, excretion, and body pool size. Urinary excretion occurred at approximately 60 mg daily.

Based on this latter study[6], and others similar to it[3,4], catabolism was chosen as a third parameter of vitamin C requirements[5]. Catabolism was estimated to be approximately 60 mg daily; ingestion of 60 mg would maintain a body pool size of 1500 mg. A larger body pool size was deemed unnecessary by the US Food and Nutrition Board[5].

Thus, the RDA is based on preventing signs and symptoms of scurvy, achieving the urinary threshold for vitaming C excretion, and replacing catabolized vitamin C. At 60 mg daily, scurvy will be prevented for at least one month if vitamin C ingestion suddenly ceased. At 60 mg daily, catabolic losses will be replaced, and a body pool size is maintained which is considered to be adequate. In other countries such as Australia and Greece, 30 mg is chosen as a dietary intake, and this too will prevent scurvy but without as large a body pool size.

Unfortuantely, this approach to vitamin C requirements is unreliable. There are a number of problems with the experiments themselves. In the Iowa prisoner studies[1-4], the assay for ascorbic acid was neither specific nor sensitive. The assay used will overestimate ascorbic acid at low concentrations[7]. Consistent with this problem, signs and symptoms of scurvy were reported at plasma concentrations $\leq 12\mu m$. This is unusually high compared to more recent information, where concentrations of $5\mu M$ were reported without signs or symptoms of scurvy[8]. The time reported to achieve plateau plasma concentrations at the repletion dose of 66 mg was substantially prolonged for a water-soluble substance and is very difficult to explain, suggesting an experi-

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mental artifact. The use of radiolabel as an index of ascorbic acid excretion could be misleading, as the radiolabel could degrade either during administration or during assay. In the later studies with tracer[5], outpatient volunteers were studied whose diets were not known with certainty. If ingestion is not clear, interpretation is very difficult since it is not certain what amount of vitamin C the volunteers really received. The identity of the radiolabel excreted in urine was not verified as ascorbic acid, making it difficult to truly calculate body pool size and to calculate catabolism. In addition, catabolism estimates and body pool size can be functions of the ingested dose. Estimates may be misleading if low but not high doses are investigated, as occurred in these studies[9].

Even more important, the conceptual approach to the current RDA may be incorrect[9]. First, the RDA and RDI area based on a minimum amount to prevent a deficiency disease, scurvy. But it may well be a flawed concept that a minimum amount is equivalent to an ideal amount. Second, it is only hypothesis that, when urinary excretion of ascorbic acid occurs, body pools are of satisfactory size. Since urinary excretion indicates saturation for some substances but not others, it may be inappropriate to assume urinary excretion is equivalent to saturation.

There are other suggestive, although less compelling, reasons to question current recommendations. Our paleolithic forbears were estimated to have ingested

Table 1. Ascorbic acid toxicities and misconceptions of ascorbic acid toxicities[9,11].

Vitamin C Toxicity

● Diarrhea: >3-4 grams at once orally

● Iron absorption: Hemochromatosis, thalassemia major, sideroblastic anemia.

● False negatives for occult blood in stool

● ??? Oxalate hypersecretion and kidney stones; Possible for doses greater than 4 grams daily

● ??? Hyperuricosuria: Intravenous doses greater than 2 grams at once

● ??? Hyperoxalemia in dialysis patients

Misconceptions of Vitamin C Toxicity

● Rebound scurvy (conditioning)

● Hemolysis

● Mutagenesis

● Venous stasis

● Destruction of vitamin B_{12} in food

approximately 400 mg ascorbate[10]. Ingestion of ascorbate can be as much as 500 mg on a modern diet rich in fruit and vegetables, without supplements. Thus, past and present, the vitamin is readily available in foods. Ingestion of 60 mg but not higher amounts would be good if the vitamin were toxic. However, vitamin C is remarkably safe. There are few concerns for toxicity, as shown in Table 1[9,11-15]. The greatest concern is for excess iron absorption in patients with iron overload, as in thalassemia, transfusion overload, or hemochromatosis. Diarrhea can occur at single doses greater than 3-4 grams; however, there are no sound reasons to ingest such doses. Although other toxicities have been suggested, the evidence doesn't substantiate the claims (Table 1). Therefore, vitamin C does not have a narrow window from therapeutic safety to toxicity window as is true for many drugs and for fat-soluble vitamins A and D.

Except for primates, most mammals synthesize ascorbic aid. Adjusted to the weight of a 70 kg human, these synthetic rates have been claimed to correspond to 500-6000 mg per day. These data have been used to suggest that the 60 mg human requirement is very much underestimated. However, the numbers were obtained by measuring synthetic rates in liver homogenates containing the enzymes for ascorbate biosynthesis; these rates were calculated for 24 hours, and then adjusted for liver weight in different species. Unfortuantely, calculations based on tissue homogenates are not a reliable basis for whole animal biosynthesis nor for human vitamin requirements[9]. Nevertheless, when all of the issues above are considered, there is suggestive evidence that the RDA is too low.

The dietary recommendations will prevent deficiency, but do not address an amount for ideal health. How can this amount be determined? Until recently, techniques to measure optimal vitamin C ingestion have been indirect. For example, endpoints measured have been resistance to temperature change, resistance to infection, resistance to 'stress', or ability to perform exercise[9]. None of these endpoints provided information to indicate optimal vitamin C consumption. Recent epidemiologic evidence has suggested that vitamin C could be involved in prolonging life or in prevention of atherosclerosis, cancer, and cataracts[16-25]. This information is provocative. However, it is also indirect, or circumstantial. Vitamin C could prevent illness or prolong life, but something else associated with vitamin C ingestion and not vitamin C itself might explain the findings. In addition, for many of them the association is not striking. Future epidemiologic studies could provide more useful information about optimal ingestion, but current work is not sufficient.

In situ kinetics

What is missing from all of these approaches to determining vitamin C requirements is biochemical function of the vitamin. Optimal vitamin requirements could be determined by knowledge of how much vitamin is required for a specific biochemical function. For this approach to be useful for requirements, many functions of vitamin C must be understood. Knowledge of functions for isolated systems is not enough, however. Functions must be studied where they occur in cells or
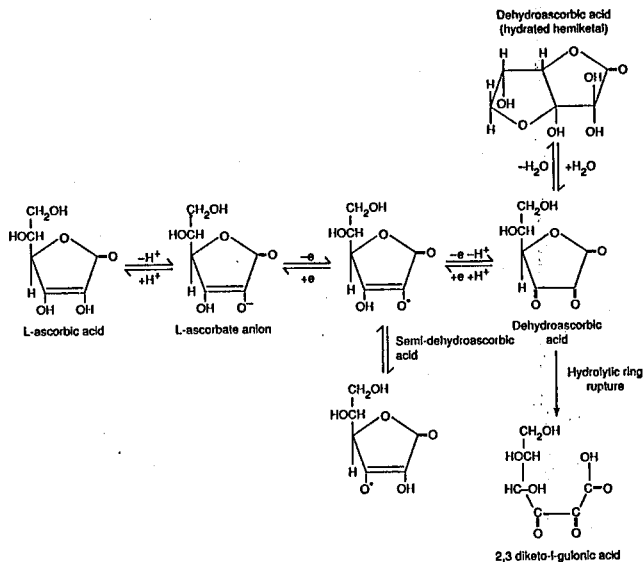
Dehydroascorbic acid
(hydrated hemiketal)

Figure 1. Ascorbic acid's structure and its oxidation products. Interconversion between ascorbate, semidehydroascorbic acid and dehydroascorbic acid is reversible. Once the ring is broken, diketogulonic acid cannot revert to dehydroascorbic acid. Dehydroascorbic acid may exist in multiple forms; two are shown here for clarity[28].

tissues in situ (translated directly: in position), not simply for isolated reactions and/or proteins[9,26]. Thus, as a novel approach to requirements, we can learn how much vitamin is needed for each different function to occur, in situ; for this reason, the approach is called in situ kinetics.

In situ kinetics has two major components. The first is to determine how specific biochemical functions are regulated by different ascorbic acid concentrations in situ. The second is to learn how ascorbic acid concentrations are achieved in normal humans which will permit the biochemical reactions to occur. These principles are described in detail elsewhere[27].

Ascorbic acid is an electron donor, or reducing agent. As such all of its known biochemical actions involve transferring its electrons; something else is reduced as ascorbate is oxidized. Transfer of one electron from ascorbic acid yields the unstable free radical intermediate semidehydroascorbic acid (half time of 10^{-5} seconds); transfer of a second electron results in dehydroascorbic acid[28]. The ascorbate:dehydroascorbic acid couple is reversible; for example, dehydroascorbic acid can be reduced back to ascorbic acid by sulfhydryl reagents. However, hydrolysis of dehydroascorbic acid to diketogulonic acid is irreversible (see Figure 1).

The function of vitamin C as a reducing agent can be intracellular or extracellular. Intracellular function can further be divided into enzymatic and non-enzymatic function. Enzymatic intracellular function occurs when a specific reaction is mediated by an enzyme and ascorbic acid. Ascorbic acid donates electrons to the enzyme, in most cases a metal-enzyme complex, which then catalyzes the reaction[9]. Non-enzymatic intracellular function is simply the chemical function of ascorbate as an electron donor, commonly described as anti-oxidant function.

Likewise, extracellular function can also be divided into enzymatic and non-enzymatic function. However, while extracellular enzymatic functions for ascorbate can theoretically exist, none have been described to our

knowledge. Therefore extracellular function is non-enzymatic or anti-oxidant. A summary of vitamin C's functions is shown in Table 2 (see[9,11] for details).

Table 2. Ascorbic acid's functions[9,11,42,64,68-70] (see text for details).

Ascorbic Acid and Biochemical Function

Intracellular Enzymatic Function	
Proline hydroxylase (EC 1.14.11.2)	Collagen synthesis
Procollagen-proline 2-oxoglutarate 3-dioxygenase (EC 1.14.11.7)	Collagen synthesis
Lysine hydroxylase (EC 1.14.11.4)	Collagen synthesis
Gamma-butyrobetaine, 2-oxoglutarate 4-dioxygenase (EC 1.14.11.1)	Carnitine synthesis
Trimethyllysine-2-oxoglutarate dioxygenase (EC 1.14.11.8)	Carnitine synthesis
Dopamine beta-monooxygenase (EC 1.14.17.3)	Catecholamine synthesis
Peptidyl glycine alpha-amidating monooxygenase (EC 1.14.17.3)	Peptide amidation
4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)	Tyrosine metabolism

Intracellular Non-enzymatic Function
?Quenching of reactive oxidants (?neutrophils, monocytes)

Extracellular Non-enzymatic Function
?Prevention of LDL oxidation ?Quenching of reactive oxidants Iron absorption in gastrointestinal tract

Function Uncertain: tissue contains high concentrations
Host defense B lymphocytes, T lymphocytes, platelets
Endocrine Adrenal cortex, testes, ovaries, pancreas
Vision Lens, Retina

How these vitamin functions are used for in situ kinetics is displayed in Figure 2, in which different functions of the vitamin are expressed in relation to vitamin concentration. Each curve in the figure can represent a different function from Table 2. The curves can be thought of as dose response curves, or biochemically as substrate velocity plots. Toxicity is indicated by a decrease in some functions at higher vitamin concentrations. The ideal vitamin concentration may be that which yields maximal function but without toxicity, as shown in the figure. Optimal vitamin ingestion would be that amount which provides this vitamin concentration[26,27].
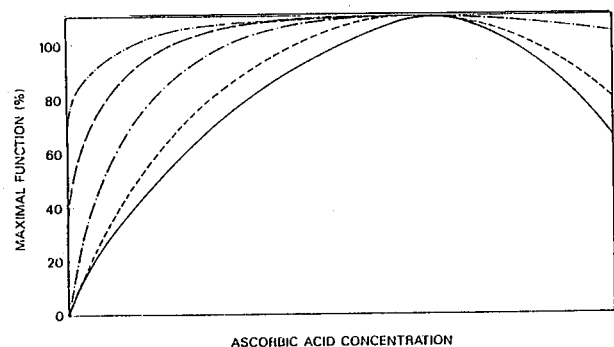

Figure 2. Ascorbic acid's functions in relation to its concentration. Each curve represents a distinct function dependent on ascorbate. Toxicity for some functions is indicated by decreasing activity at higher ascorbate concentrations. The curves are theoretical curves.

For in situ kinetics, there is a major advantage to studying intracellular function of ascorbate, especially enzymatic function. The advantage is that enzyme function can be directly and specifically related to ascorbate concentration. Ascorbate can be a co-substrate or co-factor for eight different enzymatic reactions, all of which occur intracellularly. For these enzymes to have maximal activity, ascorbate must be the specific electron donor, not other reducing agents[9]. Specificity is essential for studying reaction kinetics. Specificity allows meaningful conclusions about how much vitamin is necessary for a particular function in situ.

Intracellular non-enzymatic (antioxidant) function may or may not be specific for ascorbate. If specificity for ascorbate exists, the same advantages as for enzyme reactions apply. Even if there is no specificity for ascorbate, the reaction can still have meaning if it is physiologic. Physiologic relevance can be determined when the reaction is studied in place within cells (in situ).

However, if isolated rather than in situ reactions are studied, results can be obtained which might not occur in situ. This is because vitamin C donates electrons easily; many in vitro conditions which favor electron transfer may simply have no in vivo counterpart of physiologic relevance[29,30]. Vitamin C may act as an electron donor for isolated non-enzymatic reactions, but the same reactions may not occur in situ or vitamin C may not be the electron donor in situ. Similar problems are encountered when the effects of other reducing substances are tested on isolated reactions or proteins.

Extracellular function of vitamin C is non-enzymatic, where vitamin C acts as an antioxidant. It has been very difficult to study these reactions in situ, and isolated reactions are studied instead. But as just noted, antioxidant function is difficult to interpret if only isolated reactions are studied. Vitamin C might act as an electron donor for isolated reactions but have no relevance for such reactions in situ. For these reaons, we will concentrate here on intracellular function, where specificity and physiologic relevance can be addressed.

Catecholamine biosynthesis

The catecholamines dopamine, norepinephrine, and epinephrine are synthesized from the amino acid tyrosine. The enzyme dopamine beta monooxygenase is a mixed function oxidase that catalyzes the formation of norepinephrine from dopamine. The isolated enzyme requires ascorbate for maximal activity[31].

In situ norepinephrine formation is more complex. Catecholamines are stored in secretory vesicles; the most thoroughly investigated are chromaffin vesicles (granules) from the adrenal medulla. Dopamine beta monooxygenase is exclusively localized to the inside of the vesicle membrane in soluble and membrane bound forms[32,33]. Ascorbic acid is found inside and outside vesicles in mM concentration, which is as much as 100 fold more than plasma. Surprisingly, ascorbic acid does not cross the vesicle membrane[34]. How, then, is a high concentration achieved in vesicles? Although unknown, the answer may be that as vesicles are assembled, vitamin C is trapped inside. Since ascorbic acid is present throughout these cells in high concentration, newly synthesized vesicles contain a similar high concentration as found in chromaffin cell cytosol.
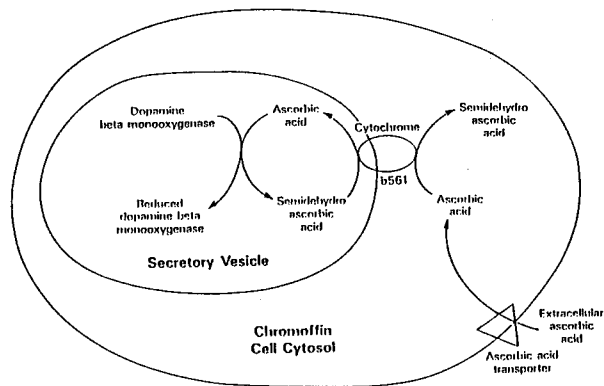

Figure 3. Ascorbic acid and catecholamine biosynthesis in situ. Cytosolic ascorbate transfers an electron to cytochrome b651, a secretory vesicle transmembrane protein. Cytochrome b561 donates an electron to intravesicular (intragranular) semidehydro-ascorbic acid, which forms as intragranular ascorbate reduces dopamine beta-monooxygenase. Reduced dopamine beta-monooxygenase catalyzes morepinephrine synthesis. The putative protein responsible for cytosolic accumulation of ascorbate is indicated by a triangle. To explain the mechanism of electron transfer, the secretory vesicle is not in proportion to cell size. See text and references[27,35,39] for details.

In light of this knowledge, it was unclear how vitamin C was involved in norepinephrine biosynthesis in situ. Since vitamin C does not have free access to the enzyme that is supposed to use it, is vitamin C really used by the enzyme in situ? If vitamin C is used by the enzyme, yet vitamin C cannot cross the vesicle membrane, what happens when all of the vitamin in the vesicle is consumed? The solution to these problems is that ascorbic acid within vesicles is used by the enzyme within vesicles. As ascorbic acid is consumed by the enzyme, the free radical semidehydroascorbic acid forms within vesicles[35-39]. The radical is reduced back to ascorbic acid by electrons from ascorbic acid on the outside of the granule membrane, in the cytosol. Electrons from vitamin C in cytosol are transferred to the protein cytochrome b561 in the granule membrane[38,39]. This protein shuttles the electrons across the granule membrane so that semidehydroascorbic acid inside is reduced. Thus, ascorbic acid within granules is maintained by ascorbic acid in cytosol, cytochrome b561, and semihydroascorbic acid[35-39]. Although cytosol ascorbic acid is consumed, it is replaced by ascorbic acid transported from plasma across the cell membrane. The mechanism is displayed in Figure 3.

The effect of ascorbic acid on norepinephrine biosynthesis can be seen from plots of in situ reaction kinetics, or dose response curves. The first curve is the effect of vitamin C within vesicles on norepinephrine biosynthesis (Figure 4). New norepinephrine biosynthesis is displayed on the y axis as a function of internal ascorbic acid, on the x axis. These data are the first in situ dose response curve for the effect of an endogenous vitamin on synthesis of a physiologic substance[35]. The data indicate that norepinephrine biosynthesis proceeds at V_{max} in situ. This finding is important for validating in situ kinetics, which proposes that ideal function is equivalent to maximal synthesis. At
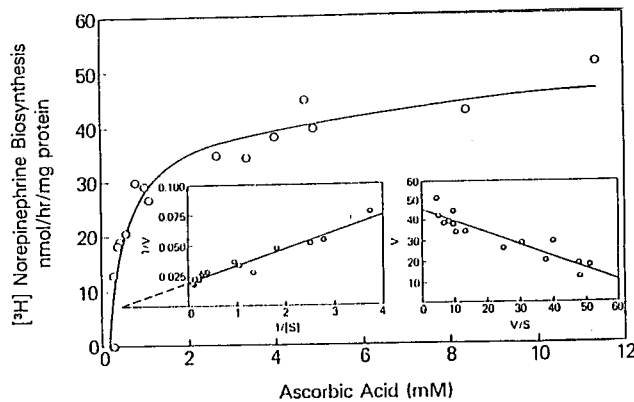
Figure 4. Norepinephrine biosynthesis in chromaffin granules as a function of intragranular ascorbate concentration. Intragranular in situ ascorbate concentration is shown on the x-axis; [^{3}H] norepinephrine biosynthesis is indicated on the y axis. Insets are Lineweaver-Burk and Eadie-Hofstee plots. Reference[35] for details.

least one function should occur at V_{max} in situ; these data demonstrate that this occurs. The data also show that ascorbic acid is a true co-substrate for norepinephrine biosynthesis; one ascorbic acid molecule is consumed for each molecule of norepinephrine formed.

The second dose response curve is the effect of external vitamin C, in cytosol, on regenerating oxidized vitamin C within vesicles (Figure 5). The external concentration of vitamin C is shown on the x axis and regeneration of internal vitamin C is shown on the y axis. The normal concentration of vitamin C in this cytosol is 5–10 mM. Thus, regeneration of vitamin C also proceeds at V_{max} in situ[36]. This finding provides additional support for the idea that optimal may be equivalent to maximal rate for at least some reactions which need vitamin C. These data also show that norepinephrine biosynthesis in situ is different from norepinephrine biosynthesis for the isolated enzyme. For vitamin requirements, in situ reactions should be studied.

These studies were performed on animal tissue from adrenal medulla. Although human tissue should show similar characteristics, these experiments have not been performed because of difficulty in getting fresh human adrenal tissue. The principles of in situ kinetics should continue to be formulated using animal tissue, since these experiments are often easier to perform. However,
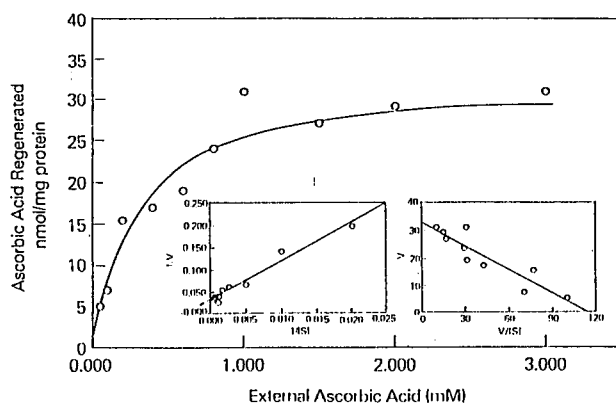

Figure 5. Regeneration of intragranular ascorbate as a function of extragranular ascorbate. External ascorbate is on the x axis; measured intragranular ascorbate is on the y axis. Insets are Lineweaver-Burk and Eadie-Hofstee plots. Reference[36] for details.

for applying the principles of in situ kinetics to vitamin requirements in humans, the key experiments should be performed using human tissue if possible.

Collagen biosynthesis in fibroblasts

Ascorbic acid is required by three isolated enzymes for hydroxylation of procollagen. Hydroxyl groups are inserted on proline to form 3-hydroxyproline or 4-hydroxyproline, and on lysine to form hydroxylysine[40-42]. Only when procollagen is hydroxylated can cross-linking of procollagen occur; cross linking is an essential part of collagen stability. Ascorbate action on catecholamine biosynthesis is to hydroxylate a single amino acid derivative. In contrast, for collagen synthesis ascorbate action is to modify a large polypeptide. The messenger RNA for this peptide must be transcribed and the message translated for ascorbate to be effective; ascorbate therefore plays a role in post-translational modification of procollagen. Ascorbate may have additional effects on message transcription independent of a role on post-translational modification[43,44].

The action of ascorbic acid has been described for three enzyme activities for hydroxylation (Table 2). Isolated prolyl (proline) hydroxylase requires alpha-keto glutarate and ferrous (Fe^{2+}) iron in addition to ascorbate. The mechanism of hydroxylation may be via an iron-oxygen complex. Iron is oxidized to Fe^{3+}; ascorbate may be necessary to reduce iron[42,45].

These enzymes are found in fibroblasts, and the effect of external ascorbate on proline hydroxylation in fibroblasts has been verified by many investigators (see 42 for review). External ascorbic acid is added to fibroblasts which have incorporated [^{3}H]proline. Incorporation of [^{3}H]proline into collagenase-sensitive protein is measured. In the presence of ascorbate, proline incorporation is increased several fold.

With respect to vitamin requirements and in situ kinetics, several key issues remain unknown. One central issue is how much external and internal ascorbate are required to form specific amounts of hydroxyproline in fibroblasts. The answer to this issue has clear implications for collagen biosynthesis in vivo. For example, a high intracellular concentration of ascorbate might be required for collagen biosynthesis. To achieve this concentration, ingestion of a certain amount of ascorbate above the current RDA may be necessary to yield the correct extracellular vitamin concentration to favor accumulation and subsequent hydroxylation. Another possibility is that collagen biosynthesis requires only low intracellular ascorbate concentrations; these concentrations may be achieved with ascorbate ingestion much less than the RDA. In either case, the amount of ascorbate which regulates collagen synthesis can be determined, to give a functional basis to vitamin C requirements.

To determine in situ kinetics for proline hydroxylation, it is essential to characterize intracellular ascorbate mass and distribution in normal human fibroblasts as a function of external concentration. This work is in progress[46]. It is also necessary to measure hydroxyproline formation. Previous assays based on [^{3}H]proline incorporation are indirect and do not measure true hydroxyproline mass. In addition, unlabelled proline

may also be present in cells; determining the ratio of unlabelled proline to [^{3}H]proline (specific activity) could be essential for measuring correct hydroxyproline formation. Thus, direct assays are required to measure proline and subsequent hydroxyproline for in situ kinetics. A new ultrasensitive and specific assay for hydroxyproline has recently been developed[47]. Using this assay and information about intracellular ascorbate concentrations, it will be possible to determine in situ kinetics for hydroxyproline formation in human fibroblasts.

Neutrophils/monocytes

Ascorbic acid was detected in crude fractions of white blood cells more than 50 years ago, during studies of scurvy in humans[48]. Its function, however, is not known. Using oxygen as a starting material, neutrophils and monocytes generate oxidizing compounds to kill bacteria. Ascorbate was proposed to be involved in oxidant generation[49]. Unfortunately, the evidence is inconclusive. Although neutrophils form oxidants to kill bacteria, neutrophils must be protected from these same oxidants. Ascorbate was proposed to be part of the protective mechanisms, by reducing otherwise toxic oxidants[50]. Although an attractive hypothesis, evidence to support it was also inconclusive. Ascorbate was also proposed to be involved in chemotaxis, or neutrophil movement. Paradoxically, ascorbate inhibits one of the reactions associated with chemotaxis, the addition of tyrosine to the microtubule protein tubulin (tubulin tyrosination)[(5)].

In situ kinetics in fibroblasts is straightforward because the function of ascorbic acid is known for isolated proteins. To apply in situ kinetics in neutrophils, ascorbate function must be determined first. Some principles of in situ kinetics can be used to learn ascorbate function. One goal is to be able to measure intracellular ascorbate concentration and then regulate it across a wide range, without subjecting animals or humans to scurvy[27]. Putative functions, such as oxidant quenching or oxidant generation, could then be investigated in neutrophils with widely varying intracellular ascorbate concentrations.

To regulate ascorbate concentration in neutrophils, we studied ascorbate transport in neutrophils freshly isolated from normal humans. These neutrophils were 'resting', or not producing oxidants. Ascorbic acid was accumulated against a concentration gradient in neutrophils; extracellular ascorbate in micromolar concentration was accumulated in millimolar concentrations by a high and a low affinity transport activity. Each was saturable and temperature-dependent[52].

Although intracellular ascorbate concentration was increased by they action of the transport activities, initial endogenous ascorbate concentration was almost always > 1 mM. To learn ascorbate function, it was necessary to deplete ascorbate in neutrophils. Unlike other cell types from which ascorbate is rapidly depleted, ascorbate was not lost from neutrophils. To deplete ascorbate, we tested whether substances of similar structure would exchange with intracellular ascorbate by reverse transport once intracellular saturation occurred. D-glucose and >-ascorbic acid have a similar ring structure and

chair conformation. We investigated the effects of D-glucose on ascorbic acid accumulation and exchange. D-glucose inhibited the high affinity activity noncompetitively and the low affinity activity competitively. Inhibition was completely reversible for each activity. Both transport activities were almost completely inhibited by glucose concentrations found in diabetics. Despite competition at the transport sites between ascorbate and glucose, glucose did not exchange with intracellular ascorbate, and intracellular ascorbate did not decrease[53].

As part of these experiments we made the accidental discovery that neutrophils making oxidants (activated neutrophils) accumulated far more ascorbate than the already high concentrations in resting neutrophils. The mechanism of this accelerated accumulation is shown in Figure 6. Extracellular ascorbate is oxidized to dehydroascorbic acid by oxidants from activated neutrophils; dehydroascorbic acid is preferentially transported and immediatley reduced intracellularly to ascorbate[54].

These observations may be a clue to the function of ascorbate in neutrophils. As shown in Figure 6, extracellular ascorbate could protect host tissues from oxidant damage by quenching destructive oxidants. Extracellular ascorbate is oxidized to dehydroascorbic acid. Preferential transport and immediate reduction to intracellular ascorbate occurs, with the result that neutrophils contain much more intracellular ascorbate than they started with. The increase in intracellular ascorbate occurs at precisely the time when ascorbate is needed to protect against oxidants, many of which diffuse freely. Thus, extracellular ascorbate may protect host tissues; oxidized ascorbate is then recycled to protect neutrophils from their own oxidants.

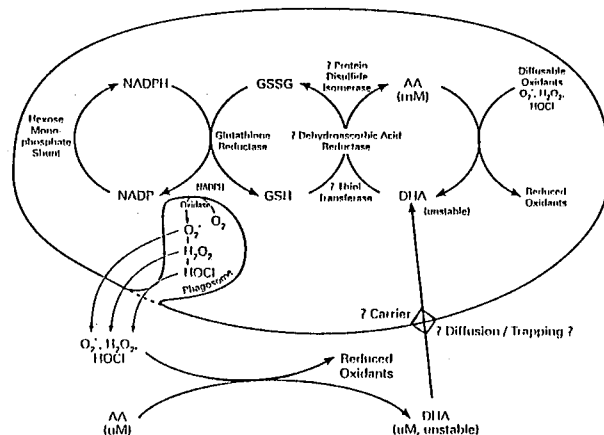Although oxidant quenching by antioxidant recycling

Figure 6. Antioxidant recycling in human neutrophils: a model. Extracellular ascorbic acid in micromolar concentration is oxidized to dehydroascorbic acid by oxidants generated by activated neutrophils. Dehydroascorbic acid in micromolar or sub-micromolar concentration preferentially enters neutrophils and is immediately reduced back to ascorbic acid; the result is accelerated accumulation of ascorbate. Extracellular ascorbate functions to quench radicals. Dehydroascorbic acid formed in this fashion is recycled intracellularly to protect the neutrophil from the oxidants it generates. The proposed mechanism of reduction is unknown but could require glutathione, NADPH, and the proteins shown. The mechanism of dehydroascorbic acid entry is unknown. Abbreviations: AA = ascorbic acid; DHA = dehydroascorbic acid; GSH = reduced glutathione; GSSG = oxidized glutathione. Reference[54] for details.

is an attractive hypothesis, antioxidant recycling (Figure 6) could have other functions. As some examples, increased intracellular ascorbate could be used for regulating chemotaxis and/or phagocytosis, or increased oxidant generation. The last possibility may seem especially attractive since the isolated enzyme myeloperoxidase, necessary for hypochlorous acid formation, utilizes ascorbate[55]. However, the intracellular distribution of ascorbate is the opposite of that predicted if myeloperoxidase requires ascorbate in situ[52]. Myeloperoxidase is found in neutrophil granules; these granules fuse with the phagosome, and hypochlorous acid is formed in the phagosome. Ascorbic acid is not expected in the phagosome for myeloperoxidase to use. Ascorbic acid in the phagosome would either be transported directly into the cytosol or oxidized to dehydroascorbic acid and then preferentially transported into the cytosol. It remains possible that electrons from ascorbate in cytosol are transferred to myeloperoxidase in phagosomes via electron transfer proteins[38,39]. Although monocytes/macrophages do not have myeloperoxidase, ascorbate function could be any of the others described above.

Lymphocytes

Since it was known for many years that ascorbic acid was found in high concentration in crude preparations of white blood cells, it was assumed the vitamin was accumulated in lymphocytes. Ascorbic acid accumulation in mM concentration was recently shown in purified B and T lymphocytes[56]. Unlike neutrophils, ascorbic acid concentrations can be both increased and decreased easily by culturing lymphocytes[56].

The function of ascorbic acid in lymphocytes is unknown. Ascorbic acid was proposed to regulate antibody production. However, no consistent effects were found on serum IgG, IgA, or IgM[57,58]. There is no clear effect of ascorbic acid on lymphocyte proliferation in response to mitogens[58]. There are inconsistent effects of ascorbate on other parameters such as T helper cell to T suppressor cell ratios; natural killer functions and mitogen-induced protein synthesis. Many other mechanisms for ascorbate action have been proposed[59], but none have substantial supporting evidence. As noted by Anderson and colleagues[58], the problem is the lack of a clear biochemical/molecular mechanism for ascorbate action in lymphocytes. The problem is compounded by conflicting data in animal model systems, species variations, and non-specificity of some assays used to determine immune function. Additional complications are due to difficulties in measuring ascorbic acid and its metabolite, dehydroascorbic acid. The problems are assay non-specificity, instability of ascorbate and especially dehydroascorbic acid, assay interference from other reducing/oxidizing substances, and assay insensitivity[7].

Since the concentration of ascorbate can be varied widely in lymphocytes, lymphocyte function can be investigated over a wide range of intracellular ascorbate concentrations. Which functions should be investigated? To learn ascorbate's action, specific biochemical/molecular reactions rather than general lymphocyte function should be studied. New techniques using molecular genetics should make these studies possible. Such experiments are now underway in our laboratory.

Carnitine biosynthesis

L-Carnitine is a quaternary amino acid necessary for lipid metabolism. Carnitine acts with two enzymes, carnitine palmitoyltransferase and carnitine acylcarnitine translocase, to allow long chain fatty acids to enter mitochondria. Once inside, lipids are oxidized to generate ATP. Carnitine, in conjunction with the enzymes carnitine acetyltransferase and carnitine-acylcarnitine translocase, also facilitates exit of short organic acids from mitochondria, which may be necessary for efficient mitochondrial function[60].

Carnitine is maintained in humans by dietary ingestion and by endogenous synthesis from lysine and methionine. There are two hydroxylation steps in carnitine biosynthesis mediated by the enzymes epsilon-N trimethyllysine hydroxylase and gamma – butyrobetaine hydroxylase. Both enzymes are monooxygenases which require reduced iron and ascorbic acid for maximal activity[61-63]. Thus, these enzymes are similar to those needed for proline and lysine hydroxylation discussed above.

While these isolated monooxygenases require ascorbate for maximal activity, investigation of the requirement in vivo faces several complex obstacles. This is because the endogenous pool of carnitine precursors, including substrates for the ascorbate dependent enzymes, may be difficult to regulate in vivo. Control of these substrate concentrations may be required to test in situ enzyme activity. Readers are referred to two thorough excellent reviews of the function of ascorbic acid in carnitine biosynthesis[64,65].

Peptidyl alpha monooxygenase

Many neuroendocrine peptides and peptide hormones are synthesized as larger molecules that are then modified before they become the final active compound. One modification is replacement of the carboxy terminus with an alpha amidated peptide. For the amidation to occur, the precursor peptide at the carboxy terminus must be glycine. Examples of hormones that undergo amidation like this are corticotrophin releasing hormone, growth hormone releasing hormone, thyrotropin releasing hormone, oxytocin, vasopressin, gastrin, cholecystokinin, vasoactive intestinal peptide, calcitonin, secretin, and substance P[66].

The enzyme that catalyzes peptide amidation is called peptidyl alpha monooxygenase (PAM). Similar to dopamine beta-monooxygenase, PAM is dependent on oxygen, copper, and ascorbate[67]. In recent studies PAM has been found to be two enzymes[68-70]. The first is peptidyl alpha hydroxylating monooxygenase (PHM), which requires ascorbate, oxygen, and copper. The second is peptidyl alpha hydroxyglycine alpha amidating lyase (PAL). PHM and PAL are encoded together sequentially on a PAM precursor mRNA. The two enzymes remain intact as a bifunctional protein (PAM) in some tissues such as the atrium. In other tissues, such as the neuroendocrine pituitary gland, the protein is cleaved and the two enzymes PAL and PHM are formed.

PAM requires ascorbate for maximal activity in pituitary tissues. In most other tissues, however, little is known about the dependence of peptide amidation on ascorbate. The need of PAM and/or PHM for ascorbate

is difficult to study because peptide biosynthesis involves many steps, many enzymes, and multiple cellular compartments. PAM and/or PHM act towards the end of peptide biosynthesis. Regulation of substrate concentration at these steps is not straightforward. Nevertheless, since small alpha amidated peptides may have many physiologic roles, regulation of their biosynthesis should be understood. These issues have been reviewed in detail by the leaders in this field[70].

Summary

Ascorbic acid is required for at least eight intracellular enzymatic reactions, and for other intracellular non-enzymatic reactions. Extracellular reactions may also be dependent on ascorbate as an electron donor. Many of these reactions can be studied in relation to ascorbate concentration using the principles of in situ kinetics. By understanding vitamin C dependent reactions in situ as a function of vitamin C concentration in situ, vitamin requirements can be developed with a biochemical and functional basis.

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Cellular functions of ascorbic acid: a means to determine vitamin C requirements

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抗壞血酸的細胞功能、測定維生素C需要量的一種手段
摘　要

人體抗壞血酸（維生素C）最理想的需要仍未明確，就地動態平衡（IN SITU KINETICS）是一種測定維生素C和其它維生素需要量的生化方法，就地動態平衡需要以特別的方式標明抗壞血酸的細胞功能。依賴維生素C的細胞反應是直接地與細胞內外的維生素C濃度有關，把抗壞血酸細胞內外的功能和維生素濃度聯系起來，將提供一個就地動態平衡的測定維生素C需要量的新穎方法。