

# Anitra C. Carr<sup>1</sup>, Principles of Nutritional Assessment: Vitamin C

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## Abstract

Vitamin C (ascorbic acid) is involved in a variety of biological processes, acting primarily as an electron donating enzyme cofactor and reducing agent (antioxidant). Clinical deficiency of vitamin C results in scurvy, a relatively rare disease in high-income countries. Nevertheless, moderate vitamin C deficiency (hypovitaminosis C) has been described in selected vulnerable groups such as the institutionalized elderly, with gingival inflammation and fatigue appearing to be the most sensitive clinical markers for moderate vitamin C deficiency. Excess intakes of vitamin C may produce osmotic diarrhea in some people, and although oxaluria has been reported, this measurement is prone to errors in the preparative or investigative procedure. There are currently no reliable functional indices of vitamin C nutriture. Static biochemical indices such as plasma or leukocyte ascorbic acid concentrations are most frequently used. The former reflect recent dietary intake within a limited range (30–80mg/d). Plasma ascorbic acid concentrations increase linearly with dietary intake but only up to a plateau of about 70μmol/L, and thus are unsuitable for detecting excessive intakes of vitamin C. A number of non-nutritional factors, including sex, body weight, smoking, and severe infection, affect plasma ascorbic acid concentrations. Plasma concentrations are a more reliable marker of vitamin C status than dietary intake assessments. Urinary excretion of vitamin C occurs when the renal reabsorption threshold had been reached and plasma “saturation” occurs (between about 60 and 70μmol/L). Urinary excretion is influenced by recent dietary intake. Moreover, the sensitivity and specificity of this test is low, and 24h urine samples are generally required. Leukocyte ascorbic acid concentrations are considered a relatively reliable index of tissue ascorbic acid stores in healthy people; they are less responsive than plasma to short-term fluctuations in vitamin C intakes, but their assay is difficult and requires a larger blood sample. Furthermore, leukocyte ascorbic acid concentrations may not be a reliable marker of vitamin C status in some conditions, such as severe infection. The body pool size of vitamin C has been estimated using isotope dilution techniques. More research is needed to identify useful functional tests of vitamin C status.



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## 19.1 Vitamin C

For centuries, the consumption of citrus fruit has been associated with the prevention of the vitamin C deficiency disease scurvy, but it was not until 1932 that the active compound was isolated ([King & Waugh, 1932](#); [Svirbely & Szent-Györgyi, 1932](#)). A year later, ascorbic acid was characterized and became the first vitamin to be chemically synthesized. Humans are unable to synthesize vitamin C *in vivo* from glucose due to random genetic mutations, which occurred at an early stage in our evolution, and have resulted in loss of the terminal enzyme in the biosynthetic pathway, L-gulonolactone oxidase ([Nishikimi & Yagi, 1991](#); [Drouin et al., 2011](#)).

The term “vitamin C” is used here to embrace both L-ascorbic acid and its oxidised form, L-dehydroascorbic acid; both have approximately equal vitamin C activity as dehydroascorbic acid can be readily reduced back to the biologically active ascorbic acid in the body. Ascorbic acid is the enolic form of an  $\alpha$ -ketolactone (2,3-didehydro-threo-hexano-1,4-lactone), which in solution can be oxidized to the diketo form— dehydro-L-ascorbic acid; their structures are shown in [Figure 19.1](#).

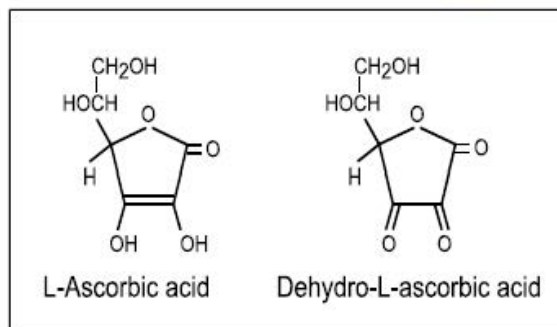


Figure 19.1. Structure of L-ascorbic acid and dehydroascorbic acid.

Under physiological conditions, vitamin C is present predominantly as the mono-anion, ascorbate.

## 19.2 Functions of Vitamin C

Ascorbic acid acts as an electron donating cofactor for a family of biosynthetic and regulatory metalloenzymes that carry out important hydroxylation reactions

([Englard & Seifter, 1986](#); [Kuiper & Vissers, 2014](#); [Young et al., 2015](#)).

These include copper-containing mono-oxygenases and iron-containing dioxygenases. Of the latter, the most well-known is hydroxylation of proline and lysine residues during synthesis of collagen, the major connective tissue protein in the body. Ascorbic acid acts in these hydroxylation reactions by reducing the metal catalytic sites of procollagen

## Contents:

- [19.1 Vitamin C](#)
- [19.2 Functions of Vitamin C](#)
- [19.3 Absorption and metabolism of vitamin C](#)
- [19.4 Deficiency of vitamin C in humans](#)
- [19.5 Food sources and dietary intakes of vitamin C](#)
- [19.6 Nutrient Reference values for vitamin C](#)
- [19.7 Indices of vitamin C status](#)
- [19.8 Plasma ascorbic acid](#)
  - [19.8.1 Ascorbic acid pharmacokinetics](#)
  - [19.8.2 Factors affecting plasma ascorbic acid](#)
  - [19.8.3 Interpretive criteria for plasma ascorbic acid](#)
  - [19.8.4 Measurement of plasma ascorbic acid](#)
- [19.9 Ascorbic acid in leukocytes and specific cell subsets](#)
  - [19.9.1 Leukocyte ascorbic acid](#)
  - [19.9.2 Ascorbic acid in specific cell subsets](#)
  - [19.9.3 Interpretive criteria for leukocytes](#)
  - [19.9.4 Measurement of leukocyte ascorbic acid](#)
- [19.10 Ascorbic acid in erythrocytes and whole blood](#)
- [19.11 Urinary excretion of ascorbic acid and metabolites](#)
  - [19.11.1. Measurement of urinary ascorbic acid](#)



proline 4-dioxygenase (EC 1.14.11.2) and procollagen lysine 5-dioxygenase (EC 1.14.11.4). Decreased activity of these two enzymes can lead to the defects in connective tissue that in turn cause the characteristic clinical features of scurvy. These include joint pain, tooth loss, bone and connective tissue disorders, and poor wound healing. Ascorbic acid is also involved in the hydroxylation of dopamine to noradrenaline, by acting as a cofactor for the enzyme dopamine- $\beta$ -monooxygenase (EC 1.14.17.1). Noradrenaline is a neurotransmitter, and deficient dopamine hydroxylation may be associated with the mood changes, depression, and hypochondria that can occur with scurvy. The early symptoms of scurvy — fatigue, lethargy, and muscle weakness — are related to the role of ascorbic acid as a cofactor for maximal activity of two dioxygenases involved in carnitine biosynthesis (N-trimethyllysine dioxygenase; EC 1.14.11.8, and  $\gamma$ -butyrobetaine dioxygenase; EC 1.14.11.1). Carnitine is essential for the transport of activated long-chain fatty acids into the mitochondria, where they are converted to energy by way of  $\beta$ -oxidation ([Englard & Seifter, 1986](#)).

[19.12 Ascorbic acid in other cells and fluids](#)

[19.13 Body pool size](#)

[19.14 Functional tests of vitamin C status](#)

[Acknowledgements](#)

Several other enzymes depend on ascorbic acid as a cofactor, although their specific role in the development of scurvy is still unclear. These include peptidylglycine  $\alpha$ -amidating monooxygenase (EC 1.14.17.3), an enzyme required for the synthesis of numerous amidated peptide hormones which have multiple functions throughout the body ([Englard & Seifter, 1986](#)). More recent discoveries have included the hydroxylases that regulate the concentration and activity of the transcription factor hypoxia-inducible factor-1 $\alpha$  (i.e. hypoxia-inducible factor — proline dioxygenase; EC 1.14.11.29, and hypoxia-inducible factor — asparagine dioxygenase; EC 1.14.11.30) ([Kuiper & Vissers, 2014](#)), and the ten-eleven translocation (TET) hydroxylases and histone demethylases that modify epigenetic marks on DNA and histones ([Young et al., 2015](#)). Together, these epigenetic and transcription regulating enzymes have the potential to up- and down-regulate thousands of genes in the body, likely contributing to vitamin C's pleiotropic functions in human health and disease.

Ascorbic acid also has several nonenzymatic functions based on its role as a reducing agent and antioxidant (i.e. electron donation). For example, ascorbic acid enhances the gastrointestinal absorption of nonheme iron, through its ability to reduce intraluminal iron to its more absorbable ferrous form ([Hallberg et al., 1987](#)). Ascorbic acid is a potent antioxidant, with the ability to quench a wide range of reactive oxygen species, including neutralising phagocyte-derived oxidants, thus attenuating tissue damage ([Carr & Frei, 1999a](#)). Ascorbic acid provides important antioxidant protection to plasma lipids and lipid membranes through direct scavenging of reactive oxygen species and through the regeneration of vitamin E from its oxidised form ( $\alpha$ -tocopheroxyl radical). Ascorbic acid can also regenerate the enzyme cofactor tetrahydrobiopterin from its oxidised form (dihydrobiopterin), thus potentially aiding synthesis of dopamine, serotonin and nitric oxide. At high intakes, vitamin C has been associated with protection against cardiovascular disease, cataracts, and cancer ([Carr & Frei, 1999b](#)), and a variety of immune-related functions ([Carr & Maggini, 2017](#)). Additional research, however, is needed to establish causal relationships between vitamin C and these degenerative diseases and immunocompetence.

### 19.3 Absorption and metabolism of vitamin C



Vitamin C is absorbed in the small intestine by an active sodium-dependent vitamin C transporter (SVCT-1) that **can become saturated and is dose dependent** ([Savini et al., 2008](#)). About 70%–90% of

vitamin C is absorbed when daily intakes range from 30 to 180mg, but absorption falls to  $\leq 50\%$  at intakes above 1g/d ([Kallner et al., 1979](#); [Levine et al., 1996](#)). Indeed, the osmotic diarrhea and intestinal discomfort sometimes experienced by persons ingesting large gram doses of vitamin C is due to the presence of a large amount of unabsorbed vitamin C. Regulation of the whole body vitamin C content is achieved in part by this dose-dependent intestinal absorption of vitamin C. Renal action to conserve vitamin C (via SVCT-1) or excrete unmetabolized vitamin C also plays an important part. Tissue uptake occurs primarily by the SVCT-2 isoform ([Savini et al., 2008](#)). Different tissues uptake and retain vitamin C to variable extents, with neuronal and glandular tissues containing the highest concentrations ([Hornig, 1975](#)), indicating vital roles for vitamin C in these tissues. Catabolism of ascorbic acid in humans occurs through oxidation to dehydroascorbic acid and, in the absence of sufficient reducing equivalents, subsequent hydrolysis to diketogulonic acid. This can then be oxidised to smaller molecules, such as oxalate, that are excreted in urine. However, it should be noted that dehydroascorbic acid is readily taken up via cell membrane glucose transporters (GLUTs) and rapidly reduced back to ascorbic acid via various chemical and enzymatic pathways ([Rumsey et al., 1997](#); [Washko et al., 1993](#)), thus limiting dehydroascorbic acid concentrations in vivo. With large intakes, most of the vitamin C is excreted in its unmetabolized form ([Levine et al., 1996](#)).

#### 19.4 Deficiency of vitamin C in humans

Clinical manifestations of scurvy, such as weakness, petechial hemorrhages, gingival bleeding, ecchymoses and subperiosteal hemorrhage, anemia, and defects in bone development in children, are relatively rare in high-income countries. Nevertheless, vitamin C deficiency has been observed in institutionalized elderly subjects, likely arising from inadequate intake of vitamin C ([Carr & Rowe, 2020](#)). Alcoholic patients, those on restrictive diets and people in refugee camps are also vulnerable to vitamin C deficiency. Infantile scurvy is rare because breast milk provides adequate amounts of ascorbic acid and infant formulas are fortified with sufficient ascorbic acid to prevent the disease.

Subclinical vitamin C deficiency may develop secondarily to some disease states such as severe respiratory infections, cancer, gastrointestinal disorders, cardiometabolic disorders and inflammatory diseases. This is likely partly due to the enhanced oxidative stress and inflammation associated with many of these diseases, as well as the use of certain drugs (e.g. aspirin) or chemotherapeutic agents that appear to affect the bioavailability or metabolism of ascorbic acid ([Basu, 1982](#); [Carr & Cook, 2018](#)).

The functional health consequences of both mild and moderate vitamin C deficiency are not well characterized. Gingival inflammation, fatigue, and irritability have been reported in moderate vitamin C deficiency induced experimentally ([Leggot et al., 1986](#); [Levine et al., 1996](#)).

#### 19.5 Food sources and dietary intakes of vitamin C

Vitamin C is synthesised in plants. However, the vitamin C content of different foods is highly variable ([Carr & Rowe, 2020](#)). Values can also depend on the growing conditions, season of the year, stage of maturity, location, and storage time prior to consumption. Exposure to high temperatures, oxidation, or cooking in large amounts of water all reduce the vitamin C content of foods.

Major food sources of vitamin C in the diets of most high-income countries are fresh fruits and fruit juices and some fresh vegetables; they can contribute up to 90% of the vitamin C intake. Citrus and

kiwifruit are particularly rich sources of vitamin C. Of the vegetables, potatoes can be one of the most important sources of vitamin C in the diets of adults, for example in the U.K. ([Gregory et al., 1990](#)).

Most staple foods, e.g. rice, wheat, maize and other grains, contain negligible amounts of vitamin C and meat, fish, eggs, and dairy products are also poor sources ([Carr & Rowe, 2020](#)). In low-income countries, the supply of food sources of vitamin C is often seasonal, so intakes may vary widely. In The Gambia, for example, intakes ranged from zero to 115mg/d in one community, depending on the seasonal availability of mangos, oranges, and grapefruit ([Bates et al., 1994](#)).

In humans, the bioavailability of vitamin C from food sources is comparable to that from supplements and is not affected by the type of food consumed ([Carr & Vissers, 2013](#)).

## 19.6 Nutrient Reference values for vitamin C



Several expert groups including the Institute of Medicine (IOM) in the U.S. and the European Food Safety Authority (EFSA) have derived values for Average Requirements (ARs) and Recommended Intakes (RIs) for vitamin C. The AR (U.S. equivalent, Estimated Average Requirement) is set for a particular group of individuals and is used to assess the prevalence of adequate intakes of vitamin C within that group. The RIs (U.S. equivalent, **Recommended Daily Allowance**; EFSA equivalent, **Population Reference Intake**) are derived from the ARs and its distribution and are used to assess intakes and plan diets for individuals.



Recommendations for vitamin C intakes vary greatly around the world, with recommended intakes (RIs) for individuals ranging from as low as 40mg/d in the U.K. to 110mg/d by EFSA (EFSA, 2017). In the U.S., the **RDAs** established by the IOM in 2000 are 75mg/d and 90mg/d for women and men, respectively ([Levine et al., 1996](#); [IOM, 2000](#)). The large discrepancies in the RIs have resulted in calls for harmonization ([Carr & Lykkesfeldt, 2020](#)). Higher vitamin C intakes are recommended by all expert groups for pregnant and lactating women due to the needs of the developing fetus and **losses of vitamin C in breast milk**. Furthermore, some countries (e.g., the U.S.) recommend higher intakes for smokers due an enhanced oxidative burden and resultant increase in turnover of vitamin C. Consequently, RIs for smokers may be increased by +20mg/d to +80mg/d, depending on country. **No increase has been set for passive smokers.**



Upper levels of intake (ULs) have also been derived for vitamin C by some expert groups. The ULs represent daily intakes that if consumed chronically over time will have a very low risk of causing adverse effects. Intakes from all sources of vitamin C are considered: food and nutrient supplements. Because vitamin C is water soluble, however, any excess not required by the body is readily excreted, therefore, there is no **known** upper toxic **concentrations**. Nevertheless, some countries have set ULs ranging from 1–2g/d for adults based on potential gastrointestinal disturbances at very large (> 4g/d) doses. For more details on the application of these nutrient reference values, see Chapter 8b.



In addition to diarrhea and other gastrointestinal disturbances, possible adverse effects of excessive intakes include an increase in the absorption of dietary iron potentially leading to iron overload in people with hemochromatosis, and hemolytic anaemia in people with glucose-6-phosphate dehydrogenase deficiency due to an inability to attenuate hydrogen peroxide generation. Current evidence is insufficient to implicate high intakes of vitamin C as a risk factor in oxalate stone formation. Neverthe-



less, individuals with hemochromatosis, glucose-6-phosphate dehydrogenase deficiency, and renal disorders, may be susceptible to adverse effects from excessive intakes of vitamin C

## 19.7 Indices of vitamin C status

There are no reliable functional tests of vitamin C status. Instead, static biochemical tests, particularly the measurement of plasma or leukocyte ascorbic acid concentrations, are most frequently used to assess vitamin C status. These static biochemical tests are discussed in detail below.

## 19.8 Plasma ascorbic acid

Ascorbic acid is transported in the plasma but is not bound to any protein; almost all is present as the ascorbate monoanion. Plasma (or serum) ascorbic acid concentrations are the most frequently used and practical index of vitamin C status in studies of individuals and populations. Concentrations are influenced by recent intake of the vitamin, making fasting blood samples essential.

### 19.8.1 Ascorbic acid pharmacokinetics

Plasma ascorbic acid concentrations exhibit a characteristic sigmoidal relationship with intake, as shown in [Figure 19.2](#). In adults, the steepest change is evident at intakes between about 30 and 90mg/d of vitamin C. When intakes are below about 20mg/d, most of the vitamin C enters the tissues, so there is very little available for circulation in the blood. For intakes greater than 100mg/d, plasma concentrations tend to plateau between 60 and 70 $\mu$ mol/L, concentrations representing the renal threshold and plasma saturation ([Figure 19.2](#)). When intakes exceed about 200mg/d, intestinal absorption of vitamin C becomes the limiting factor ([Levine et al., 1996](#)).

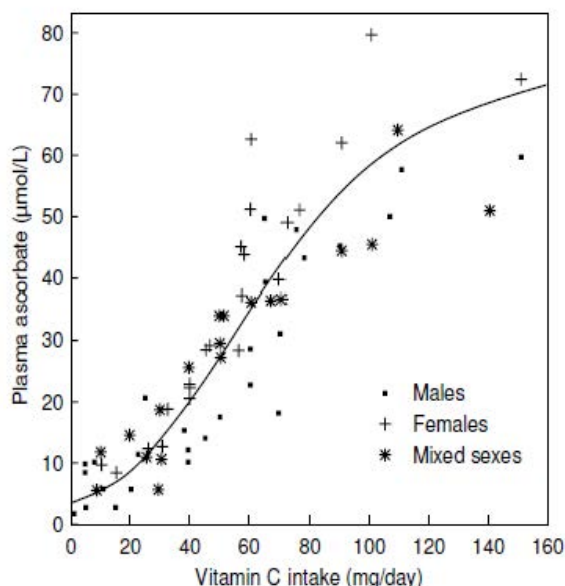


Figure 19.2. The relationship of plasma ascorbate relative to daily vitamin C intake. Redrawn from Bates et al. ([1997](#)).

([Bates et al., 1979](#)).

As a result of this threshold effect with only limited intestinal absorption at intakes > 200mg/d, and the excess circulating ascorbic acid being excreted in the urine, plasma ascorbic acid concentrations cannot identify people who are regularly consuming excessive amounts of vitamin C. Hence, it is not surprising that in the U.S. National Health and Nutrition Examination Survey (NHANES II), a correlation between plasma ascorbic acid and vitamin C intakes was only reported for people not taking vitamin C supplements ([Loria et al., 1998](#)). Vitamin C is one of the best markers for fruit and vegetable intake, particularly fruit intake ([Block et al., 2001](#); [Drewnowski et al., 1997](#)). However, strong correlations between dietary intakes of vitamin C and plasma ascorbic acid concentrations have only been reported when habitual dietary intakes of ascorbic acid are relatively modest (30–80mg/d)

People consuming chronically low intakes of vitamin C likely have plasma ascorbic acid concentrations that reflect the ascorbic acid content of the body ([Jacob et al., 1987](#)). Indeed, in such circumstances,

plasma concentrations are probably as accurate an indicator as concentrations in leukocytes. However, the relationship may be obscured in epidemiological studies by other factors that affect the absorption or metabolic turnover of vitamin C. In NHANES III, for example, only 50% of adult men had serum ascorbic acid concentrations  $> 38\mu\text{mol/L}$ , although more than 75% had dietary intakes higher than the estimated average requirement (i.e.,  $75\text{mg/d}$ ) ([IOM, 2000](#)). Such a discrepancy may be due, in part, to the high proportion of U.S. adult males who smoke or who are exposed to environmental tobacco smoke.

### 19.8.2 Factors affecting plasma ascorbic acid

**Cigarette smoking** has a marked effect on plasma ascorbic acid concentrations. Many studies report lower plasma ascorbic acid concentrations in smokers ([Carr & Rowe, 2020](#)), as well as in those regularly exposed to environmental cigarette smoke ([Figure 19.3](#)). The mechanism controlling the

influence of cigarette smoking on plasma ascorbic acid concentrations appears to be related to the higher metabolic turnover of vitamin C in smokers than in nonsmokers ([Kallner et al., 1981](#)), which is probably induced by increased oxidative stress from substances in smoke. Certainly, studies using isotopically labeled ascorbic acid show an increased metabolic turnover of ascorbic acid in smokers ( $70\text{mg/d}$  versus  $36\text{mg/d}$  for nonsmokers), resulting in a 40% greater requirement for vitamin C ([IOM, 2000](#)). Smokers also tend to have lower dietary intake of vitamin C, further contributing to a poor vitamin C status ([Schechter et al., 1989](#)).

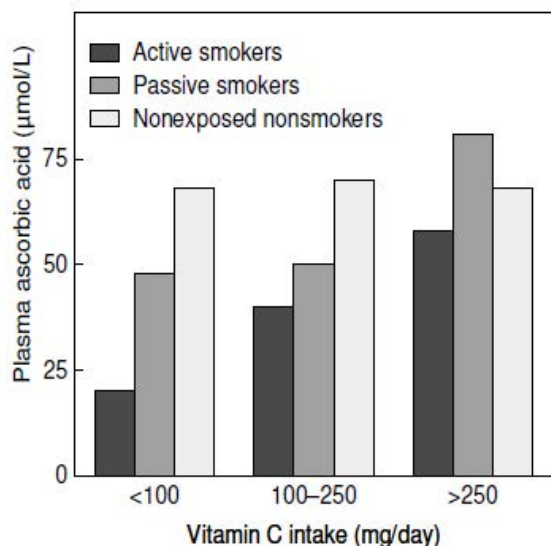


Figure 19.3. The effects of cigarette-smoke exposure on plasma ascorbic acid concentrations in 141 premenopausal women who were active or passive smokers, or nonexposed nonsmokers. Three concentrations of dietary vitamin C intake are represented in the results. Data from Tribble et al. ([1993](#)).

**Sex** influences plasma ascorbic acid concentrations; women have higher plasma ascorbic acid concentrations than men who consume similar intakes of vitamin C. However, this is likely partly explained by volumetric dilution due to differences in fat-free mass ([Jungert & Neuhauser-Berthold, 2015](#)), a premise supported by a lack of sex differences in plasma ascorbic acid concentrations before adolescence. For example, in NHANES III, higher serum ascorbate concentrations in women than in men

were only apparent in those aged  $> 19\text{y}$ ; no sex-related differences were reported in younger age groups ([IOM, 2000](#)). Thus, sex differences in lean body mass are likely to be a contributing factor ([Blanchard, 1991](#)).

**Age**-differences in plasma ascorbic acid concentrations have been reported. The elderly, especially those who are institutionalized or housebound, may have lower concentrations than those of younger persons ([Carr & Rowe, 2020](#)). This trend has been attributed in part to lower dietary intakes because of problems of poor dentition or chronic diseases that may influence absorption or renal functioning. Nevertheless, at present, there is no evidence that the elderly have higher vitamin C requirements than those for young adults. Indeed, NHANES IV data has indicated a U-shaped curve for circulating vitamin C concentrations by age ([Schleicher et al., 2009](#)).

**Pregnancy** lowers plasma ascorbic acid concentrations due to increasing body weight and hemodilution, as well as active transfer to the fetus, especially during the last trimester. Lactation results in even higher requirements due to active transfer of an average of 40mg/d vitamin C through breast milk to the growing infant ([IOM, 2000](#)).

**Higher body weight** is typically associated with lower vitamin C status in epidemiological studies. This is likely due to volumetric dilution, whereby an identical vitamin C intake will result in lower vitamin C status in a person of higher bodyweight relative to a person of lower bodyweight ([Block et al., 1999](#)). People with central obesity are likely to have even higher requirements due to enhanced oxidative stress and inflammation ([Carr et al., 2022](#)). Despite the obesity pandemic in many regions of the world, and the use of bodyweight to estimate vitamin C recommendations for women and children, there are as yet no vitamin C recommendation categories for large or overweight individuals.

**Acute or chronic infection** lowers circulating ascorbic acid concentrations. The more severe the infection, the lower the vitamin C status, as evidenced by the high prevalence of hypovitaminosis C and deficiency in hospitalised patients with pneumonia and sepsis ([Carr, 2020](#)).

### 19.8.3 Interpretive criteria for plasma ascorbic acid

Protracted intakes of vitamin C < 20mg/d cause plasma ascorbic acid concentrations to decline rapidly to deficient concentrations (i.e. 11µmol/L or less) ([Hodges et al., 1971](#); [Jacob et al., 1987](#)). At the lower plasma ascorbic acid concentrations, clinical signs of scurvy such as follicular hyperkeratosis, swollen or bleeding gums, petechial hemorrhages, and joint pain occur ([Hodges et al., 1969](#)). Therefore, plasma ascorbic acid concentrations ≤ 11µmol/L are taken as indicative of vitamin C deficiency.

The cut-off values for plasma ascorbic acid concentrations used to distinguish different categories of deficiency are poorly defined. NHANES II defined “clinically based categories” of plasma ascorbate concentrations in terms of mg/dL, i.e. deficient < 0.2, low 0.2–0.39, normal 0.4–0.99, and saturated ≥ 1.0mg/dL (equating to < 11, 11–22, 23–56, and ≥ 57µmol/L, respectively) ([Loria et al., 1998](#)). Of note, the low (hypovitaminosis C) cut-off is when the “sub-clinical” signs and symptoms of vitamin C deficiency start to be observed, e.g. changes in mood and energy levels.

More recently, the European Food Safety Authority (EFSA) has defined ≥ 50µmol/L as “adequate” ([Table 19.1](#); [EFSA, 2013](#)). Nevertheless, well-conducted pharmacokinetic studies in men and women suggest that 70µmol/L is a more accurate cut-off for “saturation”, although there is variation between individuals ([Levine et al., 1996](#); [Levine et al., 2001](#)). These higher criteria have been used in more recent NHANES analyses ([Crook et al., 2021](#); [Narayanan et al., 2021](#)), that indicated that smoking, male sex and higher body weight/BMI were common indicators for lower vitamin C status.

Status	Plasma Ascorbic Acid (µmol/L)
Deficient	≤ 11
Hypovitaminosis C	≤ 23
Inadequate	< 50
Adequate	≥ 50
Saturating	≥ 70

Of note, the mean vitamin C status of many high-income populations globally is typically around 50µmol/L, with the noted sex differences ([Rowe & Carr, 2020](#)). The prevalence of hypovitaminosis C and outright deficiency also tends to be relatively low in these countries. In contrast, many low-middle income populations typically have lower mean vitamin C status, and a higher prevalence of hypovitaminosis C and deficiency, largely explained by lower dietary





Table 19.1. Current interpretive criteria for plasma ascorbic acid concentrations.

intakes. Vitamin C status and intake data from national surveys from high-income countries (e.g. U.S. NHANES and U.K. National Diet and Nutrition Survey) can be found in the review of Rowe and Carr ([2020](#)) and the cited references.

#### 19.8.4 Measurement of plasma ascorbic acid

Fasting blood samples are required for plasma ascorbic acid analysis. The samples need to be preserved immediately after collection to avoid degradation of the ascorbic acid. Metaphosphoric acid (or trichloroacetic acid) is typically used to precipitate protein and to stabilize the ascorbic acid in the samples prior to analysis. Additionally, a reducing agent, such as dithiothreitol (DTT), can be added to preserve the ascorbic acid in a reduced state. The reducing agent TCEP (tris(2-carboxyethyl)phosphine) is versatile in that it can also work in acidified solutions with longer incubation times. A metal chelator, such as DTPA (diethylenetriaminepentaacetic acid), can be added to attenuate metal ion-dependent oxidation of ascorbic acid (particularly important if samples are hemolysed). It should be noted that EDTA (ethylenediaminetetraacetic acid), although a commonly used metal-chelators still redox-active and can catalyse the oxidation of ascorbic acid if the samples are not handled appropriately ([Pullar et al., 2018](#)).



Plasma samples for ascorbic acid analysis that have been prepared appropriately and frozen at  $-20^{\circ}\text{C}$  are stable for several weeks and at  $-70^{\circ}\text{C}$  for at least 1y ([Margolis & Duewer, 1996](#)). If the samples cannot be processed immediately, whole blood can be stored for up to 8h at  $4^{\circ}\text{C}$  before processing ([Galan et al., 1988](#)). Care needs to be taken with choice of anti-coagulants for collection of blood samples as the anti-coagulant EDTA, although a metal-chelator, remains redox active and can result in loss of ascorbic acid if the blood or plasma samples are not kept at  $4^{\circ}\text{C}$  ([Pullar et al., 2018](#)).

Several methods are available for measuring vitamin C in both the reduced form or as total ascorbic acid. The older colourimetric or fluorometric assays use predominantly 2,4-dinitrophenylhydrazine or o-phenylenediamine. These methods, however, have a number of limitations ([Washko et al., 1992](#)). Their sensitivity and specificity is low, and they are often subject to interference by other biological substances and yield falsely high readings at low ascorbic acid concentrations. In some circumstances, inadvertent oxidation of ascorbic acid or hydrolysis of dehydroascorbic acid may occur.

The method of choice for measuring ascorbic acid in plasma samples is high-performance liquid chromatography (HPLC); only  $50\mu\text{L}$  of plasma is required for the measurement. Of the HPLC methods available, electrochemical detection is preferred, with its high selectivity and sensitivity. However, this method does require a dedicated instrument and an experienced operator ([Washko et al., 1989](#)). Other simpler methods use HPLC coupled with an ultraviolet detector, but these methods can have a lower sensitivity and specificity ([Washko et al., 1992](#)).



Analysis of dehydroascorbic acid by HPLC is more difficult, because direct electrochemical or UV detection of dehydroascorbic acid is not possible. Instead, samples must first be analysed for ascorbic acid and then reduced for measurement of ascorbate plus dehydroascorbic acid. Dehydroascorbic acid is then determined by subtraction. *In vivo* concentrations of dehydroascorbic acid are generally very low (e.g.  $< 2\mu\text{mol/L}$ ) due to rapid uptake (via GLUTs) and intracellular reduction to ascorbic acid by cells of the vasculature. Therefore, reports of higher concentrations of dehydroascorbic acid in



different disease states are likely due to artefactual *ex vivo* oxidation often associated with use of the older spectrophotometric methods and/or inappropriate handling and processing of the samples prior to analysis ([Pullar et al., 2018](#)).

## 19.9 Ascorbic acid in leukocytes and specific cell subsets

Leucocytes include lymphocytes, monocytes, and three classes of granulocytes: polymorphonuclear leukocytes or neutrophils, eosinophils, and basophils. These cell types differ in their concentration of ascorbic acid and possibly in their response to supplemental vitamin C ([Levine et al., 1996](#); [Levine et al., 2001](#)), thus complicating the interpretation of ascorbic acid concentrations in leukocytes.

### 19.9.1 Leukocyte ascorbic acid

Concentrations of ascorbic acid in plasma and leukocytes correlate relatively well ([Figure 19.4](#);

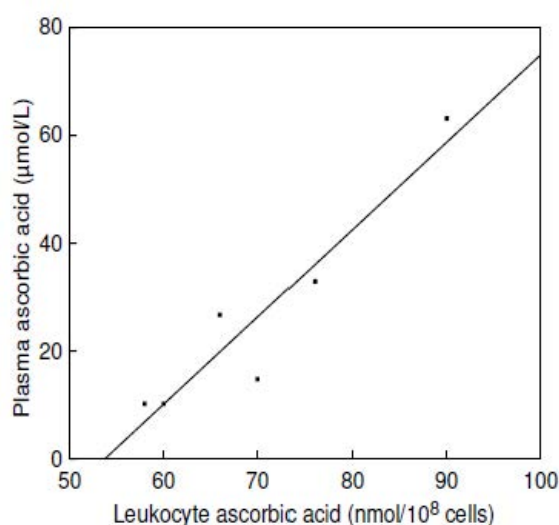


Figure 19.4. The relationship of mean plasma and leukocyte ascorbic acid concentrations measured at various stages during a depletion and repletion study of a group of six adult females. Data from [Sauberlich et al., 1989](#)),

[Sauberlich et al., 1989](#)), even though ascorbic acid concentrations in leukocytes are at least 14 times greater than those in plasma ([Levine et al., 1996](#); [Levine et al., 2001](#)). The ascorbic acid concentrations of mixed leukocytes range from 90–300nmol/10<sup>8</sup> cells in adults ([Omaye et al., 1979](#)), and depend in part on the heterogeneous nature of the leukocytes, and the methods used to isolate and analyse them.

Leukocyte ascorbic acid concentrations are commonly believed to be a more reliable index of tissue stores of ascorbic acid than are the corresponding concentrations in the plasma, erythrocytes, or whole blood ([Turnbull et al., 1981](#)). Leukocyte ascorbic acid concentrations are less responsive than plasma to short-term fluctuations in recent vitamin C intakes. In the study shown in [Figure 19.5](#), leukocyte ascorbic acid concentrations decreased about 33%, compared with the much larger and more rapid decline in plasma. Similar trends have been reported by others ([Jacob et al., 1987](#)). Thus, they provide a less

sensitive measure of lower intakes.

Concentrations of ascorbic acid in leukocytes reflect changes in tissue ascorbic acid concentrations and the ascorbic acid body pool, but not dietary intakes. Animal studies (i.e., monkeys and guinea pigs) have confirmed that leukocyte ascorbic acid concentrations provide an accurate reflection of ascorbic acid concentrations in the liver and body pool ([Omaye et al., 1987](#)).

Several factors can influence the concentrations of ascorbic acid in leukocytes. It has been proposed that the platelet/leukocyte ratio can influence leukocyte ascorbic acid values ([Vallance, 1986](#)). Platelets have comparable ascorbic acid concentrations to mononuclear cells ([Levine et al., 2001](#)), and should ideally be separated from leukocytes prior to leukocyte ascorbic acid assays ([Vallance, 1986](#)). Choice of the anticoagulant used may influence binding of platelets to leukocytes ([Healy & Egan, 1984](#)).

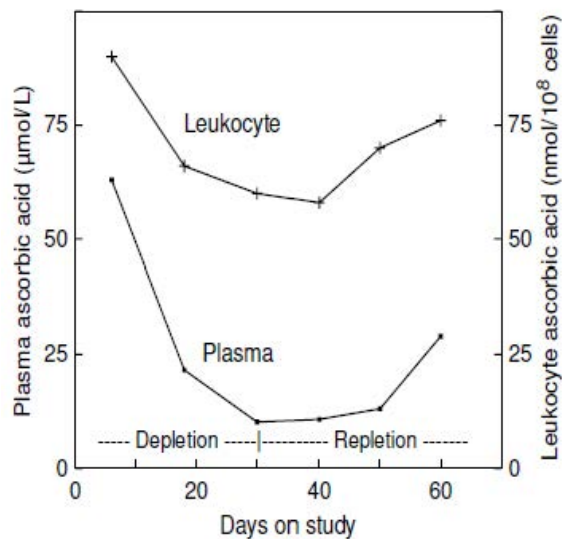


Figure 19.5. Mean plasma and leukocyte ascorbic acid concentrations during a depletion and repletion study of a group of six adult females. Ascorbic acid intakes were ad libitum for days 1–6; 0mg/d for days 6–30; 30mg/d for days 31–40; 60mg/d for days 41–50; and 90 mg/d for days 51–60. Data from [Sauberlich et al., 1989](#)),

Some additional non-dietary factors that influence concentrations of ascorbic acid in leukocytes are smoking, sex, infection, and certain drugs, as discussed for plasma ascorbic acid concentrations. In some cases, these effects are due to alterations in cell populations (e.g., acute infection) or to differences in ascorbic acid uptake ([Lee et al., 1988](#)).

Finally, concentrations of vitamin C in phagocytic cells, such as neutrophils, can be influenced by activation of their oxidative burst. This results in the generation of reactive oxygen species which oxidise extracellular ascorbic acid to dehydroascorbic acid that is readily transported into the cells via membrane GLUTs, where it is then rapidly reduced back to ascorbic acid ([Washko et al., 1993](#)). Higher than anticipated concentrations of vitamin C have been observed in leukocytes isolated from patients with severe sepsis, which is a condition characterised by systemic oxidative stress and activation of neutrophils ([Carr et al., 2021](#)).

### 19.9.2 Ascorbic acid in specific cell subsets

The specific cell type that is most useful for assessing vitamin C status is presently uncertain. As noted earlier, both ascorbic acid concentrations in individual leukocytes and their response to vitamin C supplementation vary. Mononuclear cells (lymphocytes and monocytes) have concentrations that are two- to threefold higher than granulocytes (e.g., neutrophils). Furthermore, the ascorbic acid pool in mononuclear cells is depleted more slowly than that from other blood compartments ([Jacob, 1990](#)). In addition, it can be difficult to obtain homogeneous and reproducible fractions of specific cell types, so that their reported ascorbic acid concentrations can vary widely.

Lymphocyte ascorbic acid concentrations of 120–250nmol/10<sup>8</sup> cells have been reported for healthy adults not consuming supplemental vitamin C ([Evans et al., 1982](#); [Yew, 1984](#)). Jacob et al. ([1991](#)) measured lymphocyte ascorbic acid concentrations in a depletion-repletion study of healthy men that was designed to induce moderate vitamin C deficiency. The mean baseline lymphocyte ascorbic acid concentration in the men was 209nmol/10<sup>8</sup> cells. After 60d of depletion, lymphocyte ascorbic acid concentrations fell significantly and consistently to a mean concentration of 87nmol/10<sup>8</sup> cells. Moreover, the concentrations distinguished between the group of participants receiving 5, 10, or 20mg ascorbic acid (74–145 nmol/10<sup>8</sup> cells) versus those participants receiving 60 or 250mg/d (182–261nmol/10<sup>8</sup> cells). Strong correlations of plasma and lymphocyte ascorbic acid concentrations were noted within the individual subjects when ascorbic acid intakes ranged from 5–250mg/d ([Table 19.2](#)). There was no evidence of scorbutic symptoms in the depletion phase of this study, but there was evidence of greater oxidative damage, based on alterations in indices of oxidant status ([Jacob et al., 1991](#)).

Metabolic Period	Length (d)	Ascorbic acid intake (mg/d)	n	Plasma (μmol/L)	Mononuclear leukocyte (nmol/10 <sup>8</sup> cells)
1	4	250†	8	59.6 ± 3.3 <sup>a</sup>	209 ± 9 <sup>a</sup>
2	32	5	8	6.6 ± 0.3 <sup>b</sup>	117 ± 7 <sup>‡b</sup>
3	28	10	4	5.3 ± 0.3 <sup>b</sup>	87 ± 9 <sup>b</sup>
4	28	20	4	5.9 ± 0.5 <sup>b</sup>	95 ± 5 <sup>c</sup>
5	28	60	3	26.7 ± 8.8 <sup>c</sup>	201 ± 2 <sup>a</sup>
6	28	250	5	64.2 ± 4.5 <sup>a</sup>	217 ± 11 <sup>a</sup>

Table 19.2. Ascorbic acid concentrations of healthy men after periods of various ascorbic acid intakes. Ascorbic acid concentrations are means ± SEM at the end of each period, n = number of participants. Means within vertical columns not sharing the same subscript letter are significantly different (< 0.05) by t-test. † Participants consumed a supplement of 250mg ascorbic acid in addition to their free-living diet for 1–2wk before entering the study. ‡ n = 7, one value deleted because of platelet contamination. Data From Jacob et al., (1991)

In later ascorbic acid depletion-repletion studies in men and women, Levine et al. (1996, 2001) measured concentrations of ascorbic acid in purified neutrophils, monocytes and lymphocytes. Neutrophil ascorbic acid concentrations increased markedly in response to supplemental vitamin C intakes of between 30 and 100mg/d but showed little further increase at higher doses. This trend is similar to that observed for plasma ascorbic acid concentrations (Figure 19.2), although the latter reach a plateau at higher daily doses (about 200mg/d). The concentrations of ascorbic acid in purified monocytes and lymphocytes followed a similar trend, again reaching a plateau at doses of 100mg/d.

### 19.9.3 Interpretive criteria for leukocytes

Confusion exists over the interpretive criteria used for leukocyte ascorbic acid concentrations, in part because they can be expressed in different ways: per 10<sup>8</sup> cells, per mL per DNA concentration, or per unit of protein. This makes comparisons among laboratories difficult. The most common method is in terms of cell numbers (nmol/10<sup>8</sup> cells). The heterogeneous nature of the leukocytes and various technical difficulties with their analyses, are further complicating factors.

Table 19.3 presents the interpretive criteria used by Jacob (1994) and given in Sauberlich (1999), expressed in terms of cell numbers. When expressed in this way, clinical signs of scurvy, such as swollen or bleeding gums or petechial hemorrhages, have been associated with leukocyte concentrations of about 11nmol/10<sup>8</sup> cells. Even at concentrations as high as 50nmol/10<sup>8</sup> cells, scorbutic changes including inflammation, tenderness, bleeding of the gums, and petechiae have sometimes been described (Sauberlich et al., 1989). For this reason, the cut-off shown in Table 19.3, indicative of deficiency, is < 57nmol/10<sup>8</sup> cells.

Status	Mixed leukocytes (nmol/10 <sup>8</sup> cells)	Mononuclear leukocytes (nmol/10 <sup>8</sup> cells)
Deficient	< 57	< 114
Low	57–114	114–142
Adequate	> 114	> 142

Table 19.3. Interpretive criteria for leukocyte ascorbic acid concentrations. From Jacob (1994).

Cut-off values for ascorbic acid concentrations in specific cell types such as neutrophils, monocytes, and lymphocytes are less certain. Levine et al. (1996, 2001) showed that intra-cellular ascorbic acid concentrations in neutrophils were less than half of corresponding mononuclear leukocyte (lymphocyte and monocyte) concentrations. The interpretation of leukocyte ascorbic acid concentrations may be particularly difficult in surgical patients or in cases of severe infection; these conditions are often associated with leukocytosis, activation of neutrophils, and systemic

oxidative stress (Schorah et al., 1986; Vallance, 1988; Carr et al., 2021).

#### 19.9.4 Measurement of leukocyte ascorbic acid

The isolation and assay of leukocytes for ascorbic acid is technically difficult, and presently requires relatively large blood samples (2–5mL), making the assay unsuitable for serial measurements on infants. Leukocytes can be isolated with density-gradient sedimentation ([Boyum, 1968](#)). Monoclonal antibodies to various cell types can also be used ([Field, 1996](#)). Analysis of ascorbic acid in leukocytes or specific cell types is best performed by HPLC with electrochemical detection, after deproteinization ([Washko et al., 1989](#)).

#### 19.10 Ascorbic acid in erythrocytes and whole blood

Erythrocyte ascorbic acid concentrations are not widely used as an index of ascorbic acid status. Concentrations are roughly comparable to plasma but only respond to changes in vitamin C intake over a narrow range. Hence, they are not as sensitive as plasma ascorbic acid concentrations ([Hodges et al., 1971](#)). Because erythrocyte concentrations are less responsive to recent dietary intake, they could potentially be used to assess ascorbic acid status in non-fasting individuals ([Pullar et al., 2020](#)). However, both within- and between-variation and analytical variance can be greater than for plasma ascorbic acid ([Jacob et al., 1987](#)). Erythrocyte ascorbic acid is technically more challenging to measure due to potential for oxidation of ascorbic acid by hemoglobin-associated iron; the hemoglobin is ideally removed via centrifugal filter units ([Li et al., 2012](#)). It should also be noted that conditions of vitamin C deficiency result in enhanced erythrocyte fragility and hemolysis ([Tu et al., 2015](#)).

Whole blood ascorbic acid concentrations have also been investigated in ascorbic acid depletion-repletion studies. Again, concentrations in whole blood are not as sensitive as plasma for assessing ascorbic acid depletion ([Jacob et al., 1987](#)). Concentrations < 17µmol/L are considered deficient because they have been associated with scorbutic signs ([Sauberlich et al., 1989](#)); those of 17–28µmol/L are considered low. Concentrations > 28µmol/L are interpreted as acceptable ([Sauberlich, 1999](#)). Presently, whole blood ascorbic acid concentrations are rarely used.

#### 19.11 Urinary excretion of ascorbic acid and metabolites

Urine is the major excretory route for absorbed ascorbic acid. Most ascorbic acid is excreted in its un-metabolized form ([Levine et al., 1996](#)). When intakes are > 1g/d, there is some increase in oxalate excretion ([Olson & Hodges, 1987](#)). However, reports of high oxalate excretion are likely due to artefactual *ex vivo* oxidation of excreted ascorbic acid prior to analysis ([Chalmers et al., 1985](#)), therefore careful handling of urine samples is required.

Urinary excretion of ascorbic acid reflects recent dietary intake. Experimental depletion-repletion studies have demonstrated that concentrations in the urine decline progressively with increasing depletion of vitamin C until, in persons with scurvy, concentrations are undetectable ([Jacob et al., 1987](#)). Nevertheless, urinary excretion is not a very sensitive index of ascorbic acid status; differences between persons with adequate or deficient intakes of ascorbic acid are small. For example, in the depletion-repletion study of 11 young men shown in [Figure 19.6](#), although urinary ascorbic acid excretion was significantly lower during the depletion periods (weeks 3–6) than in the initial 2wk 65mg/d baseline period, the differences were small.



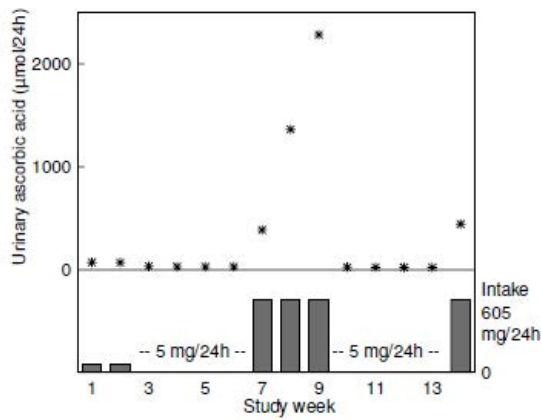


Figure 19.6. Mean urinary ascorbic acid during times of varied ascorbic acid intake. Data from a study of 11 adult men by Jacob et al., *American Journal of Clinical Nutrition*, 46, 818–826, 1987 © Am J Clin Nutr. American Society for Clinical Nutrition.

More recent pharmacokinetic investigation has indicated a renal threshold of about 60μmol/L plasma ascorbic acid for a majority of healthy individuals ([Ebenuwa et al., 2022](#)), a concentration attained by intakes of ≥ 100mg/d ([Levine et al., 1996, 2001](#)). Higher concentrations of ascorbic acid are observed in urine when plasma “saturation” is attained (i.e. intakes of ≥ 200mg/d). The vitamin C requirements of an individual can potentially be determined by administering a test dose of vitamin C and monitoring the resultant excretion.

The specificity of urinary ascorbic acid, however, is also low. The drugs aminopyrine, aspirin, barbiturates, hydantoins, and paraldehyde can all increase urinary ascorbic acid excretion ([Saubertlich, 1981](#)). An additional disadvantage of this test as a measure of ascorbic acid status in humans is the requirement for 24h urine specimens. The latter are impractical in field studies and more

useful in clinical or research settings.

#### 19.11.1. Measurement of urinary ascorbic acid

Ascorbic acid is unstable in urine and samples should ideally be kept at 4°C and stabilized with a metal chelator (e.g. DTPA) and acidification (e.g. with metaphosphoric acid) as soon as possible after collection. HPLC with electrochemical detection is the preferred method of analysis. Values can be presented as amount excreted per hour or day, or relative to urine creatinine concentrations for spot collections, to account for urine concentration.

#### 19.12 Ascorbic acid in other cells and fluids

Ascorbic acid has been detected in a number of other body fluids. The concentrations in saliva and alveolar lining fluid are generally relatively low and do not appear to correlate with concentrations of ascorbic acid in plasma, leukocytes, or dietary intakes ([Leggott et al., 1986](#); [Jacob et al., 1987](#); [Bui et al., 1992](#)). In contrast, ascorbic acid concentrations in cerebrospinal fluid are typically higher than those found in plasma ([Tallaksen et al., 1992](#)). Ascorbic acid is able to bypass the blood-brain barrier via SVCTs in the choroid plexus ([Angelow et al., 2003](#)), and is believed to have important functions in the central nervous system ([May, 2012](#)). Of note, concentrations of ascorbic acid in seminal fluid can be up to 10-fold higher than those in plasma ([Thiele et al., 1995](#)). Lower seminal ascorbic acid concentrations were observed in infertile men and this appeared to be related to elevated oxidative stress ([Thiele et al., 1995](#); [Lewis et al., 1997](#)).

Buccal cell ascorbic acid concentrations have been investigated as a potential biomarker of vitamin C status. In a vitamin C depletion-repletion study in healthy men, buccal cell ascorbic acid concentrations were significantly lower in subjects receiving low ascorbic acid intakes (5, 10, 20mg/d) compared with repletion intakes (60 or 250mg/d) ([Jacob et al., 1991](#)). Nevertheless, the investigators concluded that buccal cells are probably not suitable as a marker of ascorbic acid status over a broad

range of population intakes. In contrast, muscle tissue obtained by needle biopsy has been shown to correlate relatively well with plasma concentrations and dietary intake ([Carr et al., 2013](#)). Muscle biopsy is a highly invasive procedure and is not typically undertaken outside of research settings.

### 19.13 Body pool size

Isotope dilution techniques can be used to determine the size of the body pool of vitamin C. The method involves administration of an oral dose of  $^{14}\text{C}$ -labeled or  $^{13}\text{C}$ -labeled ascorbic acid, followed by the measurement of the specific activity of blood or urine ascorbate within 24–48h ([Baker et al., 1971](#); [Kallner et al., 1977](#)). Of the two radioactive isotopes,  $^{14}\text{C}$  has a long half-life, so  $^{13}\text{C}$ , a shorter-lived isotope, is preferred.

In young healthy male adults, the pool size of ascorbic acid has been estimated to be about 1500mg (i.e., 20mg/kg body weight) ([Baker et al., 1971](#); [Kallner et al., 1977](#)). In adult men, the total body pool of vitamin C ranges in size from < 300mg to about 3000mg, depending on the daily intake of L-ascorbate. When pool sizes are < 600mg of vitamin C, psychological abnormalities have been reported ([Kinsman & Hood, 1971](#)), whereas at concentrations < 300mg, scurvy symptoms have been observed ([Baker et al., 1971](#)).

### 19.14 Functional tests of vitamin C status

There are currently no specific functional tests for vitamin C status. Bleeding into the skin to form petechia, purpura and ecchymoses are a sign of the vitamin C deficiency disease scurvy. Capillary fragility has been used in the past as a functional test of vitamin C deficiency ([Vilter, 1967](#)). However, this test can produce inconsistent results in individuals with vitamin C deficiency and is not specific to vitamin C deficiency states as other diseases may also increase capillary fragility ([Vilter, 1967](#)). Erythrocyte fragility could be an alternative functional test of deficiency ([Tu et al., 2015](#)), however, once again, this is not specific for vitamin C deficiency. Consequently, alternative functional tests of ascorbic acid status need to be developed.

Specific markers of ascorbic acid's enzyme cofactor functions have been proposed. An example relates to collagen crosslinking ([Munday et al., 2005](#)). In this study, urinary excretion of specific crosslink ratios were higher in British children versus Gambian children, and in Gambian children during the dry season (characterised by high vitamin C intake and status) versus the rainy season. A supplementation study (100mg/d of vitamin C) in Gambian children during the rainy season did not, however, alter the crosslink ratio. This may have been due to the supplementation period (7wks) being insufficient, as collagen can have a long turnover in some tissues.

Another possibility is assessment of vitamin C-dependent epigenetic marks ([Young et al., 2015](#)), for example in leukocytes. One preliminary study has demonstrated positive correlations between vitamin C status and hydroxymethylcytosine and hydroxymethyluracil marks in leukocyte DNA ([Starczak et al., 2018](#)). Participants with vitamin C concentrations > 40 $\mu\text{mol/L}$  exhibited significantly higher concentrations of these epigenetic marks than those with plasma concentrations < 20 $\mu\text{mol/L}$ . As such, more research in this area appears warranted.

### Acknowledgements

