Vitamin C: prospective functional markers for defining optimal nutritional status

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Most species of plants and animals synthesize ascorbic acid, but human subjects cannot, making vitamin C an essential component of our diet. Relationships between vitamin C intake and status, and between status and health are not yet clear. There is evidence, however, that higher intake of vitamin C is associated with lower risk of disease, supporting the concept that optimal intake is needed for optimal vitamin C status, and that both factors are required for optimal health. Vitamin C has low toxicity in healthy subjects, but a clear definition of optimal status and the dietary intake required to meet and maintain this status is needed before a change in the current recommended intake can be considered. Available evidence suggests that intake of 200 mg vitamin C/day saturates tissues and maintains fasting plasma levels above the proposed threshold (50 µmol/l) for minimum risk of CHD. However, the issue of whether or not these levels produce ‘optimal vitamin C status’ awaits the clear and accepted definition of the term. This definition in turn awaits the development of reliable functional markers capable of assessing the effects of varying levels of vitamin C nutriture. In the present paper the relationship between intake and body stores of vitamin C and the role of vitamin C in human health are reviewed briefly. The requirements of a reliable functional marker of human vitamin C status are defined, three classes of functional markers (molecular, biochemical and physiological) are described, and possible candidate markers are examined.

Vitamin C: Ascorbic acid: Antioxidants: Micronutrient status: Functional marker

Vitamin C comprises two biologically-active vitamers, L-ascorbic acid and its two-electron reduction product dehydro-L-ascorbic acid. Most species of plants and animals synthesize ascorbic acid from glucose, but human subjects cannot, making vitamin C an essential dietary component (Levine, 1986; Padh, 1990). Relationships between vitamin C intake and status, and between status and health are not yet clear (Hennekens et al., 1994; Weber et al., 1996; Halliwell, 1997; Hemila, 1997; Strain & Benzie, 1998). There is evidence, however, that higher intake of vitamin C is associated with lower risk of disease (Block, 1991; Diplock, 1994; Riemersma, 1994; Bendich & Langseth, 1995; Gey, 1995; Machlin, 1995; Benzie, 1998), supporting the concept that optimal intake is needed for optimal vitamin C status, and that both are required for optimal health (Levine & Hartzell, 1987; Halliwell, 1996). Vitamin C has low toxicity in healthy subjects (Diplock, 1994; Hathcock, 1997), but a clear definition of optimal status, and the dietary intake required to meet and maintain this status, is needed before a change in the current recommended intake can be considered (Young, 1996). However, assessing vitamin C status is problematic, and there are currently no established functional markers of status. In the present paper the relationship between intake and body stores of vitamin C, and the role of vitamin C in human health are reviewed briefly, and the requirements of a reliable functional marker of human vitamin C status and possible candidate markers are examined.

Vitamin C status: relationship between intake and body stores

In simple terms, vitamin C status describes the amount of vitamin C within target tissues and fluids. Status reflects the balance between various factors controlling supply and turnover of the vitamin (Fig. 1). These controlling factors are far from constant and are difficult to assess. In terms of supply, vitamin C is destroyed during storage, processing and cooking of foods, and by interaction with other dietary components (Halliwell, 1996; Bates, 1997). Thus, estimates...
Factors controlling vitamin C status. GI, gastrointestinal tract.

- **Dietary intake**: Affected by storage, processing, cooking, and composition of food.
- **GI absorption**: Affected by dose, rate of transit, biochemical environment (glucose levels, pH, presence of trace metals, nitrosoamines).
- **Urinary levels**: Affected by plasma levels, glomerular filtration rate, renal threshold.
- **Plasma and tissue levels**: Affected by dietary intake, absorption, utilization, cellular uptake, recycling, renal loss.

### Fig. 1. Factors controlling vitamin C status. GI, gastrointestinal tract.

### Fig. 2. Plasma ascorbic acid response to ingestion of vitamin C; peak post-ingestion (●) and fasting (○) levels at different intakes of vitamin C. Points are mean values and standard deviations represented by vertical bars. (From Benzie & Strain, 1997a.)

The concentration of vitamin C in fasting plasma is in the reduced form, ascorbic acid (Levine et al. 1993). There appears to be rapid uptake by erythrocytes of dehydro-α-ascorbic acid formed within the circulation, dehydro-α-ascorbic acid being immediately reduced to ascorbic acid by intracellular GSH (Mendiratta et al. 1998). Plasma ascorbic acid levels are reported to be lower in men than in women and to decrease with age (Garry et al. 1987; Blanchard et al. 1990; Bailey et al. 1993). But there is no clear physiological rationale for these differences, and they are not always found (Benzie et al. 1998). Plasma ascorbic acid concentrations peak at 1–2 h after ingestion, and response is related to the dose but not to the fasting plasma concentration (Benzie & Strain, 1997b). However, the relative amount absorbed decreases as the dose increases (Mayersohn, 1972; Kallner et al. 1981; Melethil et al. 1986; Levine et al. 1993; Benzie & Strain, 1997a). The upper limit of plasma ascorbic acid concentration is controlled by the gastrointestinal absorption and renal re-absorption mechanisms, and fasting plasma concentrations rarely exceed 100 μmol/l, even with dietary supplementation (Graumlich et al. 1997); however, following a large (≥500 mg) oral dose plasma levels can increase several fold, and may approach 200 μmol/l (Benzie & Strain, 1997a). Fig. 2.

Intracellular vitamin C levels can exceed eighty times that of plasma (Evans et al. 1982). At stable intakes, vitamin C concentrations in fasting plasma correlate directly and significantly with platelet, erythrocyte and leucocyte concentrations (Jacob et al. 1987; Levine et al. 1995; Graumlich et al. 1997), and fasting plasma concentrations of >60 μmol ascorbic acid/l indicate tissue saturation (Graumlich et al. 1997). However, there is no established plasma threshold defining deficiency, and plasma ascorbic acid concentrations of >11·4, >17, and >22·7 μmol/l have been regarded as acceptable (see Ortega et al. 1998). In depletion studies of healthy young men plasma vitamin C concentrations decreased to an average of 7 μmol/l without clinical signs of scurvy (Jacob et al. 1987; Graumlich et al. 1997). However, clinical signs indicate the late stage of chronic and severe deficiency. Moreover, it is likely that demand for vitamin C in the study populations of young healthy non-smoking and otherwise well-nourished men was low. In contrast, demand may be high in certain situations or disorders. Pregnancy, smoking, inflammation, diabetes mellitus and pre-eclampsia increase the input...
required to maintain ‘normal’ plasma levels (Schorah, 1992; Smirnoff & Pallanca, 1996; Bates, 1997; Halliwell, 1997; Hubel et al. 1997); exercise, environmental pollution, plasma concentrations of vitamin E, uric acid and glucose also influence vitamin C metabolism (Levine, 1986; Schorah, 1992; Benze & Strain, 1996a; Marangon et al. 1998). Thus, plasma levels may indicate functional reserves of vitamin C but may not reflect functional status; functional markers of vitamin C status are needed for this.

Measurement of vitamin C can also be problematic in analytical terms, as most simple methods of measurement are non-specific. Sensitive and specific methods are available (Bates et al. 1994; Benze, 1996a); however, vitamin C is rapidly destroyed *ex vivo*, particularly at alkaline pH and in the presence of transition metal ions (Baetner & Jurkiewicz, 1996). Thus, special handling of samples is required, with acid stabilization of ascorbic acid before storage at low temperature, or immediate testing of the sample (Bates et al. 1994; Benze, 1996a).

Stable-isotope studies using 13C-labelled ascorbic acid and GC-mass spectrometry (Block et al. 1996) offer a powerful means of monitoring distribution and turnover of vitamin C *in vivo*. This highly sophisticated and demanding technique is still, however, in the developmental stage. Currently, therefore, and despite its limitations, measurement of fasting plasma ascorbic acid concentration is the most commonly used method of assessing vitamin C status.

**Vitamin C and health**

The reference nutrient intake of 40 mg/d (Department of Health, 1991) is aimed at prevention of the clinical deficiency state, scurvy (Bates, 1997). However, no obvious deficiency does not necessarily indicate adequacy (Levine et al. 1995), and subclinical or marginal deficiency of vitamin C owing to insufficient intake and/or to increased utilization may be common. The wide range of signs and symptoms of scurvy indicate the many and varied metabolic systems with which vitamin C interacts. These systems include collagen synthesis, steroid and peptide metabolism, endocrine function, the immune system, blood pressure control, haemostasis, Fe and Cu balance and mitochondrial fatty acid catabolism (Levine, 1986; Padh, 1990; Smirnoff & Pallanca, 1996; Bates, 1997; Hemila, 1997). However, while the systems themselves are diverse, the biochemical role played by vitamin C in each system appears to be mediated via its antioxidant properties (Friel et al. 1989; Padh, 1990).

Increased risk of chronic disease, including cancer, cataracts and CHD, is associated with low intake or plasma concentrations of vitamin C (Block, 1991; Riemersma, 1994; Gey, 1995; Machlin, 1995; Maxwell & Lip, 1997; Benze, 1998). Supplementation with vitamin C is reported to decrease blood pressure and blood lipids, improve glucose metabolism and endothelial function, and to increase resistance of lipids and DNA to oxidative damage (Paolisso et al. 1994; Benze, 1996b; Friel et al. 1996; Halliwell, 1996; Levine et al. 1996; Weber et al. 1996; Sweetman et al. 1997). However, the contribution of high intake or plasma levels of vitamin C to lowered risk of disease is difficult to assess, as other health-promoting habits generally accompany high vitamin C intake, and clinical trials have shown inconsistent and inconclusive results (Hennekens et al. 1994; Halliwell, 1997; Benze, 1998).

Nonetheless, based on results of depletion-repletion and observational studies, an optimal vitamin C intake of 200 mg/d (Graumlich et al. 1997) and a threshold ‘potential protective plasma level’ of 50 μmol/l (Gey, 1995) have been proposed. These values have yet to be endorsed in terms of increased reference nutrient intake, as changes in nutritional public policy require that unequivocal benefit ‘in terms of health-related outcomes’ of increased vitamin C intake is established (Young, 1996; Blanchard et al. 1997; Halliwell, 1997; Shane, 1997). Results of long-term vitamin C supplementation studies will resolve the question of whether the reference nutrient intake should be changed, but such studies are costly and time-consuming. Reliable functional markers of vitamin C status could serve as early surrogate health-related end points, providing information to help plan and monitor clinical trials while helping to guide more immediate nutritional requirements.

**Functional markers of vitamin C status: criteria and prospects**

Diet controls but does not completely define body stores of vitamin C; and demand may outstrip an apparently adequate supply. Fasting plasma levels of ascorbic acid reflect but do not equate to body stores, and there may be islands of need within oceans of plenty. In order to define and differentiate between optimal, adequate, marginal, inadequate and deficient categories of vitamin C status, reliable markers of vitamin C function are needed (King, 1996; Young, 1996; Bates, 1997). Such markers will complement methods of assessing intake, distribution and body stores, and help map links between the immediate effects and eventual outcome of different levels of vitamin C nutrition.

A reliable functional marker of microminutrient status in human subjects must respond sensitively, specifically and predictably to changes in the concentration and/or supply of the micronutrient, i.e. there must be a measurable dose–response relationship; be accessible for measurement, i.e. the marker must be present in body fluids or cells which can be sampled or imaged in some way; be in a form and quantity which can be measured objectively and reproducibly, i.e. suitable analytical tools and methods must be available; reflect a change in the target tissue or fluid which has a direct impact on health, i.e. must relate to a physio-logical or pathological end point.

Vitamin C acts on various biological systems, and while no single specific marker of vitamin C function has been identified, three classes of functional markers can be described:
(1) molecular markers which relate to effects on function or activity of specific molecules; these effects are likely to be mediated directly by vitamin C;
(2) biochemical markers which relate to effects on levels of biochemical constituents in body fluids; these effects are likely to be the result of molecular changes;
(3) physiological (biological) markers which relate to effects on homeostatic processes or organ systems resulting in physiological (or pathological) change; these effects are likely to be the result of biochemical changes.

**Molecular markers**

Specificity and sensitivity can be high for this class of marker which relates to, for example, oxidative changes in enzymes. Ascorbic acid affects the activity of at least ten oxidoreductases or hydroxylating Fe- or Cu-containing enzymes, including dopamine-β-monooxygenase (EC 1.14.17.1; Levine & Hartzell, 1987; Ginter, 1989; Padh, 1990). This Cu-containing enzyme is involved in noradrenaline biosynthesis and, while there is no absolute requirement for vitamin C, ascorbic acid is a specific and dose-dependent enhancer of enzyme activity. Conceptually, this marker meets the first requirement, and in theory vitamin C functional status could be assessed by measuring the rate of noradrenaline production by target cells. However, sampling these cells (chromaffin cells from the adrenal medulla) is not a practical option in human subjects, and the ascorbic acid-related enzyme kinetics reportedly differ depending on whether the enzyme is isolated or in situ (Levine & Hartzell, 1987). In addition, there is no evidence that ascorbic acid-dependent maximum activity of this enzyme is required for health, questioning its use as a functional marker (Young, 1996).

The activities of two hepatic enzyme systems, cholesterol 7α-monooxygenase (EC 1.14.13.17) and the microsomal cytochrome P450 detoxification system, may be useful functional markers of vitamin C status (Ginter, 1989). These enzymes are also dependent on vitamin C for maximal activity, and in human subjects this may be optimal for cholesterol metabolism and detoxification of xenobiotics. While perhaps sensitive and specific, and related directly to health, these intracellular markers of vitamin C function are, however, difficult to access and evaluate. Their biochemical effects in terms of cholesterol concentration and detoxification can be measured, but cannot be related specifically to vitamin C status.

Mitochondrial glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) is needed for glucose-coupled ATP-dependent insulin release from pancreatic B cells, and in guinea-pig tissue ascorbic acid is needed as a stimulatory cofactor (Jung & Wells, 1997). This role could help explain the modulating effect of vitamin C on B cell function and glucose homeostasis (Paulisson et al. 1994). More effective insulin response to a glucose load after vitamin C supplementation was reported in a study of idiopathic Ca stone-formers (Swille et al. 1997) and, therefore, study of pre- and post-vitamin C supplementation insulin sensitivity could be useful, although currently speculative, functional marker of status.

Ascorbic acid has been reported to prevent NADPH-initiated free Fe-dependent cytochrome P450-dependent microsomal lipid peroxidation (Ghosh et al. 1997). This specific inhibition is thought to be mediated by the reaction of ascorbic acid with the perfferryl radical form of cytochrome P450 (P450Fe–O2·−). Ascorbic acid neutralizes the perfferryl radical and prevents abstraction of H from a polyunsaturated fatty acid. In studies of guinea-pigs it was reported that lipid peroxidation occurred in association with ascorbic acid deficiency, even at subclinical levels and despite adequate amounts of other antioxidants such as vitamin E and GSH (Ghosh et al. 1997). This effect of ascorbic acid is related directly to health, as lipid peroxidation increases atherogenesis and risk of CHD (Steinberg et al. 1989; Machlin, 1995; Benze, 1996b; Maxwell & Lip, 1997), but has yet to be demonstrated in human subjects. Currently, the use of molecular markers is restricted owing to the invasive nature of obtaining the tissue samples required, and to the lack of suitable analytical or imaging tools. Future studies of molecular markers may target DNA mutagenesis and cytokines, adhesion molecules, heat-shock proteins and other gene products regulated by the redox state of the cell (Jackson et al. 1998; Reilly et al. 1998). These tests may prove to be very sensitive indicators of oxidative change; however, their specificity in relation to vitamin C status is questionable.

**Biochemical markers**

Currently these markers offer a more practical option than molecular markers. However, biochemical changes are removed from the direct action of ascorbic acid, and indeed the direct effect may be unknown. This factor lowers their specificity and sensitivity in relation to vitamin C function. Ascorbic acid is a cofactor in the biosynthesis of carnitine, which is required in the cytosolic–mitochondrial transfer of fatty acids (Feller & Ridman, 1988). Lack of carnitine could account for the weakness and fatigue experienced in scurvy. However, results of human studies have not shown the anticipated decrease in plasma carnitine with low vitamin C intake, and one study of marginally-deficient subjects reported an inverse relationship between plasma ascorbic acid and free carnitine concentrations, perhaps due to impaired carnitine transport (Johnston et al. 1996). Excretion of carnitine has been reported to be increased in vitamin C deficiency, and a significant inverse correlation (r = 0.727; P < 0.05) between leucocyte ascorbic acid concentrations and 24 h urinary carnitine was reported in eight healthy men given different amounts of vitamin C over 13 weeks (Jacob & Pianalto, 1997). Increased excretion of carnitine did not appear to compromise carnitine status, at least within the time frame of the study, and it was concluded that plasma carnitine measurements did not offer a useful functional measure of human vitamin C status. Nevertheless, the fairly strong inverse correlation seen between mononuclear leucocyte vitamin C concentrations and carnitine excretion is interesting, and measurement of free carnitine may offer a more sensitive index of vitamin C status (Johnston et al. 1996).

Vitamin C deficiency has a well-established consequence in altered collagen metabolism (Bates et al. 1972). Collagen...
is rich in hydroxyproline, and changes in hydroxyproline have been investigated as an index of vitamin C-related collagen turnover (Bates, 1977; Johnston et al. 1985; Hevia et al. 1990). One study showed an inverse relationship between leucocyte ascorbic acid levels and hydroxyproline excretion, but only in women (Bates, 1977). However, a study of eleven men receiving low, normal and high vitamin C intakes over 14 weeks reported significant inverse correlations between ascorbic acid concentrations in plasma, erythrocytes and leucocytes, and 24-h hydroxyproline excretion (Hevia et al. 1990). Hydroxyproline excretion decreased significantly within a few days of change from low (5 mg/d) to high (65 mg/d) vitamin C intake. However, hydroxyproline excretion was the same at moderate (65 mg/d) and high (605 mg/d) intakes of vitamin C and inter-individual responses were variable. Hydroxyproline excretion is not, therefore, a sensitive marker of mild vitamin C deficiency (Hevia et al. 1990).

A potentially powerful biochemical marker is deoxypyridinoline: pyridinoline collagen cross-links (Tsuchiya & Bates, 1997). Deoxypyridinoline synthesis requires lysine, while synthesis of pyridinoline requires hydroxylysine. Ascorbic acid is a cofactor for the hydroxylation of lysyl- to hydroxylysyl- residues in collagen. Vitamin C deficiency is known to affect the hydroxylation of lysine, inducing a lack of hydroxylysine for pyridinoline cross-linking (Bates et al. 1972). Thus, in suboptimal vitamin C status an increase in lysine-derived collagen cross-links might be expected. A study in guinea-pigs showed that deoxypyridinoline (lysine-derived) : total collagen cross-links in the femur shafts of animals fed on an 'adequate' ascorbic acid diet was virtually twofold that of animals on a high-ascorbic acid diet (Tsuchiya & Bates, 1997; Fig. 3). The same pattern, though less pronounced, was seen in urine. This potential functional marker meets the requirements of sample (urine) accessibility, suitable analytical tools are available, and there is a clear health-related link. However, questions of sensitivity and specificity remain, and human dose-response studies have yet to be reported.

Ascorbic acid is a physiological antioxidant of major importance (Frei et al. 1989), and contributes up to 24 % of the 'total antioxidant power' of plasma (Wayne et al. 1987; Benze & Strain, 1996b). Measuring the absolute and relative contributions of ascorbic acid to the total antioxidant power of biological fluids could be a useful marker of reserve for this defensive role (Benz & Strain, 1997b). A more functional approach is the measurement of the ascorbate free radical (Hubel et al. 1997). Increased levels of ascorbate free radical may indicate increased oxidative turnover of ascorbic acid and therefore, ascorbate free radical: ascorbic acid may be a useful marker of vitamin C antioxidant utilization or function.

Other potential markers of the antioxidant function of vitamin C relate to lack of effect. Ascorbic acid is the only scavenging antioxidant which can prevent initiation of lipid peroxidation (Frei et al. 1996). Thus, lipid peroxides in biological fluids could act as a sign of inadequate vitamin C status. While commonly-used methods of measuring lipid peroxides are insensitive and non-specific (Benze, 1996b), recently-developed methods such as the measurement of F₂ isoprostanes by HPLC and the flow cytometric analysis of lipid peroxidation in cell membranes (Roberts & Morrow, 1994; Makrigiorgos et al. 1997; Reilly et al. 1998) may be useful. However, the levels of F₂ isoprostanes or lipoperoxides and ascorbic acid are unlikely to correlate significantly, as once initiated the rate and extent of propagation of peroxidation is related to various factors such as vitamin C concentration and type and amount of fatty acids within lipid structures (Benze, 1996b).

**Physiological markers**

This type of marker relates to changes in homeostatic processes or organ and tissue function. However, cellular and subcellular changes are generally well advanced before detectable symptoms and measurable signs develop, and it is difficult to isolate or identify one specific cause. Nonetheless, identification of physiological markers is necessary as molecular and biochemical effects are only as important as their ultimate biological consequences.

There is strong and consistent epidemiological evidence of a protective role for vitamin C against cancer of various sites (Block, 1991; Machlin, 1995). Cancer is caused by mutational changes in DNA, and vitamin C may help prevent these changes by scavenging reactive oxygen species. Measuring DNA oxidation products (Dizdaroglu, 1994; Makrigiorgos et al. 1997; Reilly et al. 1998) may be useful. However, the levels of F₂ isoprostanes or lipoperoxides and ascorbic acid are unlikely to correlate significantly, as once initiated the rate and extent of propagation of peroxidation is related to various factors such as vitamin C concentration and type and amount of fatty acids within lipid structures (Benze, 1996b).
& Langseth, 1995; Levine et al., 1996; Weber et al., 1996). However, these physiological markers are affected by many factors. Monitoring changes in response to changing vitamin C intake could reveal useful information regarding vitamin C-specific functions in relation to these systems and the way in which vitamin C may modulate disease risk. However, a single measurement of any of these physiological markers cannot be regarded as a reliable functional marker of vitamin C status.

Conclusion

In a complex system, small initial changes can lead to large differences in outcome. This ‘butterfly effect’ which is fundamental to mathematical chaos theory relates to vitamin C status, as molecular changes and their ultimate consequences are likely to have a critical and sensitive dependence on initial conditions. Molecular changes are difficult to measure, and their eventual outcome may be unknown or highly speculative. However, molecular changes cause larger and diverse effects in the prevailing biochemical milieu. Effects become more easy to measure, but their specific origin becomes less clear, and their relevance to distant events may remain obscure. The ultimate health-related consequences may be obvious, but their cause is difficult to trace. Reliable functional markers will enable molecular, biochemical and physiological changes associated with differences in vitamin C status to be mapped. There are currently no reliable functional markers of vitamin C status; however, measurement of free carnitine and deoxypyridinoline: total collagen cross-links appear promising. Assessing the contribution of ascorbic acid to antioxidant defence capacity, measuring ascorbate free radical: ascorbic acid, and investigating oxidative damage to DNA and lipid may also provide insight into the functional effects of vitamin C. Available evidence suggests that intake of 200 mg vitamin C/d saturates tissues and maintains fasting plasma levels above the proposed threshold (50 μmol/l) for minimum risk of CHD (Gey, 1995; Graumlich et al., 1997). The issue of whether or not these produce ‘optimal vitamin C status’ awaits the clear and accepted definition of the term. This definition in turn awaits the development of reliable functional markers capable of assessing the effects of varying levels of vitamin C nutrition.

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