Vitamin C

New Biochemical and Functional Insights

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Chapter 1

A "C Odyssey"

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CHAPTER ONE

A "C Odyssey"

RECOMMENDED DIETARY ALLOWANCES AND OPTIMAL HEALTH: PARADIGM AND PROMISE OF VITAMIN C

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INTRODUCTION: RECOMMENDED DIETARY ALLOWANCES AND THEIR LIMITATIONS

The nutritional biochemist Alfred E. Harper was a member of the Food and Nutrition Board (FNB) of the National Research Council/ National Academy of Sciences for many years, and chair of the Food and Nutrition Board from 1978 to 1982. In a number of articles, he described the original intent of Recommended Dietary Allowances (RDAs) in the United States, briefly summarized as follows [1–3]. In 1940, to guide the U.S. government concerning national defense, a Committee on Food and Nutrition was established under the National Research Council, U.S. National Academy of Sciences, to advise the government on problems concerned with national defense. In 1941, the committee name was changed to the FNB. The allowances for specific nutrients from the FNB were intended to serve as a guide for planning adequate nutrition for U.S. civilians. Specifically, there was no intent to have RDAs as guides to perfect health, nor were they designed to attain ideal intakes. The RDA was stated to be not just "minimal sufficient to protect against actual deficiency disease" but sufficient "to ensure good nutrition and protection of all body tissues," and in the 1953 edition they are stated to be "nutrient allowances suitable for the maintenance of good nutrition in essentially the total population." The scientific bases for many RDAs were prevention of deficiency with a margin of safety, often determined from depletion-repletion studies or balance experiments. As

Harper wrote, "The RDA has been adopted and adapted by various organizations for many purposes, but they were devised for the planning and procurement of food supplies that would be nutritionally adequate for population groups. Therefore, any assessment of the adequacy, accuracy, and reliability of the RDA will be meaningful only if it is done in relation to their use for this primary purpose. To base such an assessment on their adequacy for other purposes would be like judging the adequacy of the design of the family car for use as a snowplow" [3].

As nutritional science has grown, and policy needs have changed, limitations of RDAs were recognized. Beginning in the 1990s, the FNB expanded nutritional intake concepts in the form of dietary reference intakes, commonly known as DRIs [4–6]. Unfortunately, because of data limitations, scientific bases for many dietary reference intakes remain as prevention of deficiency with a margin of safety, because these are the only data available.

To paraphrase Alfred E. Harper, when there is heavy snow, you need a snowplow. To achieve a nutrition goal, we have to be thoughtful in defining the nutrition problem that we are addressing, and if necessary, to think outside the box to solve the problem. Prevention of deficiency with a safety margin is not the only means to determine nutrient intake, nor, from a clinical vantage point, is it the preferred one. If there truly were a means to realize goals of ideal nutrient intake, there would be unprecedented possibilities to optimize health, prevent disease, and even treat disease. But specific methods and measures are essential to realize such lofty possibilities. Such specifics have taken decades to formulate, evolve, and solidify. What is so simple in concept has been so difficult to bring to fruition.

CONCENTRATION-FUNCTION HYPOTHESIS: PHYSIOLOGY/ PHARMACOKINETIC APPROACHES

Fundamental biochemical kinetics concepts can be the foundation for nutrient recommendations. Such concepts derive from work of Tatum and Beedle [7], David Perla and Jesse Marmorston [8], Roger Williams [9,10], and, perhaps surprisingly and independent of his later involvement with ascorbic acid in colds and cancer, Linus Pauling [11–13]. With these biochemical and experimental supports, a new hypothesis was proposed: bases of vitamin recommendations could be concentration-function relationships, or kinetics relationships, in cells, tissues, animals, and healthy humans [14–17]. Approaches were to conduct physiology and pharmacokinetic studies in healthy humans. Stated in another way, the overarching concept was that kinetics *in situ* would underlie vitamin recommendations in healthy people. Physiology and pharmacokinetic studies would provide essential data for the *x*-axis, those concentrations found *in vivo*, preferably in humans. With concentration data describing an *x*-axis, function, or the *y*-axis, could follow in relation to concentrations *in vivo* [18].

VITAMIN C AS A MODEL VITAMIN

For such work to proceed, a vitamin was selected arbitrarily: ascorbic acid. Initial efforts were focused on assay development and proof of concept. Accurate assay of ascorbic acid was an essential prerequisite, a foundation on which everything else was based. Since its discovery and isolation, ascorbic acid measurements had many uncertainties, due to limitations of sensitivity, specificity, stability, and confounding substance interferences [19]. The emergence of high-performance liquid chromatography (HPLC) techniques coupled to electrochemical detection provided a path forward [20]. A new assay was developed that addressed and solved these issues, using HPLC specifically coupled to coulometric (flow-through) electrochemical detection [21,22]. With this assay, the concept was tested and verified that kinetics relationships could be determined *in situ* for ascorbic acid. The experimental system was ascorbic acid–dependent norepinephrine biosynthesis in chromaffin granules, the secretory vesicles of adrenal medulla, isolated from bovine (cow) adrenal glands [23–25]. Norepinephrine synthesis is mediated by the enzyme dopamine β-monooxygenase [26,27]. Using this system, *in situ* kinetics were described for norepinephrine biosynthesis from dopamine, mediated by ascorbic acid as a cosubstrate [28]. To our knowledge, this was the first demonstration of kinetics *in situ* for any vitamin with the physiologic enzymatic substrate. Notably, the findings showed that the mechanisms of ascorbic acid action on norepinephrine biosynthesis *in situ* were more complex than its direct *in vitro* action as a cofactor/cosubstrate for

isolated dopamine β-monooxygenase [22,27–29]. One strong implication of these findings was that an *in vivo* system, rather than an *in vitro* system, was the preferred basis for determining concentration-function relationships, on which vitamin recommendations could ultimately be based. An equally strong implication of these findings was that data from humans, rather than animals (i.e., cows), constituted the holy grail for determining concentration-function relationships.

VITAMIN C PHYSIOLOGY AND PHARMACOKINETICS IN HEALTHY HUMANS: TIGHT CONTROL AND UNDERLYING MECHANISMS

But, before proceeding with such clinical experiments, a key precondition was to know the *x*-axis range for ascorbic acid concentrations in humans. From a clinical perspective, this is not much different from having normal limits on a basic metabolic panel, a common test in clinical care. However, data were limited or unavailable that described whether and how a wide range of different doses of ascorbic acid modulated plasma and tissue concentrations: pharmacokinetic data [30–33]. Comprehensive pharmacokinetic data of this kind were unavailable not just for ascorbic acid but for all vitamins. Without pharmacokinetic information as a foundation, it would be impossible to consider biosynthetic consequences and clinical outcomes in relation to any ascorbic acid concentration. For clinicians, an analogy would be to try to manage diabetes without prior knowledge of normal and abnormal blood glucose concentrations.

For ascorbic acid, specific pharmacokinetic goals were to learn how seven different ascorbic acid doses over an 80-fold range impacted steady-state blood and tissue concentrations in men and women, while simultaneously characterizing concentration relationships and normal physiology as extensively as possible [34,35]. Approaches were to conduct pharmacokinetic experiments in healthy humans, using oral and intravenous ascorbic acid. Data were obtained using a depletion-repletion design, with correction for all possible unintended vitamin and mineral deficiencies [36]. Subjects were hospitalized as inpatients at the National Institutes of Health (NIH) Clinical Research Center for approximately 6 months, to facilitate dietary control, compliance, and pharmacokinetic samplings over each of the seven different ascorbic acid doses. Subjects were first safely depleted of ascorbic acid using a vitamin C–restricted diet, with correction of any possible deficiencies in other vitamins [34–36]. When plasma concentrations were less than 10 μM, subjects were dose repleted in a stepwise manner. Subjects had to achieve steady state at each dose before advancing to the next highest dose. Daily doses were from 30 to 2500 mg. Half of the total daily dose was administered twice daily: before dinner, at least 4 hours after the past meal, or before breakfast in the morning, after overnight fasting. Ascorbic acid for oral administration was in a water solution, pH adjusted, with individualized doses that were routinely monitored for stability. Administration in this manner eliminated confounding effects of interferences from capsules or food components [37–39]. Ascorbic acid for intravenous injection was in individualized sterilized vials, pH adjusted, and routinely monitored for sterility and stability. The coulometric HPLC electrochemical measurement technique described earlier was utilized so that ascorbic acid could be measured in clinical samples. Measurements provided necessary sensitivity and specificity, without interferences from compounds in biological samples, and sample stability was accounted for during sample processing, assay, and long-term storage [21,40].

Inpatient studies were completed by 7 healthy men and 11 healthy women between ages 18 and 30 at the Clinical Research Center, NIH. Subjects were hospitalized for approximately 6 months each (Figure 1.1a). Both sex-specific and combined pharmacokinetic data showed that ascorbic acid concentrations were tightly controlled as a function of dose in all subjects [34,35] (Figure 1.1b). At a dose of 200 mg, there was nearly complete saturation of steady-state plasma and tissue concentrations, with higher oral doses having minimal additional effects. As doses increased above 100 mg, the fraction of the absorbed dose decreased, and the excess was excreted unchanged in urine. These pharmacokinetic data have subsequently been used by many countries to calculate intakes for ascorbic acid, including dietary reference intakes and recommended dietary allowances [6,41,42].

Some might conclude the following: mission accomplished, end of the story, time to investigate another vitamin. In contrast, our view is that these data unmasked possibilities that are the true

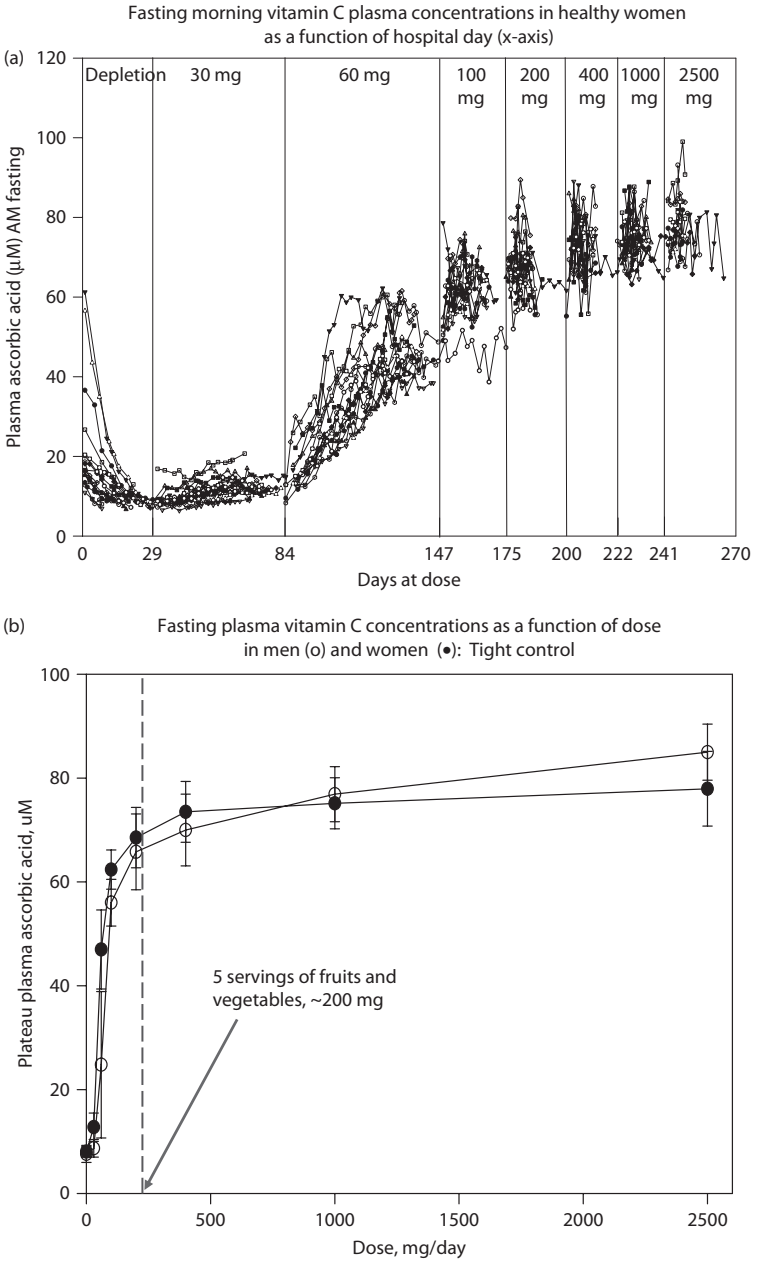

Figure 1.1. Fasting steady-state ascorbic acid plasma concentrations as a function of dose. (a) Concentrations in plasma in healthy women as a function of hospital day (*x*-axis) [35]. Number of days at each dose can be seen by comparing hospital day (*x*-axis) to each dose, listed at the top of the figure. Similar findings were observed for men [34,131]. (b) Fasting steady-state plasma concentrations as a function of dose for men and women: summary data. (From Levine, M. et al. 1996. *Proc. Natl. Acad. Sci. USA* 93, 3704–3709; Levine, M. et al. 2001. *Biofactors* 15, 71–74.)

beginning of understanding ascorbic acid, based on the clinical physiology and pharmacokinetic findings. The aggregate data showed that ascorbic acid concentrations were tightly controlled in humans as a function of oral doses. An analogy is again apparent based on glucose concentrations in humans. Glucose concentrations are tightly controlled as a function of glucose ingested, pancreatic hormones insulin and glucagon, gut hormones, gluconeogenesis in the liver, and insulin

responsiveness of muscle and fat [43]. Plasma and tissue ascorbic acid concentrations appeared to have an analogous tight control. What were the mechanisms? In addition to dose ingested, the clinical data implicated that at least four potential physiologic processes were involved in tight control: intestinal absorption, tissue transport, renal reabsorption/excretion, and utilization. Each of these mechanisms can and has been probed, in varying degrees, for clinical relevance.

Bioavailability and Unexpected Consequences

In animals, intestinal absorption of ascorbic acid is mediated by sodium-dependent transporter SLC23a1, which is localized to the small intestine [44–46]. There may be additional mechanisms because knockout mice for SLC23a1 still absorb ascorbic acid, dehydroascorbic acid (oxidized ascorbic acid) is nearly as effective as ascorbic acid in preventing deficiency in guinea pigs, and dehydroascorbic acid appears to be absorbed in humans [47–49].

In humans, intestinal absorption of ascorbic acid was measured as part of the inpatient pharmacokinetic studies at the NIH. True bioavailability, or fractional absorption, was determined for each of seven different doses. Subjects were at steady state for each dose, which was administered one day by mouth and the following day by vein. Plasma samples were collected continuously over 36 hours. These data showed that fractional absorption declined as doses rose, especially above 100 mg daily [34,35,50] (Table 1.1).

TABLE 1.1
Bioavailability of oral ascorbate: Nonlinear tissue distribution model

Dose (mg)	Bioavailability (median%)
15[a]	89
30[a]	87
50[a]	85
100[a]	80
200	72
500	63
1250	46

[a] Amounts in foods.

Bioavailability experiments showed plasma and tissue concentrations were tightly controlled with oral administration of ascorbic acid. At doses at and above 100 mg, intravenous administration bypassed tight control of ascorbic acid concentrations in plasma that were seen with oral dosing, until the dose was excreted in urine. These data indicated that intravenous administration of ascorbic acid could produce pharmacologic concentrations that were not otherwise possible with oral dosing (Figure 1.2a–c). Using another clinical analogy, these findings are similar to plasma concentrations that are achieved with oral versus intravenous administration of many antibiotics.

Why might it matter that only intravenous administration produces pharmacologic ascorbic acid concentrations? Concentrations produced only with intravenous administration could have pharmacologic effects not found with oral dosing [51,52]. Many clinical trials open now have been designed to investigate precisely such effects in cancer treatment and in sepsis (see ClinicalTrials.gov for full listings).

Ascorbic acid in cancer treatment has a long and convoluted history, described elsewhere [17,51,53,54]. Briefly, ascorbic acid was proposed as a cancer treatment agent because of its effects on maintaining tissue collagen. The hypothesis was simple: cancers metastasized via collagen breakdown, and collagen could be strengthened, or its breakdown prevented, with ascorbic acid. In comparison to 200 mg of ascorbic acid, which produces near saturation of plasma and tissues, 10,000 mg of ascorbic acid was administered to patients with a variety of cancers by Ewan Cameron and his colleagues [13,55–57]. They reported treatment effects, improved well-being, and prolonged survival in some cases. Cameron's studies were criticized because of their retrospective design, lack of pathology confirmation, lack of controls, and potential confounding effects of endemic deficiency in the treatment population [51]. Ascorbic acid at the same dose had no effect on cancer treatment in two double-blind placebo-controlled trials, both performed at the Mayo Clinic, and ascorbic acid was dismissed as a cancer treatment agent [58–60]. The physiology and pharmacokinetic studies in healthy people provided a straightforward potential explanation. Ascorbic acid was administered only by mouth in the studies at Mayo Clinic, but both by mouth and

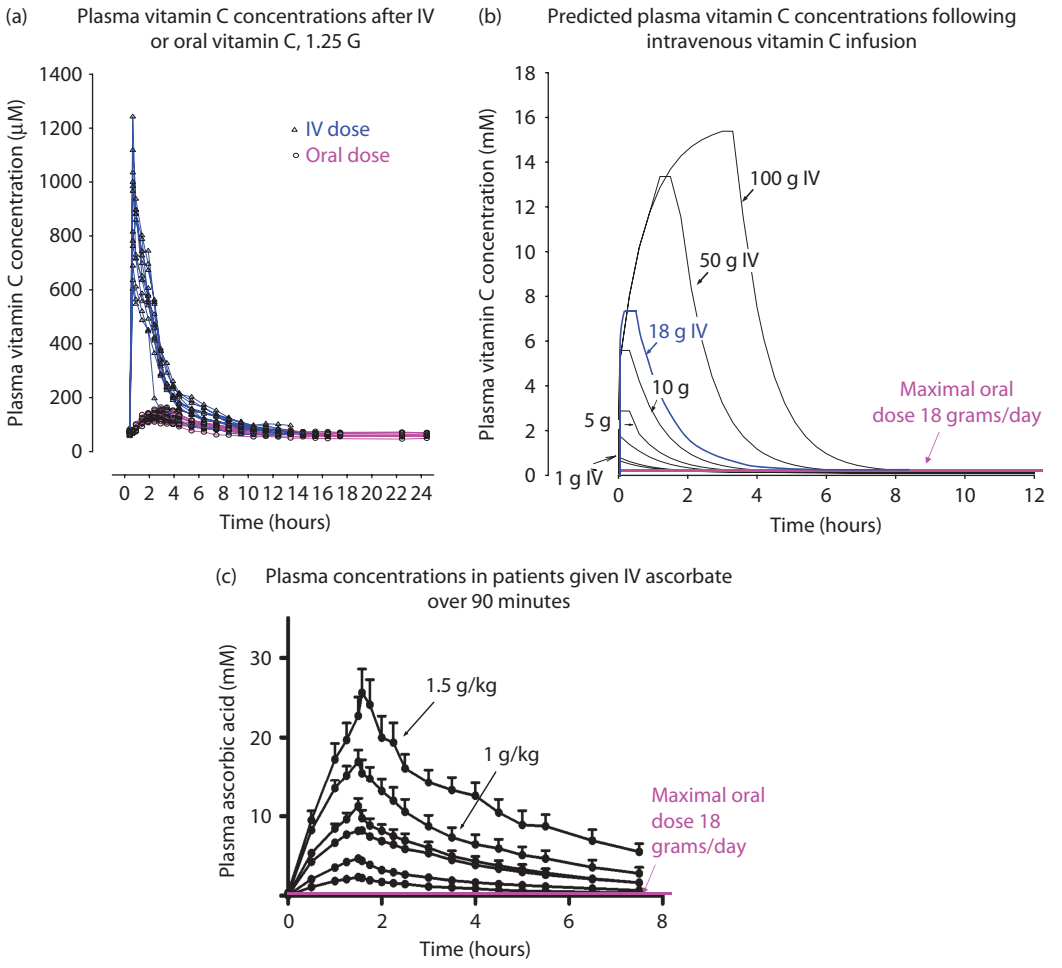

Figure 1.2. Plasma ascorbic acid concentrations as a function of oral or intravenous dosing. (a) Plasma concentration as a function of time in women who received 1,250 mg (1.25 G) of ascorbic acid either by mouth or by vein (intravenously). (b) Observed or modeled plasma ascorbic acid concentrations as a function of time at doses from 1 to 100 grams by mouth or by vein (intravenously) in healthy people. (c) Observed plasma ascorbic acid concentrations as a function of time in patients with cancer at doses approximately 0.7–100 g administered intravenously. ([b] Padayatty, S. J. et al. 2004. *Ann. Intern. Med.* 140, 533–537; [c] Hoffer, L. J. et al. 2008. *Ann. Oncol.* 19, 1969–1974.)

intravenously by Ewan Cameron and colleagues. Of note, no ascorbic acid concentrations in patients were measured in any of these cancer studies. The profound differences in ascorbic acid concentrations from oral versus intravenous dosing provided one rationale to reopen the investigation of ascorbic acid in cancer treatment [51,52]. A second rationale was provided from clinical case reports of three patients with aggressive, pathologically confirmed cancers who were cured with intravenous ascorbic acid [61]. Despite limitations of these cases, they provided firm incentive to investigate pharmacologic ascorbate. These studies revealed that pharmacologic ascorbate concentrations killed cancer cells *in vitro* and *in vivo* without affecting normal cells and normal tissues [62]. Pharmacologic ascorbate acted by generating hydrogen peroxide in extracellular fluid [63] (Figure 1.3). Hydrogen peroxide diffuses into cells and is a prodrug for reactive oxygen species that are toxic to cancers but not normal tissues. The central role of hydrogen peroxide was shown because cancer killing effects are negated with catalase, which dismutates hydrogen peroxide to water and oxygen [62,64]. Reactive oxygen species are likely to form in the setting of hydrogen peroxide, pharmacologic ascorbate, and trace iron found intracellularly and/or on domains of proteins facing the extracellular fluid or in extracellular

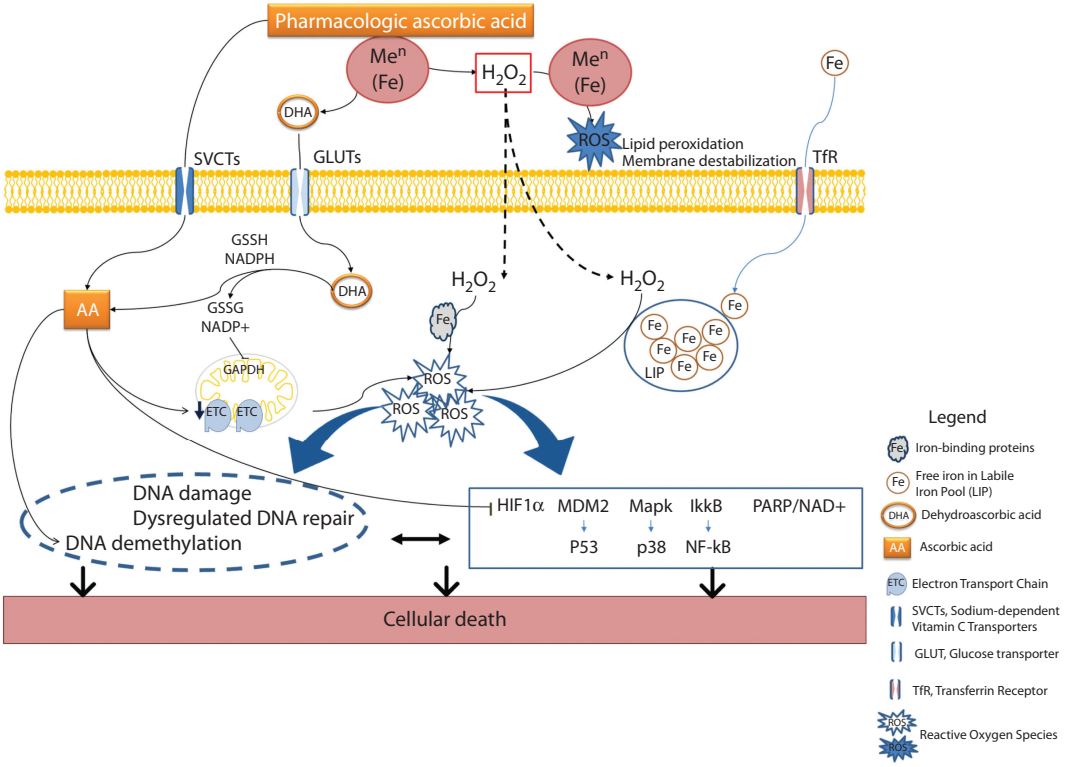

Figure 1.3. Multiple actions of pharmacologic ascorbic acid in cancer treatment. (Modified from Levine, M. and Violet, P. C. 2017. *Cancer Cell* 31, 467–469.)

fluid [63,64]. Subsequently, additional mechanisms have emerged that could explain ascorbic acid action in cancer treatment, including effects on hypoxia-inducible factor (HIF), regulation of DNA methylation, and effects of dehydroascorbic acid on ATP production in cancer cells [65–74,133] (Figure 1.3). Pharmacologic ascorbic acid has an exceptional safety profile and produces few adverse events in patients who are appropriately screened [54,75–77,132]. Small clinical trials indicate promise for pharmacologic ascorbate in metastatic pancreatic cancer, ovarian cancer, non-small cell lung cancer, glioblastoma, metastatic colon cancer, and metastatic gastric cancer [76–82]. Multiple clinical trials are open worldwide to test pharmacologic ascorbic acid in cancer treatment (see ClinicalTrials.gov for full list).

For more than 30 years, it has been described that ascorbic acid concentrations are low in critically ill patients [83–88]. Organ injury in sepsis is attenuated by ascorbic acid in animal models [88–90]. In one small single-center trial, requirement for vasopressors was decreased by pharmacologic ascorbic acid, and 28-day survival was improved [91]. In another small single-center trial, which utilized corticosteroids and thiamine as well as pharmacologic ascorbate, in-hospital survival was increased [92], with a trend to increased survival in a third study [93]. Unfortunately, in sepsis studies, there can be inadvertent bias in single-center unblinded observational studies with relatively few participants [94–97]. These trials were single-center small-scale trials. Larger and prospective multiple-center clinical trials are warranted and are in progress to test whether ascorbic acid alone or ascorbic acid with corticosteroids and thiamine improve morbidity/mortality in septic patients (examples include NCT03389555, NCT03509350, NCT03258684, NCT03422159, and NCT03338569) and separately, whether ascorbic acid alone improves outcomes in patients with adult respiratory distress syndrome (NCT02106975). Although many possibilities exist, mechanism(s) of ascorbic acid efficacy are uncertain. Nevertheless, these trials were launched and are proceeding because of existent poor treatment options. Use of ascorbic acid pharmacologically is the foundation of these trials.

Tissue Transport and Unexpected Consequences

Included in the NIH pharmacokinetic studies were isolation from subjects of circulating neutrophils, monocytes, lymphocytes, and platelets, utilizing apheresis or specific cell purification techniques. Evaluation of ascorbate concentrations served as a proxy for tissue transport and accumulation. These data showed that tissue transport was a function of ingested dose and that all isolates achieved near-maximal ascorbic acid concentrations at the dose of 100 mg daily (Figure 1.4). The plasma concentration at which tissues saturated was similar to or lower than that concentration at which plasma saturated. Isolated cells and platelets had ascorbic acid concentrations approximately 20- to 50-fold greater than plasma across the range of plasma concentrations. The data show that tissue transport was a second fundamental mechanism contributing to tight control of ascorbate in healthy people.

Ascorbic acid is a charged molecule at physiologic pH. Ascorbic acid pK_a is approximately 4.2, and physiologic pH is 7.4. Because of its charge, transport would be predicted as an essential requirement for ascorbic acid movement across the intestine, into cells, and, as discussed later, for renal reabsorption. Candidate transported substances are ascorbic acid as such, or its oxidized product dehydroascorbic acid [98,99]. However, only ascorbic acid is detectable intracellularly. For dehydroascorbic acid to be a candidate substrate, it would be predicted to undergo near-instantaneous intracellular reduction to ascorbate, the observed findings [99,100]. With these points as background foundation, we can address a fundamental issue in ascorbate physiology. Which substrate is essential for transport and activity: dehydroascorbic acid, ascorbic acid, or both? Endocrinology has several analogies. Both T4 (tetraiodothyronine, or levothyroxine) and T3 (triiodothyronine) are found in blood, the former approximately 100-fold higher than the latter, but only the latter is biologically active to regulate thyroid response elements in DNA. Similarly, in blood, 25-hydroxy vitamin D (calcidiol) is found at approximately 1000-fold higher concentrations than 1,25-dihydroxy vitamin D (calcitriol), but the latter is the active hormone. In comparison to ascorbic acid, dehydroascorbic acid concentrations in plasma are estimated as nearly two orders of magnitude less, and it is unclear whether they are reliably distinguished from zero [40,101]. To determine the biological importance of ascorbic acid and dehydroascorbic acid, transport characterization is an essential prerequisite (Figure 1.5).

Ascorbic acid is transported by two sodium-dependent vitamin C transporters, SVCT1 and SVCT2 [44,102,103]. SVCT1 was originally labeled as SLC23A2, and SVCT2 as SLC23A1, but these labels were later reversed, and the current nomenclature is SVCT1 as SLC23A1, and SVCT2 as SLC23A2. Neither of these transporters function as efflux transporters that allow ascorbate to exit cells on a basolateral surface, including in the intestine, the liver, and the kidney [104]. It is likely that there is either at least one additional SLC23 that functions as an ascorbic acid efflux

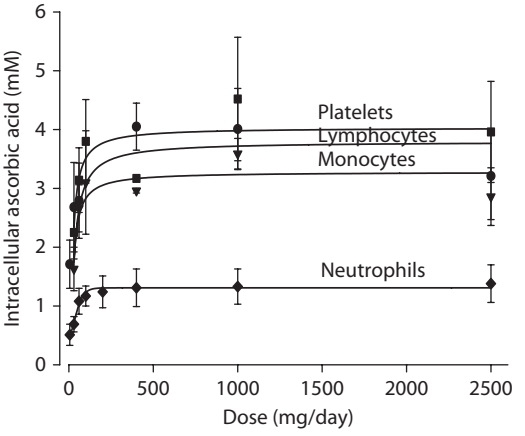

Figure 1.4. Ascorbic acid concentrations in circulating cells and platelets from healthy women as a function of dose. (From Levine, M. et al. 2001. *Proc. Natl. Acad. Sci. USA* 98, 9842–9846.)

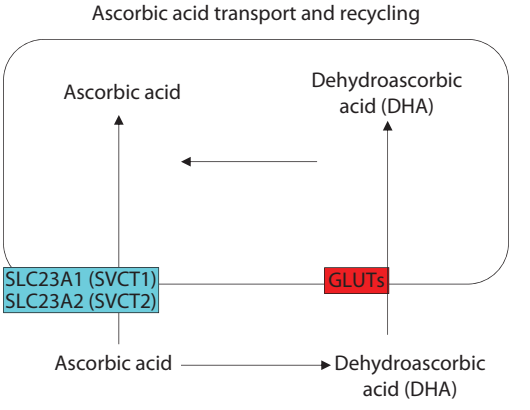

Figure 1.5. Mechanisms of ascorbic acid and dehydroascorbic acid transport and accumulation in cells.

transporter or a distinct solute carrier that is an efflux transporter, but efflux transporter activity coupled to a gene/protein has not yet been clearly demonstrated. SVCT2 knockout mice show that this transporter is essential for ascorbate uptake in many mouse tissues [105]. These mice have plasma ascorbate concentrations similar to those expected in wild-type mice, consistent with the existence of a distinct efflux transporter from the liver. SVCT1 knockout mice lose their renal threshold and ability to reabsorb ascorbic acid in the kidney [47]. However. SVCT1 knockout mice easily absorb ascorbic acid or an ascorbic acid halogen analog administered by gavage or in water. These data imply the existence of another distinct intestinal ascorbic acid transporter. It is likely that this intestinal absorption activity is distinct from dehydroascorbic acid and glucose transporters (GLUTs), because ascorbate halogen analogs and their halogenated dehydroascorbic acid products are not transported by glucose transporters [106], as discussed later. Consistent with findings for thousands of other genes and their protein products in humans, it is likely that there are mutations in SLC23A1, SLC23A2, putative efflux transporters, or an as yet unidentified intestinal transporter, any of which could produce aberrant protein function. Such cases have not yet been described but are likely to exist as yet undiagnosed. Humans with such mutations would be predicted to require massive (gram) doses of oral ascorbate or parenteral ascorbate to maintain either plasma or tissue concentrations. Predicted findings in people with such putative mutations include severely compromised bioavailability, an aberrant renal threshold, or specific cell populations with much lower ascorbate concentrations than predicted from pharmacokinetic data.

Dehydroascorbic acid in its hydrated form has a structure similar to that of glucose (Figure 1.6). Dehydroascorbic acid was predicted and found to be transported by GLUTs [98,107]. Of the 14 identified GLUTs [108], many transport dehydroascorbic acid [109]. GLUT1, expressed in many cell types, has an affinity for dehydroascorbic acid that can be estimated to be approximately three orders of magnitude more than glucose [107]. Multiple mechanisms have been proposed to explain GLUT transport activity [110]. These mechanisms are based on using glucose or glucose analogs as substrates. A confirmation model is believed to be the best explanation of the mechanism of GLUT1 transport [110], but it is unclear whether dehydroascorbic acid transport fits any of the molecular mechanistic transport models for GLUTs.

Because ascorbic acid and dehydroascorbic acid have distinct transport mechanisms, the contribution of each mechanism to ascorbic acid physiology *in vivo* can and has been explored. Before knockout mice were created, there was evidence supporting either a sole role for dehydroascorbic acid or a contribution by both mechanisms for cell accumulation [99,100,111]. Evidence supporting dehydroascorbic acid as the sole mechanism was based on much higher rates of transport compared to ascorbic acid, in cell systems and an animal model [112–114]. There were several flaws in this approach including use of nonphysiologic concentrations of dehydroascorbic acid, short time courses, inability to account for ascorbic acid transport under conditions where dehydroascorbic acid would not be present, and absence of explanation for sodium dependence of cell accumulation of ascorbate. A central limitation was the absence of techniques to measure dehydroascorbic acid with sensitivity, with specificity, and without interference from ascorbic acid [19,115].

Identification of distinct genes and transporters for each substrate was the necessary advance that allowed for the creation of knockout mice for ascorbate transporters. If dehydroascorbic acid transport was the primary mechanism of ascorbate accumulation, then absence of an ascorbate transporter should not affect ascorbate accumulation in knockout mice. Alternatively, if an ascorbate transporter was required for accumulation of the vitamin, knockout mice would have severe tissue deficiency, perhaps coupled to lethality. Mice lacking SVCT2 had severe generalized ascorbate deficiency and died within hours postpartum [105]. In theory, it is possible to test the converse hypothesis, for consequences of GLUT knockouts on dehydroascorbic acid transport and ascorbic acid accumulation. However, data from such knockouts would be confounded by effects of absence of glucose transport due to unintended multiple downstream consequences.

At first interpretation, data from SVCT2 knockout mice indicate the absence of a biological role for dehydroascorbic acid in cellular accumulation of ascorbate. A more balanced view is necessary. Not every mouse tissue was isolated and measured for

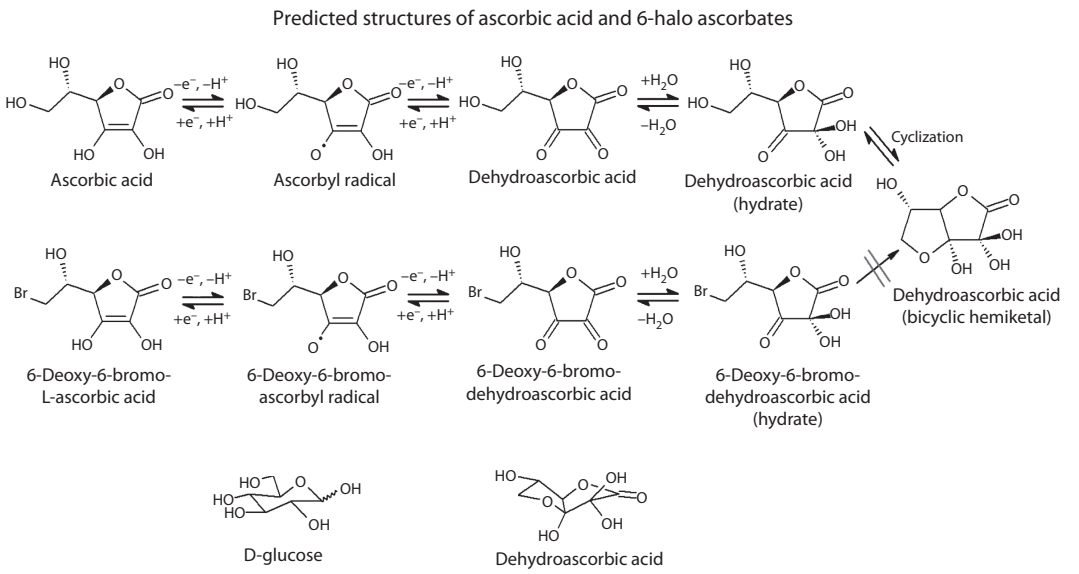

Figure 1.6. Structures of ascorbic acid, dehydroascorbic acid, and 6-halogen ascorbic acid. (From Corpe, C. P. et al. 2005. *J. Biol. Chem.* 280, 5211–5220.)

ascorbate accumulation, and identification could have been missed of a tissue that specifically required only dehydroascorbic acid transport. Obviously, mice and humans are different, and what is relevant for the mouse may not be relevant for humans, and vice versa. We do not have to look any further than endogenous ascorbate synthesis, found in most rodents but not in humans and nonhuman primates [116]. Because of the structural similarity of glucose and dehydroascorbic acid, and the difficulties in prevention and treatment of diabetes complications, there is an obligation to be thorough about the role of dehydroascorbic acid in ascorbate biology.

For these reasons, we pursued characterization of a possible role of dehydroascorbic acid in ascorbate accumulation. As earlier, knockout mice for GLUTs had unacceptable confounders. Therefore, we pursued a chemical knockout strategy. This path is based on the structure of dehydroascorbic acid and its oxidation and formation from ascorbic acid (Figure 1.6). Dehydroascorbic acid has multiple forms, based on whether it is hydrated. The hydrated form is the one that is structurally similar to glucose. The hydrated form requires that the sixth carbon on ascorbic acid has a hydroxyl group. The hydroxyl group is essential for formation of the cyclized hydrated structure of dehydroascorbic acid, that is, in its bicyclic hemiketal form [106]. Based on transport studies of ascorbate analogs, we hypothesized that substitution of a halogen for the OH group on the sixth carbon of ascorbic acid (6-halo-ascorbic acid) would prevent cyclization of dehydroascorbic acid [99,106,111]. If correct, halo-ascorbic acid analogs when oxidized would be predicted to not be recognized by GLUTs and not transported. We synthesized 6-bromo-6-deoxy ascorbic acid (bromo ascorbic acid, or BromoAA) and tested transport of reduced and oxidized (BromoAA and 6-bromo-6-deoxy dehydroascorbic acid [BromoDHA]) by SVCTs and GLUTs. The findings were that BromoDHA was a complete chemical knockout for GLUTs: no BromoDHA was transported by GLUTs. Conversely, BromoAA was as good as or better than ascorbic acid as a substrate for SVCT1 and SVCT2 [106].

These findings were based on studies using expressed transporters in *Xenopus laevis* oocytes and in cells. To advance to an *in vivo* system, we utilized gulo$^{-/-}$ mice, those whose ability to synthesize ascorbate has been knocked out by disruption of gulonolactone oxidase, the last enzyme in the *in vivo* biosynthesis pathway for ascorbic acid [117]. In this regard, gulo$^{-/-}$ mice are similar to humans, where the gulonolactone oxidase gene has multiple mutations so that no enzyme is produced [116]. Both gulo$^{-/-}$ mice and humans require ascorbic acid for survival. To test whether dehydroascorbic acid had a physiologic function, gulo$^{-/-}$ mice were raised on either ascorbic acid or BromoAA for periods as long as 1 year. The findings were that red blood cells

from mice raised on BromoAA visibly hemolyzed when plasma was prepared from whole blood by low speed centrifugation [118,119]. These findings were recapitulated when ascorbate repletion to mice was low. These findings pointed to an essential requirement for dehydroascorbic acid by red cells to them to maintain their ascorbic acid. Advancement of these experiments required development of a technique to measure ascorbic acid reliably in red blood cells, accomplished by HPLC with coulometric electrochemical detection [120]. Together, these experiments confirmed that only dehydroascorbic acid, and not ascorbic acid, is required for ascorbic acid in red blood cells in mice and humans.

The dependence of red cells on dehydroascorbic acid and intracellular ascorbate was characterized in some detail [118,119]. One mechanism was characterized by red cell fragility, mediated by β-spectrin. Under a threshold of approximately 10 μM in plasma and red cells, red blood cells become more rigid and osmotically fragile, producing hemolysis with centrifugation. Red cell fragility was reversed *in vivo* when ascorbic acid concentrations were increased above 20 μM, indicating that ascorbic acid was responsible (Figure 1.7). Similar findings were obtained in red cells from healthy humans and those with diabetes. The transporters mediating dehydroascorbic acid were identified GLUT4 in mouse red cells and GLUT1 in human red cells. Dehydroascorbic acid was competed by glucose, at concentrations similar to those in diabetes.

These findings in red cells have revealed unexpected potential roles of dehydroascorbic acid and ascorbic acid in diabetes. It has been known for more than 40 years that red cells from diabetic patients are more rigid, leading to slower flow

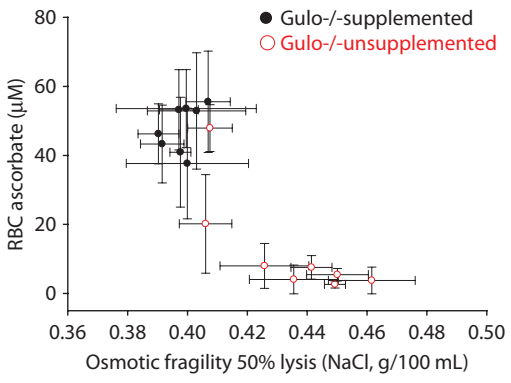

Figure 1.7. Osmotic fragility as a function of red blood cell ascorbic acid concentrations. (From Tu, H. et al. 2015. *EBioMedicine* 2, 1735–1750.)

per unit time [121,122]. Rigid red cells in diabetes would be expected to deliver less oxygen per unit time in capillaries. Clinically, this problem is all too apparent in diabetic subjects, manifested as microvascular disease. These findings with dehydroascorbic acid in red cells open new and exciting paths, with potential consequences for patients. It is possible that a contributing factor to pathogenesis of microvascular disease in diabetes is relative deficiency of ascorbic acid in red cells and perhaps plasma, deficiencies that may be reversible. Testing these possibilities will require time, great effort, and clinical experiments. Nevertheless, potential positive outcomes may be new and unanticipated means to prevent or delay microvascular disease in diabetes. What at first glance may appear to be convoluted paths are in fact open and direct. They derive linearly from pursuing findings from physiology and pharmacokinetic experiments, with concurrent studies of ascorbic acid and dehydroascorbic acid transport.

Findings with BromoAA in gulo$^{-/-}$ mice revealed that the red cell was dependent solely on dehydroascorbic acid transport for its internal ascorbate [118,119]. Mice raised on BromoAA for 1 year had minimal pathologic changes compared to controls. Nevertheless, it is possible that there are other tissues dependent on dehydroascorbic acid transport and that these were missed. The clue for red cells was visible hemolysis. Another tissue or tissues may be dependent on dehydroascorbic acid, without as clear a phenotype as found in red cells. As one example, dehydroascorbic acid has been proposed as the essential substrate to cross the blood-brain barrier [113,123]. Findings in BromoAA mice were not consistent with this possibility, as bromoAA was found in brain tissue of mice raised on BromoAA. However, dehydroascorbic acid transport remains as a possibility under some conditions, for example, with pharmacologic ascorbic acid concentrations in blood, when it is conceivable there would also be higher dehydroascorbic acid concentrations due to simple chemical equilibrium between pharmacologic ascorbic acid and its oxidation products.

Renal Reabsorption

Data from the NIH clinical physiology and pharmacokinetic studies showed that ascorbic acid was not excreted in urine at doses less than 100 mg daily in men and 60 mg in women [34,35]. When

ascorbic acid was administered intravenously, so that limitations of intestinal absorption were bypassed, all of the administered doses were excreted at the highest doses of 500 and 1250 mg. From these data, it can be inferred that there is a renal threshold for ascorbic acid, and that when plasma and renal tubule ascorbic acid concentrations exceed this threshold, then ascorbic acid will be found in urine. A renal threshold is analogous to that found for phosphate and for glucose. A precise renal threshold has not been published, but it is anticipated that such a threshold can be calculated.

Ascorbic acid, as a water-soluble vitamin, would be expected to be freely filtered at renal glomeruli. Reabsorption would be predicted in proximal renal tubules, concurrent with SVCT1 localization. SVCT1 as a necessary reabsorptive ascorbic acid transporter was shown in studies from SVCT1 knockout mice [47]. These mice lose their ability to reabsorb ascorbic acid, and clear ascorbic acid similarly to inulin, which is filtered at the glomerulus but not reabsorbed. As previously mentioned, a basolateral ascorbic acid transporter should exist but has not been identified.

Once a renal threshold for ascorbic acid is described, it becomes possible to search for aberrant reabsorption. As earlier, aberrant reabsorption is predictable in people with mutations in some regions of SVCT1, or in the as yet unidentified efflux transporter. Renal tubule disease would also be predicted to result in a shift in reabsorption. One example is from a rare disease, Fabry disease, with known damage to renal tubules in humans. Another example is from a common disease, diabetes mellitus, where again renal tubular damage occurs. Similarly, drugs that have nephrotoxicity in the renal tubule may also induce inappropriate ascorbic acid appearance in urine.

Aberrant renal reabsorption of ascorbic acid might be a sign of tubular disease, as is microalbuminuria for glomerular disease in diabetes. Separately, aberrant reabsorption could produce quantitative losses in ascorbate, leading to lowered plasma and tissue concentrations. These possibilities are worth pursuing, having as a prerequisite the accurate characterization of ascorbic acid renal threshold in humans.

Utilization

The NIH clinical physiology and pharmacokinetic data show that ascorbic acid depletion occurs in approximately 30 days in healthy people. Utilization must be responsible and appears to occur at varying rates depending on plasma concentration.

Ascorbic acid concentrations have been reported to be near or at deficiency concentrations ($<$10 μM, or 0.2 mg/dL) in patients with critical illness of many types [83–87,124–126]. Low concentrations could have multiple causes, including absent dietary ingestion, aberrant absorption or renal excretion, and increased utilization secondary to disease-associated oxidant production leading to accelerated ascorbate oxidation. Clinical studies in sepsis suggesting improved clinical outcomes are based on replacing ascorbic acid, using pharmacologic concentrations. It is unknown whether replacement is simply correcting existent deficiency [127] or whether pharmacologic ascorbic acid has another role, analogous to that of pharmacologic ascorbic acid in cancer treatment by distinct or even similar mechanisms [63,88,128,129].

Accelerated ascorbic acid utilization in disease states may be a new frontier that can lead to discoveries for roles of ascorbic acid in disease treatment. Technologies are now available to drive this field forward. The foundation for this work is characterization of expected plasma and tissue concentrations in healthy people. With knowledge of normal ranges obtained from physiology and pharmacokinetic studies in healthy people, we can then learn when the abnormal occurs, why it occurs, and whether restoration of concentrations improves outcomes.

PARADIGM AND PROMISE

Physiology and pharmacokinetic experiments in humans for ascorbic acid are foundational for dietary recommendations, for treatment possibilities in cancer and sepsis, and perhaps for delay or even prevention of microvascular disease in diabetes. Renal threshold characterization and utilization studies may reveal new therapeutics and prevention possibilities.

Physiology and pharmacokinetic experiments with ascorbic acid utilized a depletion-repletion design, which is time consuming, labor intensive, and limited to only some vitamins. With the advent of liquid chromatography/mass spectrometry (LC/MS) and deuterated or 13C carbon molecules, advances for many vitamins are feasible with the same foundational principles. As one example, we created and utilized deuterated α-tocopherol

preparations to characterize the physiology and pharmacokinetics of vitamin E in healthy people and those with hepato-steatosis [130] (manuscript submitted). Studies in ascorbic acid have shown us new ways forward. We believe that physiology and pharmacokinetic experiments in healthy people for many vitamin are not only warranted but necessary to advance nutritional principles and to give full meaning to what is optimal. The visionaries who wrote about optimal nutrition were unable to move forward because key tools were missing. Now, they are becoming available or are already here. Again, to paraphrase Alfred Harper: We have a blizzard of diseases, and we have a snowplow: let's go plow snow!

ABBREVIATIONS

BromoAA: 6-bromo-6-deoxy ascorbic acid
BromoDHA: 6-bromo-6-deoxy dehydroascorbic acid
DHA: dehydroascorbic acid
FNB: Food and Nutrition Board
GLUT: facilitated glucose transporter
RDA: recommended dietary allowance
SVCT: sodium-dependent vitamin C transporter

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