Vitamin C is a Versatile Tool for Teaching Introductory Biology Courses

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Were students on my campus trying to ward off colds or other disorders by taking large amounts of vitamin C? I conducted an informal study among nearly 200 students in introductory biology courses for nonscience majors and found that yes, indeed, nearly half of them are either currently taking or have taken more vitamin C than the current recommended dietary allowance of 60 mg daily (Food and Nutrition Board 1980). Their purpose: to prevent illness. Instructors can grasp onto this widespread interest among students in vitamin C as a therapeutic agent and use it as a mechanism for promoting interest in biology courses.

Motivation is known to play an important role in learning (Smith & Rohrman 1970). When the subject matter is more relevant to the students' experience, and thus more motivating, educators can generate greater interest in their courses, especially among nonscience majors, whose initial interest level is apt to be somewhat lower than that of majors. This concept may apply to many high school students, no matter what their future academic plans may be.

In this article, I will provide background information on the role of vitamin C in human nutrition and also address the on-going controversy concerning the value of high-dosage vitamin C usage. Finally, some methods of determining the vitamin C content of urine, small amounts of blood plasma and food substances, will also be considered. Instructors are invited to utilize pertinent material for their specific course needs and to secure some of the references cited to broaden their familiarity with this controversial nutrient. A number of comprehensive reviews on vitamin C have been published within the past few years (Basu and Schorah 1982; Counsell and Hornig 1981).

An Overview of Vitamin C Function

Vitamin C is a biologically active reducing agent whose presence in the diet is essential for survival of a very small number of mammalian species. These include guinea pigs, apes and human beings, all of whom lack the capacity to synthesize the vitamin (Chatterjee 1978). Specifically, they all lack L-gulonolactone oxidase, the last in a sequence of four enzymes needed for biosynthesis of ascorbic acid from glucose (Sato and Undenfriend 1978). This metabolic defect is responsible for the appearance of scurvy, a fatal deficiency disorder which develops when the diet is lacking in the vitamin for periods of several weeks to several months. So effective is ascorbic acid that less than 10 mg daily will both prevent and cure scurvy in virtually all patients (Hodges, Hood, Canham, Sauberlich & Baker 1971).

Ascorbic acid is the reduced form of vitamin C, the oxidized form being dehydroascorbic acid. The latter can be converted to the reduced form by the body but further oxidation of dehydroascorbic acid results in the formation of diketogulonic acid and is irreversible. Ascorbic acid is essential for the hydroxylation of the amino acids proline and lysine to form hydroxyproline and hydroxylysine, respectively (Barnes & Kodicek 1972). It is believed that ascorbic acid maintains iron in its reduced ferrous form in hydroxylation reactions. Hydroxyproline is a major constituent of the ubiquitous body protein, collagen. Most of the signs of scurvy, e.g. brittle bones, failure of wound healing, loose teeth, internal bleeding, are attributed to failure of normal connective tissue formation.

Numerous additional functions of vitamin C have been cited (Sauberlich 1984). It is used in the biosyn-

thesis of epinephrine, corticosteroids and carnitine, a compound important in fatty acid metabolism. The vitamin also regulates cholesterol metabolism and is involved in immune system function, wound healing, non-heme iron absorption, drug and toxicant metabolism and inhibition of carcinogenic nitrosamine formation. Its function in glycosamino-glycan formation may have important implications in prevention of atherosclerosis (Turley, West & Horton 1976).

Dietary Sources

Careful food selection can provide consumers with several hundred milligrams of ascorbic acid daily. Eight ounces of fresh orange juice, for example, contains over 100 mg. Table 1 lists the vitamin C content of some common foods.

It is noteworthy that food groups other than fruits and vegetables are mostly devoid of vitamin C, except when fortified with the vitamin. It should also be noted that vitamin C is one of the most labile of the nutrients as it is readily destroyed by exposure to air, heat, metals and an alkaline pH. It is also lost in cooking water that is discarded.

Historical Perspective

Examples of the devastating effects of severe, prolonged vitamin C deficiency are often described in history textbooks. Frequently, two thirds of the sailors on long sea voyages prior to the 19th century had succumbed to scurvy. The term "scurvy" is a contraction of the phrase "scourge of the navy" (Chenault 1984). It is perhaps one of the greatest ironies of history that most of these fatalities were preventable by proper use of provisions on board many of these vessels. Dry legumes, if allowed to sprout prior to their consumption, would have provided sufficient amounts of the vitamin to prevent or even cure the disorder.

Virtually all introductory nutrition textbooks describe the experiment by James Lind which led to a cure for scurvy. This was also significant in that it represented the first controlled clinical therapeutic trial. Unfortunately, four decades elapsed between Lind's discovery that citrus fruits can prevent and cure scurvy and its implementation by the British navy, a delay believed to have resulted in some 100,000 casualties.

A British sea captain named James Cook defeated scurvy for a decade on sailing ships under his command by instituting dietary changes years before the British admiralty mandated the lemon juice ration (Villiers 1969). This enabled him to complete the three longest ocean voyages of discovery up to that time without losing a single sailor to scurvy. In order to persuade seamen to consume his vegetable soup regimen which included sauerkraut, he resorted to subterfuge by instructing his officers to consume the concoction with enthusiasm. Soon afterward, the sailors demanded the previously detested anti-scorbutic dish!

The Megavitamin C Controversy

Linus Pauling (Pauling 1976) and other authors (Stone 1972) have asserted that intakes of vitamin C far in excess of those currently believed to be adequate, exert many desirable physiological effects. While claims for the efficacy of massive doses in preventing colds have not been supported by subsequent research (Chalmers 1975), these claims have led to many investigations on the role of ascorbic acid in human pathophysiology (King 1975). Publicity in the mass media accruing from Dr. Pauling's status as a Nobel Laureate has promoted widespread use of this nutrient in pharmacological doses among laymen.

One rationale for massive doses of ascorbic acid has been described by biochemist Irwin Stone (Stone 1972). He views loss of the capacity to synthesize this vitamin as a hereditary disorder requiring replacement therapy with ascorbic acid. Extrapolation from species which can produce the vitamin suggests to him that quantities in excess of one gram daily are required by humans, especially during periods of physiological stress. Several other investigators have questioned the adequacy of the currently recommended 60 mg daily for adults but none of them ap-

Table 1. Vitamin C Content of Some Common Foods

Item	*Quantity*	*Mg of vitamin C*
Whole milk	1 cup	2
Beef liver*	3 ounces	23
Apple	1 large	8
Apple juice	1 cup	2
Banana	1	12
Grapefruit	½	44
Orange	1	66
Pineapple juice	1 cup	80
Lima beans*	1 cup	22
Broccoli*	1 stalk	162
Brussels sprouts*	1 cup	135
Cabbage*	1 cup	48
Kale*	1 cup	102
Iceberg lettuce	1 cup	3
Baked potato	1	31
Baked sweet potato	1	25
Cucumber	6 slices	3
Spinach*	1 cup	50
Tomato	1	28

* cooked

Source: Agricultural Research Service. (1977). *Nutritive value of foods.* Home and Garden Bulletin No. 72. U.S. Department of Agriculture.

proaches the amounts viewed as optimal by Stone and Pauling (Goldsmith 1961; Kallner, Hartman & Hornig 1981).

In contrast, other evidence suggests that the currently recommended allowance for vitamin C is more than adequate (Jukes 1975) and that ingestion of large amounts of this vitamin may not be safe (Hodges 1980). Contradictory reports on massive dose vitamin C therapy are the rule, rather than the exception, in the medical literature. Accordingly, biased selection of specific studies can be used to support either side of the controversy. Inconsistent results are sometimes caused by the use of different experimental procedures. For example, differences in dosage and duration of vitamin C administration, failure to determine the initial ascorbic acid status of the subjects, and variation in their exposure to stressors could account for some of the contradictory findings (Levine 1986). In this context, a productive activity would involve analysis of clinical research papers in which antithetical conclusions have been drawn (Cameron and Pauling 1978; Creagan, et al. 1979). The importance of modern scientific methodology which requires implementation of double-blind, prospective, placebo-controlled experimental protocols could thus be emphasized to students.

Simple Assay Techniques

Vitamin C is readily measured in small amounts of blood plasma, in urine and in various foods, thus permitting a variety of experimental analytical procedures to be carried out in introductory biology laboratories. Equipment required for this purpose is minimal. Moreover, estimation of vitamin C intake is greatly facilitated by the fact that appreciable amounts of the vitamin generally are limited to fruits and vegetables, fortified products and liver. Processed foods to which the vitamin has been added have the amounts contained per serving on the package label. Thus correlation of plasma or urinary vitamin C levels with estimations of dietary intake of the vitamin are feasible. Differences of vitamin C nutriture between the sexes, during different phases of the menstrual cycle, and in students taking either prescription or nonprescription medication may provide interesting experimental studies. Moreover, smoking is associated with reduced levels of vitamin C in blood plasma (Kallner, et al. 1981). Consequently, measurement of vitamin C nutriture of smokers and nonsmokers should interest members of each group.

Determination of vitamin C in plant juices can be carried out by titration with the dye, 2,6-dichloroindophenol (Association of Official Analytical Chemists 1980). This method, however, is not entirely favorable, because at the titration endpoint, excess unreduced dye appears as a pale rose-pink which slowly fades and must be timed to last for five seconds. Fruit and vegetable juices contain color after filtration which may mask the end-point. Also, the method is not sensitive for juices of low vitamin C content.

Rymal (1983) described a micro method for quantitative determination of vitamin C in juices. In this procedure, chromatographic paper is impregnated with 2,6-dichloroindophenol by immersion in 95 percent ethanol solution and sprayed with a protective starch solution. This method has the dual advantages of being more accurate than titration and is also portable. An interesting activity incorporating this procedure is to test the ascorbic acid content of orange juice from various sources, eg. fresh oranges, reconstituted, frozen, canned and juices packaged in carton containers. It may also be informative to compare the results with those listed in tables of food composition found in the appendices of most introductory nutrition textbooks.

An adaptation of the titration method to determine the degree to which body tissues are saturated with vitamin C involves estimation of urinary excretion of ascorbic acid. Since collection of urine is less invasive than collection of blood plasma, this method will be presented in detail. For further details, refer to Bauer (1982) and also to Wooton and Freeman (1982).

An oral dose of 11 mg per kg body weight of ascorbic acid is administered. All of the urine produced during the period 4-6 hours after ingestion of the vitamin is collected. This corresponds to the interval of maximal excretion. Urine produced prior to that time interval is discarded. A bottle containing 20 ml of glacial acetic acid, a preservative, is used for urine collection.

The reagents required for this analysis are as follows:

1. Stock dye solution. Dissolve 100 mg of the sodium salt of 2,6-dichlorophenolindophenol (available from Eastman Organic Chemicals, Rochester, New York) in water to make 1 deciliter (dl) of solution. If refrigerated, this 3.4 moles per liter (1) solution will remain stable for several months.
2. Working dye solution. Just prior to use, dilute 5 ml of the stock dye solution to 50 ml with water. Lack of stability of this solution precludes the possibility of its storage.
3. Sodium citrate solution (0.15 mole per l). Dissolve 4.4 g sodium citrate dihydrate in water to make 1 dl of solution.
4. Mercuribenzoate solution (5.5 millimoles per l). Dissolve 200 mg of the sodium salt of p-hydroxy mercuribenzoate in water to make 1 dl.
5. Metaphosphoric acid (0.38 mole per l). Dissolve

3 g reagent grade metaphosphoric acid containing 35% HPO_3 in water to make 1 dl. If refrigerated, this solution will be stable for one week.

6. Stock standard ascorbic acid (2.3 millimoles per l). Dissolve 40 mg of ascorbic acid in 40 ml deionized water and dilute to 1 dl with the metaphosphoric acid solution. If refrigerated, it will retain its stability for about one month.
7. Ascorbic acid working solution (45.4 micromoles per l). Just prior to use, dilute 1 ml of the stock solution to 50 ml with a mixture of 3 volumes metaphosphoric acid solution and 2 volumes deionized water which must be free of even small amounts of metals. This solution contains 0.8 mg per dl.

Analytical Procedure

1. Mix 3 ml metaphosphoric acid solution with 2 ml urine and centrifuge.
2. To 3 ml of the supernatant, add 1 ml of the mercuribenzoate solution, mix and allow to stand for 10 minutes.
3. Centrifuge and collect 2 ml of the supernatant in a cuvette. To this add 0.5 ml sodium citrate solution.
4. Prepare reagent blank by combining 1.2 ml metaphosphoric acid solution, 0.8 ml water and 0.5 ml sodium citrate solution in a cuvette and mix well.
5. Prepare standard by combining 2.0 ml of working standard and 0.5 ml of sodium citrate solution in a cuvette and mix well.
6. To each of the three cuvettes (from steps 3, 4 and 5) add 1 ml of the working dye solution, mix, allow to stand for 30 seconds, and read against a water blank at 520 nm.
7. To each of the same 3 cuvettes add a few crystals of ascorbic acid until the solution is completely decolorized. Repeat reading at 520 nm.
8. For each of the 3 cuvettes, subtract the absorbance (O.D.) obtained after the addition of excess ascorbic acid (step 7) from that obtained initially (step 6) to correct for turbidity. These corrected readings are used in the following formula:

$$\frac{\text{O.D. of reagent blank} - \text{O.D. of sample}}{\text{O.D. of reagent blank} - \text{O.D. of standard}} \times 3.33 \times \text{conc. of standard} = \text{conc. of sample}$$

The normal excretion of ascorbic acid after loading as described above should be about 50 mg or approximately 0.8 mg per kg body weight. If previous intake of ascorbic acid has been suboptimal, the tissues will take up most of the vitamin so that less is excreted in the urine. In severe deficiency, states less than 10 mg may be excreted.

Several methods for spectrophotometric analysis of small amounts of blood plasma are available. One sensitive colorimetric micromethod employs dipyridyl and ferric iron (Zannoni, Lynch, Goldstein & Sato 1974). Ascorbic acid reduces the ferric to ferrous iron which, in turn, forms a complex with dipyridyl. As little as 0.01 ml of plasma can be used for analysis. An even more sensitive colorimetric method using the complexing reagent, ferrozine, has recently been described (McGown, Rusnak, Lewis & Tillotson 1982). Again ferric iron is reduced to ferrous by ascorbic acid. Trichloroacetic acid is used to deproteinize extracts in both procedures.

Another recent method permits measurement of the oxidized form of vitamin C, dehydroascorbic acid (Okamura 1980). First, dipyridyl is used to determine ascorbic acid. Then dehydroascorbic acid is reduced to ascorbic acid by incubation with dithiothreitol. N-ethylmaleimide is used to remove excess dithiothreitol. Total ascorbic acid is determined by dipyridyl. Subtraction of reduced ascorbic acid from total ascorbic acid gives the oxidized form. This determination may ultimately prove to be of value as an early indicator of diabetes mellitus (Banergee 1982).

To conclude, instructors of introductory biology courses have an opportunity to capitilize on the interest in vitamin C shown by many college and, perhaps, high school students by incorporating into the syllabus some of the techniques described above. Thus, the wide publicity surrounding the use of vitamin C supplements can be constructively exploited.

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