Chapter 11

Integrating Introductory Biology and Chemistry Laboratories: Human Metabolism of Vitamin C and Fruit Juice Analysis—an Example

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Contents

Introduction

This protocol is part of a three and a half week module called the *Examination of Vitamin C (ascorbic acid), its Sources, Properties, and Metabolism*. In turn the Vitamin C module is the first of four modules which compose an entire semester of integrated Introductory Biology and Chemistry laboratories. Vitamins are of interest to most people for personal as well as intellectual reasons. Vitamin C has been chosen as the beginning subject for a three-four week study as it provides an obvious connection between the disciplines of Biology and Chemistry. Presented here are protocols for the module components *Analysis of Vitamin C in Fruit Juices* and *Human Metabolism of Vitamin C*.

Students are separately enrolled in the lecture components of General Chemistry and the Cell and Molecular portion of Introductory Biology. Their laboratories are combined. Integrated Introductory Biology and General Chemistry laboratories require facilities and equipment and scheduling flexibility that enable students to perform both the chemistry and biology of the laboratory during the same time slots. Moreover, it depends on the support of the faculty of both departments to foster this effort successfully.

This module represents a general progression from measurement to analysis to design of experimentation. Students proceed from measurement of ascorbic acid in (a) inanimate fruit juices to (b) a living plant tissue, cabbage (presented at 1986 ABLE conference by Carol Reiss, Cornell University) to (c) human metabolism and finally to (d) manipulation of ascorbic acid to maximize its

availability in a substance. A written report (Appendices B & C) of the maximization protocol and a team oral report (Appendix D) of the human studies is required.

In the study of fruit juices, students gain experience with quantitative analysis and pooling data. They then progress to measure human metabolism of Vitamin C, where they begin to apply their skills and the knowledge they have acquired in the fruit juice work. Working in teams, students design their own experiments to monitor Vitamin C intake and excretion as measured against various lifestyles. These protocols allow students to appreciate the complexity of a) experimental design and b) the control of variables in human studies and c) readily lets them then observe the complications that arise in data collection and interpretation of results.

Example of Timetable for Module I Examination of Vitamin C

Date	*Topic*
Day 1:	Introduction
	Group Formation
	Guidelines for Written Report
	Guidelines for Oral Report
	Laboratory Prep
Day 2:	Analysis of Ascorbic Acid Content of Fruit Juices
Day 3:	Continue Analysis of Fruit Juices
Day 4:	Analysis of Vitamin C in Cabbage (see ABLE workshop Proceedings Vol. 8, Carol Reiss, Cornell University)
	This workshop is not included here but is part of the Boston University laboratory program.
Day 5:	Student Design-Human Metabolism of Vitamin C protocol
Day 6:	Data Collection of Vitamin C Excretion
Day 7:	Maximizing Ascorbic Acid Availability
	This module is conducted over a three - four week period.
Two Weeks Later:	Laboratory Report on Maximizing Ascorbic Acid Availability is due
Last Week Of Course:	Oral Report with Team Leader on Human Metabolism Of Vitamin C

Materials

Protocol 1: Analyses of Ascorbic Acid Content of Fruit Juices

Equipment and Supplies

Analytical balance
Glass wool
Drying oven (>110°c)
Suction filter flask
Weighing bottle with cap
Water aspirator or vacuum
Burette, 50 ml
Amber storage bottles, 0.5 liter or larger
Volumetric flask, 500 ml

Graduated cylinder, 100 ml
Volumetric pipet, 50 ml
Erlenmeyer flask, 250 ml, three
Buchner funnel assorted glassware

Reagents

Activated carbon, powdered
Celite filter aid
Oxalic acid, $C_2H_20_4 \cdot 2H_20$
Potassium iodate, KIO_3
Sodium carbonate, Na_2CO_3
Sodium thiosulfate pentahydrate, $Na_2S_2O_3 \cdot 5H_2O$
Sulfuric acid, H_2SO_4, 0.3M and 0.5M
Starch

Protocol 2: Human Metabolism of Vitamin C

Materials/Equipment

Pipet-pi pump
Cuvettes
Urine collection vessels with covers, containing 20 ml glacial acetic acid
Vitamin C tablets
Spectrophotometers

Solutions

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 100 ml of solution. If refrigerated, this 3.4 moles per liter 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 liter). Dissolve 4.4 g sodium citrate dihydrate in water to make 100 ml of solution.
4. Mercuribenzoate solution (5.5 millimoles per liter). Dissolve 200 mg of the sodium salt of p-hydroxy mercuribenzoate in water to make 100 ml.
5. Metaphosphoric acid (0.38 moles per liter). Dissolve 3 g reagent grade metaphosphoric acid containing 35% HPO_3 in water to make 100 ml. If refrigerated, this solution will be stable for one week.
6. Stock standard ascorbic acid (2.3 millimoles per liter). Dissolve 40 mg of ascorbic acid in 40 ml deionized water and dilute to 100 ml 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 liter). 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 100 ml.

Protocol I. Quantitative Analysis of Ascorbic Acid Content of Fruit Juice

Notes for the Instructor

1. Sodium thiosulfate solution:
   - must be prepared under nearly sterile conditions to eliminate sulfur-eating bacteria which decompose thiosulfate
   - best to standardize and use the same day as the juice analysis; solution must be prepared 24 hours before it is needed. Initial decomposition is the most rapid. Standardization will be good for one week only and after that must be restandardized.
2. Starch solution (indicator):
   - to prepare, bring distilled water to boil; then add a paste made from soluble starch and cold water to make a 5% solution
   - add paste to boiling water and boil one minute; solution must boil after the starch is added.
   - solution does not store for more than two days. One student can prepare enough for entire lab section.
   - unlike other indicators, starch solution is not added at the start of a titration, but near the end; otherwise the iodine will oxidize the starch, leading to errors.
3. Technique
   - Reading a buret: Despite, or because of, previous instruction, many students do not know how to use a buret properly and do not read it correctly. They usually know to read the meniscus at the point of greatest curvature, but often do not read the volume with sufficient accuracy. Most burets can be read to the nearest 0.2 of the smallest scale division; for a 50 ml buret graduated in 0.1-ml increments, this means that volumes can be accurately read to the nearest 0.02ml. Give this specific instruction and check to see that students are actually reading to two decimal places and not just rounding to the nearest graduation mark, then adding a zero.
   - Insist that students do a pilot titration, both for the standardization of thiosulfate and the juice analysis. In addition to familiarizing one with the titration technique, a pilot gives information for taking a sample of appropriate size for the remaining titrations, i.e. a sample that can be titrated with about 80% of the total volume of the buret, or 40 ml of titrant of a 50-ml buret.

Student Outline

Introduction

Today you will begin a three week examination of several aspects of the chemistry of Vitamin C (ascorbic acid) and its metabolism in humans. In Protocol I of the project you will quantitatively analyze fruit juices for ascorbic acid and share your results with classmates in order to compare various sources of this important vitamin. You will then look at Vitamin C in living tissue[1]. During Protocol II you will design a protocol to assay its metabolism in humans. You will also evaluate your assays and fine-tune your analyses and design methods to maximize ascorbic acid availability[2].

I. Analysis of Ascorbic Acid Content of Fruit Juice
II. Analysis of Ascorbic Acid of the Metabolism in the Human

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[1]Presented by Carol Reiss in the Proceedings 7, Cornell University 1986. Not presented at this conference.

[2]Maximizing Ascorbic Acid Availability (Not presented at this conference)

Background

Oxidation-Reduction Processes:-Analysis Of Vitamin C In Consumer Items (Samuel, Rahman, and Margolina, 1995)

Many familiar oxidation-reduction processes involve metals and other inorganic materials. Corrosion of metals such as iron and aluminum, and the action of bleaches such as hypochlorite ion (OCl^-, the active ingredient in chlorine bleaches) and hydrogen peroxide are two examples. Oxidation-reduction processes are also important in organic and biochemical systems, although this chemistry may not be as familiar to you. Analysis for vitamin C, which is a reducing agent, is the subject of this laboratory. Not only is vitamin C an essential nutrient, but it can be quantitatively determined by a reaction which is easily followed.

Vitamins are organic compounds which are essential for growth and health, but cannot be synthesized by a given animal. Ascorbic acid is synthesized by plants and most animals, except primates, guinea pigs, the Indian fruit bat, and certain fishes. Thus, in these latter animals, including humans, ascorbic acid is a vitamin. The synthetic pathway (Figure 1) is a multi-step reaction which begins with glucose, after several steps leads to L-gulonic acid, and finally, after three more steps, produces L-ascorbic acid. The second of these last three steps is blocked in primates, who must obtain ascorbic acid, Vitamin C, in their diet.

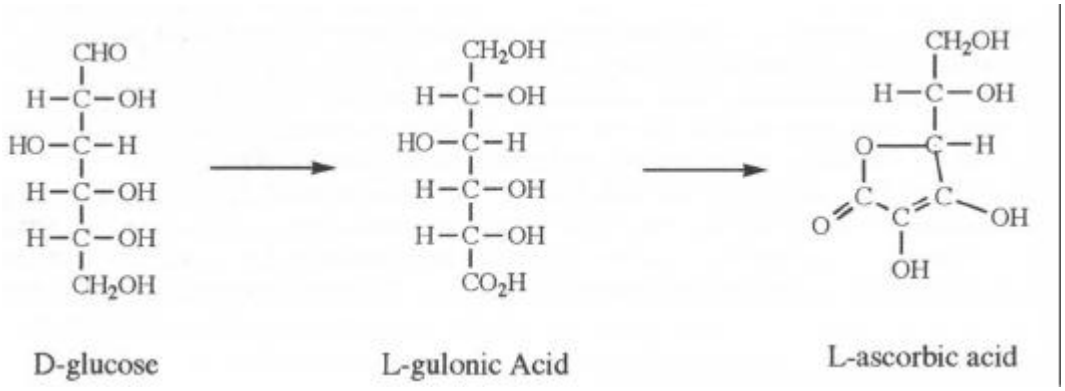


Figure 11. Structural formulas of D-glucose, L-gulonic acid, and L-ascorbic acid.

The necessity of vitamin C in the human diet has been known at least since 1700, and possibly earlier. Acute deficiency of this vitamin leads to the disease scurvy, while milder deficiency produces fragility of the capillaries (resulting in easy bruising) and possibly other effects. Ascorbic acid is a reducing agent. It can complex with metal ions and reduces iron from Fe(III) to Fe(II). Only Fe(II) can be incorporated into heme in red blood cells as they form in bone marrow. Vitamin C is very important in the synthesis of the protein collagen, where it promotes hydroxylation reactions, i.e. conversion of a C-H group to a C-OH group. Insufficiently hydroxylated collagen cannot form fibers, which results in the skin lesions and blood vessel fragility observed in scurvy. The complete biochemical function of ascorbic acid is not yet understood. More information about vitamin C is found in the literature cited by Lehninger *et al* (1993), Stryler (1995), and Marsh *et al* (1973).

Fruit juices are among the most common consumer items that are regularly taken to meet our daily requirements of ascorbic acid. It would, therefore, be useful to analyze some juices to determine their vitamin C content. Of the various methods available for the analysis of vitamin C,

titrimetic techniques are most frequently used. This laboratory exercise will employ an oxidation-reduction titrimetric method called iodometry, as recommended by the US Pharmacopeia. Since ascorbic acid is a very strong reducing agent, it is easily oxidized. In the analysis described here, a known quantity of molecular iodine (I_2) is generated from iodide ion (I) and a portion of it is used to oxidize the ascorbic acid in the sample. The remaining iodine is titrated with thiosulfate ($S_2O_3^{2-}$), a reaction which is easily monitored. Starch solution is an excellent indicator for molecular iodine, producing a definitive dark blue color.

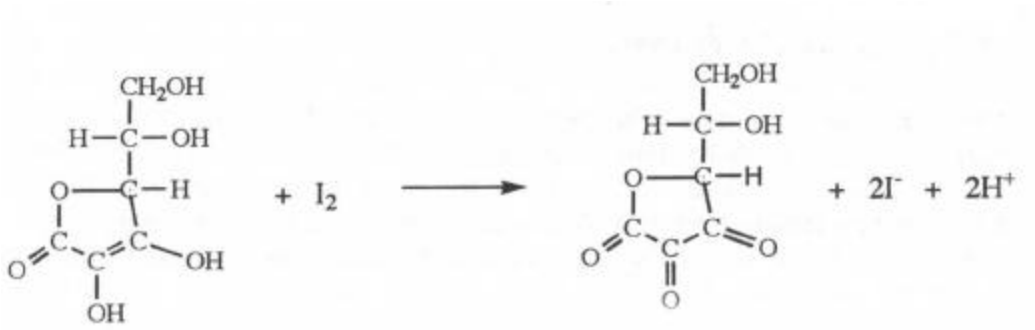
Iodide ion, a weak reducing agent, reduces iodate ion (IO_3), a strong oxidant, producing iodine in an amount equivalent to the iodate:

$IO_3^- + 5I + 6H+ \rightarrow 3I_2 + 3H_2O$

Molecular iodine can be conveniently titrated with thiosulfate ion:

$I_2 + 2S_2O_3^{2-} \rightarrow 2I + S_4O_6^{2-}$

Ascorbic acid can be oxidized by iodine as well. If sufficient iodine is generated (equation 2), then a portion of it can react with ascorbic acid in the sample, and the remainder is titrated with thiosulfate.



The reaction of iodine with ascorbic acid is shown in the following equation:

As described above, the iodine oxidizes the adjacent alcohol (C-OH) groups in ascorbic acid to carbonyl (C=0) groups in dehydro-ascorbic acid; the oxidation involves a two-electron change. You should demonstrate to yourself that these equations do indeed represent oxidation-reduction reactions.

Procedure

In order to collect useful data for comparison you must first design the experiment. The directions for analyzing an individual sample of juice are given in this document, so the important question remaining is, "What samples should be chosen?" Here are some sampling parameters:

- Type of fruit source, e.g. orange, grapefruit, pineapple, cranberry, grape; note, apple is a poor source
- Type of processing, e.g. fresh squeezed, fresh, frozen concentrate, prepared from concentrate before sale, canned
- Variety of fruit, e.g. pink vs. white grapefruit, navel vs. temple oranges
- Style of juice, e.g. Minute Maid Country Style vs. Regular
- Brand of juice, e.g. Minute Maid, Tropicana, Star Market house brand

Two groups of four people should work together to design this experiment. You may find the following schedule helpful.

First Period: Project design and preparative work

- Form groups, choose samples, assign analysis of particular samples to each group member
- Prepare a sodium thiosulfate solution
- Prepare a potassium iodate primary standard solution
- Clean and store volumetric glassware

Note: Each person will prepare and standardize a solution of sodium thiosulfate and use it to analyze juice.

Second Period: Data collection and analysis

- Standardize the sodium thiosulfate solution
- Analyze the juice sample(s), in quadruplicate
- Share raw data with group members via computer

Your chemistry professor and your teaching fellows will be available to discuss results. You should use a spreadsheet for data analysis. As you do these analyses think ahead to Part C of the module in which you will be designing a protocol to accurately measure vitamin C metabolism.

Report: Turn in a copy of your calculations and results the week following the second lab period. The narrative report will be written at the end of the project and will incorporate all the sections of it.

Preparation of $Na_2S_2O_3$ and KIO_3 Solutions

Sulfur-eating bacteria love thiosulfate solutions! It is important, therefore, to use nearly sterile technique when preparing the thiosulfate solution. The distilled water must be boiled, and the solution stored in a scrupulously clean bottle. After washing the bottle, rinse it three times with small portions of the boiled water. Although freshly boiled distilled water should be used if time permits, the water may be boiled a day or two before the analysis is performed, as long as it is stored in a properly cleaned bottle.

Prepare 0.07 M $Na_2S_2O_3$ (sodium thiosulfate) by dissolving 8.7 g of $Na_2S_2O_3\ 5H_2O$ in 500 ml of (freshly) boiled distilled water containing 0.05 g of Na_2CO_3 (sodium carbonate). Store this solution in a clean, tightly-capped amber bottle in order to protect it from light and air. (Why should the solution be so protected?)

Sodium thiosulfate cannot be used as a primary standard. The exact concentration of this sulfate solution must be determined experimentally, each time that it is used. A thiosulfate solution that has been standardized will hold its titre for one week (no longer), provided it is stored under the conditions described here.

To prepare a solution of the primary standard potassium iodate, dry 1.5 g KIO_3 (potassium iodate) for 1-2 hours at 110° C, then store it in a desiccator until it is cool, and until it is ready to be weighed out. Prepare 0.01M standard KIO_3 solution by accurately weighing (analytical balance) 1.0 g of the dried solid reagent and dissolving it in distilled water in a 500 ml volumetric flask. Calculate the molar concentration of the solution from the exact weight of the reagent used.

Standardization of Sodium Thiosulfate

Pipet 50.0 ml of KIO_3 solution into a 250 ml Erlenmeyer flask. Add 2 g of solid KI (potassium iodide) and 10 ml of 0.5M H_2SO_4. Immediately titrate this solution with $Na_2S_2O_3$ solution from the buret until the initial red-brown color of the solution turns to pale yellow. Then add 2 ml of starch indicator and complete the titration just at the disappearance of the blue color, marking the end-

point. Repeat the titration with two additional 50 ml aliquots of KIO_3 solution. Record the data and calculate the molarity of the $Na_2S_20_3$ solution.

The principal reactions involved in the standardization process are:

$$IO_3^- + 5I^- + 6H^+ \rightarrow 3I_2 + 3H_2O$$

$$I_2 + 2S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-}$$

Analysis Of Fruit Juices

Preparation of Sample

In this section of the laboratory exercise a modified version of the procedure described by Paul Haddad (1977) is used.

Take three 100 ml samples of orange or other fruit juice (measure accurately using a 100 ml flask if pulpy). To each sample add 0.5-0.7 gm of solid oxalic acid ($C_2H_20_4 \cdot 2H_2O$) and swirl until the oxalic acid has dissolved. The purpose of the oxalic acid is to stabilize the ascorbic acid in the juice, which could otherwise be oxidized by air during the filtration process.

If analyzing orange juice, add sufficient filter aid (Celite) to the orange juice to form a thick slurry, and filter this slurry through a Buchner funnel fitted with a pad of glass wool (not filter paper) into a clean flask. A little of the Celite may pass through the filter. It will not interfere with the titration. The resulting filtered solution should be only pale yellow in color. It is now ready for titration and should be titrated immediately.

For analyzing dark, clear juices, such as cranberry or grape, to each 100 ml aliquot add 0.5-0.7 g oxalic acid and 0.7 g activated carbon (charcoal), swirl for a few minutes, and filter, using a Buchner funnel and coarse filter paper (or gravity, if no suction is available). Do not heat, since heat decomposes vitamin C. The juice should be light brown and clear. Note: Activated carbon does not decolorize many of the commercial fruit punches.

Light-colored juices such as lemon, grapefruit, or apple, need neither Celite nor charcoal treatment before titration.

Titration of the Juice

To each sample of filtered juice add 30 ml of 0.3M H_2SO_4, 1 g of solid KI and 25.00 ml of standard KIO_3. Titrate the resulting red-brown solution with standard $Na_2S_2O_3$, adding 2-3 ml of starch indicator just before the end-point. Repeat the titration with the two remaining samples, calculate the mg vitamin C/100 ml of orange juice and compare your results with the values listed on the manufacturers' labels.

Note The letters "L" or "D" in the name of a compound indicate that the molecule is optically active; the D and L forms of a molecule are mirror images of each other. Optically active molecules contain structural features that cause plane polarized light to rotate, either to the left or to the right. Optical activity is important in biological systems because in most cases, only one form of the molecule, L or D, can be used by the organism.

Protocol II. Human Metabolism of Vitamin C - Design of an Experiment

Notes for the Instructor

1. Clearance by your Institutional Review Board for Human Studies is required.
2. Make sure all participating students and the instructor(s) sign the Informed Consent form (Appendix E) before beginning protocol.
3. All reagents should be prepared a few hours before the laboratory begins. This is especially important for the working standard ascorbic acid and the dye DCPIP. Both tend to give poor results if they are left overnight.
4. Before the laboratory starts, the instructor can discuss how the laboratory is important for testing a large number of variables that affect human metabolism of a vitamin.
5. Students must be asked to take notes for monitoring variables.
6. Urine samples must be handled carefully and the cup must remain covered at all times. A plastic disposable pipet can be inserted into the lid (where normally a straw would go) for use in the laboratory.
7. Both the test and the standard will appear colorless or almost colorless, which causes panic among students.
   - The blank should be light colored because there is no ascorbic acid at first. It decolorizes only on adding ascorbic acid crystals.
   - The standard should be colorless because it has a "standard" or known concentration of ascorbic acid. It does not change further on adding ascorbic acid crystals.
   - The test sample depends upon the individual's concentration of ascorbic acid in the urine.
8. Students can convert their weight in pounds to weight in kilograms by dividing their weight by 2.2: mass (kg) = [mass (lb)] / 2.2 lb/kg
9. Ask students to have three separate test tubes for sample, blank, and standard. They can then transfer samples from each to the same cuvette. The cuvette should be washed after every trial with distilled water.
10. At no point should pipets be intermixed while using metaphosphoric acid and the dye.

Student Outline

Introduction

Although the data may be easier to collect from inanimate objects or from dead specimens, we ultimately want to know how a nutrient affects the living human. Thus, much of biological and medical research is based on the study of processes in whole organisms. Even when your research focuses on a specific process in an organism, ultimately it becomes significant only when viewed in the context of the total organism. This is especially evident in the studies of biological and chemical processes in the human. A researcher is now faced with factors not completely in his/her control. This portion of Module I attempts to focus your attention on the need to develop protocols, with as many controls as possible for a whole organism, in this case the human. To do this you will continue your assay of Vitamin C. Keep in mind the observation and data skills you learned in the preceding protocols.

Today will be devoted primarily to designing the mechanisms to study ascorbic acid metabolism in young adults. You should discuss with your team of four, information from the library search. Use this information to plan your own protocol. Remember your team is part of a larger study involving

all students in the integrated labs, divided into several teams. You are free to work with other teams to design a significant report.

Your planning for experimental protocols must include:

1. Dosage levels for members of the class - based on height/weight
2. Mechanisms to monitor diet
3. Provision for accurate urine collection
4. Signed Informed Consent Form
5. Identification and control of variables
6. Other
   - Will you accomplish more if one member takes a "Project Director" role?
   - How will you accomplish equity among all members of the team?
   - Who will represent the group during your oral presentation of the study?

Background—modified from Barry S. Kendler (1986) Manhattan College, Riverdale, NY

Vitamin C is a biologically active reducing agent whose presence in the diet is essential for the survival of the mammalian species. Members include guinea pigs, apes, and human beings, all of whom lack L-gulonolactone oxidase, the last in a sequence of four enzymes needed for biosynthesis of ascorbic acid from glucose (Sato and Udenfriend, 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).

As we have seen, 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 oxidization 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 and 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 biosynthesis of epinephrine, corticosteroids and carnitine, a compound important in fatty acid metabolism. The vitamin also regulates cholesterol metabolism and is involved in the 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).

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. The optimal amount of Vitamin C required by the human is quite controversial. In the last twenty years many researchers have investigated Vitamin C intake in the human. The most well known of these investigators was the Nobel laureate, Linus Pauling. There is extensive literature both supporting and disclaiming supplemental use of this vitamin. Biased selection of specific studies can be used to support the use of mega vitamin dosages or to reveal the mega dosage dangers.

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.

Vitamin C is easily measured in small amounts in blood plasma, urine and foods. In today's laboratory you will determine the degree to which body tissues are saturated with Vitamin C by estimating urinary excretion of ascorbic acid.

Pre-laboratory questions

1. To prepare for analysis of Vitamin C metabolism in humans it is helpful to do a library search to determine various contradictory studies published during the last six years concerning the affects of Vitamin C on the human.
2. Write a 1-3 page summary of five different studies of Vitamin C metabolism in the human during the last six years. State the number of subjects, how the vitamin was measured, the results, and the interpretation of the results. Clearly indicate how they agree. Where do they disagree?
3. Begin thinking of a laboratory study for human metabolism of Vitamin C. List at least seven variables to be considered in its design. Be sure to include factors other than those mentioned in this manual.
4. Use information you learned in other components of this module to help you trouble shoot possible problems or variables that may arise.
5. Hand in the "Informed Consent Form" to your instructor (person obtaining consent) at the beginning of the laboratory period.

First Laboratory Meeting of the Semester

You will:
a) record who regularly takes Vitamin C
b) record the amount each person takes
c) pool data from all lab sections for future reference
Students should not stop performing or change their normal daily activities.

Now that you have measured the ascorbic acid content of various substances, you may appreciate differences in data collection. In performing experiments, humans continually make errors. Moreover, in experiments with humans, not only does the experimenter err, but subjects often give incorrect information such as data about their weight, age, foods eaten, etc. Thus, there must be a constant effort to reduce error as much as possible by collecting data accurately. There is an old axiom: “Data don’t change, interpretations do.”

Read the following protocol and answer questions 1-4 below to make sure you understand possible sources of error. Then continue with question 5.

Over a period of a week, five students in four different laboratories each measured the height of the same laboratory instructor with the standing scale bar (seen in doctors’ offices), i.e. 20 different students measured the same instructor.

Students	Lab 1	Lab 2	Lab 3	Lab 4
1	177.00 cm	177.00 cm	176.98 cm	177.01 cm
2	166.99 cm	177.00 cm	176.99 cm	177.01 cm
3	177.11 cm	177.00 cm	177.01 cm	177.02 cm
4	177.10 cm	177.00 cm	177.02 cm	177.03 cm
5	177.12 cm	177.00 cm	177.00 cm	177.03 cm

Questions

1. Give explanations for: (a) differences between results of Lab 2 and other laboratories, (b) differences within a laboratory.
2. If you had to use a figure from a range of figures in the calculation of a metabolic process, which figure would seem most accurate?
3. State basis for choice.
4. How would you standardize the measurements if performing the measurements again?
5. In consultation with other members of your team, write out your protocol in your laboratory notebook before starting the experiment.
6. What is an aliquot and how is it useful?

Procedure

Urine collection

CAUTION: At all times you are to handle ONLY YOUR OWN URINE.

1. Four-six hours prior to attendance in the laboratory, take an oral dose not to exceed 11 mg Vitamin C/ kg body weight.
2. Set up experimental protocol.
   A. Determine controls such as
      1/ Standarizing the amount of urine collected per person.
      2/ Time of day collected.
      3/ Type of metabolic activity prior to collection (eating, sleeping, running etc.)
      4/ Other controls?
   B. Some possible variables to monitor can be:
      1/ Differences among women during menstrual cycles
      2/ Amount of exercise
      3/ Students on prescription drugs vs. those who are not
      4/ Smokers vs. non-smokers
      5/ Source of Vitamin C- a) juice; b) a vegetable (see chart)
      6/ Other.

When planning urine collection, remember that if Vitamin C is taken when going to bed, maximum excretion of Vitamin C occurs upon awakening and must be collected first thing in the morning. The containers holding urine must be *COVERED at all times*

3. If using awakening collection time (preprandial) bring urine sample between 8:30-9:30 am to the laboratory and pour 2.0 ml of the urine, as specified by your instructor, into a styrofoam cup,

covered with a lid. With a pipet add 20 ml glacial acetic acid[3]. through the designated opening in the lid. Keep urine samples covered. Store until your laboratory section meets.

4. Data must be in black ink and written clearly so that photo-copies can be made for distribution. (The undergraduate assistant will record all the data after the students fill in the table on the blackboard. Data will be pooled from all lab sections.)
5. "Comments" might include what their dietary sources of Vitamin C were, whether they are a smoker, if they are on prescription drugs, if they exercised before taking the vitamin.

Chemical Analysis

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[2] 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[3] .
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 (of known concentration) and 0.5 ml of sodium citrate solution in a cuvette and mix well.
6. To each of three test tubes (from steps 3, 4 and 5) add 1 ml of the working dye solution, mix, allow to stand for 30 seconds. In a single cuvette read against a water blank at 520 nm. Wash cuvette between each reading.
7. To each of the same 3 test tubes add a few crystals of ascorbic acid until the solution is completely decolorized. Repeat reading at 520 nm.
8. For each of the 3 test tubes, 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:

$$\frac{\Delta A \text{ of reagent blank} - \Delta A \text{ of sample}}{\Delta A \text{ of reagent blank} - \Delta A \text{ of standard}} \times 3.33 \times \text{conc. of standard} = \text{conc. of sample (mg/100 ml)}$$

Derivation of the constant 3 33

During the preparation of the urine sample, 2 ml urine are diluted with phosphoric acid to 5 ml so the concentration is now 0.4× the concentration in the original urine. Three ml of this solution is then diluted to 4 ml with mercuribenzoate so that the relative concentration is now 0.75×4 or 0.3 × the original concentration in the urine. Two ml of this solution is now processed identically as the standard. The constant 3 33 in the equation corrects for this dilution, so the unknown concentration is the concentration of the original urine, not the sample already diluted with phosphoric acid and mercuribenzoate. ($3.33 \times 0.3 = 1$) (Kendler).

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[1]Glacial acetic acid is added to the urine sample when it has to be stored for a short time to prevent further oxidation of Vitamin C and slow microbial growth. Dichloroindophenol (DCIP), as you will recall from earlier work, is a pH sensitive dye which turns pink in acid and blue in base and can be reduced by ascorbic acid to a colorless form.

[2] Mecuribenzoate has antimicrobial activity because it reacts with and denatures proteins through the amino acid cysteine. When added to the urine sample, it removes the protein in the form of a precipitate. The supernatant is then saved. Removing the protein minimizes light scattering, which would interfere with the spectrophotometric assay. Metaphosphoric acid will inactivate oxidase to prevent loss of ascorbic acid (Kendler).

[3] Sodium citrate can chellate (bind) metal ions but its role seems to be to mix with the phosphoric acid to create a mixed citrate buffer to stabilize the pH for the assay.

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 as been suboptimal, the tissues will take up most of the vitamins so that less is excreted in the urine. In severe deficiency states, less than 10 mg may be excreted.

Table 2*. Vitamin C Content of some Common Foods

Item	Quantity	Vitamin C (mg)
Whole milk	1 cup	2
Beef liver**	3 ounces	23
Apple	1 large	8
Apple juice	1 cup	2
Banana	1	12
Grapefruit	1/2	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	45
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

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

**cooked

Post Laboratory Questions to consider

1. The roles of mercuribenzoate and sodium citrate are stated. What is the significance of their use in this study?
2. Has your literature search of ascorbic acid research over the last five years contradicted any of the background information presented in this module? Explain.
3. Has your current study here at Boston University contradicted either the early or late literature? Explain.
4. Are you confident to submit this for publication to a journal of nutrition?
5. Can you really answer the question, "Are you ingesting the right amount of Vitamin C?" What problems arise as you attempt to do this?
6. Case Study:

Susan lay on the floor dead. At death, all active transport ceases; muscles stop working; diffusion takes over; fluids flow from orifices. An analysis of her body fluids show the content of Vitamin C 500 times greater than average. Six cartons of unopened orange juice were found in her

refrigerator, a seventh was nearly empty. Two dirty glasses of orange pulp were knocked over on the floor.

George, the person accused, was seen leaving the house at about the estimated time of death. He was arrested an hour later and his blood and urine were tested. It was about 300 times above normal.

Defend George as not guilty based on chemical evidence.

Prosecute George as guilty based on chemical evidence.

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APPENDIX A
Record Keeping in the Laboratory Notebook

Always record data, interpretations and answers to questions in pen. Number your answers in a consecutive, orderly fashion. Diagrams may be made in pencil. All questions to be answered or observations to be noted or discussed, if located in the body of instructions, are numbered in brackets (#). Be sure to answer these and graph results in an orderly fashion in your laboratory notebook.

Always write in clear and simple language, using words as precisely as you can. Avoid ambiguity. When you can express data quantitatively, do so as precisely as possible, using the metric system; but avoid the appearance of greater precision than is real. To state that something weighed 1.002 grams when your scales will weigh no more precisely than 0.1 gram is dishonest. In averaging results, to carry the average out to several decimal places when measurements were made to only the first decimal place, leads to a false impression of accuracy. This again is unscientific. It is permitted to round off an average only to the same degree involved in the measurements taken, no more.

When applicable, make an outline drawing with a firm pencil and continuous outlines. Label structures neatly with names all parallel. Labeled lines, running horizontally to the structures to be designated, should not cross. Be sure to give the whole drawing a title, preferable placed below it. Avoid shading (except by stippling) since it obscures more than it reveals. In general, your drawings should look like diagrams in a book.

Graphs of quantitative data are important in scientific communication. Use them whenever you can, and when you are graphing a process, take pains to plot the points accurately and to connect them with smooth lines. Label the quantities plotted on the **vertical axis (ordinate)** and **horizontal axis (abscissa)** along the scales, and provide the entire graph with a concise title.

Always remember that communication is a most important part of science. In publishing scientific work, the results must be stated in such a way that another person can repeat the procedures exactly. S/he must know what material was used (if living, that means the species used and the genetic strain if that is known), and be able to comprehend every detail of the work as well as the analysis and interpretation of the results. By reading your work, scientists in a laboratory, anywhere in the world, given access to the facilities, would be able to duplicate it. Part of your education in this course will be to learn clarity of expression.

APPENDIX B
Writing Narrative Laboratory Reports

Because some of the laboratory work you are performing is original your findings and interpretation could be a candidate for publication in a professional journal. Thus, from the very outset of this course, you should begin recording your results in the format accepted by scientific journals. This may differ slightly among different journals. Because the journal *Science* is internationally respected and moreover encompasses all the sciences, the authors have selected its format as a model for you to follow.

An Appendix in your laboratory manual clearly outlines the method of presentation of your data and its analysis. So that there is no misunderstanding Appendix C indicates how your report will be graded.. Attach one blank copy of this page to the top of each laboratory report you submit. Because the written word is still the most powerful tool of communication for scientists, we hope from the very start the written reports should he well thought out, data presented clearly, credit given to colleagues of former researchers, and interpretations logical and clearly presented.

You can, and often should, collaborate with other team members in the preparation of laboratory materials. However, you must *acknowledge collaborations* and write the names of people with whom you collaborate in the text and in the references in your written narrative reports. Reports must be written *individually*. Write your observations in your own words. Report what you saw, not what you thought you should see. When you use another written source you must cite references for credit in your write-up

Because of the variety of problems you will be studying in each module, you will be expected to write your report on one part only, A, B , C, or D. Moreover, because the module represents an integration of work performed in both the Biology and Chemistry facilities, you are required to refer to findings from associated work in that particular module.

Because Module I Part II a) involves your design of an original project and also b) to allow the maximum amount of time for data collection from the total student cohort, interpretation of data, and preparation for the oral presentation, it has been chosen as the focus for your oral presentation. Because *Maximizing Ascorbic Acid* also is a student designed project, your first written report on Module I will be focused on this protocol.

Your instructors feel very strongly that you must get the *writing for a science journal skills* correct from the very beginning. Thus, if necessary, you will be allowed to correct your first narrative laboratory report and resubmit it for a maximum perfect score of 7.0 rather than 10.

All Narrative Written Reports must be compiled on a word processor and are expected to be handed in on time. You will automatically be deducted 2 points for the first day late and 1 point for each succeeding day

APPENDIX C
Lab Report Grade Sheet

Include Title Page and Grade Sheet

Title and Abstract

____/0.5 pt	Title accurately describes paper contents
____/0.5 pt	Abstract briefly describes what was performed (methods) and adequately describes the results obtained

Introduction

____/0.5 pt	Justifies experiment and states hypothesis
____/0.25 pt	Presents relevant background information
____/0.25 pt	All statements supported by references

Comments:

Materials and Methods

____/0.5 pt	Clear and concisely written in text format
____/0.5 pt	Source (lab manual) appropriately cited
____/1 pt	Methods for all topics clearly outlined and use is rationalized

Comments:

Results

____/2 pts	All necessary data included
____/1 pt	Tables/graphs/figures easily illustrate results
____/0.5 pt	Tables/graphs/figures are properly titled
____/0.5 pt	Tables/graphs/figures have correct units of measure
____/1 pt	Test explains relevance of data and figures

Comments:

Discussion

____/1 pt	Restates Hypothesis
____/1 pt	Describes relevant background
____/1 pt	Predicts lab results
____/1 pt	Correct interpretation/analysis of results
____1 pt	Addresses objective questions
____/0.5 pt	Concluding remarks contain suggestions for future research
____/0.5 pt	Concluding remarks bring paper to an appropriate closure
____/2.0 pts	Ability to convey knowledge of topic

Comments:

References

____/0.25 pts	Minimum references included (3)
____/0.25 pts	Correct Format
____/0.5 pts	All literature cited in body of paper

From: Joseph Walsh

APPENDIX D
Presenting the Oral Report

One member of your team of four collaborators will give a twelve minute oral presentation on the results of your study, *Human Metabolism of Vitamin C.* The other three members will write the paper to be presented and prepare the transparencies of relevant graphs and significant points. Moreover, the non-presenting team members will each be expected to ask well-thought out questions during the discussion time following each talk. The presenter will be expected to give intelligent answers. Nonpresenting students will submit at least three questions ahead of time to their instructor. A student may change the question at the presentation if someone has already asked that one or the student thinks of a better one. Both the team's speaker and listeners will be graded on the content of the paper presented and its clarity.

Modern research today is usually due to both team and individual efforts. Thus, the grade given for the oral presentation with a perfect score of 15 will be based on

9 points - team grade
6 points - individual effort.

APPENDIX E
Informed Consent Form

Title: Human Metabolism of Vitamin C - Design of an Experiment

Name: Professor _____

(1) The following laboratory protocol outlines in detail the experiment that you will be performing in the next few weeks. Your participation involves possible assignment to ingestion of Vitamin C, collection of urine over a 24 hour period, and the subsequent analysis of urine specimens for excretion of Vitamin C. The amount of Vitamin C to be administered is 11 mg of Vitamin C/kg body weight one time only. Assignment will be random. You have the option of not participating in the experimental group, if assigned.
As a member of a laboratory team, you will decide what aspects of your daily activity might affect your Vitamin C metabolism. You and your team may collect data on your normal daily nutritional intake, menstrual status, level of exercise, amount of sleep, etc. You with other members of your team will agree on a protocol to gather this information which is consistent to the group.

(2) There are no foreseeable risks or discomforts to the subject. The amount of Vitamin C ingested is below safe levels for the individual.

(3) Because of this study, you can begin to appreciate the complexity of performing studies on the human. It will introduce you to the many variables one has to consider when designing a study or experiment for humans.

(4) The laboratory section of 24 students will be divided into 4 teams of 6 students each. Because you are a student you are expected to participate in the conduct of the experiment. If you have any reservations about taking the Vitamin C at 11 mg/kg body weight, you may choose to be part of the control group that does not ingest it in this experimental form.

(5) You will be given a random number by your laboratory instructor. This number will be used to identify your results by other members of the class, who will not know your identity. Your instructor will destroy this number once all the data is collected and pooled by members of the class.

(6) You are free to contact Mr. David Berndt, of Institutional Review Board (353-4365), if you have any concerns regarding your participation in this laboratory study. Moreover, you may contact Professor Godrick if you have additional questions at 353-2472.

I,_____(print) understand the purpose of this project and the extent of my participation. I have had my questions answered and agree to participate in the test or control group only.

Signatures _____(student) Date _____

_____(person obtaining consent) Date _____

APPENDIX F
Student Data Sheet

				Vitamin C Intake			**Vitamin C**	
Student #	**Sex (m/f)**	**Weight (lbs)**	**Sleep (hrs.)**	**Diet (mg)**	**Tablets (mg)**	**Total (mg)**	**Excreted (mg)**	**Comments: (exercise, smoker, dietary source, other)**