Lab III-3

Proteins, Enzymes, and Vitamins

Other than collecting household items, no advance preparation is required for any of the procedures in this lab session. Procedure 2 requires at least one urine specimen, which you should obtain immediately before you begin the session. If there will be a significant delay between obtaining the specimen and doing the lab session, refrigerate the specimen in a full, tightly-capped container until shortly before you begin the session.

> We'll assume that you're healthy and using your own urine, which presents no biohazard to you. In fact, urine obtained from any healthy person is sterile unless it is contaminated during collection. If you obtain urine specimens from others, wash your hands with soap and water thoroughly after handling the containers. In any event, wash the outsides of the containers after collecting the specimens.

Equipment and Materials

You'll need the following items to complete this lab session. (The standard kit for this book, available from *www.thehomescientist.com*, includes the items listed in the first group.)

Materials from Kit

- □ Goggles
- □ Ascorbic acid tablet
- □ Beaker, 100 mL
- □ Beaker, 250 mL
- □ Biuret reagent
- □ Centrifuge tubes, 15 mL
- □ Gelatin (unflavored)
- □ Gram's iodine stain
- □ Graduated cylinder, 10 mL
- □ Graduated cylinder, 100 mL
- □ Hydrochloric acid
- □ Lead(II) acetate
- □ l-Glutamine
- □ pH test paper
- □ Pipettes
- □ Reaction plate, 24-well
- □ Reaction plate, 96-well
- □ Sodium hydroxide
- □ Spatula
- □ Stirring rod
- □ Test tubes
- □ Test tube clamp
- □ Test tube rack

Materials You Provide

- □ Gloves
- □ Marking pen

- □ Blood from uncooked meat
- □ Desk lamp or other strong light source
- □ Egg white, raw
- □ Freezer
- □ Hydrogen peroxide, 3%
- □ Isopropanol, 99%
- □ Microwave oven
- □ Paper or cloth, black
- □ Paper towels
- □ Starch water (see text)
- □ Urine specimen(s) (see text)
- □ Water, distilled

Background

In this lab session we'll investigate three more classes of biologically-important molecules, proteins, enzymes, and vitamins.

Proteins

A *protein* is a member of a class of organic chemical compounds that are made up of *amino acid* building blocks. An amino acid is an organic chemical compound that includes a *carboxyl group* (COOH) and an *amino group* (NH_2). In proteins, amino acids link together in chains, with the carboxyl group from one amino acid in the chain connected to the amino group of the next amino acid by a *peptide bond*.

There are a huge number of different amino acids, but only 20 of them (22 in some lifeforms) are used as protein building blocks. Simple proteins may include just a few amino acids in the chain, while complex proteins may include hundreds or thousands of individual amino acid building blocks. The sequence (order) of the amino acids in a protein is defined by the gene that is responsible for producing that particular protein. Small changes in that sequence can result in huge differences in the characteristics and functioning of the resulting proteins.

Although proteins can be thought of as simple chains of amino acids, the physical reality is different. Rather than existing as long linear chains, during production most actual proteins fold in upon themselves to produce molecular structures that have the form of globes or fibers. These structures, called *conformations*, may be relatively rigid and fixed or they may be flexible and therefore subject to conformational changes. Proteins of the first sort may be found as structural elements in cells and other applications where their fixed conformations are essential; those of the second sort are often used for signaling and are important elements of the *cell cycle*.

> Most proteins can be *denatured* by heat, acids or bases, heavy metals, or other *denaturants*. For example, when you cook liquid egg white, the albumin protein present in the egg white is denatured and precipitates as a white solid.
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> The process of *denaturation* unfolds the conformation of the protein, disrupting its three-dimensional structure and inactivating it. Depending on the protein and the denaturant used, denaturation may or may not be reversible.
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> The term denatured is used in two distinct ways. For example, changing the pH of a solution slightly may inactivate a particular protein, but returning the pH of that solution to the range in which that protein is active may reactivate the protein. Conversely, making a large change of pH in that solution may inactivate the protein irreversibly. Some people use the term denaturation to refer to either reversible or irreversible inactivation; others use it to refer only to irreversible inactivation.

Like carbohydrates and lipids, proteins play a key role in life processes, participating in nearly every cellular process. Some proteins fill structural roles at the cell and organ levels. Others are

enzymes that catalyze biochemical processes, including metabolism. Still others play key roles in cell signaling, immune system responses, and most other critical life processes.

Biologists use numerous chemical and instrumental tests to detect, identify, and quantify the types and amounts of amino acids and proteins present in a specimen. The oldest of these is the *biuret test*, which is a general test for proteins and can be run qualitatively or, with a colorimeter or spectrophotometer, quantitatively. Other common tests include the *Bradford protein assay*, which is more sensitive than the biuret test but does not respond to all proteins, the *cystine test*, which detects the presence of cysteine residues in proteins, and the *xanthoproteic acid test*, which is specific to the two amino acids tyrosine and tryptophan. (We'll use the biuret test and the cystine test in this lab session.)

Enzymes

An *enzyme* is a protein that functions as a *catalyst*. (A catalyst is a chemical that increases the rate of a chemical reaction but is not consumed during the reaction.) In the presence of a catalyst, a reaction may proceed millions of times faster than it would in the absence of that catalyst. Nearly all of the biochemical reactions that occur in cells require enzymes to proceed at useful rates. About 4,000 different biochemical reactions are known to be catalyzed by enzymes.

Most enzymes are specific to one reaction. For example, the enzyme *invertase* catalyzes the hydrolysis (splitting) of the disaccharide sucrose (table sugar) into the monosaccharides glucose and fructose, but has no effect on DNA, while the enzyme *DNA polymerase* plays an essential role in DNA replication, but has no effect on sucrose. Because most enzymes are so selective, the types of enzymes made in a cell determine the types of biochemical reactions (*metabolic pathways*) that occur in that cell.

Enzyme activity is affected by many factors, including concentration, pH, and temperature. It can also be increased or decreased by the presence of other organic molecules. Molecules that increase enzyme activity, including some vitamins, are called *enzyme activators*. Molecules that decrease enzyme activity, including many drugs and poisons, are called *enzyme inhibitors*. As proteins, most enzymes can be denatured, reversibly or irreversibly.

In this lab session, we'll investigate some of the properties of *peroxidase*, an enzyme that speeds up the decomposition of hydrogen peroxide millions of times. (That's why hydrogen peroxide foams when you pour it on a cut; the peroxidase enzyme in your blood catalyzes the decomposition of hydrogen peroxide into water and oxygen gas, which causes the foaming.) Hydrogen peroxide occurs naturally, and is a strong oxidizing agent that damages cells. In the absence of a catalyst like peroxidase, hydrogen peroxide decomposes very slowly, and continues damaging cells until it has completely decomposed. In the presence of peroxidase, hydrogen peroxide breaks down very quickly into harmless water and oxygen gas.

Vitamins

A *vitamin* is an organic compound or group of related compounds that is a required *trace nutrient*, but either is not produced by or is produced in insufficient quantity by an organism and so must be obtained from the diet. (Many dietary minerals—such as iron, copper, zinc, and iodine—are also required trace nutrients, but no organism can produce these from simpler precursors, so by convention they are considered essential trace minerals rather than vitamins.)

For example, vitamin C (ascorbic acid) is required by all forms of plant and animal life. Nearly all species produce sufficient ascorbic acid for their own needs, so in these species ascorbic acid is not considered a vitamin. Conversely, a few species—including bats, guinea pigs, some birds and

fish, and some primates, including humans—produce little or no ascorbic acid, and so must obtain it from their diets. For these species, ascorbic acid is a vitamin.

As is true of some proteins, some vitamins must be obtained from the diet only in some circumstances. For example, humans require vitamin D, but we also make it ourselves when our skin is exposed to the ultraviolet wavelengths in sunlight. Under some conditions, we make sufficient vitamin D to meet our needs, but in other conditions we may need supplemental vitamin D from our diets.

Skin color and sunlight intensity have significant effects on vitamin D production. The ancestors of light-skinned people originated at high latitudes, where sunlight is weak, while the ancestors of dark-skinned people originated at equatorial latitudes, where sunlight is intense. Dark skin contains large amounts of the pigment melanin, which blocks UV light, preventing skin damage and melanomas but at the same time reducing production of vitamin D. Light skin contains little melanin, which maximizes production of vitamin D, but does not protect against damage from intense sunlight. Accordingly, someone with light skin exposed to the strong equatorial sunlight may overproduce vitamin D (not to mention suffering a bad sunburn), while someone with dark skin exposed only to the weak sunlight at high latitudes may produce insufficient vitamin D.

Unlike the other biologically-important molecules we've looked at so far, vitamins are classified by their biochemical activity rather than by their structures. The biochemical functions of vitamins are diverse. Some regulate metabolism or cell and tissue growth. Others function as free-radical scavengers (anti-oxidants). But most function as *enzyme cofactors*, which assist enzymes in their catalytic activities.

What constitutes a “trace” also differs from vitamin to vitamin. For example, a multivitamin tablet might contain 100,000 micrograms (µg) of vitamin C, but only 750 µg of vitamin A and only 15 µg of vitamin D3.

In this lab session, we'll use iodometric titration to determine the concentration of vitamin C in urine, how that concentration varies from person to person and over the course of time, and the importance of timeliness in assaying concentrations of some organic compounds in living organisms.

We will attempt to resolve the following:

- The presence or absence of proteins in the various specimens.
- Whether or not general tests for proteins yield positive results for amino acids.
- The denaturation of proteins by various mechanisms.
- Whether an enzyme is denatured by extreme temperatures or heavy-metal ions.
- The effect of pH on the activity of an enzyme and whether that effect is reversible.
- The presence and concentration of vitamin C in a urine specimen.
- Whether the concentration of vitamin C in a urine specimen decreases spontaneously over time.

Procedure III-3-1: Investigating Proteins

Before you begin, prepare a hot water bath by filling the 250 mL beaker about half full of tap water and heating it in the microwave until it comes to a gentle boil. Alternatively, simply bring a pot of water to a gentle boil on the stove, and use that pot as your source of boiling water during this procedure.

Warning: USE EXTREME CAUTION. A microwave oven can actually heat water above its boiling point without causing the water to boil, a phenomenon called superheating. The slightest disturbance can cause superheated water to boil violently, expelling it from the container.

The water needn't be actually boiling, as long as its temperature is close to 100 °C. As the hot water bath cools during these procedures, periodically replace the water with boiling water to maintain the temperature near 100 °C.

Preparing specimens

1. If you have not already done so, put on your goggles, gloves, and protective clothing.
2. Label a centrifuge tube for each of your specimens.
3. Transfer about 2 mL of raw egg white and 10 mL of distilled water to a labeled centrifuge tube. Cap the tube and agitate the contents to mix. This solution contains about a 2% concentration of albumin, the primary protein present in egg white.
4. Transfer one rounded microspatula spoon of unflavored gelatin to a labeled centrifuge tube that contains about 10 mL of cold distilled water. Cap the tube and agitate the contents to mix. Remove the cap and place the tube in the hot water bath. Allow the tube to heat until the gelatin dissolves. Remove the tube, cap it, place it in the rack, and allow it to cool to room temperature.
5. Transfer one rounded microspatula spoon of L-glutamine to a labeled centrifuge tube that contains about 10 mL of cold distilled water. Cap the tube and agitate the contents to mix.

Detecting proteins with the biuret test

The biuret test is a general color test for the presence of proteins. If a specimen contains a protein or proteins, adding a few drops of the specimen to a small amount of biuret reagent causes the color of the reagent to change from blue to violet. The test is specific for proteins, as opposed to the amino acids that are the building blocks of proteins. Testing a pure amino acid with biuret reagent usually results in no obvious color change or at most a slight darkening of the light-blue color of the reagent itself.

1. If you have not already done so, put on your goggles, gloves, and protective clothing.
2. Place the 96-well reaction plate on a sheet of white paper under a strong light.
3. Transfer 8 drops of biuret reagent to each of wells A1, A3, A5, and A7 of the reaction plate.
4. Add 2 drops of distilled water to well A1, 2 drops of the egg-white solution to well A3, 2 drops of the gelatin solution to well A5, and 2 drops of the L-glutamine solution to well A7. Note any color changes, and record your observations in your lab notebook.

The time required for the biuret reagent to react varies with the concentration of the protein(s) present in the specimen and other factors. If no immediate reaction occurs, place the reaction plate aside and recheck it after 10 to 15 minutes. You can be working on other parts of this lab session while you wait.

Testing for the presence of cystine

Cystine is a dimeric amino acid that is formed by the oxidation of two cysteine residues linked via a disulfide bond. Cystine is significant because the disulfide bonds it forms within and between protein molecules are major determinants in the conformation of most proteins. At basic pH,

cystine reacts with lead(II) ions to form an insoluble black precipitate, which is the basis of the cystine test.

1. If you have not already done so, put on your goggles, gloves, and protective clothing.
2. Label two test tubes A and B.
3. Transfer about 1.0 mL of albumin solution to tube A and 1.0 mL of gelatin solution to tube B.
4. Transfer about 0.25 mL of 6 M sodium hydroxide to each of the two tubes and swirl the tubes to mix the solutions.
5. Transfer about 0.25 mL of lead(II) acetate solution to each of the two tubes and swirl the tubes to mix the solutions.
6. Using the test tube clamp, place the tubes in the hot water bath and allow them to remain for about 5 minutes.
7. Carefully remove the tubes from the hot water bath and place them in the rack.
8. Observe the tubes for the presence of a black precipitate, which indicates the presence of cystine in the specimen. Record your observations in your lab notebook.

Denaturing proteins with alcohol, acids, bases, and heavy-metal ions

Denaturing a protein changes it into a different conformation (physical form). We've already seen that heat is a denaturant for some proteins. For example, when you fry an egg the albumin protein in the egg white is denatured, changing from a colorless liquid to a white gel. There's no way to un-fry the egg and convert the denatured albumin back into native albumin. But proteins can be denatured by many other mechanisms, including strong acids or bases, heavy metals, and alcohols. Some proteins are more resistant than others to being denatured by any particular means, and a means that is very effective for denaturing one protein may be less effective or ineffective in denaturing a different protein.

One sure sign that a protein has been denatured is that it coagulates (clumps) and precipitates from solution. The converse is not true; *denaturation*, sometimes called *denaturization*, may occur without coagulation and precipitation also occurring.

1. If you have not already done so, put on your goggles, gloves, and protective clothing.
2. Place the 24-well reaction plate on a black cloth or sheet of paper and illuminate it with a desk lamp or other strong light source.
3. Transfer about 0.5 mL of the albumin solution to each of wells A1 through A6.
4. Transfer about 0.5 mL of the gelatin solution to each of wells C1 through C6.
5. Transfer 5 drops of 6 M hydrochloric acid to each of wells A1 and C1.
6. Transfer 5 drops of 6 M sodium hydroxide to each of wells A3 and C3.
7. Transfer 5 drops of 0.1 M lead(II) acetate to each of wells A4 and C4.
8. Transfer 5 drops of isopropanol to each of wells A6 and C6.
9. After 30 seconds have passed, observe each of the wells carefully for any visual indication that a reaction has occurred. Record your observations in your lab notebook.
10. If a visible change occurred in wells A1 and/or C1 (the wells to which you added acid), the protein in question is denatured at low pH. Determine if that denaturation is reversible by transferring 5 drops of 6 M sodium hydroxide to each well, A1 and/or C1, in which a reaction occurred. Record your observations in your lab notebook.

11. If a visible change occurred in wells A3 and/or C3 (the wells to which you added base), the protein in question is denatured at high pH. Determine if that denaturation is reversible by transferring 5 drops of 6 M hydrochloric acid to each well, A3 and/or C3, in which a reaction occurred. Record your observations in your lab notebook.

Procedure III-3-2: Investigating Enzyme Catalysis

In this procedure, we'll investigate the phenomenon of enzyme catalysis and examine the effects of heat, pH, and heavy-metal ions on the activity of an enzyme catalyst. We'll observe the catalyzed breakdown of hydrogen peroxide solution into water and oxygen, which is evident as bubbles in the solution. Our catalyst is the enzyme peroxidase, which is present in human and animal blood.

One molecule of peroxidase catalyzes the breakdown of millions of peroxide molecules per second, so it's important to use a peroxidase solution of the appropriate concentration. It there is too little peroxidase present, the catalytic breakdown will proceed too slowly; too much, and the peroxide solution will foam uncontrollably. We'll use uncooked meat juice as a source of peroxidase enzyme, and adjust the concentration of the solution to provide a usable level of activity.

Prepare a standard peroxidase solution

1. Transfer about 1 mL of uncooked meat juice to a 50 mL centrifuge tube and fill the tube nearly full with tap water. Cap the tube and invert it several times to mix the solution thoroughly.
2. Transfer about 5 mL of drugstore 3% hydrogen peroxide to a test tube, add one drop of your peroxidase solution, and swirl the tube to mix the solutions. Observe the intensity of the bubbling that occurs.
3. If the concentration is correct, numerous individual bubbles will form rapidly in the peroxide solution. If the peroxidase concentration is too high, rapid foaming occurs, and part of the solution may actually be ejected from the tube. If the peroxidase concentration is too low, bubbles form slowly, if at all.
4. If your solution is too concentrated, empty half the contents of the centrifuge tube and add tap water to bring up the volume to about 50 mL. Retest and repeat the dilution if necessary until you have a peroxidase solution with a usable activity level. If your solution is too dilute, add additional uncooked meat juice and repeat the testing until the peroxidase concentration is usable.

Prepare modified peroxidase solutions

1. Transfer about 5 mL of the standard peroxidase solution to each of six 15 mL centrifuge tubes labeled A through F.
2. Tube A is the unmodified peroxidase solution.
3. Cap Tube B and place it in the freezer until the solution freezes. When it has frozen, remove the tube and allow the peroxidase solution to melt and come to room temperature.
4. Place Tube C in a hot water bath at boiling or nearly so, and allow it to remain in the bath for 10 minutes or so. Remove the tube and allow the contents to come to room temperature.
5. Add 5 drops of 0.1 M lead(II) acetate solution to Tube D. Cap the tube and invert it several times to mix the solutions.
6. Add 5 drops of 6 M sodium hydroxide solution to Tube E. Cap the tube and invert it several times to mix the solutions.

7. Add 5 drops of 6 M hydrochloric acid solution to Tube F. Cap the tube and invert it several times to mix the solutions.

Prepare a neutral solution of hydrogen peroxide

The pH of hydrogen peroxide solutions varies significantly, depending on concentration and the type of stabilizer present. We want a hydrogen peroxide solution that is at neutral pH or nearly so for our tests.

1. If you have not already done so, put on your goggles, gloves, and protective clothing.
2. Transfer about 80 mL of 3% hydrogen peroxide solution to the 100 mL beaker.
3. Use the stirring rod to transfer 1 drop of the solution to a piece of pH test paper. After a few seconds, compare the color of the test paper to the color key.
4. If the solution is acidic, add 6 M sodium hydroxide solution dropwise, with stirring, and continue testing the pH until it is approximately neutral (approximately pH 7). If the solution is basic, use the same procedure, but substituting 6 M hydrochloric acid solution.

Work quickly. Adding acid or base to the hydrogen peroxide solution destroys the stabilizer present in the solution. Unstabilized peroxide quickly breaks down into its components (water and oxygen).

Complete this procedure as quickly as possible after you prepare the neutral peroxide solution. As a final step, re-test some of the peroxide solution with unmodified peroxidase solution to verify that oxygen bubbles are still produced. If they are not, that peroxide solution has already broken down, invalidating your results.

Test the activity of peroxidase solutions

1. If you have not already done so, put on your goggles, gloves, and protective clothing.
2. Label six test tubes, A through F, transfer about 5 mL of the freshly-prepared hydrogen peroxide solution to each tube, and place them in the rack.
3. Transfer one drop of the unmodified peroxidase solution from centrifuge tube A to test tube A, and return any unused peroxidase solution to the centrifuge tube. Swirl the test tube, and observe the formation of bubbles. Record your observations in your lab notebook.
4. Using a clean (or well-rinsed pipette), transfer one drop of the modified peroxidase solution from centrifuge tube B to test tube B, return any unused peroxidase solution to the centrifuge tube, swirl the test tube, and observe the formation of bubbles. Record your observations in your lab notebook.
5. Repeat step 4 for for the remaining four peroxidase solutions, C, D, E, and F.
6. If the peroxidase solutions in centrifuge tubes E (strongly basic) and F (strongly acidic) were about as active as the unmodified peroxidase solution, further adjust the pH of those tubes to even more strongly basic and acidic by adding 10 more drops of 6 M sodium hydroxide solution to Tube E and 10 more drops of 6 M hydrochloric acid solution to Tube F. Swirl the tubes and then retest their contents with fresh hydrogen peroxide solution. Record your observations in your lab notebook.
7. If the peroxidase solutions in centrifuge tubes E (strongly basic) and F (strongly acidic)—either originally or after adding additional base and acid—were inactive or showed noticeably lower activity than the unmodified peroxidase solution, adjust the pH of those tubes to near neutral by adding 5 (or 15) drops of 6 M hydrochloric acid solution to Tube E and 5 (or 15) drops of 6

M sodium hydroxide solution to Tube F. Swirl the tubes and then retest their contents with fresh hydrogen peroxide solution. Record your observations in your lab notebook.

Procedure III-3-3: Assaying Vitamin C Concentration in Urine

Vitamin C, also called *ascorbic acid*, is an essential nutrient, the lack of which causes the horrible disease scurvy. Most mammals produce sufficient vitamin C for their needs; primates (including humans) and guinea pigs do not.

Humans are doubly unfortunate. Not only do we not produce as much vitamin C as we need; we don't store it, either. We must obtain the necessary amount of vitamin C from our diets, and we waste most of the vitamin C we consume. Very little vitamin C is metabolized in the human body; most is excreted unchanged. About 3% of excreted vitamin C is found in the feces, with the remainder excreted in the urine.

The concentration of vitamin C in human urine can vary dramatically, from less than 10 milligrams (mg) per liter (mg/L) to several thousand mg/L. The concentration varies from person to person and from hour to hour for the same person, depending on the amount of vitamin C consumed, frequency and volume of urination, time of day, state of health, and so on. For healthy people, the normal concentration of vitamin C in fresh urine ranges from about 100 mg/L to about 1,000 mg/L.

Vitamin C is a strong reducing agent. We'll use this fact to do a quantitative assay of vitamin C in urine specimens using a procedure called *iodometric titration*. An aqueous or alcoholic solution of iodine is brown. Vitamin C reacts quickly and quantitatively with iodine, reducing the brown elemental iodine to colorless iodide ions and oxidizing the vitamin C to dehydroascorbic acid, which is also colorless. We'll start with an iodine solution and slowly add urine until all of the iodine has been decolorized. By measuring how much urine is required to reach that point, and comparing that value with the amount of a vitamin C solution of known concentration needed to decolorize the same volume of the iodine solution, we can determine how much vitamin C is present in a known volume of that urine specimen.

One molecule of vitamin C reacts with one molecule of iodine, producing one molecule of dehydroascorbic acid and two iodide ions. The gram molecular weight of vitamin C, $C_6H_8O_6$, is 176.126 g/mol, and that of iodine, I_2, is 253.809 g/mol. Because we know that one molecule of vitamin C reacts with one molecule of iodine, we also know that 176.126 grams of vitamin C reacts with 253.809 grams of iodine. Simplifying this ratio, 176.126:253.809, tells us that this reaction consumes about 0.6939 milligrams (mg) of vitamin C per mg of iodine. Or, another way of looking at it, 1.4411 mg of iodine reacts with 1.0000 mg of vitamin C. We'll titrate a known volume of iodine solution with a solution of urine of unknown vitamin C concentration, and use this ratio to calculate the concentration of vitamin C in the urine specimen.

Make up a standardized vitamin C solution

In order to determine the unknown concentration of vitamin C in a urine specimen, we need a comparison standard with a known concentration of vitamin C. The easiest way to obtain such a standard is to dissolve a vitamin C tablet (which has a known mass of vitamin C) in a known volume of water.

Recall that the normal range for vitamin C in human urine is about 100 mg/L to 1,000 mg/L. If we make up our standard solution to a concentration near the center of this range, say 500 mg/L, we can expect that it will contain a roughly similar concentration of vitamin C as our urine sample and that roughly similar volumes of the standard solution and the urine should be needed to neutralize a specific volume of iodine solution. For example, if a given volume of iodine solution is

neutralized by 20 drops of our 500 mg/L standard solution, we'd expect that same volume of iodine solution to be neutralized by anything from 10 drops of urine that contains 1,000 mg/L to 100 drops of urine that contains 100 mg/L.

> Try to obtain a vitamin C tablet that includes only vitamin C as an active ingredient. If your tablet contains an amount of vitamin C other than 500 mg, such as 100 mg or 1,000 mg, adjust the volume of water accordingly.

1. If you have not already done so, put on your goggles, gloves, and protective clothing.
2. Transfer a 500 mg vitamin C tablet to a 100 mL beaker that contains about 50 mL of distilled water. Swirl or stir the contents to dissolve the vitamin C tablet. (Don't worry if some solids remain undissolved; the tablet may contain starch or other insoluble binders.)
3. Pour the contents of the beaker into the 100 mL graduated cylinder. Rinse the beaker two or three times with a few mL of distilled water and add this to the cylinder to ensure that all of the vitamin C present in the beaker is transfered to the graduated cylinder. (This is called doing a *quantitative transfer*.)
4. Fill the graduated cylinder to the 100.0 mL mark with distilled water and stir to mix the contents thoroughly. At this point, the solution contains 500 mg of vitamin C per 100 mL, or 5,000 mg/L, which is 10 times more concentrated than we want.
5. Allow any solids in the graduated cylinder to settle, and then carefully pour 10.0 mL of the solution into the 10 mL graduated cylinder.
6. Rinse the 100 mL graduated cylinder thoroughly, and then transfer the 10.0 mL of solution from the 10 mL graduated cylinder to the 100 mL graduated cylinder. Rinse the 10 mL graduated cylinder two or three times with distilled water, and transfer the rinse water to the 100 mL graduated cylinder.
7. Fill the 100 mL graduated cylinder to the 100.0 mL mark with distilled water, and stir to mix the solution. At this point, the solution contains 50 mg of vitamin C per 100 mL, or 500 mg/L, which is the concentration we want for our standard solution.
8. Fill a 15 mL centrifuge tube with the standard vitamin C solution and cap it. (Vitamin C in solution quickly breaks down, particularly when exposed to air and light, so try to minimize such exposure over the course of this experiment.) Discard the vitamin C solution remaining in the 100 mL graduated cylinder.

> Preparing starch water
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> Even tiny amounts of free iodine react with a starch solution to produce an intensely colored blue-black complex. This phenomenon is used as an indicator for the presence of iodine (or starch). You can use any starch solution for this purpose. (We used some water that we'd cooked pasta in for dinner the previous evening; it keeps for a day or so in the refrigerator.) To prepare starch solution on the fly, simply boil a small amount of potato, rice, or pasta briefly in half a test tube of water.

Assay vitamin C concentrations

1. If you have not already done so, put on your goggles, gloves, and protective clothing.
2. Transfer 2.0 mL of water to each of two clean test tubes, and place those tubes in the rack.
3. Add 10 drops of Gram's iodine to each of the two test tubes and swirl to mix the contents.
4. Working as quickly as possible, draw up a full pipette of the standard vitamin C solution and recap the centrifuge tube.

5. Add the vitamin C dropwise to the first test tube with swirling, keeping track of the number of drops you've added. As you continue adding the vitamin C solution, the color of the contents of the tube gradually fades from brownish to a pale yellow.
6. Once the tube contents appear pale yellow, add a few drops of starch water to the tube. The solution should immediately turn dark, indicating that some free iodine is still present.
7. Continue adding standard vitamin C solution with swirling until the solution in the test tube turns colorless (or very light blue). This may require only one or two more drops of solution.
8. Record the total number of drops of standard vitamin C solution required to neutralize the 10 drops of Gram's iodine in your lab notebook.
9. Repeat steps 4 through 8 using the urine specimen. Given the known volume and concentration of the standard vitamin C solution needed to neutralize 10 drops of Gram's iodine and the known volume of urine needed to neutralize the same amount of Gram's iodine, calculate the concentration of vitamin C in the urine specimen in mg/L and record that value in your lab notebook.

The color of pee

Urine may be anything from nearly colorless to a fairly deep yellow. Urine voided early in the morning is often darker in color and urine voided during the day is usually paler. Yellow urine presents an obvious problem. As you near the endpoint of the titration, the iodine solution fades to a pale yellow color, which can easily be masked by the color of the urine itself. In this situation, the best option is to add the starch indicator solution before you begin the titration. The intense blue color of the complex formed by iodine and starch causes the yellow urine to appear dark blue black. As the color changes to blue-green and then to green, the titration is very near the endpoint. You'll need to add only a drop or two more titrant to complete the titration.

So why not always add the starch indicator at the beginning of the titration? Because the color change from dark blue to colorless is extremely sharp. One drop of titrant may suffice to turn the solution from dark blue to colorless, so you have to add titrant very slowly and carefully to avoid missing the endpoint. If the urine is colorless, you can watch the color of the solution gradually change from dark brown-yellow to pale yellow, so you can titrate much faster until the endpoint approaches.

Review Questions

??? RBT: review questions/answers and add additional material after completing the labs.

??? Production: Please strip the answers (formatted as comments) from the final layout. RBT

1. When you tested the reaction of biuret reagent with the various specimens, what was the purpose of adding only distilled water to well A1? What color changes did you observe with the biuret reagent, and from those observations what do you conclude about the makeup of the various specimens?

   Well A1 served as a control well. We need a control well to determine the appearance of the reagent in the absence of any specimen that contains a protein. Well A1 remained light blue, indicating that nothing was present to react with the biuret reagent. Well A3 (egg white) and well A5 (gelatin) assumed a violet color, indicating the presence of protein(s) in those specimens. Well A7 (L-glutamine) showed a change to slightly darker blue relative to A1, which is characteristic of a specimen that contains amino acid(s) but not a protein.

2. In the denaturation tests, what purpose did wells A2, A5, C2, and C5 serve?

   These were control wells, which contained only the proteins in question. Using columns 2 and 5 meant that each tested well (columns 1, 3, 4, and 6) was adjacent to a control well for easy comparison.

3. Why did we adjust the pH of the drugstore 3% hydrogen peroxide to near neutral?

   One of the variables we tested was the effect of pH on enzyme catalysis activity. We wanted the peroxide solution to be near neutral to establish a baseline activity before testing activity at low and high pH values.

4. What activities did you detect with the standard and modified peroxidase solutions? What do you conclude about each modification?

   Tube A (unmodified peroxidase solution) provided our benchmark activity level. Tube B (frozen and thawed) had an activity level identical to tube A, indicating that freezing has no effect on peroxidase activity. Tube C (heated) was inactive, indicating that high temperatures irreversibly inactivate peroxidase. Tube D (lead ions) showed no change in activity level, indicating that peroxidase is not inactivated by the presence of these heavy metal ions, at least at the relatively low concentration we tested. Tubes E (basic) and F (acidic) were inactive, but activity returned when the peroxidase solution was neutralized, indicating that peroxidase is temporarily denatured at high or low pH, but not permanently denatured.

5. What key assumption do we make in using iodometric titration to determine the concentration of vitamin C in urine?

   That nothing other than vitamin C that is present in the urine reacts with the iodine.

6. Many texts recommend using a dilute solution of sodium thiosulfate to remove iodine stains. What alternative can you propose?

   A vitamin C solution, of course. Like sodium thiosulfate, vitamin C reacts with iodine to produce colorless iodide ions.

7. What implication does the high concentration of vitamin C typically present in urine have for the human diet?

   Vitamin C is eliminated quickly. Its absence over a period of days to months causes increasingly serious and eventually fatal health problems. Humans cannot store vitamin C, so it's essential that it be a regular part of our diet.

8. We may have been mistaken about the stability of vitamin C in solution. Design an experiment to determine if vitamin C in urine in fact degrades when exposed to air and light and, if so, how quickly. Or perhaps the vitamin C concentration in urine decreases over time, regardless of exposure to air or light. State your hypothesis, the results you expect, and your proposed experimental procedure.

   Answers will obviously vary in details, but here is one possible design.

   Obtain urine specimens first thing in the morning by filling a foam cup and several centrifuge tubes with urine. Cap the tubes and store them in the dark. Immediately titrate the specimen to determine the concentration of vitamin C present in the urine initially.

   Allow the urine specimen to sit in the open foam cup, exposed to air and light. After 30 minutes, withdraw another aliquot from the container and titrate it and one of the specimens that has been stored in the dark to determine the concentrations of vitamin C remaining in the specimens. Repeat the titration after total elapsed times of 1 hour, 2 hours, 4 hours, and 8 hours.

We were not mistaken. Vitamin C in solution does degrade when exposed to air and light. How fast it does so depends on many factors, so your experimental results will no doubt differ from ours, but should show significant degradation, particularly after 8 hours. The specimens stored in the dark should show little or no reduction in vitamin C concentration.

9. Chemicals that are excreted in the urine (whether unchanged or as metabolytes) are said to have a biological half-time (BHT). That is, after one BHT has passed, half of the material has been excreted, after two BHT's have passed, three quarters has been excreted (the original half plus half of a half, or a quarter), and so on. This is important for many pharmaceuticals. For example, in order to maintain effective serum levels of an antibiotic, the rate at which that antibiotic is excreted must be known and taken into account. An antibiotic with a BHT of 24 hours might be administered every twelve hours, while one with a BHT of twelve hours might be administered every six hours, twice as often.

   Design an experiment to determine an approximate BHT for vitamin C.

   Once again, answers will obviously vary in details, but here is one possible design.

   Use iodometric titration to obtain a baseline value for the concentration of vitamin C in your own urine. Take as large a dose of vitamin C as you are comfortable taking, with plenty of water. Note the time when you swallow the dose. After 30 minutes has passed, obtain a urine specimen and titrate it to determine the vitamin C concentration. Obtain additional urine specimens every 30 minutes—timed as closely as possible; it helps to drink a lot of water initially and as the experiment progresses and to urinate as little as possible when you produce each specimen. Record the time of each specimen and titrate it immediately. Continue obtaining specimens and titrating them for several hours, or until the vitamin C concentration in the most recent specimen approaches the baseline figure. Graph concentration against time to determine whether vitamin C concentration in urine follows the BHT half-life law and, if so, what that half-life is.