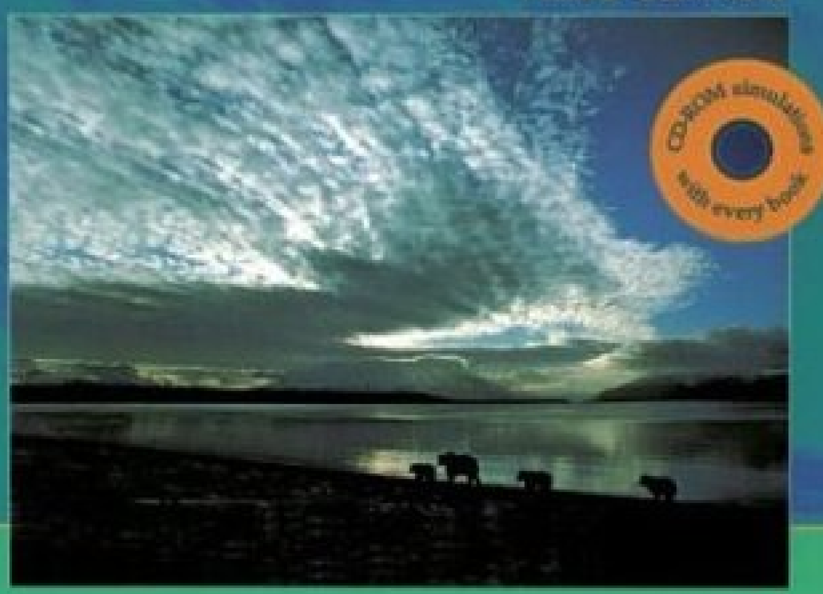
Biology in the Laboratory

THIRD EDITION



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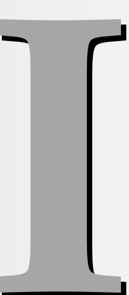
LABORATORY



Science—A Process

OVERVIEW

Science is a way of examining and finding order in the natural world. It is a dynamic process of asking questions and then seeking answers. Observations lead us to formulate questions and, with our limited knowledge, we may offer tentative explanations or make educated guesses about the answers to our questions.

Scientists call a tentative explanation a **hypothesis.** Experimentation follows, providing information that may support or refute a hypothesis. From data, often reinforced by statistics, conclusions can be made about what it is we wish to know.

In this laboratory, you will investigate reaction time—the length of time it takes to react to a stimulus. If available, a computer-generated reaction time program will be used. A less complex series of tests can be conducted without the use of a computer. By formulating hypotheses, designing experiments, and analyzing data, you will engage in the process of science.

> **Please Note:** During your laboratory course, you will often be required to formulate hypotheses and design investigations in the Procedure section of an exercise. You will also have the opportunity to design your own experiments to apply what you have learned. These experiments are designated as Extending Your Investigation. Some of these are designed as class experiments; others are intended to be carried out at home.

Laboratory I provides you with the basic tools to carry out scientific investigations and the statistical tools to evaluate your results.

STUDENT PREPARATION

Before coming to the laboratory, complete Exercise A and steps 1 and 2 of Exercise F.

EXERCISE A The Scientific Method

The scientific approach is a powerful method for understanding the natural world because it is based on observations of how the world works. However, not just any observations will do: the observations must be systematic and objective. The method for making these observations is sometimes broken down into a series of steps referred to as the **scientific method.**

The scientific method of inquiry is an important part of everything we do in our daily lives; we simply do not recognize the steps because we are so used to them. Scientific inquiry involves the steps outlined below.

Problem You want to find out whether a combination of anti-cholesterol drugs X and Y is more effective in reducing high cholesterol levels than either of the drugs given separately. You might proceed as follows.

Step 1 Make **observations** that lead to the formulation of a question.

> You observe that drugs X and Y, used independently, lower LDL (low-density lipoprotein) levels in the blood. You question whether drug X plus drug Y, given in combination, would be even more effective.

Step 2 The question leads to a tentative explanation or educated guess—a **hypothesis.** Prior knowledge or research or even intuition can contribute to the formulation of a hypothesis. The hypothesis must be tested in a way *that allows it to be proven false.* We can never prove that a hypothesis is true, but we can support the hypothesis if repeated experiments do not falsify it.

> You formulate the following hypothesis: If individuals with high cholesterol levels are treated with a combination of drugs X and Y, their cholesterol levels will be lowered more than for similar groups treated with drug X alone or drug Y alone.

For statistical reasons (to be covered later), you also devise a **null hypothesis** or prediction of what would happen if the experimental treatment has *no* effect.

> The null hypothesis is that cholesterol will be lowered by the same amount with all three drug treatments.

Step 3 Make **predictions** about the results you would expect if the hypothesis is correct. In this way, scientists begin to formulate an experimental design. Hypotheses are often stated in the form of predictions.

> You predict that treating individuals with drugs X and Y in combination will be more effective than treatment with either drug alone. As part of your experimental design, you know that you will need to compare at least three experimental groups, and you may begin to plan how to identify participants for your study.

Step 4 Clearly define the experiment's independent, dependent, and standardized **variables.** The **independent variable** is the factor that is being manipulated in the current experiment.

> The independent variable is the type of drug treatment. (In the experiments you will be performing in this laboratory, you will usually deal with only one independent variable at a time.)

The **dependent variable** is the aspect of the system that is showing some response to the manipulations of the independent variable.

> The dependent variable could be any of the many aspects of an individual's condition that define the difference between life-threatening high cholesterol levels and lower levels typical of healthy individuals.

The **standardized variables,** or **controlled variables,** are all the variables that are held constant between the treatments.

> The way the drugs or drug mixture are administered, the frequency with which the subjects are checked, and the average ages and general health characteristics of the individuals assigned to the treatments are all standardized variables.

Step 5 Define the **experimental treatments.** A treatment is a test group of individuals that are subjected to the same levels of the independent variable.

> The group that gets drug X alone is one treatment, the group that gets drug Y alone is another treatment, and the group that gets both drug X and drug Y is the third treatment.

Step 6 Select materials and identify experimental methods and methods of data collection and analysis, as part of a well-planned **experimental design.** These are incorporated into a **procedure** that tests whether the predicted results occur.

You identify a large group of individuals who have high cholesterol levels but are otherwise healthy. You randomly assign them to groups that will get drug X alone, drug Y alone, and drugs X and Y. You make sure that all the treatments have a fair chance against one another by ensuring that they all have subjects with a similar range of ages, health, previous treatment histories, and so forth. If you do not do this and one treatment ends up with most of the younger, healthier patients and the other with most of the older, sicker ones, it will be impossible to say whether the results of the experiment are due to the drugs or to the biased selection of people entering each treatment.

The experiment should also have a fourth **control treatment.**

In the control group, individuals with high cholesterol get *no* drug treatment but are given placebos and are held to the same standardized variables.

Step 7 **Perform experiments** and collect data.

You make sure that all participants who will be taking the drugs understand the drug dosages and administration conditions that the experiment demands, that the subjects are checked frequently, and that all medical personnel are using the same definition of high cholesterol. You should pre-plan procedures for dealing with inevitable problems such as patients who miss drug treatments or drop out of the program.

Step 8 Analyze **results** (data) from the experiments that test the hypothesis, using statistics when necessary, and interpret these results to determine whether the hypothesis is supported or falsified. This leads to a **conclusion.**

Step 2 included the statement of a null hypothesis, or prediction of what would happen if no treatment effect occurred. While the null hypothesis may seem negative and uninteresting, it is important because most statistical techniques can only test a null hypothesis. Therefore, you will probably end up concluding either that **the data allowed us to reject the null hypothesis** (meaning that there was a treatment effect), or that **the data did not allow us to reject the null hypothesis** (meaning that there was no evidence of a treatment effect). You *cannot* say, "We proved there was an effect" or "We proved there was no effect." Although it is common to talk about "experimental proof," experiments do not *prove* anything. **Experiments can only offer evidence that either supports, or fails to support, hypotheses.**

Step 9 **Repeat the process,** using a more refined question about the system.

Assume that the combination of drugs was more effective than either drug taken individually. Next, you might ask if the best results are obtained when the two drugs are given in equal or in unequal amounts. Or, you might ask if a combination of drugs X, Y, and Z is more effective than a combination of just X and Y.

The usual result of an experiment is more questions.

Objectives

- ☐ Recognize the stages of scientific inquiry as it applies to everyday experiences.
- ☐ Make observations, formulate hypotheses, make predictions, and design experiments to test hypotheses.

Procedure

You have just received a grade on your first major examination in biology, and it is not as high as you had hoped. You wanted an "A" and you earned a "C." You talk to your professor and do a lot of thinking about what might have gone wrong. Perhaps your mistake was reviewing your notes only before the exam rather than every night. Or perhaps you made some other mistakes.

1. Using this scenario, apply the steps of the scientific method to identify what you might do to raise your grade on the next exam.

Step 1 Make observations that lead to a question. ____________________

Step 2 Formulate a hypothesis. ____________________

Step 3 Make predictions based on this hypothesis. ____________________

Step 4 Define the independent and dependent variables. ____________________

Step 5 Define the experimental treatments. ____________________

Step 6 Design your experiment. ____________________

Step 7 Perform the experiment and collect data. ____________________

Step 8 Analyze your data. ____________________

Step 9 Plan a more refined experiment. ____________________

2. Now, suppose your grade on the next examination remains low. *How could this cause you to revise your conclusions about your original hypothesis?* ____________________

EXERCISE B Reaction Time Experiments: Making Observations

One of the basic features of life is that living things react to stimuli from the environment. This may be as obvious as a frog hopping away as you approach, or as subtle as a plant changing its pattern of hormone secretion in response to increasing day length in the spring.

Reaction time is the length of time it takes to begin a response to a stimulus. Any number of stimuli can evoke specific responses. For instance, seeing movement out of the corner of your eye could cause you to turn your head. Another example might be slamming on the brakes when an animal runs out in front of your car. From your normal day-to-day life, list some stimuli and their associated, observed responses. Be careful not to extend your observations into inferences (*inferences* are explanations or interpretations of observations).

In this experiment, you will use a computer program to test your reaction time. The program presents a stimulus and then uses the computer's internal clock to measure the time it takes you to respond to the

Stimulus	Observed Response

stimulus by pressing the spacebar. If the computer program is not available, you will test your ability to catch a ruler dropped between two of your fingers—can you react fast enough to catch it?

Objectives

- ☐ Make observations about responses to stimuli.
- ☐ Make observations about differences in reaction times.

Note: If you are *not* using a computer for this exercise, skip to Exercise B, Part 2.

PART 1 Making Observations (*for Laboratories Using Computers*)

Procedure

1. Your instructor will provide you with directions for loading the reaction time program.
2. After starting the program, choose *Collect some reaction time data.*
3. Choose *Use just the reaction time program.*
4. The reaction time program will present you with a stimulus and then ask you to respond by pressing the spacebar. The stimuli can be either visual or auditory, and simple or complex. Possible reaction time tests include:
   - **X at a known location** An "X" appears in the middle of the screen.
   - **Spot the dot** A period appears somewhere on the screen. The period may be either high-contrast (white on a blue background) or low-contrast (black on a blue background). The dot may appear on a small area of the screen or anywhere on the whole screen.
   - **Symbol recognition** You are given a list of 1 to 10 letters. Then letters appear in the middle of the screen and you press the spacebar *only* if the letter is on your list. For example, if your list is "A G B," you press the spacebar if you see an A, or G, or a B, but *not* if you see any other letter.
   - **Reaction to sound** The computer sounds a tone.
   - **Tone recognition** The computer sounds either a low tone or a high tone. You press the spacebar only if the *high* tone sounds.
5. Take some time to become familiar with what each test requires.
6. In the space below, record observations about your reaction time on the different tests, possible environmental effects on your reaction time, or differences in reaction times among individuals.

These observations will serve as information for formulating a hypothesis. You will work in a laboratory group of eight (four pairs), so share your observations with others in your laboratory group. Once you are familiar with the program, you and the other members of your lab group will devise a reaction time hypothesis (Exercise C) and an experiment to test your hypothesis (Exercise D). You will then collect and analyze your data (Exercises E–G).

PART 2 Making Observations (*for Laboratories Not Using Computers*)

Procedure

1. Work in pairs. Place a ruler (preferably stainless steel) in the crevice formed by the second (index) and third fingers of your partner's preferred hand (right, if right-handed; left, if left-handed). Orient the ruler so that the 1-cm mark is downward (Figure IB-1). Now ask your partner to open the two fingers as wide as possible.
2. Drop the ruler. When caught, record the millimeter marking at the top surface of the fingers.

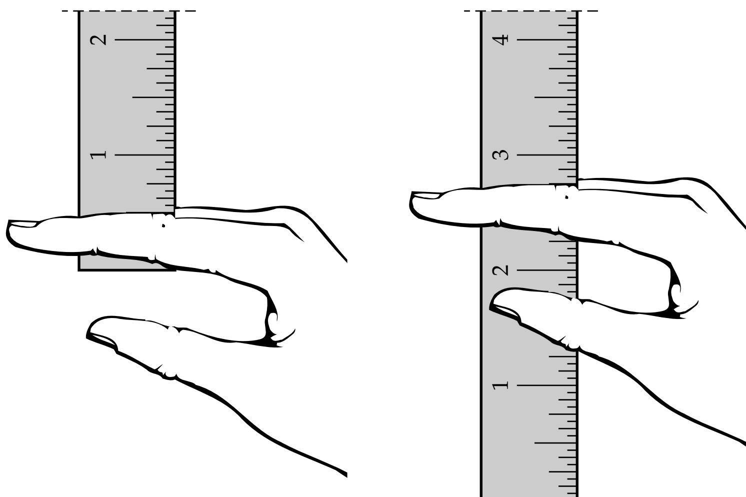


Figure IB-1 *Positioning of ruler for reaction time experiment.*

3. Try this technique several times. Make sure that the original position of the ruler, before dropping (step 2), is the same each time. In the space below, record observations about the mechanics and "times" of reactions (measured as number of millimeters) for different partners. Are there any environmental or individual differences that might be affecting reaction times?

These observations will serve as information for formulating a hypothesis. You will work in a laboratory group of eight (four pairs), so share your observations with others at your laboratory table. Once you are familiar with the mechanics of the exercise, you and the other members of your lab team will devise a reaction time hypothesis (Exercise C) and an experiment to test your hypothesis (Exercise D). You will then collect and analyze your data (Exercises E–G).

EXERCISE C The Reaction Time Experiments: Formulating a Hypothesis

Use of our general and perhaps nonsystematic observations often leads to devising a question about the observed system. A **hypothesis** is a question, often stated in the form of an educated guess or possible answer (tentative explanation) to a question. Hypotheses lead to predictions—indeed, hypotheses are often stated as predictions. Two additional criteria must be met by a hypothesis:

1. We must have a hypothesis that can be **falsified** (refuted or proven false). If there is no possibility of proving that a hypothesis is false, then it cannot be tested. Why? Because we can never "prove" that a hypothesis is "true." We can only add to the body of evidence that **supports** a hypothesis.
2. The hypothesis must focus on a limited, specific, **well-defined** problem. A hypothesis that is too broad often entails consideration of multiple variables that confuse our interpretation of results.

Formal testing of a hypothesis requires that we distinguish between two alternative possibilities:

1. The variable being manipulated has an effect.
2. The variable being manipulated has *no* effect.

The second possibility ("no effect") is often referred to as the **null hypothesis** because it states the alternative possibility—that no effect occurs. A null hypothesis is a statement of "no relationship."

A null hypothesis must also be falsifiable. If we can falsify the null hypothesis, our results support the hypothesis. If, in testing our hypothesis, we find that the data support the null hypothesis, we need to evaluate both the design of the experiment and the usefulness of the hypothesis. Scientists generally accept the null hypothesis unless they have evidence that causes them to reject it.

Objectives

- ☐ Formulate a hypothesis and null hypothesis that can be used to investigate differences in reaction times.

Note: If you are *not* using a computer for this exercise, skip to Exercise C, Part 2.

PART I Formulating a Hypothesis (*for Laboratories Using Computers*)

In this exercise, you will devise and test a hypothesis involving reaction time. For instance, you may wish to investigate whether there is a difference in response time to different stimuli or whether response times differ between two or more groups (male vs. female, athlete vs. nonathlete, etc.).

Example Seated around a laboratory table are six band members. Three of the students are woodwind players and the other three are percussionists. Both groups believe they have the fastest responses. They decide to settle the dispute like scientists, so they develop a simple investigation.

Observation: More and faster finger movement is required to play a woodwind instrument than a percussion instrument.

Hypothesis: Woodwind players will have faster reaction times than percussionists in the test "X at a known location."

Null hypothesis: There is no difference in the reaction times of woodwind players and percussionists.

Procedure

With others in your group, use your observations from Exercise B to develop a reaction time hypothesis and null hypothesis. After agreement has been reached, supply the information below.

Stimulus used: ______________________________

Observation: ______________________________

Hypothesis: ______________________________

____

Null hypothesis: ______________________________

____

PART 2 Formulating a Hypothesis (*for Laboratories Not Using Computers*)

In this exercise, you will devise and test a hypothesis involving reaction time. For instance, you may wish to investigate whether response times differ between two or more groups (male vs. female, athlete vs. nonathlete, etc.).

Example Seated around a laboratory table are six band members. Three of the students are woodwind players and the other three are percussionists. Both groups believe they have the fastest responses. They decide to settle the dispute like scientists, so they develop a simple investigation.

Observation: More and faster finger movement is required to play a woodwind instrument than a percussion instrument.

Hypothesis: Woodwind players will have faster reaction times than percussionists for catching a ruler.

Null hypothesis: There is no difference in the reaction times of woodwind players and percussionists.

Procedure

With others in your group, use your observations from Exercise B to develop a reaction time hypothesis and null hypothesis. After agreement has been reached, supply the information below.

Stimulus used: ______________________________

Observation: ______________________________

Hypothesis: ______________________________

____

Null hypothesis: ______________________________

____

EXERCISE D The Reaction Time Experiments: Developing an Experimental Design

To test a hypothesis, you have to design an experiment. The design process often begins when you make predictions from your hypothesis. Next, you must identify your experimental variables and treatments. You must also determine how to control all other factors that might influence your results. Finally, you should consider how you want to collect and analyze data.

PART 1 Identifying Variables (*for All Laboratories, Using or Not Using Computers*)

Developing a good experimental design requires the experimenter to define what factors will be varied or held constant during the test. The **independent variable** can be anything the experimenter decides to manipulate (vary)—density, temperature, light intensity, altitude, concentration, to name a few of the hundreds of possibilities. Probably the most commonly used independent variable is time: the experimenter takes measurements once a day, every 2 hours, every 2 minutes, or whatever time period is appropriate for the experiment.

Example Suppose you want to test the effect of time of day on reaction time. Do you react more quickly at 7 A.M. or at noon? The independent variable is time of day.

a. The independent variable in an experiment on the effect of time of day is ______________________________

The **dependent variable** is the aspect of the system that is showing response to the manipulations of the independent variable. It is what is measured (or counted or somehow recorded) at different "settings" or levels of the independent variable. An experiment may have several dependent variables—all of the

things that are affected and can be measured as you manipulate the independent variable. In any experiment, however, you should always strive to have only one independent variable at a time.

b. The dependent variable in an experiment on the effect of time of day is ______________________________

Since an experiment should have only one independent variable, all other things that could vary during the experiment must be kept constant or be controlled. These are the **controlled variables** or **standardized variables.**

c. What would happen if there were more than one independent variable in an experiment on the effect of time of day? (For example, suppose all your morning readings were just after breakfast, but all your noon readings were just before lunch? ______________________________

____

d. What conditions would you want to keep constant in an experiment designed to measure the effect of time of day on reaction time? ______________________________

____

Objectives

☐ Identify and distinguish between dependent, independent, and controlled variables.

Procedure

Identify the dependent, and controlled variables for your reaction time experiment.

Dependent variable(s): ______________________________

Independent variable: ______________________________

Controlled (standardized) variables: ______________________________

PART 2 Defining Experimental Treatments (*for All Laboratories, Using or Not Using Computers*)

A **treatment** is a test group (or treatment group) of individuals that are subjected to the same levels of independent variable. For instance, you might make the following comparisons:

Same group of subjects given two different tests:

Treatment 1 Women given test A.

Treatment 2 Women given test B.

Two different groups of subjects given the same test:

Treatment 1 Men given test A.

Treatment 2 Women given test A.

Same group of subjects given same test at two different times or under two different conditions:

Treatment 1 Women given test A before lunch.

Treatment 2 Women given test A after lunch.

It is also possible to have more than two treatments:

Treatment 1 Women given test A before lunch.

Treatment 2 Women given test A 1 hour after lunch.

Treatment 3 Women given test A 2 hours after lunch.

Treatment 4 Women given test A 3 hours after lunch.

In the experiments listed above, all subjects in each treatment or test group (treatment group) experience the same manipulation or level of independent variable. But how can the experimenter assure that the outcome is in fact due to manipulation of the independent variable? This is usually done by running a **control** for the experiment.

When comparing two treatments, one treatment group may serve as the *control treatment* and the other as the *experimental treatment* exposed to the independent variable. In before-and-after tests, the "before" conditions often serve as the control treatment. It *is* possible to have no true control group (for example, when you compare the reaction times of men and women).

Objectives

☐ Identify treatment groups in an experiment.

Procedure

For your reaction time experiment, define the treatment groups (try to limit your experiment to two treatment groups).

Treatment 1: ______________________________

Treatment 2: ______________________________

a. Do you have a "control" group for your experiment? ______________________________

____

b. Explain why or why not. ______________________________

PART 3 Defining Data Collection and Analysis Procedures (*for All Laboratories, Using or Not Using Computers*)

Once you know what your variables are and have identified your treatment groups, you should list the steps you will follow to test your hypothesis. You should also consider how you want to collect data; the steps you take to collect data often determine (and limit) the ways in which you can analyze the data.

For the reaction time experiments, you will need to decide what kind of analysis (for statistical purposes) you want to perform on your data. You have a choice of a **paired** or **unpaired** analysis. The following rules will help you decide this question:

Paired analysis The same individuals are used for each treatment (both treatment groups contain the same individuals)—typical of before-and-after experiments.

Unpaired analysis Different individuals are used for each treatment (each treatment group is a different group of individuals).

Example Suppose you want to look at before-and-after treatments. You are working in a group of four students: A, B, C, and D. An unpaired test would randomize the four individuals and treat them as two different treatment groups, comparing all observations in treatment 1 with all observations in treatment 2. It would be better to treat this as a paired test, comparing before and after for the same individuals (compare first observation in treatment 1 with first observation in treatment 2). For a paired test, be sure to test individuals in the same order! (See Appendix I, Part B.)

Unpaired			Paired		
Treatment 1		Treatment 2	Treatment 1		Treatment 2
A, B, C, D	← random →	C, A, D, B	A, B, C, D	← paired →	A, B, C, D

Objectives

- ☐ Design an experimental procedure.
- ☐ Determine the type of analysis appropriate for an experimental design.

Procedure

1. In the space below, list the steps of your experimental procedure.

2. Determine whether your data analysis should be conducted as a paired or unpaired test.

   Type of analysis: ______________________________

   a. *Why did you choose this form of data analysis?* ______________________________

   ____

There is no substitute for a good experimental design: formulation of a clear, falsifiable hypothesis and null hypothesis, predicting outcomes, defining variables, and identifying treatments. The following suggestions will help you design valid experiments.

Suggestions for Designing Experiments

1. **Use adequate replication.** If you want to generalize your results to a larger group, you should have at least 10 different people in each treatment (when testing one group against another), or at least 10 people (when doing a before-and-after test on the same individuals). This will probably require you to go outside your lab group to get enough people. If you *cannot* get 10 people, you will have to note that small sample size has weakened the conclusions of your experiment. Perhaps you will only be able to draw conclusions about the individuals tested, not broader groups such as all men or all women.

   Regardless of the number of people, collect at least 10 reaction times per person per treatment. For example, if you are testing men versus women, each person should do 10 reaction times. If you are testing effects of caffeine, each person should do 10 reaction times before caffeine and 10 after.
2. **Do not try to make up for a small number of individuals by having each person perform more tests.** All this will yield is increasingly precise estimates of the reaction times of these particular individuals. If Bob is the only man in a men-versus-women experiment and Bob does 100 reaction time tests, this doesn't make Bob any more representative of men as a group.
3. **Avoid bias.** Bias occurs when one treatment has an advantage or disadvantage that has nothing to do with the independent variable. For example, in a men-versus-women experiment, let's say that all the men are athletes, and athletes have faster reaction times. If the results show that men have faster reaction times, is this because these individuals are male or because they are athletes?

   A common kind of bias relates to the time the tests are done. For example, say that in a men-versus-women study, all the women do the test first, then all the men. If the men watch the women, they may learn tricks that will improve their own performance. One way to combat this problem would be to have men and women alternate as they do the tests.
4. **Do not allow fatigue to become a factor.** It's better to have each individual do several short series of reaction time tests rather than complete all tests at one long sitting. This will also help with the "who goes first" problem. For example, say a group of students is doing a men-versus-women experiment and wants to have each individual log 10 reaction times. It would be better to have men and women alternate doing five tests at each sitting rather than have each person try to complete all 10 tests at once.

EXERCISE E | The Reaction Time Experiments: Conducting the Test

Once the experimental design and procedures have been identified and materials have been gathered to conduct your experiments, you are ready to test your hypothesis. Observations and data must be collected in a systematic way. A laboratory notebook or log is a must.

Another important aspect of experimental design is replicating the experiments. If you do something only once, you cannot be sure your results are valid.

a. What is the advantage of increasing the number of replications? ______________________

____

You should *not* expect results from test to be the same—you cannot control everything. Some variation will always be present. Most experimental data give us an average result of many experiments carried out under identical—or as nearly identical as possible—conditions.

Objectives

- ☐ Conduct a reaction time test.
- ☐ Collect data in a systematic fashion.

Procedure (*for All Laboratories, Using or Not Using Computers*)

Identify and describe all parts of your reaction time experiment.

Hypothesis: ____

____

Null hypothesis: ____

____

Prediction: ____

____

Independent variable: ____

Dependent variable: ____

Standardized variable(s): ____

____

Treatment 1: ____

Treatment 2: ____

Procedure: ____

____

Type of data analysis (paired or unpaired): ______________________

Note: If you are *not* using a computer for this exercise, skip to Exercise E, Part 2.

PART 1 Conducting the Reaction Time Experiments (*for Laboratories Using Computers*)

Procedure

1. The directions that follow present some steps for using the computer to study reaction times. Your instructor will provide you with additional information for your specific computer.
2. After starting the program, choose *Collect some reaction time data.*
3. Since you have already worked with a selection of tests and have formulated your hypothesis and designed your procedure, you are ready to conduct your experiment to analyze the data.

(You will do this using a statistical package that is part of the reaction time program.) Choose *Use the reaction time program and then perform an immediate statistical analysis.*

4. Enter how many treatments you are using (usually two, but certainly more than one) and press ENTER.
5. If you want to repeatedly use *exactly* the same reaction time test, answer yes (Y) to the question asking about this. If you want to make the slightest alteration (like using high-contrast spot-the-dot one time and low-contrast another time, or using one-letter symbol recognition one time and three-letter another time), answer no (N) to the question.
6. For the purpose of data analysis, you must decide which kind of statistical analysis you want—a *paired* or an *unpaired* analysis. Indicate which you are going to use. You should use a paired analysis *only* if you have two treatments and will be using the same individuals in the same order, with the same number of observations in each treatment.
7. Choose the reaction time experiment you would like to do. As you set up each experiment, you will be asked to what treatment this group of observations belongs. There is no need to enter treatment 1 first and then treatment 2. If you want to do treatment 2 first, type in that the treatment is 2 as you set up your first experiment.
8. As you finish each set of tests in your experiment, you will be asked if you want to add the observations to the treatment. If the data are valid, indicate yes (Y). Then the next group of tests will begin. There is no need to write down the results of each test. The computer will keep track of the data for you.
9. *Do not* indicate that this is the end of the experiment unless all students (all treatment groups) have completed all of their tests and you want the final statistical analysis.
10. When all data have been collected, the program will send them for statistical analysis. Record your results in the tables below. (You must have at least two treatments.) Chi-square and probability data should be recorded for use in Exercise G.

Treatment	Average

For an Unpaired Analysis

Treatment	Below Median	Above Median

Chi-square = __________

Probability = __________

For a Paired Analysis

Treatment 1 Higher	Treatment 2 Higher

Chi-square = __________

Probability = __________

11. After completing the data tables, continue to Exercise F and Exercise G, Part 1. **You will use the data recorded above for these exercises.**

PART 2 Conducting the Reaction Time Experiments (*for Laboratories Not Using Computers*)

Procedure

1. Conduct your "drop the ruler" reaction time experiment, collecting at least 10 reaction times (trials) per person or subject. Remember, if you are conducting a before-and-after paired test, collect 10 values for "before" and 10 for "after" for each individual, keeping the individuals in the same order.
2. Record your data for treatments 1 and 2 in the tables below.

Treatment 1 (Treatment Group 1)

Trial	Subject							
	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
Mean								

Treatment 2 (Treatment Group 2)

Trial	Subject							
	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
Mean								

3. After completing the data tables, continue to Exercise F and Exercise G, Part 2. **You will use the data recorded above for these exercises.**

EXERCISE F Presenting Experimental Data

Data consist of information that can be measured or counted and recorded. Various types of tables or graphs are used for presenting data. You must decide how the data are best presented so that your results can be readily communicated to others. Scientists must be able to accurately graph data collected from their experiments, and must be able to interpret graphs showing results from experiments done by other people.

Objectives

☐ Present experimental data using appropriate formats.

Procedure

Complete steps 1 and 2 before coming to the laboratory. Step 3 requires data collected during the laboratory period.

1. Data are usually presented visually in the form of **tables, line graphs,** or **bar graphs.** To determine which type of presentation is appropriate for your data, you must first decide whether your experimental variables (dependent, independent, and standardized) are **continuous** or **discrete.** Knowing this, you can present your data in the most effective way. Review Appendix I, Part A, before proceeding to step 2.
2. The data in Table IF-1 are results from an experiment on reaction time. Subjects were exposed to various volumes (decibels) of three types of background music and, while listening, were asked to respond to observing an "X" that would appear at random intervals in the center of their computer screen (see Exercise B, Part 1, step 4).

Table IF-1 Reaction Time (in milliseconds) to "X at a Known Location" Test for Male and Female Subjects with Different Levels of Background Noise

Decibel Level	Country Music		Classical Music		Rock Music	
	Male	Female	Male	Female	Male	Female
60	226	241	259	252	245	238
	243	239	276	250	262	236
	292	237	325	248	311	234
70	218	232	251	243	241	234
	222	219	255	230	245	220
	220	224	253	235	243	225
80	187	224	220	235	215	230
	224	221	257	232	252	227
	221	223	254	234	249	229
90	233	232	266	243	264	264
	235	234	268	245	266	243
	233	241	269	252	267	250
100	246	239	279	250	286	257
	223	221	256	232	263	239
	229	271	262	282	269	289

Many different hypotheses could be tested using the above experiment. After each of the following hypotheses (*a* through *e*), write the null hypothesis and identify the format (table, bar graph, or line graph) you would use to present the data.

a. Women will have a faster reaction time than men when country music is being played.

Null: ______________________________

Presentation format: ______________________________

b. Increasing the volume of rock music will affect the time it takes to react to the stimulus.

Null: ______________________________

Presentation format: ______________________________

c. Men will be significantly affected by an increase in noise volume from 70 to 80 decibels, but women will not.

Null: ______________________________

Presentation format: ______________________________

d. Musical style affects men's reaction time, but not women's.

Null: ______________________________

Presentation format: ______________________________

e. An increasing volume of rock music will affect a woman's reaction time, but not a man's.

Null: ______________________________

Presentation format: ______________________________

3. Prepare a table, bar graph, or line graph for *your* experimental data from Exercise E. Use a separate sheet of graph paper if needed. You will use this information as part of preparing a laboratory report (see Appendix II) if requested by your instructor.

EXERCISE G | Interpretation: Conducting Statistical Analyses and Forming a Conclusion

A distinctive quality of scientific thinking is that a hypothesis can never be considered to be "proven" as a result of experiments or observations. Scientists accept ideas, theories, and hypotheses when they cannot show them to be false. They accept the null (no treatment effect) hypothesis unless they have evidence to falsify it.

Scientists often employ statistics to interpret experimental results and to determine whether the results meet a minimal level of acceptance or should be rejected. This allows us to come to a **conclusion** about a hypothesis. We might then suggest other hypotheses and test them against the null (no-effect) hypothesis.

The experimental method, designed to be replicated or repeated by others, is the process that precedes the scientific acceptance of a hypothesis. Such acceptance is always provisional. We cannot prove that a hypothesis is true because we can never be certain that we examined all the evidence or considered all possible alternative hypotheses.

Testing hypotheses leads to more questions. In this way, evidence accrues in support of a hypothesis, which may, after years of testing, gain the status of a theory. A **theory** is a generalization (based on many observations and experiments) that forms a basis for further studies.

Treating data statistically allows us to decide whether to accept or reject our null and alternative hypotheses. Say that you took the same reaction time test 10 times, then another 10 times, and then another 10 times. There is no reason to believe that your reaction time is changing, but it would be very unusual if all three average (mean) reaction times came out exactly the same. That is, even in the absence of any true treatment, successive groups of trials will have different means due simply to *random variation.* How do we distinguish between random variation and variation caused by true treatment differences? This is the purpose of experimental statistics.

Statistical tests allow us to generalize from a subset sampled during experimentation to a larger population, based on the probability that chance alone caused the difference observed between treatment groups or samples. Recall that the null hypothesis for any experiment is that there is no difference

between the treatment groups or samples. The null hypothesis is useful because it is easily testable by statistics. Statistics give the probability that the results are due to chance and not some real treatment difference between the treatment groups. Arbitrarily, we can define the level at which chance is not affecting our decision. For biologists, this is usually a probability level of $p = 0.05$. This means that we have only 5 chances in 100 of drawing the wrong conclusion for the entire population based on our sample data.

- If the computed probability is low (usually less than 5%) that the difference between two samples or treatment groups is caused by chance, *we can reject the null hypothesis* and accept the alternative hypothesis that there *is* a treatment effect.
- If the computed probability is *high* (usually greater than 5%), *we cannot reject the null hypothesis;* we accept the null hypothesis (no difference) because we do not have enough evidence to declare that there is a treatment effect.

These principles can be seen in the statistical test you will use in this lab, the **chi-square test.** The chi-square test gives us a χ^2 value which, when compared with critical values in a χ^2 table (see Table AI-2 in Appendix I, Part A), allows us to determine the probability that our assumption of no difference or a difference due to chance only (null hypothesis) is a safe assumption. Depending on experimental design, you will use chi-square to perform either an unpaired median test or a paired median test.

If you used the computer reaction time program, the computer has performed the chi-square test for you. If you performed the "drop the ruler" test, you will have to calculate χ^2 using your calculator or a computer made available by your instructor. The reaction time computer program will allow you to enter data and will perform statistical tests for you even though you did not use the computer for the reaction time experiment. (See Appendix I, Part B, for a complete description of the chi-square test.)

Objectives

☐ Use chi-square and probability values to determine whether to accept or reject a hypothesis.

Note: If you did *not* use a computer for the reaction time test, skip to Exercise G, Part 2.

PART I Analyzing Chi-Square Results (*for Laboratories Using Computers*)

Procedure

1. After reviewing the chi-square median test (Appendix I, Part B), return to your data tables in Exercise E, Part 1. Record below the chi-square and probability values for your experiment.

   Chi-square = __________

   Probability = __________

2. Determine the degrees of freedom (*df*) for your experiment (see Appendix I, Part B).

   Degrees of freedom = __________

   a. Is the computed value for probability above or below the 5% probability level ($p \geq 0.05$)?

   ____

   b. Do you accept or reject your null hypothesis? ______________ *Why?*

   ____

   ____

3. Examine the chi-square table in Appendix I, Part A (Table AI-2). If your χ^2 value is above that associated with the $p = 0.05$ level, it will be found to the right in Table AI-2; if it is below, it will be to the left. Remember that you are looking at the probability of being *wrong* if you accept the null hypothesis. So, if the probability of being wrong is higher, your χ^2 value will be *smaller* than at $p = 0.05$. In this case, you would accept the null hypothesis because the risk

of being wrong if you reject it is too great. If the probability of being wrong is lower, your χ^2 value is *higher* than at $p = 0.05$. You would reject the null hypothesis, with less than a 5% chance of being wrong to do so.

4. Continue with Exercise G, Part 3.

PART 2 Analyzing Chi-Square Results (*for Laboratories Not Using Computers*)

Procedure

1. Using your data from Exercise E, Part 2, compute your χ^2 value. This can be done using a calculator or the reaction time computer program if made available by your instructor. Written directions for calculating chi-square are summarized in Appendix I, Part B. Your instructor will provide you with a worksheet (included in the *Instructor's Guide and Preparation Manual*) for performing the χ^2 calculation appropriate to the type of test (paired or unpaired) you performed.

   Chi-square = __________

2. Determine the degrees of freedom (*df*) for your experiment (see Appendix I, Part B).

   Degrees of freedom = __________

3. Using this *df* value, determine the probability (*p*) from Table AI-2 (Appendix I, Part B).

   Probability = __________ to __________

   a. *Is your* χ^2 *value above or below the* critical value *in Table AI-2 (Appendix I, Part B) at the* $p = 0.05$ *level?* ______________

   b. *Do you accept or reject your null hypothesis?* ______________ *Why?*

   ____

   ____

4. Examine the chi-square table (Table AI-2). If your χ^2 value is above that associated with the $p = 0.05$ level, it will be found to the right in Table AI-2; if it is below, it will be to the left. Remember that you are looking at the probability of being *wrong* if you accept the null hypothesis. So, if the probability of being wrong is higher, your χ^2 value will be *smaller* than at $p = 0.05$. In this case, you would accept the null hypothesis because the risk of being wrong if you reject it is too great. If the probability of being wrong is lower, your χ^2 value is *higher* than at $p = 0.05$. You would reject the null hypothesis, with less than a 5% chance of being wrong to do so.

PART 3 Forming Conclusions

When drawing conclusions from experimental data, it is important not to "overstate" your findings. Suppose a man and a woman try the same reaction time test, and the man has a faster reaction time. Does this mean the experimenters have the evidence to reject the null hypothesis, that they should conclude that men have faster reaction times than women?

Certainly not. This particular man might be faster than the average man, and this particular woman might be slower than the average woman. Furthermore, especially if the difference in reaction times is small, tomorrow the results might be reversed. The experimenters might be able to draw conclusions about these two individuals, but drawing conclusions about men in general and women in general goes way beyond these data.

Objectives

- ☐ Draw a conclusion from your reaction time experiment.

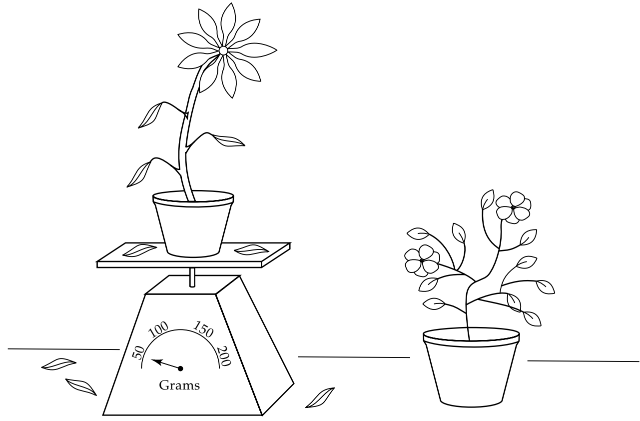
Procedure

Consider your data table or graph, the students you tested, and the computed statistical probability that your results were or were not due to chance alone. In the space below, record what you conclude from your reaction time experiment.

EXERCISE H Writing a Report

You now have all of the information necessary to write a laboratory report. Directions for writing a report and an example of a student lab report are included in Appendix II.

Laboratory Review Questions and Problems



1. For each of the following statements about the situation depicted above, indicate whether it is an observation or an inference.

a. The total mass of the plant pot and soil is 50 g.

b. The plant on the balance is not as healthy as the plant on the table.

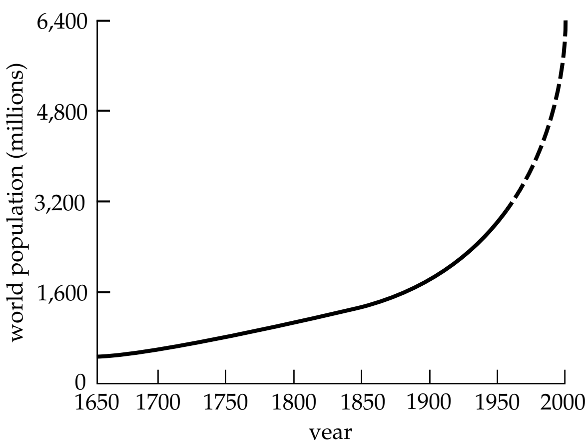
c. The larger plant on the table has a greater mass than the one on the balance.

d. The plant on the balance has three leaves.

e. The plant on the balance is smaller than the plant on the table.

f. The plant on the balance has lost some leaves.

2. From the following graph, provide the information requested below.



What was the world population in 1900? ______________

Title of graph: ____

Dependent variable: ____

Independent variable: ____

Description of information provided by graph: ______________________________

3. For a reaction time experiment, a group hypothesizes that people who wear glasses will have slower reactions than people who don't. What would be the best null hypothesis for this group?

a. There is no reaction time difference between people who wear glasses and people who don't.

b. People who wear glasses will have *faster* reaction times than people who don't.

c. People who don't wear glasses will have reaction times that are half those of people who do wear glasses.

d. Glasses slow reaction time by restricting peripheral vision.

4. The results of the experiment described in question 3 were as follows.

Treatment	Average
Glasses	0.225
No glasses	0.287

Treatment	Below Median	Above Median
Glasses	14	6
No glasses	6	14

Chi-square = 6.4

Probability = 1%

"Probability = 1%" means there is a 1% chance that:

a. there is a reaction time difference between people who wear glasses and people who don't.

b. glasses slow down a person's reaction time.

c. a difference this large between the glasses and no-glasses groups would have arisen by chance even if the two groups had the same reaction times.

d. it would be correct to reject the null hypothesis.

5. What would be the best conclusion for the group performing the experiment described in questions 3 and 4?

a. They must reject their null hypothesis and report that glasses slow reaction times.

b. The null hypothesis cannot be rejected.

c. Glasses seem to have no effect on reaction time.

d. The null hypothesis has been falsified, but the results still do not support the group's original hypothesis.

LABORATORY

Observations and Measurements: The Microscope

OVERVIEW

The success of a scientific experiment depends upon several factors: the problem must be well defined, the variables must be identified, and the experimental techniques and equipment used must be appropriate for the method of inquiry.

Understanding and properly applying the methods of scientific inquiry require that you become proficient at observing and recording data accurately. To do this, you need to be familiar with the types of instruments used for experimental work and with proper sampling techniques. During this laboratory period you will learn about the use and care of the **compound microscope** and the **dissecting microscope.** You will prepare living materials for observation and you will learn to use the microscope to measure the size of cells.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency. Review Laboratory I, "Science—A Process."

✓ EXERCISE A Identifying the Parts of the Compound Microscope

The simplest example of a microscope is a double convex lens of the type that is used as a magnifying glass. In the late 1500s, two Dutch spectacle makers developed the compound microscope. Their device had two convex lenses placed at either end of a tube and was capable of magnifying an object to 10 times (10×) its actual size. Today, developments in microscopy provide scientists with a wide selection of instruments with which to view the smallest organisms and even the components of individual cells. These microscopes range in complexity from the relatively simple models you will use in the laboratory today to highly sophisticated scanning and transmission electron microscopes.

Objectives

- ☐ Locate the optical and mechanical parts of the compound microscope.
- ☐ Discuss the function of each part of the compound microscope.

Procedure

To use the microscope properly, you must first be familiar with the care of this expensive and delicate instrument. Keep the following precautions in mind:

- Always carry the microscope in an upright position. Use one hand to grasp the arm of the microscope; use the other to support the base. The eyepiece (ocular lens) slides into the body tube and could fall out if the microscope is tilted.
- Never place the microscope close to the edge of the lab table or counter. Be sure to place the electrical cord out of the way and not in a position where it could catch and drag the microscope to the floor.
- Use only lens paper for cleaning the lenses. Using your fingers, handkerchief, or other materials could smudge or damage the lenses.
- When you are finished with your observations, turn off the illuminator and rotate the low-power objective into viewing position. Never put a microscope away with the high-power objective in the viewing position.

1. Obtain a compound microscope from your instructor.

2. Study the **optical** system of the microscope, familiarizing yourself with the location and function of each part. The letters in Figure 1A-1 correspond to the parts described below. After locating each part on the diagram, write the name of the part in the lettered space on Figure 1A-1, and identify that part on your own microscope.

Figure 1A-1 *Parts of the compound microscope. Write the names of the parts in the spaces provided.*

a Light source May be built into the base with a lens that focuses light onto the lower condenser lens or may be a separate light that is focused onto the condenser lens by a mirror.

b Condenser Contains a system of lenses that focuses light on the object (Figure 1A-2). Some microscopes may not have a condenser, particularly if they do not have a built-in light source. Others have either a movable or a fixed condenser. If your microscope is equipped with a movable condenser, locate the knob that raises and lowers the condenser and circle it on the diagram.

**Mechanical stage not shown.*

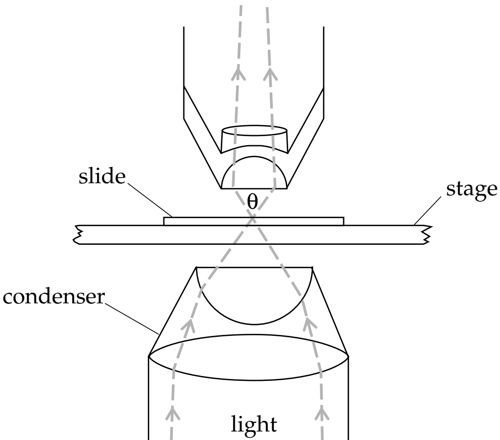


Figure 1A-2 *The microscope condenser focuses light onto the specimen on the microscope stage. Observe the point at which the dotted lines cross.*

c **Iris diaphragm** Used to adjust the amount of light striking the object. It can be opened or closed using the lever on the side of the condenser. On some microscopes this function is accomplished by a disk-aperture diaphragm. (Different-sized holes in the diaphragm are used to view objects at different magnifications.)

d **Objective lenses** Mounted on a revolving **nosepiece** or **turret e.** Most new microscopes are **parfocal;** that is, when an object is in focus with one lens, the lenses can be changed without completely losing focus. Each objective contains a complex lens system. The lens closest to the specimen produces the **magnification.** Magnification is indicated on the side of the objective (Figure 1A-3). The nosepiece usually holds the following objectives (check to see which of these are present on your microscope): scanning objective (4× magnification), low-power objective (10× magnification), high-power objective (40×, 43×, or 45× magnification), and oil-immersion objective (100 × magnification).

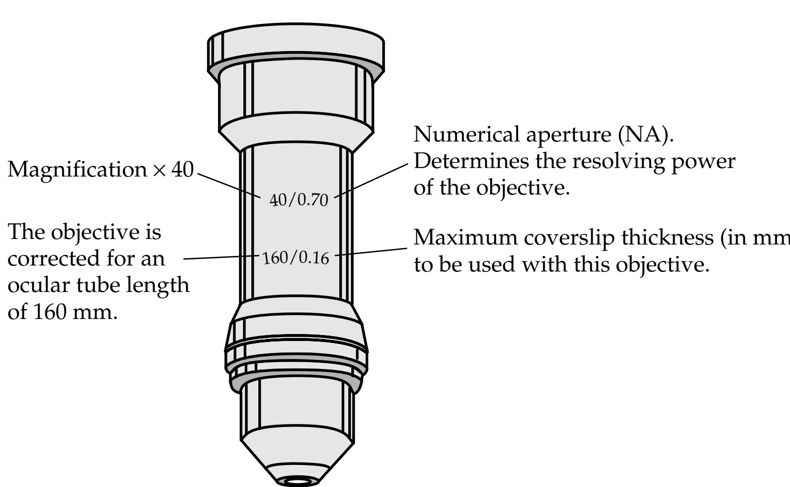


Figure 1A-3 *The power or magnification of an objective lens is engraved on the side of the objective.*

A second lens within the objective is responsible for limiting its **resolving power:** the ability to reveal detail, to distinguish two closely spaced objects as being two rather than one. The smaller the distance between two objects that can be distinguished from one another, the better is the resolving power of the instrument used to view the objects. The unaided human eye can distinguish (resolve) two objects when they are at least 0.1 mm apart, whereas with the light microscope, the human eye can distinguish two objects as separate when they are up to 1,000 times closer than that!

Resolving power (R) is dependent on three factors:

Angular aperture (θ) Examine Figure 1A-2. Note the cone of light entering the objective. The optimum value for θ is the angle that produces a cone of light whose diameter just matches the diameter of the objective. When angle θ is too small, the resolution is poor. One of the functions of the microscope's condenser is to produce the ideal angle of the cone of light.

Refractive index (n) The medium through which the light must travel will affect the shape of the cone of light and thus resolution. Air has a refractive index of $n = 1$. Oil has a greater refractive index than air ($n = 1.5$) and is often used to increase resolution of the microscope at higher powers by increasing the angle (θ) of the cone of light that passes into the objective.

Wavelength of light (λ) The shorter the wavelength of light, the greater is the resolution of the objective. The value of λ can be changed by using colored filters.

The value of R (resolving power) can be determined by the expression

$$R = \frac{\lambda}{2\,[n \sin(\frac{1}{2}\theta)]}$$

where

R = resolving power

λ = wavelength of light used

n = refractive index of the medium between the lens and object

θ = angular aperture of light cone

The expression $[n \sin(\frac{1}{2}\theta)]$ in the above equation is known as the **numerical aperture** (NA), and the equation can be rewritten as

$$R = \frac{\lambda}{2\ \mathrm{NA}}$$

Note that since λ is the only term in the equation expressed in units, the value of R will be expressed in the same units: nanometers (nm). Numerical aperture is a pure number: it is unitless. The numerical aperture is engraved on the side of all objectives next to the number indicating magnification (Figure 1A-3). The higher the NA value, the smaller R will be. And remember, the smaller R, the better will be the resolution of the objective (and the more expensive!).

As magnification increases, so does resolving power, but the relationship is not linear: resolution always increases less than magnification. Magnification without increased resolution is not advantageous for studying specimens. *a. Why?* ______________________________

____

f **Ocular lens** or **eyepiece** The lens you look through. It will usually magnify objects to 10 times their size (10×). In some cases, the body tube **g** can be rotated, making it easier for someone else to view the specimen without moving the entire microscope. If your microscope has one ocular, it is monocular. If there are two oculars, it is binocular.

3. Study the **mechanical system** in the same manner as you studied the optical system. Letters in Figure 1A-1 correspond to the parts described below. Continue to label the diagram and locate each part on your own microscope.

   h **Stage** Holds the slide to be viewed. The stage can be moved vertically by turning the **coarse adjustment knob i** and the **fine adjustment knob j.** These are located in different places on different types of microscopes, either separately or together. Coarse adjustment is used for initial focusing of specimens at low power. Fine adjustment makes very slight changes, allowing precision focusing at higher power.

k Stage clips Hold the slide so that it can be moved by hand. If your microscope does not have stage clips, it will be equipped with a mechanical stage (not shown in Figure 1A-1). Adjustment knobs are used to move the slide in the horizontal plane, that is, side to side and toward and away from you.

l Base and **arm m** Important support parts of the microscope; these also allow for easy carrying.

EXERCISE B Using the Compound Microscope

Objectives

- ☐ Learn to use the compound microscope properly.
- ☐ Learn to position specimens properly and adjust the microscope for optimum use.
- ☐ Calculate the magnification of a specimen.
- ☐ Define "resolution" and explain how it applies to obtaining maximum clarity of image.

Procedure

To get maximum performance from the microscope, you will need to adjust the illumination and focus properly.

1. Place the microscope on the table with the ocular pointing toward you. If your microscope has a binocular head (two eyepieces), you will need to adjust the distance between the two oculars to match the distance between your eyes. There is an adjustment dial between the oculars. Turn the dial to change the interocular distance until the oculars are positioned in front of your eyes. When the adjustment is correct, you can comfortably see a single, round field of view.
2. Revolve the nosepiece until the scanning objective (4×) is in line with the body tube. You will hear (or feel) a click when the objective is properly engaged (otherwise you will see only your eyelashes against a black background).
3. Turn on the light switch and adjust the illumination. Higher-power objectives require more illumination. Adjust the *condenser* if it is movable. When using the 4× or 10× low-power objective, the upper lens of the condenser should be about 5 mm below the slide, but when using a 40× (high-power) objective lens, the upper lens of the condenser should be slightly below its uppermost position or about 2 mm below the slide.

   The best way to adjust the condenser is to begin by placing the sharpened tip of a pencil directly on top of the light source. Then, using the condenser adjustment knob, move the condenser up and down until the tip of the pencil is clearly in focus. The condenser should be readjusted with each objective, but the amount of adjustment needed will be slight.

   The iris diaphragm or disk-aperture diaphragm must also be adjusted to obtain the proper balance between contrast and resolution. The amount of light that enters the microscope affects both **contrast** (ability to distinguish something from its background) and **resolution** (ability to distinguish two points as separate). Best observation with the microscope occurs when neither contrast nor resolution is maximized. To adjust the iris diaphragm, remove the ocular lens (or one of the lenses, if the microscope is binocular) and use the diaphragm lever to adjust its leaves until they are just at the edge of the circle of light seen through the open ocular. Then, adjust the leaves so that they are approximately one-third of the way in toward the center of the circle of light. At this setting, the best balance exists between contrast and resolution. Replace the ocular lens before proceeding.
4. Use the coarse adjustment knob to adjust the stage *downward* before placing the slide on the microscope stage. (*Note:* If your microscope has a movable body tube rather than a movable stage, move the body tube *upward*.)

a. Which way did you have to turn the coarse adjustment knob (toward you or away from you) to increase the distance between the objective and the stage? ____________________

5. Obtain a slide of the letter **e** (or make a wet-mount slide: cut the letter **e** from an old newspaper, add a drop of water, and cover with a coverslip). Place the slide on the microscope stage, making sure that the coverslip is facing upward toward the objective. Hold the slide in place with stage clips or the mechanical stage. If your microscope is equipped with a mechanical stage, do *not* try to place the slide beneath the arms of the slide carrier. Pinch together the two metal extensions of the carrier and its arms will open to allow for insertion of the slide. Now position the slide so that the specimen is directly over the hole in the stage. You will need to do this by hand if your microscope is equipped with stage clips. To move the mechanical stage, use the two knobs beneath the right side of the stage. The upper knob moves the stage backward and forward. The lower knob moves the stage from side to side.
6. To adjust the focus, you will need to move the objective lens using the coarse and fine adjustment knobs. Watch from the side as you adjust the stage upward (or the body tube downward) until the objective lens is almost touching the slide. While doing this, observe the direction in which you are turning the coarse adjustment knob—toward you or away from you. With experience, you will develop a feel for which way to turn the knob. Take care not to bump the slide with the objective lens because this could damage both the objective lens and the slide.
7. While looking through the eyepiece, use the coarse adjustment knob to slowly move the stage downward away from the objective lens (or move the body tube upward away from the stage). When the specimen becomes visible, turn the fine adjustment knob slowly to sharpen the focus. If you are using a binocular microscope, you may have to adjust the focus for each of your eyes. Sometimes the visual acuity of our two eyes is different. After you think you have the letter **e** in focus, close your left eye and fine-tune the focus by turning either the coarse or fine adjustment knob. Once the picture has been sharpened, open your left eye and close your right eye. Turn the binocular focus knob of the left ocular tube until the object is in sharp focus. You can determine how much your specimen is magnified by multiplying the power of the objective lens by the power of the ocular lens. If you are using a binocular microscope, consider the power of only one of the ocular lenses.

Total magnification = ocular magnification × objective magnification

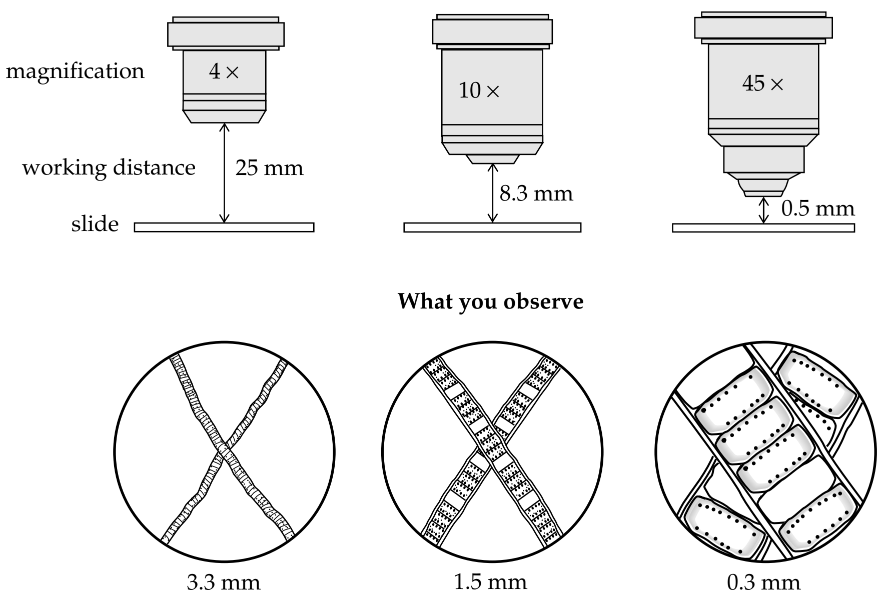
8. Move the slide to the right.

b. Which way does the letter **e** *move (as viewed through the microscope)?* ________ *c. Is it upside down?* ________ *Is it backwards?* ________ *(It is important to remember these spatial relations.)*

9. Now obtain a prepared slide of *Oscillatoria.* Place the slide on your microscope stage and repeat steps 1 through 8.
10. Rotate the nosepiece so that the 10× objective clicks into place. Again, check the space between the objective lens and your slide. The **working distance** (the space between the objective lens and the slide) decreases with higher-power objectives and increased magnification (Figure 1B-1). If your microscope is parfocal, you will probably be able to bring your specimen into focus by using only the fine adjustment. If you do need to use the coarse adjustment, remember: never turn the coarse adjustment while looking through the ocular. Always view from the side.

    The size of the **field of view** (the area you can see) varies inversely with magnification. The greater the magnification, the smaller is the field of view (Figure 1B-1).
11. Examine a slide of *Spirogyra* or *Oedogonium.* Count the number of cells you can see in one strand at 4× and 10×.

Figure 1B-1 *The field of view and the working distance change with magnification. (When each of these magnifications is used with a 10× ocular, the magnification is multiplied by 10.)*



d. What happened to your field of view as you changed from the 4× objective to the 10× objective?

____

e. Examine Figure 1B-1. What can you say about the relationship between magnification and field of view? ______________________________

12. Practice moving the slide until you can move it smoothly. Locate something in the field of view that can be followed as the slide is shifted. Attempt to move the object completely around the edge of the field without losing it (manipulations of this type will be particularly important when you view live organisms). Rotate the fine adjustment knob as you move the slide.

f. Do some parts of the specimen appear to be in focus when other parts are not? _______

If you said yes, you are probably correct. Your specimen is not of equal thickness in all places and the depth to which your microscope can focus (called the **depth of field**) is limited. The higher the power or magnification, the shallower is the depth of field. You can study thick objects by continually changing the fine focus, thereby bringing into focus different planes through the specimen. This allows you to "optically section" some materials (Figure 1B-2).

13. When you can operate the microscope successfully using 10× magnification, change to the 40× high-power objective. When using high power, the object to be viewed must be at the center of the field because the high-power objective magnifies only a small portion of the field of view observed under low power (Figure 1B-1).

14. Again, to avoid hitting the slide with the objective lens, watch from the side as you switch to the high-power objective. You may need to use the fine adjustment knob to focus the specimen. Remember: never use the coarse adjustment knob with higher power.

g. What is the total magnification of your specimen with the 40× objective in place? _______

15. On a separate sheet of paper, accurately draw what you observe at this magnification. Drawings should always be done in pencil and be labeled to indicate the name of the specimen and the total magnification.

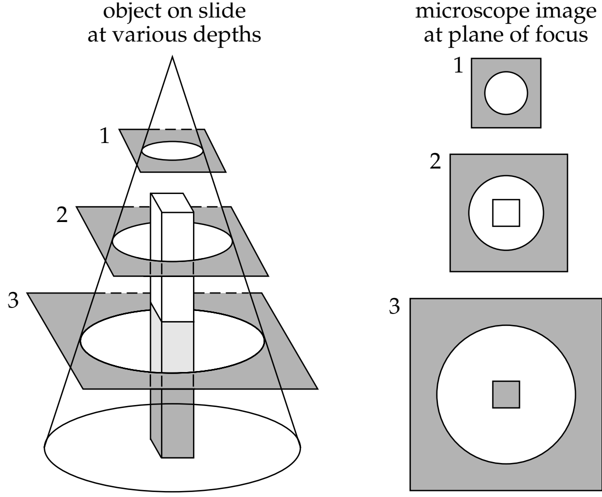


Figure 1B-2 *This schematic shows how focusing at different depths reveals the structure of an object. When viewing a thick specimen at a higher power (40× or 100×), you may use this optical sectioning process to examine the specimen's structure at various depths.*

Below are a few additional suggestions for using a microscope.

- Be certain that you have the proper amount of light.
- Try to keep both eyes open. This will take some practice on your part, but will be less tiring for your eyes.
- Always clean ocular and objective lenses with lens paper before use.
- If you wear glasses, remove them.
- Always begin by using a low-power objective lens to find the specimen. You can then turn to a higher power to make your observations.
- If you are having difficulty locating the specimen, use a systematic pattern to search the slide.
- If all else fails, ask your instructor for help.

EXERCISE C | Preparing a Wet-Mount Slide

A wet-mount slide is a temporary preparation. Specimens are mounted in a drop of liquid and are covered with a coverslip.

Objectives

☐ Learn the proper technique for making a wet-mount slide.

Procedure

1. Place a drop of water in the center of a clean microscope slide. To the drop, add a torn piece of an *Elodea* leaf.
2. Place one edge of a coverslip at the edge of the water drop and gently lower it so that the water containing the specimen completely spreads out under the coverslip (Figure 1C-1). Take care not to trap bubbles under or around the specimen. Do not press down on the coverslip. If there is too much water, draw off the excess by touching the corner of a paper towel to the edge of the coverslip.
3. Examine your wet mount under low power and then under high power. Remember, care must be taken to avoid touching the coverslip with the objective lens. Not only will it move your specimen, it might break the coverslip or scratch the objective lens.

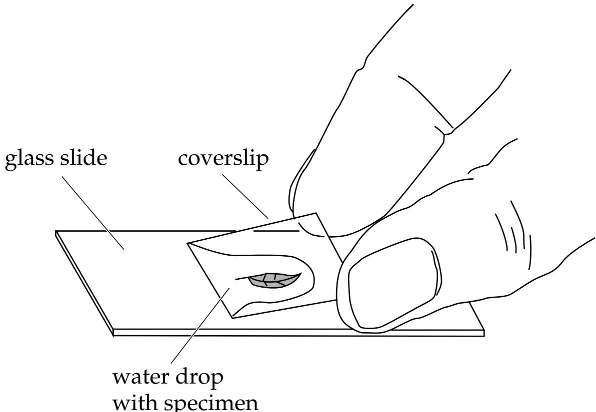


Figure 1C-1 *Technique for preparing a wet-mount slide.*

a. Give three reasons for using a coverslip when preparing a wet mount.

____

4. Use the 10× objective to focus the torn edge of the *Elodea* leaf. Notice that only part of the whole thickness of the leaf is in focus. Using the fine adjustment knob, focus on various planes throughout the thickness of the leaf. Note that the leaf is more than one cell layer thick.
5. Now switch to your 40× objective. You should notice that even less of the leaf's thickness is in focus at any one setting. What you have just accomplished is a demonstration of a principle of magnification that was discussed earlier: the higher the magnification, the shallower is the depth of field (depth of the area that is clearly focused).
6. With the *Elodea* leaf still in position, notice the movement of the little green bodies inside each cell (Figure 1C-2). These are chloroplasts, organelles responsible for photosynthesis in plant cells. The movement you observe is called **cyclosis,** or cytoplasmic streaming. As the cytoplasm moves around the large central vacuole, it carries with it dissolved substances as well as suspended organelles. Does cyclosis occur in the same direction in all cells?

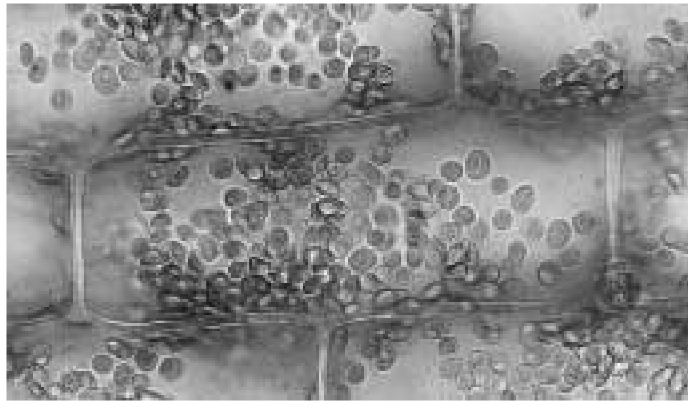


Figure 1C-2 *A cell of an* Elodea *leaf. The small round bodies in the cell are chloroplasts.*

b. What might be the practical advantage of cyclosis to the cells of the leaf?

____

7. Now replace the liquid around your specimen with a concentrated salt solution (Figure 1C-3). Place a drop of the concentrated salt solution at the edge of your *Elodea* wet mount. Draw the

solution under the coverslip by placing the corner of a paper towel at the opposite edge. Watch closely to see what happens to the cells of the leaf. You will probably see the cytoplasm shrink away from the cell walls. This phenomenon is called **plasmolysis.**

c. Does cyclosis continue or does it stop? ________

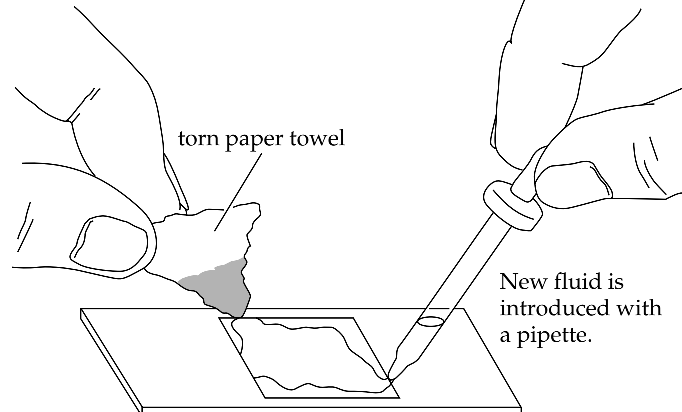


Figure 1C-3 *The solution under a coverslip can be changed without removing the coverslip by using a piece of torn paper towel to absorb the fluid at one corner edge of the coverslip, and introducing new solution with a pipette or eyedropper near the opposite corner.*

EXTENDING YOUR INVESTIGATION: MEASURING CYCLOSIS

In steps 6 and 7 of this exercise, you observed cyclosis. Do you think that environmental conditions can affect natural functions of cells, including such processes as cyclosis? *Does temperature affect cyclosis? Does light affect cyclosis?* Choose one of these questions and formulate a hypothesis to explore it. (Refer to Laboratory I, Science—A Process.)

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will happen to the rate of cyclosis for the conditions you have chosen to investigate?

Identify the **independent variable** in this experiment.

Identify the **dependent variable** in this experiment.

Design an experimental procedure to test your hypothesis. (How might you measure cyclosis? You might consider the rate of chloroplast movement as a measure of cyclosis activity.)

PROCEDURE:

Now, determine the rate of cyclosis. (How many measurements should you make?)

RESULTS:

From your results, describe what happened to the rate of cyclosis given the environmental conditions you chose. (Graphing your data may make your results easier to interpret.)

Do your results support your hypothesis?

Your null hypothesis?

Was your prediction correct?

What do you **conclude** about the effects of varied environmental conditions on cyclosis?

8. If pond water, algal, or protozoan cultures are available, make wet mounts of these by placing one drop of culture medium on a clean slide and applying a coverslip. Some of the unicellular organisms you will see are protozoans, which may move very rapidly across the microscope's field. It may be necessary to slow these organisms by adding a drop of methyl cellulose (Protoslo) to the culture drop (mix with a toothpick).

   On a separate piece of paper, draw, in as much detail as possible, one or more of the organisms you observe and insert your drawing into the laboratory manual.

9. Turn off the microscope light, coil the cord as directed by your instructor, and return your specimens and microscope to the proper place.

✔ EXERCISE D Measuring the Size of Objects Using the Compound Microscope

The microscope can be used as a tool to gather quantitative data in addition to serving as an instrument for making qualitative observations.

Objectives

- ☐ Determine the diameter of the field of view for microscope objectives.
- ☐ Estimate the size of an object from the diameter of the field of view.
- ☐ Accurately determine the size of an object using an ocular micrometer.

Procedure

The size of objects viewed with the compound microscope can be estimated by first determining the diameter of the field of view (see Exercise B) for a particular microscope objective and then estimating the size of the specimen by comparing it with the total diameter of the field of view.

1. Place a transparent ruler across the field of view under scanning power and record the diameter in millimeters: ______ What is the diameter in micrometers? ______ The diameter of the field of view using the scanning objective (A) can be used to calculate the

diameter using any other objective (B) (*Recall:* total magnification = objective magnification × ocular magnification):

$$\frac{\text{Total magnification A}}{\text{Total magnification B}} \times \text{diameter A } (\mu\text{m}) = \text{diameter B } (\mu\text{m})$$

2. Calculate the diameters of the fields of view using the other objectives on your microscope.

Objective	Diameter (μm)

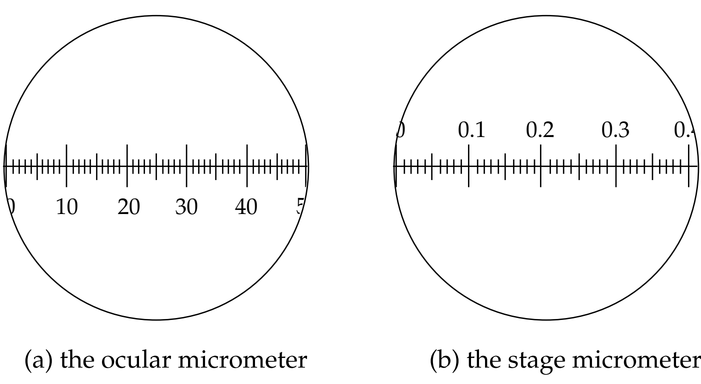
3. Obtain a prepared slide of *Nostoc,* a type of cyanobacterium. Estimate the length of one cell in micrometers (*Hint:* Use the diameter of the field of view to determine the length of a strand of cells, then divide by the number of cells): ______ μm

a. If a cell measures 10 μm at 100×, what is the length at 200×? ______

4. To determine the diameter of the field of view more accurately, you can use an **ocular micrometer,** a glass disk with a scale etched into it. It is placed in the ocular of the microscope and is visible when you look through the ocular. The ocular micrometer appears as in Figure 1D-1a. Notice there are numbered divisions, but no units per division.

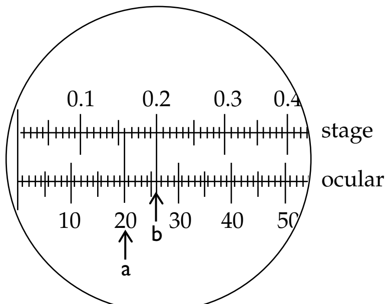
Due to variations among microscopes, an ocular micrometer must be **calibrated** for the microscope with which it will be used. A **stage micrometer** is used to calibrate an ocular micrometer. The stage micrometer (Figure 1D-1b) looks like a microscope slide but has a standard scale etched into it. The smallest divisions are 0.01 mm in length. It is just like a tiny ruler!

Figure 1D-1 *Ocular micrometer and stage micrometer.*



You can calibrate an ocular micrometer as follows. Using your scanning objective (4×), look through the ocular containing the micrometer and focus on the stage micrometer. Now move your objective to low power (10×) and refocus on the micrometer using the fine adjustment knob. The two scales should appear to be superimposed on one another. Move the stage micrometer to match up its left end with the left end of the ocular micrometer

Figure 1D-2 *Ocular and stage micrometers are aligned for calibration. (For clarity, the stage and ocular micrometers are shown separated here; as you look through the ocular, the two scales should appear superimposed.)*



(Figure 1D-2). The actual distance between the lines of the ocular micrometer can be calculated by finding a line on the ocular scale that aligns exactly with a line on the stage micrometer scale.

Example In Figure 1D-2 observe that 20 spaces (**a**) on the ocular micrometer equal 0.16 mm and that 26 spaces (**b**) equal 0.2 mm. Therefore, 1 space on the ocular scale equals:

$$\frac{0.16}{20} \text{ or } \frac{0.20}{26} = 0.008 \text{ mm} = 8\ \mu\text{m}$$

Your instructor has already calibrated the ocular micrometer (using the 10× objective) in one of the microscopes on demonstration. Note that the value for a division varies for different objectives. *b. Why?* ____________________

You will need to recalibrate the ocular micrometer for each objective.

c. 10× objective: 1 division on the ocular micrometer = _______ *mm =* _______ *μm*

d. 43× objective: 1 division on the ocular micrometer = _______ *mm =* _______ *μm*

It is now possible to measure the size of a specimen by viewing it through an ocular containing an ocular micrometer that has been calibrated.

5. Remove the stage micrometer. Using low power, locate a filament of *Nostoc* and bring it into clear focus. Switch to high power and, as accurately as possible, count the number of ocular micrometer divisions from one end of a cell to the other or the number of cells that reach from one division to the next. (Each cell is approximately the same size.) For example, if you had a cell that fit between the 0 and the 25 on your ocular, you could then determine its length. If you had calibrated the ocular at 43× and each division was equal to 2 μm, then to determine the length of the cell you would multiply the number of divisions the cell covered by the measurement of each division: $25 \times 2\ \mu\text{m} = 50\ \mu\text{m}$.

You determined that each division of the ocular micrometer equals _______ μm.

Therefore, the length of one *Nostoc* cell = _______ μm. Show your work in the space below.

✔ EXERCISE E | The Stereoscopic Dissecting Microscope

Most of the biological specimens you observe through the compound microscope are very thin and can almost be considered two-dimensional. Larger specimens, including whole organisms, can be viewed more easily using the **stereoscopic microscope.** Dissection is often done using this microscope. Thus, it is sometimes called a **dissecting microscope.**

The dissecting microscope is really two microscopes in one—each eyepiece has its own objective and ocular lenses. Each eye is presented with its own image. Human beings ordinarily see in three dimensions by virtue of receiving a slightly different image in each eye. This possibility is lost in the compound microscope with its single objective lens for both eyes, but is permitted by the design of the dissecting microscope.

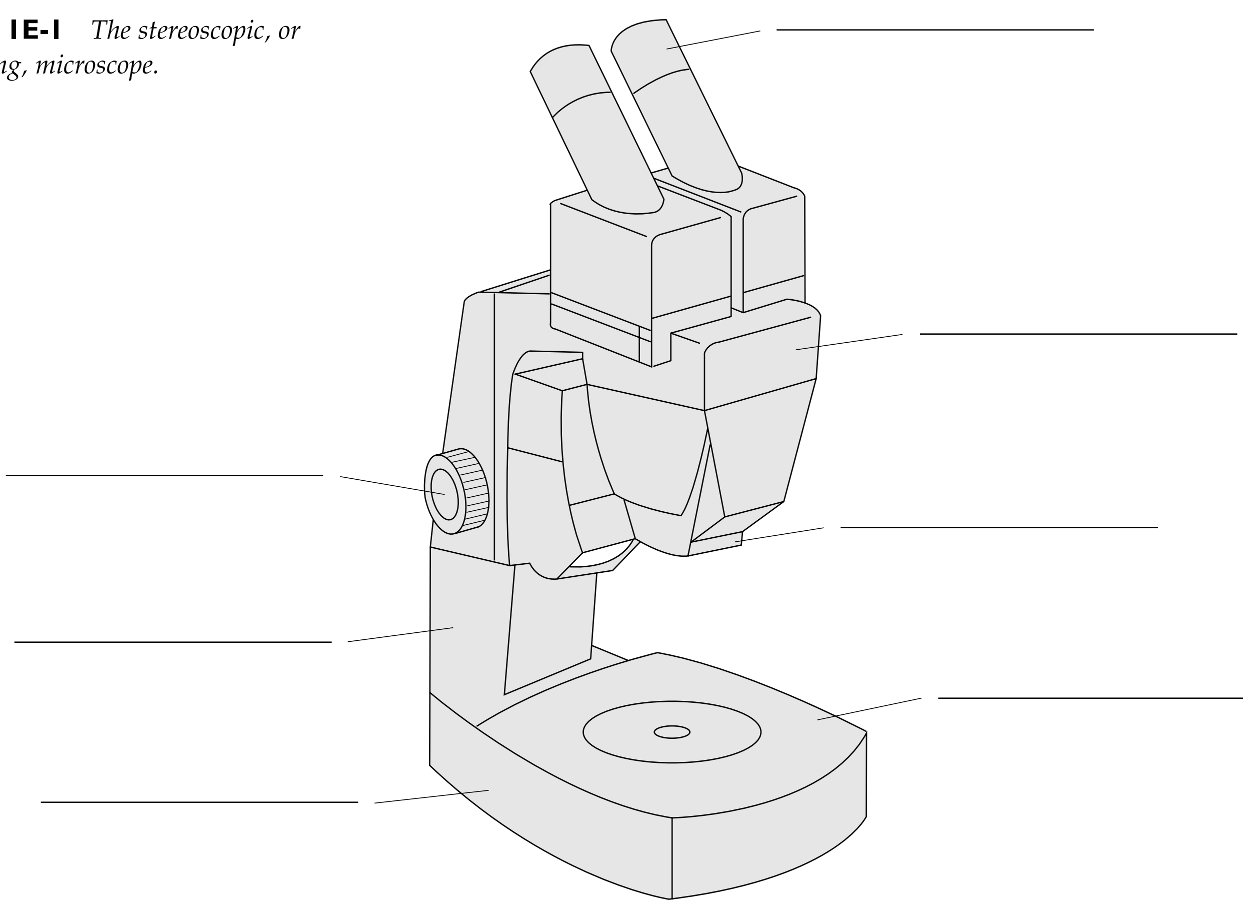
Objectives

- ☐ Locate and describe the function of each part of a dissecting microscope.
- ☐ Learn to use the dissecting microscope properly.

Procedure

1. Use your knowledge of the compound microscope to identify the parts of the dissecting microscope. Write the names of the parts on the lines provided in Figure 1E-1: **eyepiece** or **ocular, focus knob, body tube, arm, objective, stage,** and **base.**

Figure 1E-1 *The stereoscopic, or dissecting, microscope.*



2. Find the **magnification changer** on your microscope. Most, but not all, dissecting microscopes have this feature. If present, it will be located on the side or top of the microscope. Draw the magnification changer on Figure 1E-1. What final magnification can your dissecting microscope achieve?
3. The **light source** for your microscope may be a separate light. Light can be **transmitted** through a specimen on the microscope stage by proper adjustment of the mirror below the

stage. Light can be **reflected** from the surface of a specimen by positioning the light source above the specimen so that it shines downward onto the microscope stage. If your illuminator is built into the microscope, light will be adjusted by two switches, the **transmitted light** switch and the **reflected light** switch. Familiarize yourself with each of these and add them to Figure 1E-1.

4. Select a three-dimensional specimen from those provided. Center your specimen on the stage plate.
5. Grasp both ocular housings and move them together or apart to adjust the spacing until you are comfortable using both eyes simultaneously to view a single field.
6. Try using both reflected and transmitted light to illuminate your specimen.

   a. Which works better? ______________________ *Why?* ______________________

   ____

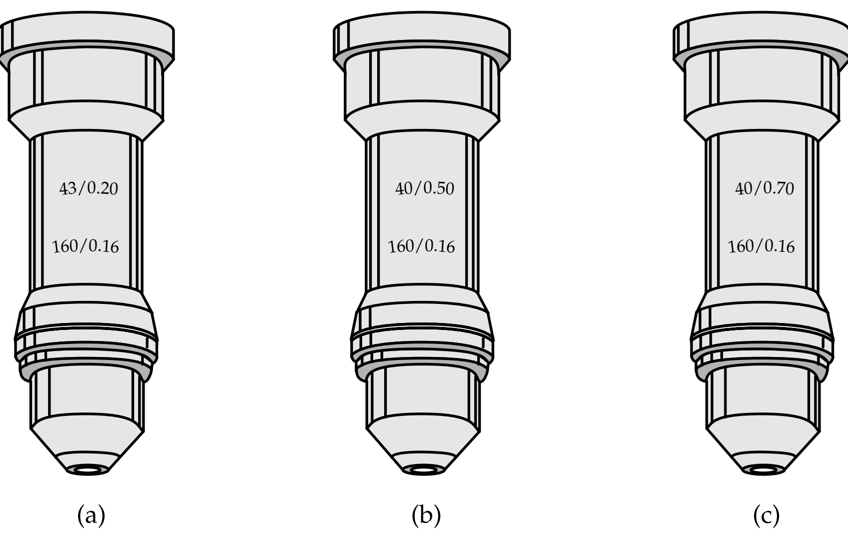
7. Use the coarse adjustment knob to focus. There is no fine adjustment knob on the stereoscopic microscope.
8. If possible, change to a higher magnification and refocus as necessary to view different portions of your specimen.
9. Sketch your specimen in the space below.

10. Turn off the microscope light, coil the cord as directed by your instructor, and return your specimens and microscope (and its light) to the proper place.

Laboratory Review Questions and Problems

1. What is the resultant total magnification of an object as seen through a microscope with 10× oculars and each of the following objectives? *a.* 4× ____________ *b.* 10× ____________ *c.* 43× ____________
2. Describe the difference between magnification and resolution.
3. Which part of the microscope is most important in determining its resolving power? Why?

4. You are interested in purchasing a microscope. The salesperson shows you several microscopes, each with one of the following objectives. Which would you want to purchase?



5. Briefly describe the function of each of the following parts of the microscope.
Objective

Ocular

Iris diaphragm

6. In which direction do you turn the coarse focus adjustment knob to move the objective *away* from your slide? ______ In which direction do you turn the fine adjustment? ______

7. What is the purpose of the condenser on a microscope? Relate this to the angle θ and to the numerical aperture (NA) of the objective.

8. The resolution of a microscope objective depends on numerical aperture. Is the resolution better with a high NA or low NA?

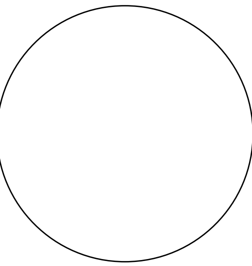
9. You are using a microscope with a 100× objective that has a numerical aperture of 0.71. You place a dot of oil ($n = 2$) on the coverslip of the slide you want to observe. What is the resolving power of the 100× objective if you are using light of wavelength 560 nm?

Which is preferable: a large or small *R?* ______ Why?

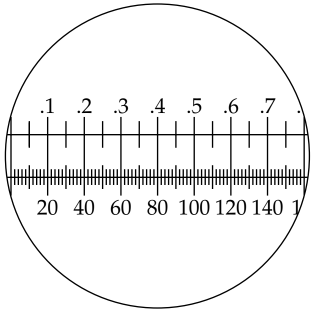
10. Suppose your 10× objective has an NA of 0.25 and you are using a green filter that limits the wavelength of light striking the objective to 560 nm. What is the resolving power for this objective? ______________ What type of dimensions does your answer have? ______________ This is the theoretical value for R. Suppose $\theta = 26°$. What would be the true resolution for your system? ______________

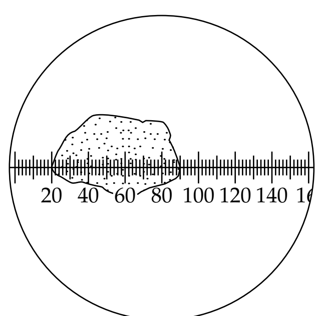
11. What is meant by *field of view?* What will happen to the field of view for each resultant magnification as you change objectives from 4× to 10× to 43×?

12. Using your 4× objective, you measure the diameter of the field of view to be 3 mm. You notice that the length of a large letter **E** takes up half the width of the diameter of your field at 10×. The oculars on your microscope are 10×. What is the size of the letter **E**? Draw what this letter would look like through the microscope.



13. You use an ocular micrometer to measure the size of a cell, as seen below. The stage micrometer is divided into 0.1-mm units. Which of the following accurately represents the size of the cell? *a.* 3.2 μm; *b.* 350μm; *c.* 35 mm; *d.* 0.0035 mm.





Observations and Measurements: Measuring Techniques

LABORATORY 2

OVERVIEW

We learn about the world by making observations and comparing them with other observations. The major difference between the types of comparisons we make in everyday life and in the discipline known as science is that science requires a more rigorous, focused, and systematic approach. By using a common system of units of measurement and by making precise and accurate measurements, scientists ensure that procedures and results can be reported and repeated anywhere in the world.

In this laboratory you will become familiar with techniques used to make accurate measurements.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

✔ EXERCISE A The Metric System

The two systems of measurement in common use are the English system and the metric system. Most scientific data are expressed in units of the metric system. The modern metric system is called the **International System of Units (SI).**

Objectives

- ☐ Identify the units of the metric system and make conversions among these units.
- ☐ Express measurements in scientific notation.
- ☐ Distinguish between accuracy and precision.
- ☐ Report data in significant figures.

Procedure

In the metric system, the basic units are the **meter** (**m**) for length, the **liter** (**l**)* for volume, and the gram (**g**) for mass.

**This has been changed to L in the SI system of measurements, but l is still the notation biologists use most frequently.*

Table 2A-1 Commonly Used Metric Units

Prefix	Meaning	Size Relative to Base Unit	Length	Volume	Mass
nano-	billionth	10^{-9}	nanometer (nm)	nanoliter (nl)	nanogram (ng)
micro-	millionth	10^{-6}	micrometer (μm)	microliter (μl)	microgram (μg)
milli-	thousandth	10^{-3}	millimeter (mm)	milliliter (ml)	milligram (mg)
centi-	hundredth	10^{-2}	centimeter (cm)		
kilo-	thousand	10^{3}	kilometer (km)		kilogram (kg)
mega-	million	10^{6}			
giga-	billion	10^{9}			

1. The prefixes used with the basic metric system units indicate either a fraction of a unit or a multiple of a unit, depending, in part, on the size of what is being measured and the degree of accuracy of the measurement. (See Exercise C, Part 4.) Metric prefixes always express a power of 10 by which the basic unit has been multiplied; see Table 2A-1.

Convert the following measurements from one unit of metric measurement to another as indicated.

1 nm = _______ m 25 μm = _______ cm

1 cm = _______ mm 10 cm = _______ mm

1 mm = _______ nm 12 l = _______ ml

1 m = _______ μm 22 μl = _______ ml

1 μm = _______ cm 250 μm = _______ nm

1 mg = _______ μg 10 μg = _______ ng

1 mg = _______ kg 9 μm = _______ m

1 l = _______ μl 50 g = _______ mg

2. Compare metric units with the English units given in Table 2A-2.

Table 2A-2 English and Metric Equivalents

English	Metric
1 inch (in)	2.54 cm
0.4 in	1 cm
39.37 in	1 m
0.62 mile (mi)	1 km
1 mi	1.6 km
1 fluid ounce (fl oz)	29.57 ml
1 quart (qt)	946.4 ml
1.05 qt	1 l
1 ounce (oz)	28.53 g
1 pound (lb)	453.6 g
2.2 lb	1 kg

a. *If you were dieting, would you rather lose 10 pounds or 5 kilograms?* _______

b. *Would you rather have 50 grams or 2 ounces of chocolate (assuming that you love chocolate and are* not *dieting)?* _______

c. Would you be likely to get a speeding ticket if you were driving 85 km/hr in a 55 mi/hr speed zone?

____

d. If 2 qt or 2,500 ml of orange juice costs $1.79, which package would be the better bargain? ________

e. If you have a cup that holds 50 ml of coffee, would it be full if you poured 50,000 μl of coffee into the cup? ________

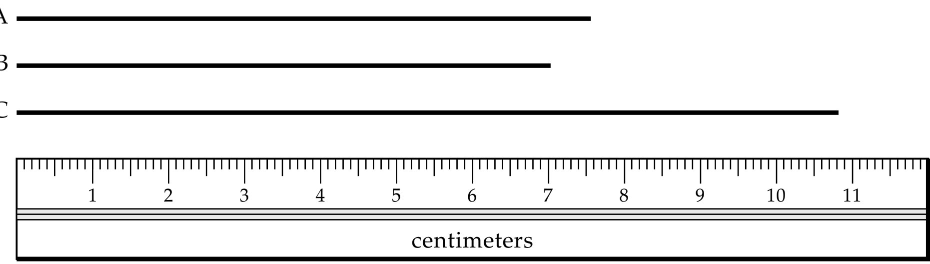
3. When measuring the number, mass, length, or volume of objects, scientists write numbers in **scientific notation.** A number is expressed in scientific notation when it is written as a product of a decimal number between 1 and 9 and the number 10 raised to the proper power. For instance, the number 100 can be written as 1×10^2, 34,698.5 can be written as 3.46985×10^4, and 0.0069 as 6.9×10^{-3}. (*Note:* When a number is larger than 10, the decimal point must be moved left. When the decimal point is moved left, the power of 10 is positive and equal to the number of decimal places moved; for example, $20 = 2.0 \times 10^1$. When the number is smaller than 1, the decimal point must be moved right, and the power of 10 is negative and equal to the number of decimal places moved; for example, $0.01379 = 1.379 \times 10^{-2}$.) Convert the numbers in the following table to scientific notation, and then convert to the metric units indicated in the second column.

	Scientific Notation	Metric Unit Conversion
0.00013 g	________ g	________ mg
0.00000625 l	________ l	________ ml
2,323,000 m	________ m	________ μm
10 μg	________ μg	________ kg
1,654 km	________ km	________ m

4. When reporting measurements, it is permissible to estimate one digit beyond the precision of the measuring device. For example, the "ruler" shown in Figure 2A-1 is marked in centimeters. The length of line A could be reported as 7.5 cm by estimating the distance covered beyond the 7-cm mark. This measurement has two significant figures, one certain (7) and one estimated (uncertain) (0.5). All certain digits, plus the next estimated digit, are called **significant figures.** Line B is exactly 7 cm long. This may be reported as 7.0 cm, again using one certain (7) and one estimated (0) digit. To report the length of line B as 7.00 would *not* be correct because only one estimated digit can be reported.

f. How many significant figures would be used in reporting the length of line C? ________ *Are both digits in front of the decimal point significant?* ________

Figure 2A-1



g. Look at a ruler. If the length of an object is to be reported in centimeters, what is the maximum number of significant figures to the right of the decimal point in any measurement made with this instrument? _______

h. If a balance can measure mass to an accuracy of ±0.01 g, what is the maximum number of significant figures if the object being measured is slightly heavier than 100 g? _______ *If, using the same balance, you find that an object has a mass of 100 mg, how many figures in this measurement can be considered significant?* _______

5. Typically, length is measured with a ruler or meter stick. Your instructor will provide a set of objects to measure in centimeters. Use the maximum number of significant figures for each measurement and record your data in the following table. When you have completed each measurement, convert it to millimeters and meters.

	Length				Length		
Object	cm	mm	m	Object	cm	mm	m
1				6			
2				7			
3				8			
4				9			
5				10			

At the end of this laboratory, you will use these data to construct a histogram.

✔ EXERCISE B | Measuring Mass

Mass is measured by using a balance or scale. Most measuring of mass (commonly called weighing) that you will do in this course will be in the range of tenths of a gram through tens of grams.

The **gram (g)** is the standard unit of mass. It is defined as 1/1,000th of the mass of a 1-kg block of platinum-iridium alloy. The commonly used subdivisions of the gram are the **milligram (mg)** (1,000 mg = 1 g) and the **microgram (μg)** (1,000,000 μg = 1 g). Measuring devices sensitive enough to measure milligram or microgram amounts may not be available in your laboratory.

Objectives

- ☐ Learn to use a balance.
- ☐ Explain what is meant by "taring" the balance.

Procedure

To protect the balance pan and make measurement of mass more convenient, a piece of paper (usually called "weighing paper"), a plastic boat (or "weighing boat"), or another container may be used. The mass of the container must be subtracted from the total mass to obtain the mass of the material itself. This can be done by **taring** the balance. With the empty container on the balance pan, the balance is set to zero with the tare knob. When the material to be measured is placed in the container, the mass of the material alone will be registered.

When using balances without a tare knob, you must first measure and record the mass of the paper or container and add it to the mass of the material you want to measure. You then add material to the container until you obtain the total amount calculated. Alternatively, subtract the mass of the container from the total mass of the container plus material.

Example The paper has a mass of 0.2 g and you wish to measure 2 g of NaCl. You must add NaCl until the balance reads 2.2 g.

Example You are given a beaker of NaCl and asked to determine the mass of the NaCl. First determine the mass of the beaker with the NaCl in it, then remove the NaCl, determine the mass of the beaker, and subtract the mass of the beaker from the first measurement.

1. Examine the balance available in your laboratory.

a. What is the maximum mass that can be measured? ______ *b. What is the maximum number of significant figures that can be used to report data?* ______

2. Place a piece of paper or a plastic boat on the balance pan and read the result. Tare the balance, or record this mass if no tare knob is present.

3. Obtain three rubber stoppers (or other objects provided by your instructor). Determine and record the mass in grams of each stopper. The manufacturer's specifications indicate the accuracy of the balance you are using. Record the possible error with each of your measurements in Table 2B-1.

Table 2B-1 Measuring Mass

Object	Mass	Possible Error	Potential Range of Values
1			
2			
3			

EXERCISE C Measuring Volume

Life, as you know, depends on water and the types and concentrations of particles dissolved in it. To determine the concentration of dissolved substances, you must be able to measure volume accurately. A number of devices are available to measure volume, but the most common are the pipette, graduated cylinder, and volumetric flask.

Objectives

☐ Measure volume and report data accurately and precisely.

☐ Select and use appropriate measuring devices.

PART I The Pipette

Pipettes are usually used to measure liquid volumes of 10 ml or less, although some types of pipettes can measure up to 100 ml or more. Pipettes are of two types: **to deliver** (TD) and **to contain** (TC). The label near the upper end of the pipette shaft indicates the type of pipette. Most pipettes are the TD type. There are two varieties of TD pipettes: "delivery" and "blow out." Examples are shown in Figure 2C-1. Notice that on the delivery pipette (Figure 2C-1a), the scale stops before the pipette narrows. When using this type of pipette, it is important to deliver liquid only down to this line. The blow-out pipette (Figure 2C-1b) is graduated to the tip, so all of the liquid must be delivered, usually by blowing out (**to**

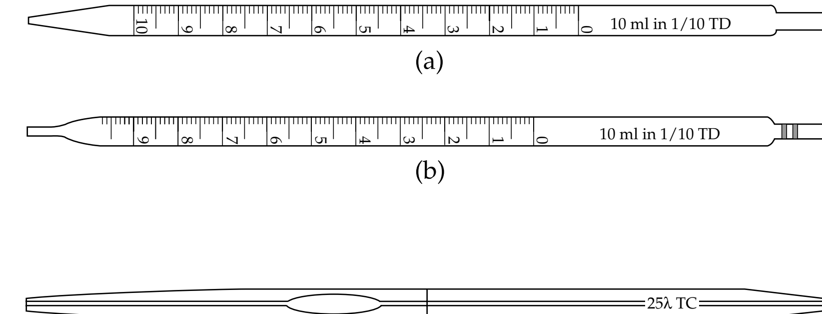


Figure 2C-1 *Types of pipettes: (a) to deliver (TD) delivery pipette, (b) to deliver (TD) blow-out pipette.*

Figure 2C-2 *To contain (TC) pipette ($\lambda = \mu l$).*

deliver/blow-out pipettes are usually distinguished from other types by two frosted rings on the upper part of the pipette shaft).

When using a to contain or TC pipette, simply allow the liquid to flow out of the pipette until the flow stops. The pipette is *not* blown out and a small amount of liquid will remain in the end of the pipette, but this has been taken into account in calibrating the pipette. Some micropipettes (Figure 2C-2) are calibrated in this manner.

Before using any type of pipette, you need to determine how the pipette is calibrated. Both the largest and smallest volumes that a pipette can measure are marked on the upper end of the pipette. The first or larger number gives the total volume and the second, smaller number indicates how the pipette is calibrated into smaller dimensions—much like a centimeter ruler. For example, "5 ml in 1/10" means that the pipette measures 5 ml from the line marked "5" to the line marked "0" (delivery pipette) or from the line marked "5" to the tip (blow-out pipette). Each large line marks 1 ml and each smaller division represents 1/10 ml.

Procedure

The **propipette** is a mechanical device used to draw liquids into a pipette. (It is particularly useful when working with hazardous materials.) There are two types: the bulb type (Figure 2C-3a) and the plunger type (Figure 2C-3b). Your instructor will demonstrate the use of one or both types. *Note:* In this laboratory you will NOT pipette by mouth. You will always use a propipette or rubber bulb.

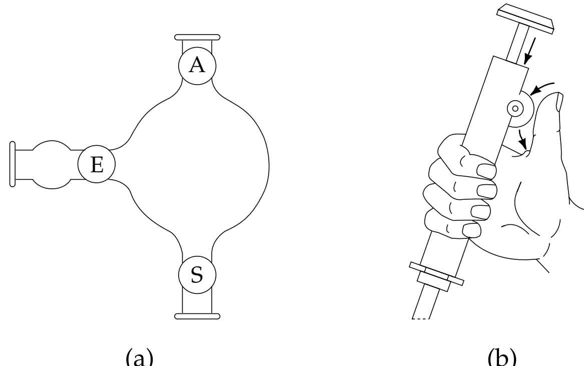


Figure 2C-3 *Types of propipettes. (a) Bulb type: (S) is pinched to suck up liquid and (E) is pinched to release the liquid. If a blow-out pipette is used, (A) must also be squeezed to remove all liquid. (b) Plunger type in which a wheel is turned to suck up or release liquid; a plunger can be used for rapid dispensing.*

1. Examine the pipettes on demonstration in your laboratory room. Notice the variety of types and scales. Before using a pipette, always examine the tip and read the markings on the upper part of the pipette shaft. **DO NOT TOUCH THE TIP WITH YOUR FINGERS!** If you are not familiar with the markings, you will end up with your pipette in a solution and have no idea of how much liquid to pull up or release.

2. Obtain a 10-ml disposable pipette and a beaker of red liquid. Determine whether the pipette is designed for delivery or blow-out. Attach the propipette.
3. Measure out 8 ml: carefully suck the red liquid up into the pipette to a point slightly above the 0 level.
4. Keep the pipette in the liquid with the tip pressing against the side of the container and slowly adjust the level to the 0 line. To read the level correctly, your eye should be directly in line with the meniscus. The bottom of the meniscus marks the correct volume. (*Note:* No meniscus is formed in plastic labware.)
5. Move the pipette to the container into which you wish to dispense the liquid and release the liquid in the proper manner, depending on the type of pipette you are using.
6. Pipette 8 ml of the red liquid into a 100-ml graduated cylinder. Repeat four more times.

   a. What volume of red liquid should the cylinder contain? ______ *What volume of liquid does it actually contain?* ______ *b. If you found a difference in volume, how do you explain it?*

   ____

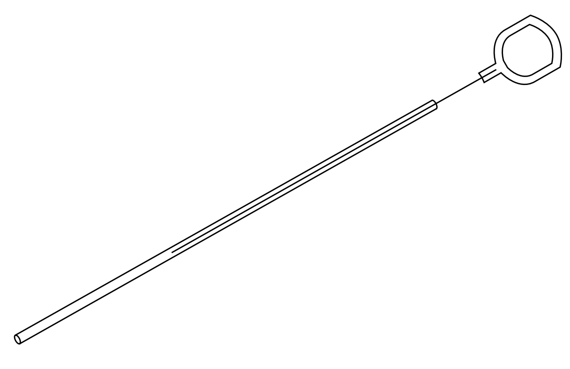
   ____

7. Practice using the propipette by measuring out 3.2 ml, 4.8 ml, and 7.3 ml of the red-dye solution.

When you have finished, place your pipettes in the pipette washer, tips upward!

8. Now obtain a **microcapillary pipette.** This is a simple glass capillary tube used for measuring microvolumes. It is calibrated in microliters (μl), often with multiple graduations. A wire plunger is used to deliver liquid from the pipette. Alternatively, a smaller version of the propipette (Accropet) can be used to fill or empty the microcapillary tube. This is done by rotating the wheel on the end of the Accropet (Figure 2C-4).

Figure 2C-4 *Types of glass microcapillary pipettes: (a) microcapillary pipette with plunger, (b) microcapillary pipette with Accropet attached.*



(a)

(b)

9. Place a drop of red-dye solution on a piece of Parafilm or wax paper. Now use a 50-μl microcapillary pipette to draw up the drop of liquid. How much liquid do you estimate is in the drop? ______ Dispense the liquid carefully onto the Parafilm at a different location.

   c. If you wanted to deliver 0.1 ml of a solution, you could use a regular 0.1-ml pipette or a ______-μl microcapillary pipette.

10. Research technicians in in biotechnology laboratories need to measure microvolumes accurately and repeatedly. To do this, **digital micropipettes** are used (Figure 2C-5). These pipettes usually have a continuous range of 1 μl to 100 μl, and even fractional volumes can be selected. A digital display allows you to set the pipette to a desired amount by turning the handle of the digital micropipette. A plastic tip (which can be sterilized if needed) is used to hold the liquid and the tip can be ejected easily. Depending on the brand of digital pipette used, there are "stops" on the plunger that allow you to fill and dispense and to eject the tip. Your lab assistant will demonstrate how to use the type of digital micropipette available in your laboratory.

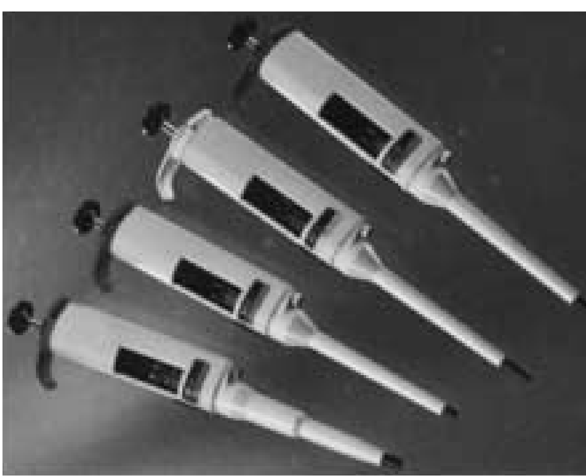


Figure 2C-5 *Digital micropipettes. Disposable plastic tips of different sizes fit on the ends of the pipettes.*

11. Use the digital micropipette to transfer 25.5 μl of red-dye solution onto a piece of Parafilm or wax paper. After dispensing the dye, pick up the drop with the same pipette and transfer it to another location on the Parafilm.

PART 2 The Graduated Cylinder

For measuring larger amounts of fluid, graduated cylinders are used. Two types are in common use: glass cylinders and cylinders made from organic polymers such as polycarbonates. Water adheres more strongly to glass than to polymers, and thus the water at the top of a column will "climb" the sides of a glass cylinder to form a bowl-like meniscus. To obtain a precise measurement, volume *must* be read at the bottom of the "bowl."

Procedure

1. Partially fill a 100-ml graduated cylinder with water and set it on a table.
2. View the cylinder and estimate volume: (a) from a standing position ______, (b) at eye level ______, and (c) from below ______ (see Figure 2C-6).

   a. How do volumes estimated from these various positions compare? ______________________

   ____

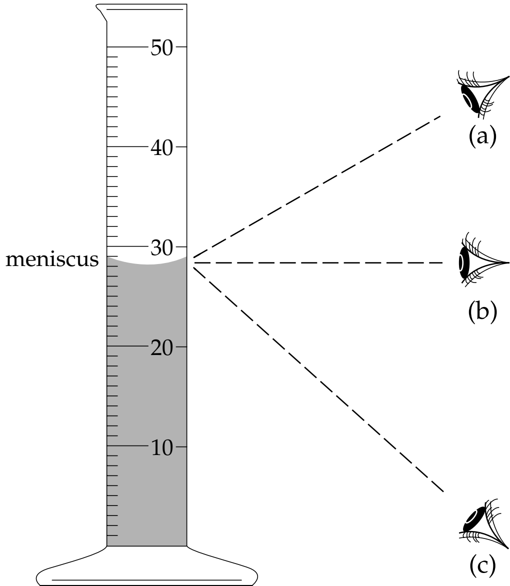


Figure 2C-6 *Reading the volume in a graduated cylinder.*

3. Repeat the procedure using a graduated cylinder made from an organic polymer. You will notice that there is much less of a meniscus and that the volume can be read more precisely from any eye level.
4. Add water to the 10-ml level in a 100-ml glass graduated cylinder. Pour the 10 ml of water into a 10-ml graduated cylinder. What does the measurement read on this cylinder?
   b. *Which cylinder would be more accurate for measuring 5 ml of solution: the 100-ml cylinder or the 10-ml cylinder?* ____________________

   ____

EXTENDING YOUR INVESTIGATION: COMPARING MEASURING DEVICES

There are two additional types of measuring devices on your laboratory table: a beaker and an Erlenmeyer flask. If you wanted to measure 50 ml of water, do you think the beaker or the flask would be as accurate as the graduated cylinder? Formulate a hypothesis to explore this question.

HYPOTHESIS:

NULL HYPOTHESIS:

Which measuring device do you **predict** will be the most accurate?

Design a procedure to test your hypothesis.

PROCEDURE:

Identify the **independent variable** in this experiment:

Identify the **dependent variable** in this experiment:

Now, accurately measure 50 ml of water using the three measuring devices available. (How many measurements should you make for each measuring device?)

RESULTS:

From your results, describe the accuracy of the beaker and the Erlenmeyer flask.

Do your results support your hypothesis?

Your null hypothesis?

Was your prediction correct?

What do you **conclude** about the type of measuring device that should be used to measure liquids?

✔ PART 3 The Volumetric Flask

Volumetric measuring devices do not have graduations. Volumetric equipment is calibrated at the factory to measure a single volume—for example, a 25-ml flask, a 10-ml pipette, or a 200-ml flask—and should be used *only* to measure that volume—25, 10, or 200 ml, respectively. Volumetric equipment is more accurate than graduated equipment.

Procedure

1. Place 100 ml of water into a graduated cylinder. Measure this as accurately as possible.

2. Pour the 100 ml of water into a volumetric flask. The water should be exactly at the line on the neck of the volumetric flask.

a. Is it? ________ A volumetric flask is a very accurate measuring device. What does this mean in terms of when you should measure with a graduated cylinder and when you should measure with a volumetric flask? ____

The greater accuracy of the volumetric flask is due to the narrowness of its neck, which extends the last few milliliters of solution over a longer distance than in the graduated cylinder, allowing greater accuracy in matching meniscus position with the etched line on the flask.

PART 4 Precision and Accuracy of Measuring Devices

In recording measurement data it is always important for a biologist to be both accurate and precise, but exactly what do we mean by these terms? **Accuracy** is the degree to which an observed value corresponds to the *true* value. **Precision** is the degree to which measurements are reproducible when repeated. Lack of accuracy or precision may be a function of the measuring device or the technique of measurement. Lack of precision may also be a function of experimental design. A measurement may be accurate but not precise, precise but not accurate, both, or neither.

As a rule, only containers designed for measuring should be used to measure. Markings on Erlenmeyer flasks and beakers are only approximate. Always select a measuring device closest in capacity to the volume to be measured. For example, small measuring devices are most accurate for small volumes. However, you should not use a device so small that the measurement must be repeated, because there is always some error in each measurement (for example, using a 1-ml pipette three times to obtain 3 ml would decrease both the accuracy and precision of the measurement).

The following experiment illustrates the difference between precision and accuracy and the degree of precision and accuracy that can be attained with different kinds of measuring devices. Recall that density equals mass per unit volume. By definition, the density of water is 1 g per cubic centimeter (cc) at 4°C. Recall that 1 cc = 1 ml. Therefore, 100 ml of distilled water at 4°C should have a mass of 100 g. You can therefore determine both the accuracy and precision of volumetric flasks and graduated cylinders by determining the mass of the water they contain.

Procedure

1. Place a volumetric flask on a balance. Tare the balance to read 0.000 g.
2. Fill the flask to a point just below the line marking 100 ml. Adjust the water level to the line by adding the remaining milliliter or so with a Pasteur pipette. If you spill any water on the flask, wipe it dry.

   a. *Why is it important that the flask be dry?* ____________________

   ____

3. Determine the mass of the water and record it below. Repeat the procedure using two other volumetric flasks of the same size.

   Flask 1 ______ g

   Flask 2 ______ g

   Flask 3 ______ g

   b. *For your measurements, what was the range of deviations (smallest to largest) from the true value of 100 g?*

   ____

   c. Accuracy *refers to the freedom from error or mistake of a measuring device. How accurate is a volumetric flask as a measuring instrument?* ____________________

   ____

   d. *The mass of the water in the filled volumetric flask was probably less than 100 g. Why? (Hint: Consider the definition for the density of water as given at the beginning of this part of the exercise.)*

   ____

   ____

   e. *What other factors could affect the accuracy of your measurements?*

   ____

4. Now fill a Nalgene graduated cylinder to the 100-ml mark.
5. Place a 150-ml beaker on the balance and tare the balance to 0.000 g.
6. Pour the water from your graduated cylinder into the beaker and record the mass below. Repeat this procedure four more times. Be sure to dry the beaker thoroughly between measurements and tare the balance before each measurement.

   Measurement 1 ______ g

   Measurement 2 ______ g

   Measurement 3 ______ g

   Measurement 4 ______ g

   Measurement 5 ______ g

   f. For your measurements, what was the range of deviations (smallest to largest) from the true value of 100 g?

   ____

   g. How accurate is the graduated cylinder compared with the volumetric flask?

   ____

   h. It is possible for a measuring device to be inaccurate but precise. *How could this be?*

   ____

   ____

   i. How precise is a graduated cylinder as a measuring device?

   ____

   j. What factors, other than the measuring instrument, could affect the precision of your measurement?

   ____

   ____

EXERCISE D | Preparing Solutions

In the biology laboratory, you will often have to prepare **solutions** to use in experiments. Solutions are homogeneous mixtures of **solute,** the dissolved substance, and **solvent,** the dissolving medium. In this exercise, you will learn to prepare the most common type of solution, a molar solution. (Molal, normal, and percent solutions may also be used in the laboratory and can be prepared according to directions in Appendix III, Preparing Solutions.) By making **dilutions** you can use a "stock" solution to prepare less concentrated solutions without wasting chemicals.

Objectives

☐ Prepare a stock NaCl solution of given molarity.

☐ Accurately prepare serial dilutions of a stock solution.

Procedure

A **molar** (1 molar, or 1 M) solution contains 1 mole of a solute in a liter of **solution.** A **mole** of a substance contains Avogadro's number (6.02×10^{23}) of molecules (or atoms or ions) of that substance. For a compound, Avogadro's number is the number of molecules contained in 1 gram molecular weight of the compound. (**Gram molecular weight** is simply molecular weight expressed in grams.) Thus for any compound, 1 mole has a mass equal to its gram molecular weight. The gram molecular weight of NaCl is

58.5 g. Thus a mole of NaCl has a mass of 58.5 g and Avogadro's number of NaCl molecules is contained in that mole (58.5 g of NaCl). (Since this ionic compound dissociates completely in solution, 1 mole *each* of Na^+ and Cl^- are produced when NaCl dissolves in water.)

A mole of $CaCl_2$ has a mass of 111 g (the molecular weight of $CaCl_2$).

To prepare a liter of a 1 M solution of NaCl you would add 58.5 g of NaCl to a 1,000-ml volumetric flask and add water to the 1,000-ml mark. Remember that a molar solution is defined as 1 mole of material per liter of **solution,** not per liter of solvent. The total volume of water and solute that make up the solution must equal 1,000 ml. If you added 1,000 ml of water to 58.5 g of NaCl, you would have more than a liter of solution (this is called a **molal** solution).

a. Define molar solution. ______________________________

b. How would you make 1 l of a 1 M solution of $CaCl_2$ (molecular weight = 111)? ______________________________

____

c. How does a molar (M) solution differ from a molal (m) solution? ______________________________

____

To calculate the amount of solute required to make a liter of 0.2 M solution, simply multiply the gram molecular weight by the molarity (0.2 M).

d. A 0.2 M solution of NaCl contains ________ *g of NaCl in 1 l of* ______________.

Often you need less than a liter of solution of a specific molarity. An easy way to determine the number of grams of solute needed is to use the following formula:

$$\text{Grams of solute} = \text{molecular weight (in grams)} \times \text{volume (in liters)} \times \text{molarity}$$

e. How would you prepare 500 ml of a 0.4 M solution of NaCl? ______________________________

____

f. How would you prepare 250 ml of a 2 M solution of NaCl? ______________________________

____

1. Your laboratory instructor will ask you to prepare 100 ml of a NaCl solution. The molarity assigned to you or your group will be selected from a range of 0.2 to 1.2 M. Molarity of NaCl solution ________. NaCl required (grams) ______________________.
2. Prepare your solution using a 100-ml volumetric flask.
3. After mixing your solution, pour it into a beaker. With your laboratory instructor, measure the specific gravity* of the NaCl solution using a hydrometer. Specific gravity ________.
4. Compare the specific gravity of your solution to known values of NaCl solutions using the standard curve (Figure 2D-1). Specific gravity is plotted on the *Y*-axis and molarity on the *X*-axis. Locate the specific gravity of your solution on the *Y*-axis and draw a horizontal line to the standard curve (a straight line in this case) on the graph. Now drop a vertical line to the *X*-axis and determine molarity. Assigned molarity ________. Measured molarity ________.

   g. How accurately did you mix your solution? ______________________________

   h. If your measured value differs from your assigned molarity, what do you think might be the cause?

   ____

**The specific gravity of a substance is the ratio of the density of the substance to the density of water.*

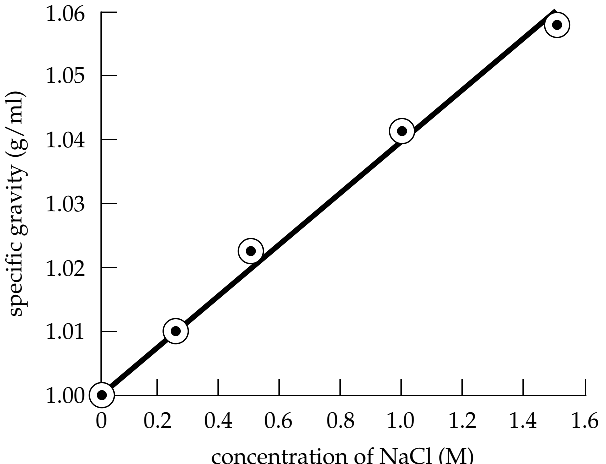


Figure 2D-1 *Standard curve of specific gravity for NaCl solutions of different molarities.*

Suppose you were asked to make 100 ml of a 0.001 M solution of NaCl. This would require 0.00585 g of NaCl in 100 ml of solution. You would need to accurately measure 0.00585 g of NaCl. This would be very difficult. To obtain this amount in solution, you could start with 5.85 g of NaCl in a 10-ml solution. Taking 1 ml of this solution (1 ml contains 0.585 g NaCl) and adding it to 9 ml of water would produce a solution of 0.0585 g/ml. Adding 1 ml of this solution (it contains 0.0585 g) to 9 ml of water would produce a solution of 0.00585 g/ml. Then adding 10 ml of this solution to 90 ml of water would produce a solution of 0.00585 g/100 ml—or 100 ml of a 0.001 M solution. This technique is one of dilution. It is called **serial dilution** because it is accomplished in a series of steps (Figure 2D-2). The original solution from which the first dilution was made is called a **stock solution**.

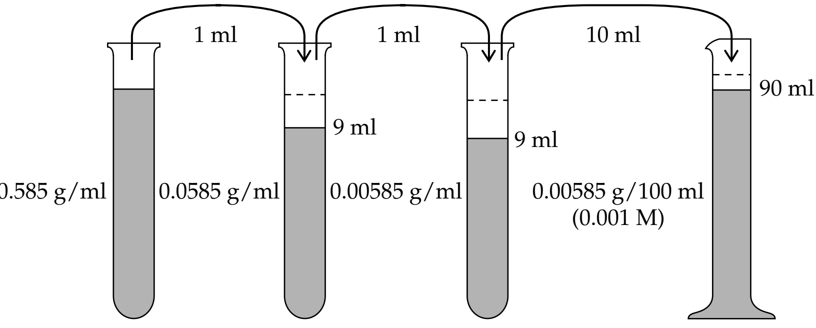


Figure 2D-2 *Serial dilution.*

i. How would you prepare 50 ml of a 0.002 M solution of NaCl? (Hint: First determine the total amount (in grams) of NaCl you need in your solution.)

____

____

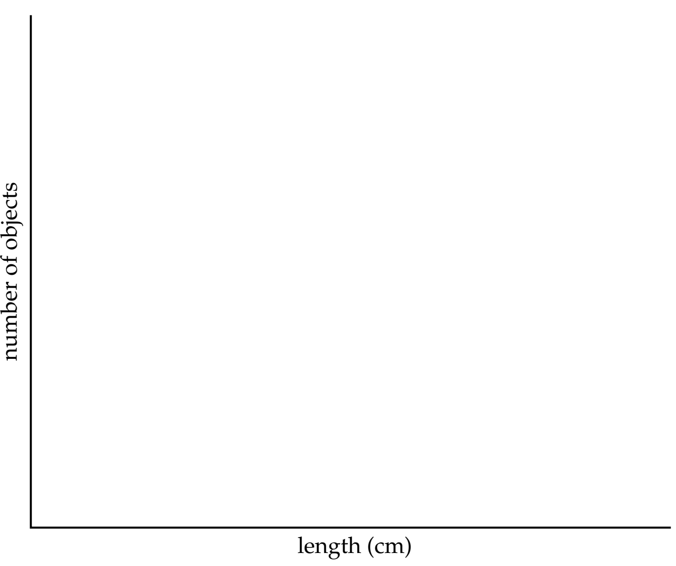
5. Your laboratory instructor has prepared a 4 M solution of NaCl. Use this to prepare 100 ml of a 0.4 M solution. Use the space below for your calculations.

6. Check the accuracy of your work by measuring the specific gravity of your solution and comparing it with those on the standard curve (Figure 2D-1).

j. How accurate was your dilution? ________________________________

Laboratory Review Questions and Problems

1. Using your measurement data from Exercise A (page 2-4), construct a frequency distribution or *histogram.* The abscissa (*X*-axis) should be labeled in centimeters. You will first need to determine the intervals to be used in your frequency distribution. This choice will depend on your data. Refer to Appendix I for assistance.

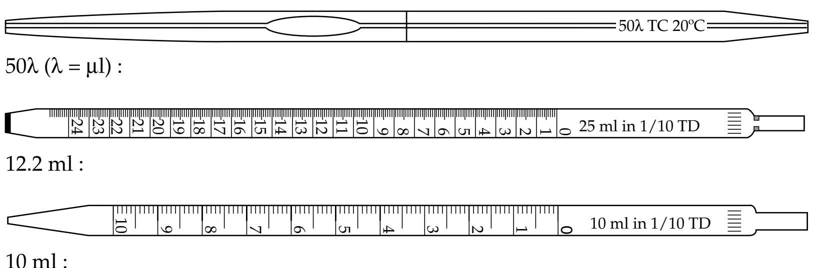


2. What is meant by "taring" the mass of a measuring vessel when using a balance?

3. You place a piece of paper on a balance and find that its mass is 0.63 g. You add a lump of NaOH to the paper and get a mass of 22.21 g. You remember that you forgot to tare the balance before taking this measurement. You tare the balance, add another lump of NaOH, and read 20.64 g. You add another lump of NaOH and read 38.98 g. How much NaOH do you have on the balance, in grams:

_______; milligrams: _______; micrograms: _______; kilograms: _______?

4. On the drawings below, label each type of pipette and indicate how you would measure out the amount of liquid specified for each type of pipette.



5. Draw a representation of the graduations you expect to see on pipettes labeled as follows (to-deliver scales):

5 ml in 1/10

1/10 ml in 1/100

6. When pipetting acids or other harmful materials, should you pipette by mouth? ________
If not, how should you pipette such materials?

7. You are using a 10-ml blow-out pipette graduated in 1/10 ml. (Your pipette reads 0 at the highest marking on the stem.) You have sucked up 8.6 ml of liquid and you cannot put any back into the bottle even though you need to measure out only 6.5 ml. At what mark on the pipette is the meniscus of your liquid now located? If you release liquid from your pipette, at what line should you stop? Draw the pipette and, using two arrows, indicate the start and stop points.

8. Put the quantities below into scientific notation and then convert to the metric units indicated in the second column.

	Scientific Notation	Convert
16 ml	________ ml	________ μl
0.0005 g	________ g	________ μg
150 μg	________ μg	________ ng
12 nm	________ nm	________ mm

9. Given a 50-ml volumetric flask, 50-ml graduated cylinder, 10-ml pipette, 100-ml beaker, and 75-ml Erlenmeyer flask, which measuring instrument would you choose to measure 25 ml of water?

10. Why is a volumetric flask more accurate than a graduated cylinder for measuring volume?

11. What is meant by precision and accuracy in making measurements?

a. What determines how precise a measurement is?

b. What determines how accurate a measurement is?

12. You measure 5 ml of a solution and determine its mass on a balance. You repeat this procedure four times. You try the experiment again. This time you have four new values for mass, as shown below. Choose the statement that best describes your measurements.

Experiment 1 5.7 g, 5.8 g, 5.7 g, 5.8 g

Experiment 2 4.9 g, 5.0 g, 4.8 g, 5.3 g

a. The measurements in both experiments are accurate and precise.

b. The measurements in both experiments are not accurate but are precise.

c. The measurements in experiment 1 are not accurate but are precise; the measurements in experiment 2 are accurate but not precise.

d. The measurements in experiment 1 are accurate and not precise; the measurements in experiment 2 are not accurate and not precise.

LABORATORY

3

pH and Buffers

OVERVIEW

The concentration of hydrogen ions in solution, expressed as pH, is of great importance to living systems. Because both cell structure and function can be affected by even small changes in pH, maintaining pH within a narrow range is a major goal of cellular homeostasis.

During this laboratory, you will learn how the concentration of hydrogen ions in a solution can be changed or maintained. You will also learn how to prepare various types of solutions and how to use several methods of determining pH.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency. After reading Exercise A, Understanding pH, you should complete the problems.

✔ EXERCISE A Understanding pH

Molecules that are dissolved in water may separate (dissociate or ionize) into charged fragments or ions. Often one of these fragments is a hydrogen ion (H^+). The pH of a solution is a measure of the concentration of hydrogen ions (written as $[H^+]$, where [ ] means "concentration of") in that solution, and pH is a measure of the **alkalinity** (*basicity*) or **acidity** of a solution.

Objectives

- ☐ Define pH.
- ☐ Define acid and base.
- ☐ Define neutralization.
- ☐ Given the molarity of a solution of an acid or base, calculate its pH.
- ☐ Given the pH of H^+ concentration of a solution, calculate its pOH or OH^- concentration.

Procedure

Water ionizes when a hydrogen atom that is covalently bound to the oxygen of one water molecule leaves its electron behind and, as a hydrogen ion (H^+), joins a different water molecule. Two ions are produced by this reaction: a hydroxide ion (OH^-) and a hydronium ion (H_3O^+). We can express this reaction as follows:

$$2H_2O \rightleftharpoons H_3O^+ + OH^-$$

Convention, however, allows us to express the ionization of water more simply as

$$H_2O \rightleftharpoons H^+ + OH^-$$

In any given volume of pure water, or in any solution, a small but constant number of water molecules are ionized. In pure water, the number of H^+ ions exactly equals the number of OH^- ions, since one cannot be formed without the other being formed. In pure water, $[H^+] = 1 \times 10^{-7}$ M and $[OH^-] = 1 \times 10^{-7}$ M (where M stands for molar concentration or moles per liter).* The product of the molar concentrations of the two ions, $[H^+][OH^-]$, in pure water is always 1×10^{-14}, a number known as the **ion product of water,** and this represents a dissociation constant (K_w) for pure water. (Note that the brackets indicate molar concentration for the substance they enclose.) So, for pure water,

$$[H^+][OH^-] = 1 \times 10^{-14}$$

The concentrations of OH^- and H^+ ions can be written in terms of the number 10 with an exponent. Recall that in a number such as 1×10^{-7}, 10 is the base and $^{-7}$ is the exponent (the power to which 10 is raised). Remember that to find the product of two numbers with exponents, you add the exponents. So, again, for pure water,

$$\mathbf{[H^+][OH^-] = 1 \times 10^{-14}} \quad \text{or}$$

$$[1 \times 10^{-7}][1 \times 10^{-7}] = 1 \times 10^{-14}$$

Numbers such as these can more easily be expressed as logarithms (base 10). A logarithm is the power to which a base, in this case 10, must be raised to give the desired number. Thus the log of 1×10^{-7} is -7, since this is the power to which 10 must be raised to give the number 0.0000001. pH is defined as the negative logarithm ($\log_{10}$) of the molar hydrogen ion concentration in a solution:

$$\mathbf{pH = -\log_{10} [H^+]} \quad \text{or} \quad pH = \log_{10} \frac{1}{[H^+]}$$

We use the negative logarithm in working with pH so that the numbers, and the pH scale, are positive—note that $-\log_{10} (1 \times 10^{-7}) = +7$, or simply, 7.

For pure water, we can also determine the pOH, based on the concentration of OH^- ions in a solution:

$$\mathbf{pOH = -\log_{10} [OH^-]} = \log_{10} \frac{1}{[OH^-]}$$

We can establish a relationship between the pH and the pOH of a solution by using the expression for the ion product of water. In logarithmic form, this can be written as

$$\log_{10} [H^+] + \log_{10} [OH^-] = \log_{10} (1 \times 10^{-14})$$

Since pH is expressed in terms of the *negative* logarithm of the H^+ concentration in solution, we will express the above equation in *negative* logarithmic form:

$$-\log_{10} [H^+] + (-\log_{10} [OH^-] = -\log_{10} (1 \times 10^{-14})$$

Since the letter "p" in pH stands for "negative logarithm of," we can write this equation as

$$p[H^+] + p[OH^-] = -\log (1 \times 10^{-14}) \quad \text{or}$$

$$\mathbf{pH + pOH = 14}$$

**In a liter of water, there are 3.34×10^{25} water molecules—55.5 moles $\times$ Avogadro's number (6.02×10^{23}). Of these, 1×10^{-7} mole per liter is ionized, so a liter of water contains 6.02×10^{16} H^+ ions. If you divide the number of H^+ ions in a liter by the total number of water molecules in a liter ($6.02 \times 10^{16}/3.34 \times 10^{25}$), you will discover that only 2×10^{-9} or 2 ten-millionths of a percent of all water molecules present in 1 l of water are ionized. That is a very small amount!*

Since for pure water, $[H^+] = 1 \times 10^{-7}$ M and $[OH^-] = 1 \times 10^{-7}$ M, then pH = 7 and pOH = 7, so pH + pOH = 14.

It is important to realize that in any solution, as is the case for pure water, the *product* of $[H^+]$ and $[OH^-]$ is *constant*. For pure water, the molar amounts of H^+ and OH^- are equal. If an ionic or polar substance is dissolved in water, it may change the relative amounts of H^+ and OH^- but the product of the two concentrations is always 1×10^{-14} because water is a source of H^+ and OH^- ions and the ion product (which reflects the tendency of water to ionize) remains constant. For instance, if a substance added to a volume of water ionizes to produce sufficient H^+ ions that the H^+ concentration of the solution increases to 1×10^{-5} M (expressed as $[H^+] = 1 \times 10^{-5}$ M), then the OH^- concentration decreases to $[OH^-] = 1 \times 10^{-9}$ M. The ion product of water for the solution will always equal 1×10^{-14} (*remember:* to find the product of two numbers written in logarithmic form, add the logarithms according to the rules for exponents):

$$[H^+][OH^-] = 1 \times 10^{-14}$$
$$[1 \times 10^{-5}][1 \times 10^{-9}] = 1 \times 10^{-14}$$

Likewise, if a substance added to a volume of water increases the $[OH^-]$, the $[H^+]$ will necessarily decrease. It is important to note, however, that adding H^+ to a solution not only increases the number of H^+ ions but *also* decreases the number of OH^- ions, since some of the added H^+ will combine with OH^- to make water. Adding OH^- to a solution can also decrease the H^+ concentration for the same reason—H^+ and OH^- combine to form H_2O! But, no matter what is added, the product $[H^+][OH^-]$ must always be 1×10^{-14}, so if we know the concentration of one ion, we always know the concentration of the other.

We now can use the expression pH + pOH = 14 to generate the pH scale. If we know the pH we can calculate the pOH (pOH = 14 − pH), and if we know the pOH we can calculate the pH (pH = 14 − pOH). The sum of the negative logarithms of $[H^+]$ and $[OH^-]$ is always 14 and the product of the molar ion concentrations, $[H^+][OH^-]$, is always 1×10^{-14}. To be sure that you understand this relationship, examine Table 3A-1 carefully.

A solution of pH = 7 is **neutral,** at the midpoint of the pH scale. Solutions with a pH value lower than 7 are said to be **acidic.** In an acidic solution, the number of H^+ ions exceeds the number of OH^- ions. Solutions with a pH above 7 are **basic** or alkaline: the number of OH^- ions exceeds the number of H^+ ions. Expressed another way, the more acidic a solution, the higher the number of H^+ ions and the lower the number of OH^- ions. The more basic a solution, the lower the number of H^+ ions and the higher the number of OH^- ions. Check Table 3A-1 to make sure this is true. Also, remember that the pH scale is logarithmic, not arithmetic: if two solutions differ by 1 pH unit, then one solution has *ten* times the hydrogen ion concentration of the other.

An **acid** is a substance that causes an increase in the number of H^+ ions and a decrease in the number of OH^- ions in solution. This increase is most often the result of an ionization that produces H^+. Some common acids and their ionization products are

Hydrochloric acid $HCl \longrightarrow H^+ + Cl^-$

Acetic acid $CH_3COOH \rightleftharpoons H^+ + CH_3COO^-$

Carbonic acid $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$

Phosphoric acid $H_3PO_4 \longrightarrow H^+ + H_2PO_4^- \longrightarrow H^+ + HPO_4^{2-}$

The more completely the acid ionizes, the more H^+ is released, and the stronger the acid is. For example, if the concentration of an HCl solution is 0.1 mole/l and the HCl ionizes completely, we have 0.1 mole/l of H^+. This could also be expressed as $[H^+] = 1 \times 10^{-1}$ M.

Of the acids listed above, HCl and H_3PO_4 are considered *strong acids* since they ionize completely. CH_3COOH, H_2CO_3, and $H_2PO_4^-$ are relatively *weak acids.* A weak acid might, for example, ionize only 10%, so a 1×10^{-2} M solution would contain only 1×10^{-3} mole/l of H^+. Once $[H^+]$ is known, pH can be calculated.

Table 3A-1 The pH Scale

$[H^+]$ M	pH	$[OH^-]$	MpOH	
10^{0} (1.0)	0	10^{-14}	14	acidic
10^{-1} (0.1)	1	10^{-13}	13	
10^{-2} (0.01)	2	10^{-12}	12	
10^{-3} (0.001)	3	10^{-11}	11	
10^{-4} (0.0001)	4	10^{-10}	10	
10^{-5} (0.00001)	5	10^{-9}	9	
10^{-6} (0.000001)	6	10^{-8}	8	
10^{-7}	7	10^{-7}	7	neutral
10^{-8}	8	10^{-6} (0.000001)	6	basic
10^{-9}	9	10^{-5} (0.00001)	5	
10^{-10}	10	10^{-4} (0.0001)	4	
10^{-11}	11	10^{-3} (0.001)	3	
10^{-12}	12	10^{-2} (0.01)	2	
10^{-13}	13	10^{-1} (0.1)	1	
10^{-14}	14	10^{0} (1.0)	0	

Example A solution with $[H^+] = 1 \times 10^{-2}$ M ionizes 10%.

$$[H^+] = 1 \times 10^{-2} \text{ M}$$

$$10\% = 0.10 = 1 \times 10^{-1}$$

$$(1 \times 10^{-2}) \times 10\% = (1 \times 10^{-2})(1 \times 10^{-1}) = 1 \times 10^{-3}$$

$$[H^+] = 1 \times 10^{-3} \text{ M}$$

$$\text{pH} = \log_{10} \frac{1}{[H^+]} = \log_{10} \frac{1}{(1 \times 10^{-3})} = 3$$

1. Fill in Table 3A-2 by calculating the pH for each acid.

Table 3A-2 Calculating pH for Acids

Molarity of Acid	Degree of Ionization	$[H^+]$ M	pH
1×10^{-3}	100%		
1×10^{-3}	10%		
1×10^{-3}	1%		
1×10^{-4}	100%		
1×10^{-2}	100%		
1×10^{-1}	100%		

A substance need not give up hydrogen ions itself to cause an increase in the [H^+] in a solution. For instance, an important molecule in biological systems is CO_2 (carbon dioxide) which combines with water to form carbonic acid (H_2CO_3), which ionizes to produce bicarbonate ion (HCO_3^-):

$$CO_2 + H_2O \longleftrightarrow H_2CO_3 \longleftrightarrow H^+ + HCO_3^-$$

The reaction of SO_2 (sulfur dioxide) with atmospheric water is, in part, responsible for acid rain. It dissolves in water to form sulfurous acid (H_2SO_3):

$$SO_2 + H_2O \longleftrightarrow H_2SO_3 \longleftrightarrow H^+ + HSO_3^-$$

A **base** is a substance that causes a decrease in the number of H^+ in solution and an increase in OH^-. In many cases this is achieved by the ionization of the molecule to produce OH^- (hydroxyl ion), which not only adds to the OH^- in solution but also *removes* H^+ from solution by combining with it to form water, thus raising the pH.

$$KOH + HCl \longleftrightarrow K^+ + OH^- + H^+ + Cl^- \longleftrightarrow KCl + H_2O$$

Thus, **neutralization** of an acid by a base produces a **salt** (an ionic compound composed of a negative ion from an acid and a positive ion from a base, such as KCl) and **water.**

Some common bases that ionize to produce OH^- are

Sodium hydroxide $NaOH \longrightarrow Na^+ + OH^-$

Magnesium hydroxide $Mg(OH)_2 \longrightarrow Mg^{2+} + 2\,OH^-$

Potassium hydroxide $KOH \longrightarrow K^+ + OH^-$

These are all *strong bases* because they ionize completely in solution.

Ammonia (NH_3), dissolved in water, is also basic. It does not produce OH^- but it can remove H^+ from solution:

$$NH_3 + H_2O \longrightarrow NH_4^+ + OH^-$$

The bicarbonate ion (HCO_3^-) is also basic. It, too, can accept H^+:

$$H^+ + HCO_3^- \longleftrightarrow H_2CO_3$$

2. Fill in Table 3A-3. Remember the relationship between pH and pOH discussed earlier.

Table 3A-3 Calculating pH for Bases

Molarity of Base	Degree of Ionization	[OH^-] M	[H^+] M	pH
1×10^{-3}	100%			
1×10^{-3}	10%			
1×10^{-2}	100%			
1×10^{-5}	100%			
1	100%			

3. Keeping in mind that $[H^+][OH^-] = 1 \times 10^{-14}$, give the following for a *neutral* solution:

[H^+] = ______

[OH^-] = ______

pH = ______

✔ EXERCISE B | Using Indicators to Measure pH

Indicators are chemicals that change color depending on the pH of the solution and are often used to determine the pH of a solution colorimetrically.

Objectives

- ☐ Use a colorimetric pH indicator.
- ☐ Use an indicator solution or alkacid test paper to determine the pH of some common solutions.
- ☐ Prepare and use a series of standards for qualitative measurements.

✔ PART I Making a pH Indicator

A solution of certain **anthocyanins,** the plant pigments responsible for red, blue, and purple colors in flowers, fruits, and autumn leaves, can be used as a pH indicator. At low pH, solutions of anthocyanins turn red; at high pH, they turn blue. In some flowers, however, soil pH affects the uptake of certain metals that can complex with the anthocyanin pigment and prevent its normal color expression. For instance, in hydrangeas, low soil pH promotes the uptake of aluminum, which then complexes with the anthocyanins and prevents the production of "normal" pink color. Instead, the flowers appear blue. Raising the pH of the soil prevents aluminum uptake and the pink color can be expressed. At more neutral pH values, the flowers appear intermediate in color.

A solution of anthocyanins extracted from red cabbage will be used as a pH indicator in this laboratory. At the beginning of class, your instructor will make an extract of anthocyanins by boiling red cabbage in water for 3 minutes and then filtering the cabbage extract through cheesecloth.

To use an indicator to determine the pH of solutions of unknown pH, you must first determine the color changes that occur when the indicator is used with substances whose pH is known. The color changes that occur in the cabbage extract indicator when mixed with substances of pH 2, 4, 6, 7, 8, 10, 12, and 14 will be used as a set of **standards.** You can then determine the pH of various substances by mixing each with cabbage extract and comparing the resulting colors with the colors of the standards.

Procedure

1. Work in pairs. Put eight clean test tubes in a rack and label them 2, 4, 6, 7, 8, 10, 12, and 14. Use a pipette to measure 5 ml of the appropriate buffer into each test tube (i.e., place the pH 2 buffer in the tube labeled "2," and so on).
2. Use a pipette to measure 3 ml of cabbage extract and add it to each test tube. Cover with Parafilm and invert to mix well.
3. Record the color of each tube below. *Note:* Record both initial and final colors at pH 12 and 14. The pigments are not stable at these pH values.

pH	Color
2	
4	
6	
7	
8	
10	
12	
14	

✔ PART 2 Measuring pH with Cabbage Indicator

Procedure

1. Obtain two clean test tubes and label them A and B.
2. In tube A, mix 2 ml of solution A with 1 ml of cabbage extract.
3. Compare the color in tube A with the colors of the standards. The approximate pH of the unknown solution is the pH of the standard whose color most closely matches the color in tube A.

   pH of A ______ $[H^+]$ ______

4. Use the same method (steps 2 and 3) to measure the pH of solution B.

   pH of B ______ $[H^+]$ ______

   a. Which solution is more acidic? ______

✔ PART 3 Using Alkacid Test Paper

The cabbage indicator method can be used only with colorless or white solutions. Alkacid test papers, which are impregnated with indicators, are another means of estimating pH and can be used with colored solutions.

Procedure

1. Hold a test paper with forceps. Use a clean stirring rod to apply a drop of solution A to the test paper.
2. While the paper is still wet, compare its color with the standard pH color scale on the label of the alkacid paper's container.

   pH of A ______

3. Measure and record the pH values of solutions B, C, and D.

   pH of B ______ pH of C ______ pH of D ______

   a. Do the results obtained with the alkacid papers match those obtained using the cabbage indicator method? ______

   b. Explain any discrepancies (alkacid paper doesn't have a standard for pH 7, so a neutral solution will be closer to either 6 or 8). ______________________________

   ____

EXERCISE C Determining the pH of Some Common Solutions (*Optional*)

Cabbage indicator and alkacid test paper can be used to determine the pH of common beverages, medicines, and cleaning solutions.

Objectives

☐ Understand the relationship of pH to the taste of common beverages and to the activity of medicines and cleaning solutions.

PART 1 pH of Beverages

Many beverages differ in their acidity or alkalinity, often due to CO_2 content (if carbonated) or organic acids.

Formulate a hypothesis that predicts the relative differences in pH values for the beverages listed in the Procedure below.

HYPOTHESIS:

NULL HYPOTHESIS:

Which beverage do you **predict** will have the lowest pH?

What is the **independent variable**?

What is the **dependent variable**?

Procedure

1. Work in pairs. Use the cabbage indicator method (see Exercise B, Part 2) to determine pH values for 7-Up and wine. Use alkacid paper (see Exercise B, Part 3) for determining the pH of coffee and apple juice. Record the values below.

Beverage	pH
Apple juice	
Coffee (black)	
7-Up	
White wine	

2. Arrange these solutions in order of increasing $[H^+]$:
   1. ________________
   2. ________________
   3. ________________
   4. ________________

Do your results support your hypothesis? _______ *Your null hypothesis?* _______

Was your prediction correct? _______

a. *Did you discover anything about the beverages that surprised you?* ______________________________

b. *Based on your results, can you think of reasons to drink or not drink any of these beverages in excess?*

____

PART 2 pH and Activity of Some Common Medicines

Many medicines, especially those used to guard against acidity in the digestive tract, are fairly alkaline, while other medicines tend to be acidic.

Formulate a hypothesis that predicts the relative differences in pH values for the medicines listed in the Procedure below.

HYPOTHESIS:

NULL HYPOTHESIS:

Which medicine do you **predict** will have the highest pH?

What is the **independent variable**?

What is the **dependent variable**?

Procedure

Work in pairs. Use the cabbage indicator method to determine pH values for the following medicines.

Medicine	pH
Aspirin	
Milk of Magnesia [$Mg(OH)_2$]	
Sodium bicarbonate (Alka-Seltzer) ($NaHCO_3$)	
Maalox	

Do your results support your hypothesis? ______ *Your null hypothesis?* ______

Was your prediction correct? ______

a. It is often recommended that aspirin be taken with a large glass of milk or water. Based on your results above, why might this be important? ____________

b. Would apple juice or citrus juice be a good accompaniment to aspirin? ______ *Explain why or why not.*

____

Enzymes function best at particular pH values. In the normal human stomach, a pH of 2.0 to 3.0 provides the environment required for the proper functioning of the digestive enzymes found there. The last three medicines in the list above are often used for treatment of "acid indigestion" of the stomach, a condition in which a reduction in pH interferes with efficient enzyme action and thus with digestion.

c. Based on your results, how would you explain the action of these medicines? ____________

d. What might happen if an excess of any of these medicines were used? ____________

PART 3 pH and the Action of Some Cleaning Solutions

Cleaning solutions vary in their chemical composition. This, in turn, determines what materials they mix with for cleaning purposes.

Based on your experience with cleaning your clothes, dishes, body, and even your sink, formulate a hypothesis that predicts the relative differences in pH values for the cleaning solutions listed in the following Procedure.

HYPOTHESIS:

NULL HYPOTHESIS:

Which cleaning solution do you **predict** will have the highest pH?

What is the **independent variable**?

What is the **dependent variable**?

Procedure

Work in pairs. Use the cabbage indicator method to determine the pH values of the cleaning solutions listed below.

Solution	pH
Drano	
Ivory Liquid	
Cascade	
Tide	

Do your results support your hypothesis? _______ *Your null hypothesis?* _______

Was your prediction correct? _______

a. *What do all these solutions have in common?* ____

____

EXERCISE D Soil pH and Plant Growth (*Optional*)

Since plants obtain most of their nutrients in the form of ions, the kinds and amounts of nutrient ions available from the soil affect plant growth and health. They also have a significant effect on the types of plants that can be grown in soils of various compositions. The chemical composition of soil varies greatly from region to region, due to differences in geology, topography, climate, and plant and animal life. In any soil, the complex equilibria among soil particles, water, and nutrient ions are influenced by soil pH. For example, phosphorus (P), an element essential for plant growth, is taken up in the form of phosphate ions, either as $H_2PO_4^-$ or HPO_4^{2-}. If the soil is acidic, the $H_2PO_4^-$ form is most abundant. HPO_4^{2-} is prevalent under neutral conditions, and PO_4^{3-} exists in alkaline soils. Plants absorb the $H_2PO_4^-$ form most readily, so phosphorus is most available in acidic soils. If the pH is too low, $H_2PO_4^-$ combines with other ions and is precipitated out of the soil. Thus phosphorus is most available at pH 5.5 to 6.5. A similar effect is seen with aluminum, as described in Exercise B, Part 1.

There are at least 13 elements plants must obtain from the soil, and each type of plant thrives within a more or less narrow range of chemical balance. There is no single soil pH that provides the perfect environment for all plants. Instead, plants have adapted to various soil chemistries, and each species has its own range of pH tolerance (Table 3D-1).

Table 3D-1 pH Preference of Selected Species

Plant	pH Range
Eastern hemlock, azalea, rhododendron, gardenia, cranberry, blueberry	4.5 to 6.0
Coleus, iris, tomato, squash, strawberry, tobacco	5 to 7
Gladiolus, cherry, pear, sugar maple, alfalfa, asparagus, yellow poplar	6 to 8

Objectives

- ☐ Relate soil pH to plant nutrition.
- ☐ Measure the pH of soil samples.

Procedure

In this exercise you will determine the pH of several soil samples. Water has been added to these samples to help release some of the ions into solution so the pH can easily be determined.

Based on your experience with gardening and your knowledge of soil and plants common to different environments, formulate a hypothesis that predicts the relative differences in pH values for the soils listed below.

HYPOTHESIS:

NULL HYPOTHESIS:

What type of soil do you **predict** would have the lowest pH?

What is the **independent variable**?

What is the **dependent variable**?

Work in pairs. Use alkacid test paper to measure the pH of each of the following soil samples.

Sample	pH
Potting mix	
Clay	
Sand	
Lime	
Peat moss	

Do your results support your hypothesis? ________ *Your null hypothesis?* ________

Was your prediction correct? ________

a. *Suppose that a soil sample from your garden has a pH of 4. You want to grow* Coleus, *which requires a pH range of 5 to 7. Which of the substances tested could be used to adjust the pH to one suitable for growing* Coleus *plants?* ________________ *Why?* ____

b. *Suppose that you want to grow azaleas, which require a pH range of 4.5 to 6.0. The soil in your garden has a pH of 7. Which of the substances tested could be used to adjust the pH to one suitable for growing azaleas?*
________________ *Why?* ____
____

c. *Your hydrangea bush produces beautiful pink flowers year after year. One spring you decide you would like to have blue hydrangea flowers instead. [Recall that certain pigments called anthocyanins are often complexed with metals abundant in the soil, and are responsible for pink, purple, and blue color in many flowers (see Exercise B, Part 1).] What should you do?* ____
____

✔ EXERCISE E | The pH Meter

In Exercises B, C, and D, you learned how the pH of a solution can be estimated by comparing experimental indicator colors with known standards. In many cases, however, higher accuracy and greater reliability are necessary. Electrometric methods of pH determination, using pH meters, give more precise results.

The standard laboratory pH meter has a **glass electrode** that is sensitive to hydrogen ion activity ("activity" is defined as the effective concentration of an ionic species in solution; it is usually expressed in moles per liter) and a **reference electrode** which completes the electrical circuit. On some pH meters, a combination electrode performs both functions. Other parts of the pH meter include the following:

Readout meter An analog meter with a pH scale for pH determinations and a millivolt (mV) scale for millivolt measurements.

Mechanical meter zero An adjustment that mechanically zeros the meter pointer.

Function selector A switch that maintains the meter on standby when measurements are not being taken and that selects the measuring mode, either pH or millivolts.

Standardize control A control that allows the meter to be set to the pH of the buffer solution used to standardize the instrument.

Temperature control A control that compensates for the temperature of the solution being measured (it is active only when the function selector is in the pH mode).

Objectives

- ☐ Use the pH meter to measure the pH of a solution.

Procedure

1. Work in pairs. Locate each of the parts (listed above) on the pH meter available in your laboratory. You instructor will calibrate and standardize the pH meters in your lab by adjusting the pH reading on the analog scale of the meter when its electrodes are placed in a buffer solution of known value (the standard). To obtain valid results when measuring the pH of a solution, the standardization buffer should have a pH close to that of the sample to be tested. In other words, if you are working with acidic solutions, you should standardize the pH meter with a known buffer solution of pH 4, *not* pH 13.

   Because of the range in pH values for the solutions being tested in this exercise, you will need to select a pH meter that has been standardized with a buffer that has a pH close to the estimated or expected pH of the solution you are testing (the cabbage extract indicator or

alkacid paper has given you an estimate of pH for each solution). Each pH meter in your laboratory is labeled to indicate the pH of the buffer with which it has been standardized.

2. At one of the pH meters, you will find a 50-ml beaker containing 20 ml of apple juice and a small magnetic stirring bar. Check the approximate pH from your previous results in Exercise C, Part 1, and select the proper pH meter. Record your estimated pH values in Table 3E-1.
3. Set the beaker on the magnetic stirring plate next to the pH meter and turn on the stirrer so that the magnetic bar revolves slowly in the solution.
4. Check to be sure that the function selector is on “standby.” Raise the pH electrode out of its storage beaker. Over a waste beaker, rinse the electrode with distilled water from a wash bottle.
5. While taking care that the stirring bar does *not* hit the electrode, immerse the electrode in the beaker of apple juice.
6. Change the function selector from “standby” to “pH.”
7. Record the pH in Table 3E-1.
8. Turn the function switch back to “standby.”
9. Raise the electrode out of the solution. Over a waste beaker, rinse the electrode with distilled water and place it into its own storage beaker.
10. Repeat this procedure for the remaining solutions listed in Table 3E-1.

Table 3E-1 pH of Common Solutions

Solution	Estimated pH	Measured pH	$[H^+]$ M	$[OH^-]$ M
Apple juice				
7-Up				
Maalox				
Tide				
Ivory Liquid				

11. Now that you can obtain a more accurate measure of pH (to tenths of pH units), you can calculate $[H^+]$ or $[OH^-]$ by using your calculator. Since pH is expressed as a negative logarithm of $[H^+]$, you need to determine the antilogarithm (“antilog”) of the pH expressed as a negative number.

Example A solution has a pH of 3.2. What is its hydrogen ion concentration? Using a Texas Instruments calculator or other scientific calculator (see your calculator’s directions if it uses reverse Polish notation—RPN),

ENTER 3.2

Change it to -3.2 (use the $+/-$ key)

Press (INV)(LOG) $= 0.000631$, or 6.31×10^{-4}

The hydrogen ion concentration is 6.31×10^{-4} M.

a. What is the hydroxide ion $[OH^-]$ concentration of this solution? ______________

12. Given $[H^+]$, you can also determine the pH.

Example A solution has an $[H^+]$ of 1.95×10^{-7} M. What is its pH? Using a scientific calculator, enter the number 1.95×10^{-7} into your calculator in scientific notation form:

ENTER 1.95

Press the exponent entry key (EE) to indicate to the calculator that the next numbers entered represent the exponent.

Enter 7 and press the +/− key to convert to −7

Press LOG and the +/− key: $-\log 1.95 \times 10^{-7} = 6.71$

The pH is 6.71.

13. Practice on the following.

Solution	$[H^+]$ M	pH
Urine	5.89×10^{-5}	
Pancreatic juice	7.95×10^{-8}	

Solution	pH	$[H^+]$ M
Lemon juice	2.8	
Milk	6.4	

14. Refer to Appendix III, Preparing Solutions, and then prepare 100 ml of the solutions listed in the table below. Work in pairs.

Solution	Estimated pH	Measured pH	$[H^+]$ M	$[OH^-]$ M
0.1 M NaOH				
0.1 *N* $Ca(OH)_2$				
0.2 M KH_2PO_4				
5% NaCl				

15. Use alkacid test paper to estimate the pH of each solution. Then use the pH meter to measure the pH of each solution. Record your results in the table.

16. Now that you know that pH + pOH = 14 and that $[H^+][OH^-] = 10^{-14}$, and you know how to use logarithms to convert from ion concentration to pH or antilogarithms to convert from (−)pH to ion concentration, you can convert from any of the following quantities to the other (see next page):

Example If the pH of a solution is 6, what is its OH^- concentration? First, find the antilog of −6. This will give you the H^+ concentration of the solution. Divide 1×10^{-14} by $[H^+]$ to give $[OH^-]$. Calculate this value. ______________________

b. Given that a solution has on OH^- concentration of 1×10^{-4} M, what is its pH? ________

c. Given that a solution has $[H^+] = 1 \times 10^{-6}$ M, what is its $[OH^-]$? ____________

$$\text{pH} \xrightleftharpoons[-\log_{10}[H^+]]{\text{antilog }(-)\text{pH}} [H^+]$$

$$\text{pOH} = 14 - \text{pH} \qquad \text{pH} = 14 - \text{pOH} \qquad [\text{OH}] = \frac{1 + 10^{-14}}{[H^+]} \qquad [H^+] = \frac{1 + 10^{-14}}{[OH^+]}$$

$$\text{pOH} \xrightleftharpoons[-\log_{10}[OH^+]]{\text{antilog }(-)\text{pOH}} [OH^+]$$

EXERCISE F Buffers

Physiological processes require that pH remain relatively constant. The pH of blood in our bodies, for example, is usually maintained between 7.3 and 7.5. However, blood returning to the heart contains CO_2 picked up from the tissues (recall that CO_2 combines with water to form carbonic acid), and our diets, as well as the normal metabolic reactions in cells, may contribute an excess of hydrogen ions. The pH must be kept constant by several *buffer systems.*

A **buffer** is defined as a solution that *resists change* in pH when small amounts of acid or base are added. Bicarbonate, phosphate, and protein buffer systems maintain our blood pH. We will use the phosphate buffer system as an illustration.

A buffer is made by mixing a weak acid with its salt in order to have in solution something that can act as an acid (give up hydrogen ions) *and* something that can act as a base (accept hydrogen ions). In a potassium phosphate buffer, the weak acid, $H_2PO_4^-$, is supplied as KH_2PO_4 (monobasic potassium phosphate) and its salt as K_2HPO_4 (dibasic potassium phosphate).

At equilibrium, these substances are ionized to some degree:

$$KH_2PO_4 \rightleftharpoons K^+ + H^+ + HPO_4^{2-}$$

$$K_2HPO_4 \rightleftharpoons 2K^+ + HPO_4^{2-}$$

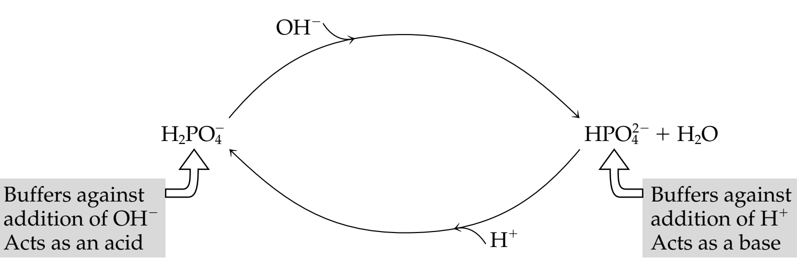
If hydrogen ions are added to the solution, they can be picked up by HPO_4^{2-}, which acts as a base:

$$H^+ + HPO_4^{2-} \longleftrightarrow H_2PO_4^-$$

If hydroxyl (OH^-) ions are added to the solution, they can be picked up by H^+:

$$OH^- + H^+ \longleftrightarrow H_2O$$

In summary,



Objectives

- ☐ Define buffer and discuss why buffers are important to organisms.
- ☐ Use a buffer system to show how buffers work.
- ☐ Explain why some substances have buffering capacity and others do not.

Procedure

In this exercise, you will use colorimetric indicators to determine which of four unknown solutions act as buffers. A change in one of the indicators, congo red or thymolphthalein, indicates a change in pH.

The four solutions, A, B, C, and D, were prepared using K_2HPO_4 or KH_2PO_4, or a combination of these, dissolved in distilled water.

1. Locate the four solutions marked A, B, C, and D on your laboratory table.
2. Pour approximately 40 ml of one of the unknown solutions into a 100-ml beaker. Insert a stirring bar and place the beaker on a stirring plate.
3. Add 1 dropperful of congo red. Record the color of the solution in Table 3F-1.
4. Measure the pH using a pH meter and record this value.
5. Using a pipette, add 2 ml of 0.1 *N* HCl. Record the color of the solution and its pH.
6. Dispose of this solution, and wash and dry the beaker and stirring bar thoroughly.
7. Pour another 40 ml of the same unknown solution into the beaker, insert the stirring bar, and return it to the stirring plate.
8. Add 1 dropperful of thymolphthalein and again measure the color and pH and record in Table 3F-1.
9. Now use a pipette to add 2 ml of 0.1 *N* NaOH. Record the color and pH.
10. Dispose of the solution and wash and dry the beaker and stirring bar thoroughly.
11. Repeat these steps for all three of the other unknown solutions, to complete Table 3F-1.

Table 3F-1 Determination of Changes in pH and Color

Unknown Solution	Initial Color	Initial pH	Solution Added	Final Color	Final pH
A Congo red			0.1 *N* HCl		
Thymolphthalein			0.1 *N* NaOH		
B Congo red			0.1 *N* HCl		
Thymolphthalein			0.1 *N* NaOH		
C Congo red			0.1 *N* HCl		
Thymolphthalein			0.1 *N* NaOH		
D Congo red			0.1 *N* HCl		
Thymolphthalein			0.1 *N* NaOH		

12. Answer the following questions based on your observations of color changes that occurred when either acid or base was added.

a. Which unknown solution buffered against both acids and bases? ______ *What did this solution contain?* ____

b. Which unknown solution buffered only against the addition of acid? ______ *What did this solution contain?* ____

Did the solution act as an acid or base? ____________ *Explain.*

____

c. Which unknown solution buffered only against the addition of base? ______ *What did this solution contain?* ____

Did the solution act as an acid or base? ____________ *Explain.*

____

d. Which solution did not buffer against addition of either acid or base? ______ *What did this solution contain?* ____

e. Which indicator would be the best to use to determine the presence of an acid?

____________________ *What color would it appear at pH = 2?* ____________

pH = 7? ____________ *pH = 11?* ____________

f. Which indicator would be the best to use to determine the presence of base?

____________________ *What color would it appear at pH = 2?* ____________

pH = 7? ____________ *pH = 11?* ____________

Use of indicators gives qualitative evidence of pH change. Qualitative observations can be further supported quantitatively by considering the actual H^+ and OH^- concentration changes.

When adding 2 ml of a 0.1 *N* acid or base, you are adding the equivalent of 5×10^{-3} M H^+ ions or OH^- ions.

$$\text{Increase in } H^+ \text{ or } OH^- = \frac{\text{volume of acid or base added}}{\text{volume of solution added into}} \times \text{normality of solution added}$$

Note: When dealing with acids and bases it is convenient to use equivalents to indicate the concentration of reacting particles. For example, a solution is 1 *N* when it contains one gram equivalent weight of reacting particles. (See Appendix III, Normal Solutions.)

In our example,

$$\text{Increase in } H^+ = \frac{0.002 \text{ l}}{0.04 \text{ l}} \times 0.1 \text{ equivalents/liter}$$

where 0.002 l = 2 ml of HCl or NaOH added to 0.04 l or 40 ml of solution. This gives

$$\text{Increase in } H^+ = 0.005 \text{ M (or } 5 \times 10^{-3} \text{ M)}$$

Therefore, if the solution you are testing does not buffer against an acid or a base, you will expect a 5×10^{-3} M increase in its H^+ or OH^- concentration. However, if your solution does buffer, the change in H^+ or OH^- concentrations will not be as dramatic.

13. Calculate the changes in H^+ and OH^- that you observed in your four unknown solutions. To calculate the change in H^+, subtract the initial H^+ concentration from the final H^+ concentration. To calculate the change in OH^-, first convert the pH into pOH. Then, subtract the initial OH^- concentration from the final OH^- concentration. Record these values in Table 3F-2.

Table 3F-2 Calculated Changes in H^+ and OH^- Concentrations

Unknown Solution	Initial $[H^+]$	Final $[H^+]$	Change in $[H^+]$	Initial $[OH^-]$	Final $[OH^-]$	Change in $[OH^-]$
A						
B						
C						
D						

g. From these results, did the indicators identify the buffering abilities of the unknown solutions accurately? ____

____

Laboratory Review Questions and Problems

1. HCl ionizes completely in water. What is the $[H^+]$ of a 0.01 M solution of HCl? What is the pH?

2. Is the hydrogen ion concentration of a pH 3.8 solution higher or lower than that of a solution with a pH of 6.2?

3. If one solution has 100 times as many hydrogen ions as another solution, what is the difference, in pH units, between the two solutions?

4. If solution A contains 1×10^{-6} M H^+ ions and solution B contains 1×10^{-8} M H^+ ions, which solution contains *more* H^+ ions?

5. Make a statement relating hydrogen ion concentration to the acidity and basicity of solutions.

6. HA is an acid that ionizes 10% in solution. What is the $[H^+]$ of a 0.01 M solution of HA? What is its pH?

7. Write the equation for the neutralization of NaOH by HCl.

8. What is the $[H^+]$ of a solution whose pH is 8? What is the $[OH^-]$?

9. Complete the following table.

$[H^+]$	$[OH^-]$	pH
	1×10^{-6}	
		4
1×10^{-3}		

10. You have made the following solutions:

Solution A Hydrogen ion concentration = 1×10^{-6} M

Solution B pH = 5

State whether each of the following is true or false. If false, explain why.

Solution A contains more H^+ ions than solution B.

Solution A contains 1×10^{-8} M OH^- ions.

Solution B contains 1×10^{5} M H^+ ions.

Solution B is acidic and solution A is basic when measured using the pH scale of 0–14.

Solution A is less acidic than solution B.

If the pH of solution A is to be raised by one pH unit, you would want to increase the OH^- concentration to 10^{-7} by adding base.

The two solutions differ in hydrogen ion concentration by a factor of 10 (i.e., one solution has 10 times the hydrogen ion concentration of the other).

11. Assuming complete ionization, what are the $[OH^-]$, $[H^+]$, and pH of a 0.01 M solution of NaOH?

12. Assuming complete ionization, what are the $[OH^-]$, $[H^+]$, and pH of a 0.1 M solution of KOH?

13. Calculate the pH of the listed solutions.

Solution		pH
Maalox	$[H^+] = 3.1 \times 10^{-9}$ M	
Saliva	$[H^+] = 1.95 \times 10^{-7}$ M	
Vinegar	$[OH^-] = 2.4 \times 10^{-12}$ M	

14. Calculate the H^+ and OH^- concentrations of the listed solutions.

Solution	pH	$[H^+]$ M	$[OH^-]$ M
Tomato juice	4.2		
Blood plasma	7.4		
Seawater	8.2		

15. State two reasons why pH maintenance is important to biological systems, and give examples of how pH may affect biological reactions.

16. You have four 1,000-ml beakers filled with four different clear solutions:

0.1 M NaH_2PO_4

0.1 M Na_2HPO_4

0.1 M phosphate buffer, pH 7.2

Distilled water

Oops! You forgot to label them and they all look alike. You get the congo red and thymolphthalein from the lab and test a sample of each solution, labeling the beakers randomly as A, B, C, and D. You use congo red when HCl is added to the sample, and thymolphthalein when NaOH is added. You get the following results.

	Color Before Addition	Add	Color After Addition
A	Red	HCl	Red
A	Colorless	NaOH	Blue
B	Red	HCl	Blue
B	Colorless	NaOH	Blue
C	Red	HCl	Red
C	Colorless	NaOH	Colorless
D	Red	HCl	Blue
D	Colorless	NaOH	Colorless

What is the identity of solutions A, B, C, and D?

A

B

C

D

17. You have buffers of pH 2, 4, 6, 8, and 10, but you need a pH 7 buffer for your experiment. Describe how you will make the pH 7 buffer.

18. A gardener planted pink hydrangeas in his yard last year, but this year when the flowers bloomed they were blue. He doesn't understand what happened. He took good care of the plants and mulched them with pine straw, just like the gardening encyclopedia said. What happened to his plants? How could he make them produce pink blooms again?

LABORATORY

Using the Spectrophotometer

OVERVIEW

Color provides us with both beauty and useful information. Color is a source of our pleasure in a sunset, in the autumn leaves, or in a beautiful bouquet of flowers. Color can also be an indicator of when vegetables or fruits are ripe, when our coffee is strong enough, or when a storm is coming.

In this laboratory, you will explore how a **spectrophotometer** uses the colors of the light spectrum to determine the concentration of light-absorbing molecules in a solution.

The visible light spectrum, like X-rays, radio waves, and infrared waves, is part of the spectrum of electromagnetic radiation. Types of electromagnetic radiation differ in both wavelength and energy level, but all types travel through space in waves. The height of a wave at its crest is called its **amplitude;** the intensity or the brightness of visible light is proportional to its amplitude. The distance from the crest of one wave to the crest of the next wave is called the **wavelength** (λ); in the visible spectrum, the color of the light we see depends on its wavelength. Wavelength is measured in units called nanometers (1×10^{-9} m). Wavelengths of 400 to 700 nm comprise the "visible light spectrum"—the part of the electromagnetic spectrum that can excite photoreceptors within the human eye.

STUDENT PREPARATION

Prepare for this laboratory by reading Exercise A, Part 1.

✓ EXERCISE A How the Spectrophotometer Works

✓ PART 1 Principles of Spectrophotometry

Molecules either absorb or transmit energy in the form of electromagnetic radiation. White light (normal daylight) is made up of all the wavelengths of electromagnetic radiation in the visible spectrum. How objects or chemical substances absorb and transmit the light that strikes them determines their color.

What we see as the color of an object, or a solution, is determined by what wavelengths of light are "left over" to be transmitted or reflected by the object after certain wavelengths are absorbed by its constituent molecules. For example, the pigment chlorophyll, present in the leaves of plants, absorbs a high percentage of the wavelengths of light in the red and violet to blue ranges (Figure 4A-1). Green light, not absorbed by chlorophyll molecules, is reflected from the surface of the leaf—thus most plants appear to be green. A solution of chlorophyll extracted from a leaf would also be green.

The spectrophotometer can be used to measure the amount of light absorbed or transmitted by molecules in a solution. The spectrophotometer operates on the following principle. When a specific

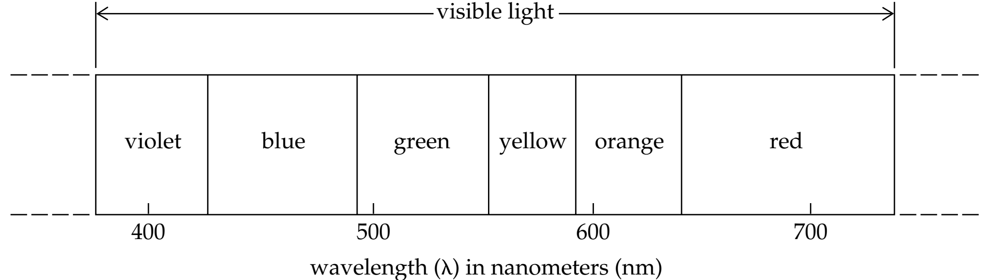


Figure 4A-1 *Electromagnetic wavelengths in the visible light spectrum. For a diagram illustrating the full range of electromagnetic radiation, see Figure 12A-2, page 12-5.*

wavelength of light is transmitted through a solution, the radiant light energy absorbed, **absorbance** (A), is directly proportional to (1) the absorptivity of the solution—the ability of the solute molecules to absorb light of that wavelength; (2) the concentration of the solute; and (3) the length of the path of light (usually 1 cm) from its source, through the solution, to the point where the percentage of light energy transmitted or absorbed is measured by a phototube.

Spectrophotometers that employ ultraviolet or visible light are the types most often used to study biological structures and reactions. The investigator selects a wavelength of light that will be maximally absorbed by a particular solute in solution. (If visible light is used and the molecule of interest does not absorb light, it is often possible to set up a chemical reaction that will yield a colored product.) After passing through the solution, the light energy received at the phototube is expressed as the ratio of transmitted light I_T (the light that passes through the sample) to incident light I_0 (the intensity of light at the source before it enters the sample). The light received at the phototube is measured as percent transmittance (T), or as the log of its inverse, absorbance (A):

$$\%T \text{ (percent transmittance)} = \frac{I_T}{I_0} \times 100$$
$$A \text{ (absorbance)} = \log \frac{I_0}{I_T}$$

By measuring the absorbance (or transmittance) it is possible to determine the concentration of the absorber (molecule) in solution. Concentration can be calculated directly if the molar absorptivity of the molecule (the amount of light at a specific wavelength absorbed by a specified concentration of solute in moles per liter) is known. Usually, however, molar absorptivity is not known and absorbance readings indicate only relative concentrations—a higher absorbance (A) resulting from a higher concentration. In such cases, the concentration can be found by locating the absorbance reading of the unknown concentration on a graph of the absorbances of known concentrations (standard curve).

✔ PART 2 Using the Spectrophotometer

The Bausch & Lomb Spectronic 20 Colorimeter (Figure 4A-2) is an extremely versatile instrument that is useful for the spectrophotometric, or colorimetric, determinations of solutions.

Within the optical system of the spectrophotometer, rotation of a prism (diffraction grating) allows the investigator to select specific wavelengths of light in a range from 375 to 625 nm. Light of a selected wavelength is passed through the sample and is picked up by a measuring phototube, where the light energy is converted to a reading on the meter of the spectrophotometer (Figure 4A-3).

Most spectrophotometers have two scales—one is a linear scale (the *transmittance scale*) given as percent transmittance, the other is a logarithmic scale with the same gradations as the percent transmittance scale (the *absorbance scale*) (Figure 4A-2). (Since transmittance is related to absorbance as the log of the inverse, values of 0.0 absorbance occur at 100% transmittance and values of infinite absorbance

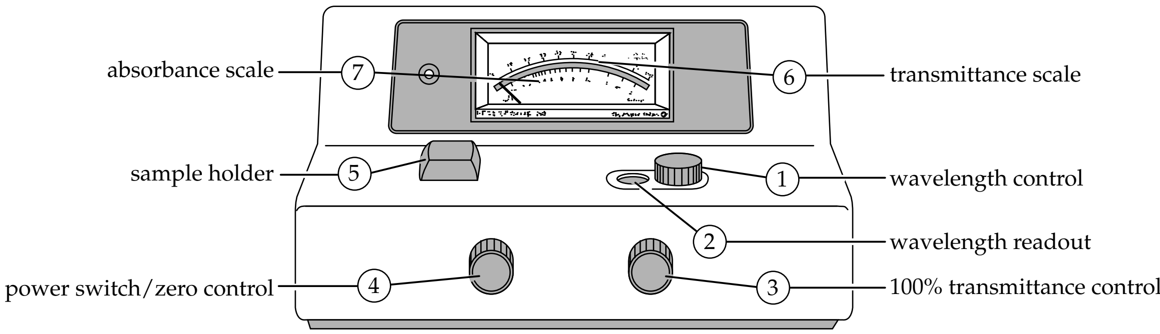


Figure 4A-2 *Features of the Bausch & Lomb Spectronic 20 Colorimeter. The transmittance and absorbance scales run in opposite directions. Why?*

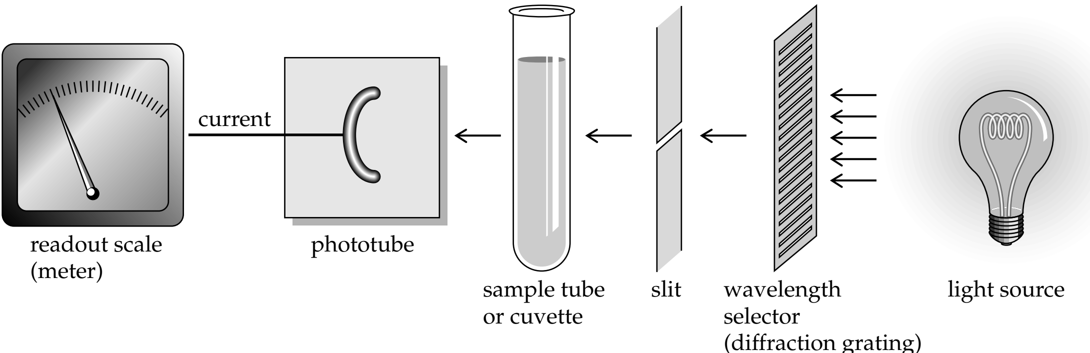


Figure 4A-3 *Operation of the spectrophotometer. Light of 340 to 750 nm is emitted from a light source and passes through a diffraction grating (wavelength selector) that generates light of a particular wavelength. Light intensity is adjusted as the light passes through a slit before reaching the sample. Some light is absorbed by the sample, and the rest is transmitted and detected by a phototube on the other side of the sample. The current generated by the phototube is proportional to the intensity of light hitting it and is registered as transmittance (or its inverse, absorbance) on the readout scale (meter) of the spectrophotometer.*

occur at 0% transmittance.) Examine the readout meter on the spectrophotometer you will be using. Note that the scales run in opposite directions.

Objectives

- ☐ Describe the relationship between absorbance and transmittance.
- ☐ Describe how absorbance and transmittance of light are related to the color of an object.
- ☐ Explain how the optical system of a spectrophotometer works.
- ☐ List the steps in the proper use of the spectrophotometer.

Procedure

1. Familiarize yourself with the parts of the spectrophotometer shown in Figure 4A-2.
2. Work in pairs. Cut a strip of white paper to just fit the diameter and length of a Spectronic 20 tube or cuvette. Slide the paper into the tube and insert the tube into the sample holder of the spectrophotometer.

3. Leave the sample holder open and place a cylinder of black paper around the opening.
4. Set the wavelength control to 620 nm and adjust the position of the tube containing the white paper until you see the maximum amount of red light on the right side of the paper.
5. Have your partner turn the wavelength dial in both directions and record the range of each wavelength at which you see a particular color. Range of wavelengths:

   Red ______________

   Yellow ______________

   Green ______________

   Blue ______________

   Violet ______________

6. Trade positions and let your partner check your observations by repeating steps 3–5. Compare your results with the wavelengths given in Figure 4A-1.

✓ PART 3 Determining Transmittance and Absorbance

To assure that spectrophotometer readings indicate only the concentration of the solute we wish to measure, a reading must first be obtained using a **blank,** a sample that contains all the components of the solution except the absorbing molecule. For instance, if you are using a reagent that changes color when mixed with a certain solute molecule, a blank should contain *all* the components of the test solution, including the colorimetric reagent, *except* the substance (solute) to be measured.

Example A 1-ml sample of substance X is mixed with 5 ml of water and 1 ml of colorimetric reagent to give a volume of 7 ml in a sample tube. A blank is prepared by mixing 5 ml of water with an additional 1 ml of water (as a substitute for substance X) and 1 ml of colorimetric reagent. The volume in the blank tube is 7 ml. Note that the volume of the blank should always be the same as the volume of the sample.

With the blank inserted into the spectrophotometer, the instrument is adjusted to 100% transmittance (zero absorbance). This step is similar to taring a balance: the transmittance of light through the blank will be less than 100% because of substances (including the colorimetric reagent) present in the blank. However, the instrument can be adjusted to accept this reading as 100% transmittance, so that when the blank tube is replaced by the sample tube to measure absorbance of the sample, the only thing absorbing light will be the sample molecule of interest (the solute that reacts with the colorimetric reagent).

Procedure

1. Prepare a sample tube. Place 10 drops of albumin solution into a spectrophotometer tube and add 1 ml of distilled water. Add 5 ml of Coomassie brilliant blue (a colorimetric reagent used to identify protein) and allow the tube to stand until a blue color develops.
2. Prepare a blank: place 10 drops of water into a spectrophotometer tube and add 1 ml of distilled water and 5 ml of Coomassie blue.
3. Turn the power switch on, and allow a 5-minute warm-up period. The on/off switch is operated by the zero control knob on the left.
4. Use the wavelength control knob to adjust the spectrophotometer to any wavelength between 550 and 600 nm. The selected wavelength is indicated on the wavelength readout in the window next to the knob.
5. When using the Spectronic 20, the meter must be adjusted to read across its full scale—0% transmittance to 100% transmittance. With *no* sample tube in the machine, use the zero (left-hand) control knob to set the scale to 0% transmittance (infinite absorbance). (With no sample tube, the light path is automatically blocked, and no light reaches the phototube; thus, 0% transmittance and infinite absorbance are simulated.) Be sure the cover on the sample holder is closed when you perform this step.

6. Insert your blank (be sure it is clean and dry on the outside) into the sample holder, and turn the right-hand control knob to set the meter scale to 100% transmittance, zero absorbance. This adjustment regulates the amount of light reaching the phototube in the absence of the absorber. *Whenever the wavelength is changed, the 100% transmittance adjustment must be reset.* Also, when operating at a fixed wavelength for an extended period of time, periodically check the 100% and 0% transmittance readouts and adjust if necessary.
7. If you are beginning an experiment, repeat steps 5 and 6 to make sure the machine is stable.
8. Insert the sample tube into the chamber; read absorbance directly on the absorbance scale (lower scale). The reading on the absorbance scale is proportional to the concentration of your sample substrate. *Note:* The absorbance scale reads from *right to left,* opposite to the direction of the transmittance scale. Record your data: wavelength _______ nm, absorbance _______, transmittance _______.

   ***Do not* discard your sample and blank tubes!**
9. The steps that follow provide a brief checklist for using the spectrophotometer during this laboratory and in later lab work.

QUICK CHECKLIST FOR USING THE SPECTROPHOTOMETER

1. Turn the power on and allow a 5-minute warm-up period before taking sample readings.
2. Select the wavelength.
3. Check that the sample holder is empty and the cover is closed.
4. Use the zero control knob to set the meter to 0% transmittance.
5. Wipe off fingerprints from the reference blank, insert it into the sample holder, and set transmittance to 100%.
6. Wipe off fingerprints from the unknown sample tube, insert it into the sample holder, and read the meter display in percent transmittance or absorbance.

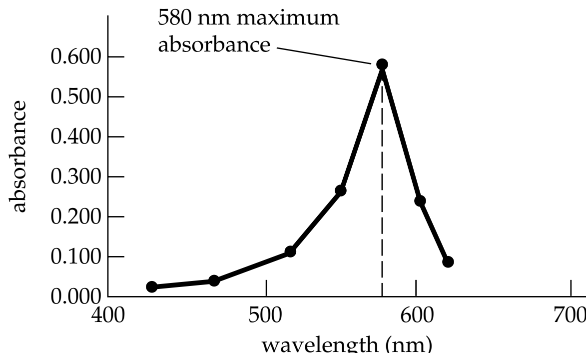
For best results when using the spectrophotometer, always remember the following:

- All solutions *must* be free of bubbles.
- All sample holders *must* be at least one-half full.
- For best performance with test tube holders, be sure that the index mark on the tube or cuvette aligns with the mark on the adapter (if tubes and cuvettes are marked).
- All sample tubes *must* be clean and free of scratches. Use lens paper to remove all fingerprints from the sample tubes and cuvettes.
- During extended operation at a fixed wavelength, make occasional checks for meter drift: use the blank to check for 100% transmittance.

EXERCISE B Determining the Maximum Absorption Wavelength

Molecules in solution (solute) will absorb light maximally within a narrow range of wavelengths. When deciding upon the wavelength to use in measuring concentration, an “absorbance spectrum” is generated in which the absorbance of a particular solute, or “absorber,” is measured at a continuous selection of wavelengths. A curve such as that shown for bromphenol blue (Figure 4B-1) is generated, and *maximum absorbance* can be determined. The most accurate measurements of absorbance are obtained by selecting a wavelength of light that is maximally absorbed by the solute of interest.

Figure 4B-1 *Absorption spectrum for bromphenol blue.*



Objectives

- ☐ Determine the maximum absorbance wavelength for a light-absorbing substance in solution.
- ☐ Relate the shape of an absorbance curve to the absorption of light at different wavelengths.

Procedure

1. Set the wavelength on your spectrophotometer to 540 nm.
2. Use the zero control knob to set the spectrophotometer to 0% transmittance.
3. Adjust the spectrophotometer to 100% transmittance using the same blank as you used in Exercise A.
4. Remove the blank tube and place the albumin sample tube (used in Exercise A, Part 3) into the chamber. Record the absorbance and transmittance readings at 540 nm in Table 4B-1.
5. Readjust the wavelength to 560 nm. Repeat steps 2–4.

   a. *Why do you need to use the blank to adjust the spectrophotometer at each wavelength used for a reading?* ____

   ____

6. Continue to increase the wavelength until you reach 640 nm, repeating steps 2–4 at each wavelength and recording the data in Table 4B-1.
7. Verify the relationship between transmittance and absorbance by calculating absorbance from the transmittance data ($T = \%T/100$). Show your calculations in the last column of Table 4B-1.

Table 4B-1 Absorbance and Transmittance at Various Wavelengths

Wavelength (nm)	Absorbance	% Transmittance	Calculations $A = \log 1/T$ (or $-\log T$)
540			
560			
580			
600			
620			
640			

8. Graph the absorbance data on graph paper.

b. At what range of wavelengths is absorbance at a maximum for Coomassie blue? ____________

c. Within this range of wavelengths, determine the maximum wavelength for absorption,

A_{max} = ______

d. Do you think the wavelength at which absorbance is maximum would change if the concentration of albumin in the sample tube were doubled? ______ *Why or why not?*

____

EXTENDING YOUR INVESTIGATION: ABSORBANCE AND TRANSMITTANCE

Your instructor will provide you with a tube of solution that appears red/red-orange. Consider the way in which light waves are absorbed or transmitted. What wavelengths of light would you expect to be absorbed by this solution? ____

____

Formulate a hypothesis that predicts what the absorption spectrum would look like for this solution.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will be the outcome of your experiment?

What is the **independent variable**?

What is the **dependent variable**?

Follow steps 1–8 to test your hypothesis, using the appropriate range of wavelengths for your sample. Collect absorbance data in the table below.

Wavelength	Absorbance
420	
470	
520	
570	
620	
670	

Graph the absorbance data on graph paper. From your results, describe the absorbance properties of the red solution. ____

____

Do your results support your hypothesis? Your null hypothesis?

Was your prediction correct?

EXERCISE C Exploring the Relationship between Absorbance and Concentration

Coomassie brilliant blue forms a colored complex with proteins. In Exercise B, you determined the wavelength at which this complex maximally absorbs. Now, using this wavelength, you can determine the concentration of protein in a solution by measuring the intensity of color developed when Coomassie blue is mixed with the solution.

Color intensity can be accurately measured by determining the amount of light absorbed by the solution. Absorbance is a function of concentration and, by comparing the absorbance of a solution containing an unknown amount of protein to a **standard curve,** a graph of absorbances plotted from a series of samples of known concentrations of the same material, the concentration of protein in an "unknown" can be determined (Figure 4C-1).

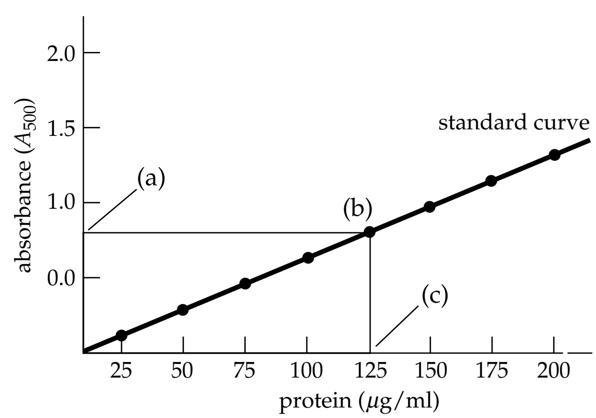


Figure 4C-1 *Absorbance measurements of known concentrations of a protein molecule are used to generate the standard curve (in this case the "curve" is linear). An absorbance reading (a) is obtained for a solution containing an unknown amount of protein. By drawing a straight line from (a) to the curve at (b) and dropping a line from (b) to (c), the concentration of the protein can be determined.* A_{500} *(Y-axis) indicates absorbance at 500 nm.*

Objectives

- ☐ Describe the relationship between absorbance and the concentration of a light-absorbing substance in solution.
- ☐ Use a standard curve to determine the concentration of a light-absorbing substance in solution.

Procedure

On your laboratory table you will find a tube marked BSA. It contains 240 μg/ml of bovine serum albumin (BSA), a more highly purified form of albumin. To develop a standard curve for bovine serum albumin, you and your laboratory partner should prepare at least five dilutions (see Appendix III or Laboratory 2, Exercise D).

1. Prepare dilutions as follows. Add 0.5 ml of distilled water to each of five test tubes and label the tubes 1 through 5.
2. To the first tube, add 0.5 ml of BSA stock solution and *mix well.* This will give a protein concentration of 120 μg/ml.
3. Take 0.5 ml of solution from test tube 1 and add it to test tube 2. Mix well.
4. Take 0.5 ml from test tube 2 and add it to test tube 3. Mix well. Repeat this procedure until you have added protein solution to each test tube.

5. Discard 0.5 ml from the last test tube. *a. Why is this necessary?* ______________________________

____

6. To prepare a blank, add 0.5 ml of distilled water to another test tube and label it "blank."

b. Why is a blank necessary? ____

____

c. What type of serial dilution have you performed—1:2, 1:5, 1:10, and so on, or some other?

____

7. Tube C on your laboratory bench contains an unknown amount of BSA. Add 0.5 ml of this solution to a test tube, label it "unknown," and set it in the test tube rack with the standards and the blank.

You should now have a series of five test tubes containing protein concentrations of 120, 60, 30, 15, and 7.5 μg/ml; a blank containing only distilled water; and a tube containing an unknown concentration of protein.

8. Add 5 ml of Coomassie blue to each of tubes 1 through 5 and to the blank. Wait at least 3 minutes, but no longer than an hour, then read absorbances at 595 nm. Follow the Spectronic 20 procedure steps outlined in Exercise A, Part 3, or refer to the quick checklist. Record your data in Table 4C-1.

Table 4C-1 Data for Determining Concentration from Absorbance

	Protein Concentration (μg/ml)	Absorbance (595 nm)
Tube 1	120	
Tube 2	60	
Tube 3	30	
Tube 4	15	
Tube 5	7.5	
Unknown		

9. Obtain a sheet of graph paper. Label the abscissa (*X*-axis) "Concentration" and the ordinate (*Y*-axis) "Absorbance." Make a graph as large as possible on the paper; plot the absorbance data for the solutions of known concentration. This is your standard curve. (Keep in mind that a "standard curve" can be used as a standard only when known and unknown concentrations have been prepared according to the same procedure. For example, for this standard curve to be useful, knowns and unknowns must be prepared by using 0.5 ml of the sample and 5 ml of reagent.)

10. Add 5 ml of Coomassie blue to your unknown. Wait 5 minutes or the same amount of time as in step 8, and read its absorbance at 595 nm using the Spectronic 20. Record absorbance in Table 4C-1. From the standard curve you have prepared, determine the concentration of your unknown:

________ μg/ml BSA (Record this value in Table 4C-1.)

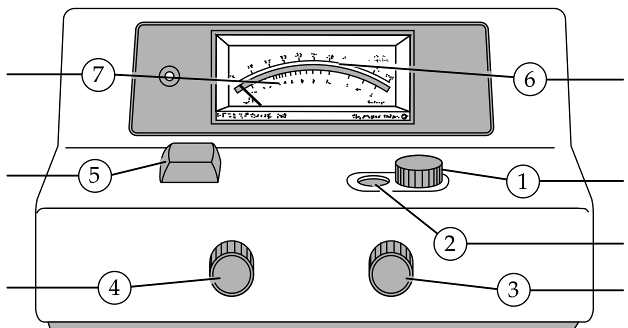
Laboratory Review Questions and Problems

1. You have a solution that appears green. What color light is being transmitted? What color(s) of light are most strongly absorbed?

2. How is absorbance related to transmittance of light through a solution?

3. List the steps in using a spectrophotometer. What is meant by "zeroing" the spectrophotometer?

4. You want to use the Spectronic 20 to find the wavelength of light you should use for determining the concentration of protein in an unknown solution. Using the following diagram, indicate the number of the dial or scale that you would use to accomplish each of the steps listed below.



________ Insert protein sample.

________ Adjust to 100% transmittance and 0 absorbance using a blank.

________ Change wavelength.

________ Adjust to maximum absorbance and 0% transmittance.

________ Read wavelength.

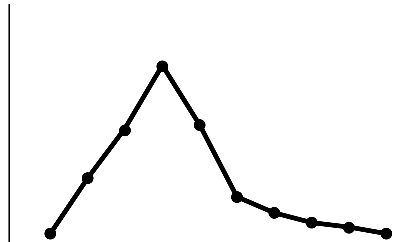
________ Read absorbance of sample.

________ Read transmittance of sample.

5. What is the purpose of a "blank" or reference tube?

6. You are making a standard to be used to measure protein concentration. You have added 2 ml of protein, 3 ml of water, and 4 ml of Coomassie blue. How do you make a blank for this experiment?

7. You generate an absorbance spectrum for a red solution as shown below. Label the axes of this graph (include units where necessary). What wavelength (or color) of light would you expect to show the maximum reading? ____________



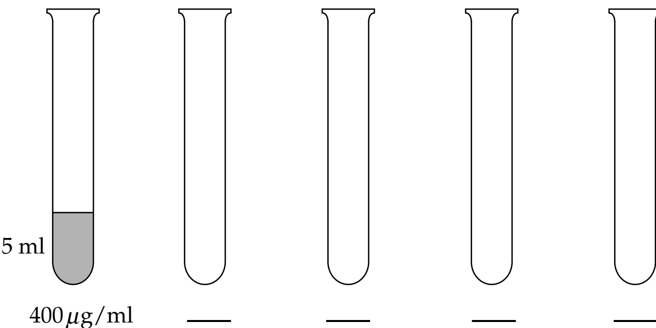
8. a. You are conducting an experiment in which an extract of cytoplasm is mixed with a red dye. When the mitochondria in the cytoplasmic extract are functioning, the red dye fades. You wish to measure the rate of the reaction that causes the decolorization. What wavelength of light might you use to make your measurements if you wanted to measure the decrease in absorbance by the red solution? ______

b. What would you use as a reference blank?

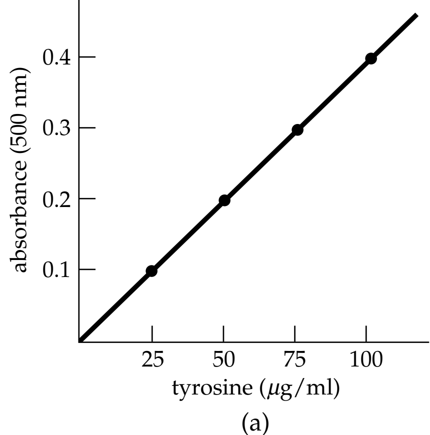
c. What would you use as a control for this experiment?

d. How might you determine the rate (decrease in concentration/time) of the reaction? (*Hint:* How would you measure concentration?)

9. In order to determine the concentration in an unknown solution, you need to prepare a standard curve. You are given a tube containing 5 ml of a 400-μg/ml solution of protein. You are told to make *four* dilutions and record final concentrations for each of the dilutions. Use the tubes below to indicate how you would make these dilutions, and record the final concentration in each tube.



10. You have developed a standard curve for several dilutions of tyrosine, as shown below (a). Tyrosine can be oxidized by an enzyme, tyrosine oxidase, found in liver. Tyrosine reacts with nitrosonaphthol. You begin with 100 μg of tyrosine. This is mixed with tyrosine oxidase and absorbance readings are obtained (b). Use the graph to complete the table.



(a)

Time (minutes)	Absorbance (500 nm)	Tyrosine (μg/ml)
0	0.40	
0.5	0.35	
2	0.2	
5	0.1	

a. How much tyrosine has been oxidized during a 5-minute period? ______________

b. At what rate does the tyrosine disappear during the first 2 minutes of the reaction?

______________ c. During the last 3 minutes? ______________

LABORATORY

5

Organic Molecules

OVERVIEW

A cell is a living chemistry laboratory in which most functions take the form of interactions between organic (carbon-containing) molecules. Most organic molecules found in living systems can be classified as carbohydrates, fats, proteins, or nucleotides. Each of these classes of molecules has specific properties that can be identified by simple chemical tests.

In this laboratory you will learn to identify three of the four major types of organic molecules: carbohydrates, fats, and proteins. The fourth major type, the nucleotide, is the basic structural unit of nucleic acids and will be studied during the isolation of the genetic material, DNA, in Laboratory 16.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

EXERCISE A Testing for Carbohydrates

The basic structural unit of carbohydrates is the **monosaccharide** (or single sugar). Monosaccharides are classified by the number of carbons they contain: for example, **trioses** have three carbons, **pentoses** have five carbons, and **hexoses** have six carbons (Figure 5A-1). They may contain as few as three or as many as 10 carbons.

Monosaccharides are also characterized by the presence of a terminal aldehyde group (Figure 5A-1a, b) or an internal ketone group (Figure 5A-1c). Both of these groups contain a double-bonded oxygen atom that reacts with Benedict's reagent to form a colored precipitate.

When two monosaccharides are joined together, they form a **disaccharide.** If the reactive aldehyde or ketone groups are involved in the bond between the monosaccharide units (as in sucrose, Figure 5A-2a), the disaccharide will not react with Benedict's reagent. If only one group is involved in the bond (as in maltose, Figure 5A-2b), the other is free to react with the reagent. Sugars with free aldehyde or ketone groups, whether monosaccharides or disaccharides, are called **reducing sugars.*** In this exercise, you will use Benedict's reagent to test for the presence of reducing sugars.

**These sugars are oxidized (lose electrons to) by the Cu^{2+} in Benedict's reagent, which then becomes reduced (gains electrons); hence the name* reducing sugar.

Figure 5A-1 *(a) Glyceraldehyde, a representative triose with an aldehyde reactive group. (b) Ribose, a representative pentose with an aldehyde reactive group. (c) Fructose, a representative hexose with a ketone reactive group. In solution, ribose and fructose occur predominantly in ring forms (d, e). When heated, sugars in the ring form interconvert to the chain form.*

Usually sugars need to be in the chain form to interact with colorimetric substances.

aldehyde group

ketone group

glyceraldehyde ($C_3H_6O_3$) (a)

ribose ($C_5H_{10}O_5$) (b)

fructose ($C_6H_{12}O_6$) (c)

original aldehyde group

original ketone group

ribose (ring form) (d)

fructose (ring form) (e)

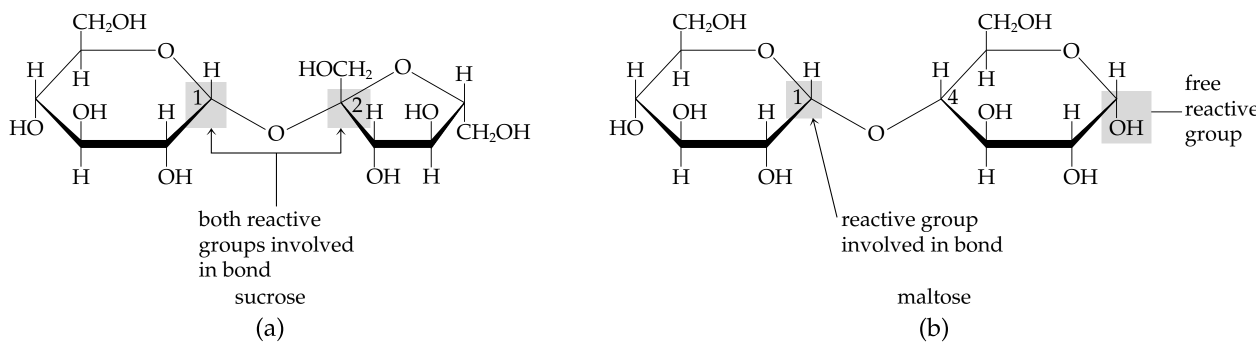


Figure 5A-2 *(a) In sucrose, both reactive groups are involved in the bond between monosaccharides, preventing a reaction with Benedict's reagent. (b) In maltose, only one reactive group is involved in the bond between monosaccharides, leaving the other group free to react with Benedict's reagent. Here the sugars are shown in ring form. The reactive group of maltose is highlighted.*

Monosaccharides may join together to form long chains (**polysaccharides**) that may be either straight or branched. Starch is an example of a polysaccharide formed entirely of glucose units. Starch does not show a reaction with Benedict's reagent because the number of free aldehyde groups (found only at the end of each chain) is small in proportion to the rest of the molecule. Therefore, you will test for the presence of starch with Lugol's reagent (iodine/potassium iodide, I_2KI).

Objectives

- ☐ Identify reducing sugars (both monosaccharides and disaccharides) using Benedict's reagent.
- ☐ Identify polysaccharides using Lugol's reagent.

PART I Benedict's Test for Reducing Sugars

When Benedict's reagent is heated with a reactive sugar, such as glucose or maltose, the color of the reagent changes from blue to green to yellow to reddish-orange, depending on the amount of reactive sugar present. Orange and red indicate the highest proportion of these sugars. (Benedict's test will show a positive reaction for starch only if the starch has been broken down into maltose or glucose units by excessive heating.)

Formulate a **hypothesis** and **predict** what you might expect to find for each of the sugars to be tested with Benedict's reagent (Table 5A-1).

Hypothesis **Prediction**

1. Water ______________________________
2. Starch ______________________________
3. Glucose ______________________________
4. Maltose ______________________________
5. Sucrose ______________________________
6. Onion juice ______________________________
7. Potato slice ______________________________
8. Milk ______________________________

What is your **null hypothesis** for this group of tests?

What is the **independent variable**?

What is the **dependent variable**?

Procedure

1. Set up a row of eight test tubes. Use a marker to number them 1 through 8.
2. To the test tubes, add 2 ml of the solutions listed in Table 5A-1, matching each number to the number on the tube.
3. Add one dropperful (approximately 2 ml) of Benedict's reagent to each tube.
4. Mix the reagent and the sample by agitating the solution in each tube from side to side. Record the original color of each tube's contents in Table 5A-1, under "Benedict's Test."
5. Heat the test tubes in a boiling water bath for 3 minutes. Record any color changes in Table 5A-1 under "Benedict's Test."

   a. *Why did you test water with Benedict's reagent?* ______________________________

   b. *Which sugars reacted with Benedict's reagent?* ______________________________

   Why? ______________________________

   c. *Which sugars did not react with Benedict's reagent?* ______________________________

   Why not? ______________________________

   d. *Explain your results for each of the following:*

   Onion juice ______________________________

   Potato slice ______________________________

   Milk ______________________________

Table 5A-1 Data Table for Benedict's and Lugol's Tests

Tube	Benedict's Test		Lugol's Test	
	Original Color Before Boiling	Final Color After Boiling	Original Color Before Adding I_2KI	Final Color After Adding I_2KI
1. Water				
2. Starch				
3. Glucose				
4. Maltose				
5. Sucrose				
6. Onion juice				
7. Potato slice				
8. Milk				

Did the results for each test support your hypotheses? ______________________

Did your results support your null hypothesis? ______________________

Did the results for each test agree with your predictions? ______________________

Explain any discrepancies. ____

PART 2 Lugol's Test for Starch

Lugol's reagent changes from a brownish or yellowish color to blue-black when starch is present, but there is no color change in the presence of monosaccharides or disaccharides.

Formulate a **hypothesis** and **predict** what you might expect to find for each of the sugars to be tested with Lugol's reagent (Table 5A-1).

Hypothesis **Prediction**

1. Water ____
2. Starch ____
3. Glucose ____
4. Maltose ____
5. Sucrose ____
6. Onion juice ____
7. Potato slice ____
8. Milk ____

What is your **null hypothesis** for this group of tests?

What is the **independent variable**?

What is the **dependent variable**?

Procedure

1. Prepare another eight test tubes as indicated in step 1 above.
2. To the test tubes, add 1 ml of the solutions listed in Table 5A-1, matching each number to the number on the tube.
3. Record the original color of each tube's contents in Table 5A-1.
4. Add several drops of Lugol's reagent (I_2KI) to each tube, mix, and immediately record in Table 5A-1 any color changes that take place. Do *not* heat the test tubes in the Lugol's test.

   a. *Which sugars reacted with Lugol's reagent?* ______

   Why? ______

   b. *Which sugars did not react with Lugol's reagent?* ______

   Why not? ______

   c. *Explain your results for each of the following:*

   Onion juice ______

   Potato slice ______

   Milk ______

Did the results for each test support your hypotheses? ______

Did your results support your null hypothesis? ______

Did the results for each test agree with your predictions? ______

Explain any discrepancies. ______

d. *From the results of the Benedict's and Lugol's tests, would you conclude that a potato stores its carbohydrates as sugars or as starch?* ______ *How do you know?* ______

e. *How does an onion store its carbohydrates?* ______ *How do you know?*

______

EXERCISE B Testing for Lipids

The word **lipid** refers to any of the members of a rather heterogeneous group of organic molecules that are soluble in nonpolar solvents such as chloroform ($CHCl_3$) but insoluble in water. Although lipids include fats, steroids, and phospholipids, this exercise will focus primarily on fats.

Triglycerides, a popular topic in discussions of diet and nutrition, are the most common form of fat. They consist of three fatty acids attached to a glycerol molecule (Figure 5B-1). Triglycerides are found predominantly in adipose tissue and store more energy per gram than any other types of compounds.

At room temperature, some lipids are solid (generally those found in animals) and are referred to as **fats,** while others are liquid (generally those found in plants) and are referred to as **oils.** Vegetable oil, a liquid fat, is a mixture of triglycerides.

Since both solid and liquid fats are nonpolar, you will test for their presence by using Sudan IV, a nonpolar dye that dissolves in nonpolar substances such as fats and oils but not in polar substances such as water.

Objectives

- ☐ Distinguish between lipid and nonlipid substances using the Sudan IV test.

Figure 5B-1 *A triglyceride is composed of three fatty acids and a glycerol molecule. Each bond is formed when a molecule of water is removed by condensation (boxed).*

Procedure

1. The familiar "grease spot" is the basis of a very simple test for fats. On a piece of unglazed paper, such as brown wrapping paper, place one drop of oil and one drop of water. Allow the drops to dry.

   a. Describe the difference between the oil spot and the water spot after a period of drying.

   ____

The Sudan IV test is a more useful laboratory test for fats. (Since the Sudan IV test is messy, your instructor may demonstrate this method.)

Formulate a **hypothesis** and **predict** what you might expect to find for each of the substances to be tested with Sudan IV (your instructor will tell you which five substances are to be tested; list them here and in Table 5B-1).

	Hypothesis	Prediction
1.	__________	______________________________
2.	__________	______________________________
3.	__________	______________________________
4.	__________	______________________________
5.	__________	______________________________

What is your **null hypothesis** for this group of tests?

What is the **independent variable**?

What is the **dependent variable**?

2. Label five tubes in sequence, 1 through 5. Add 1 dropperful (1 ml) of each substance you have listed in Table 5B-1 to the appropriate tube. Add 3 drops of Sudan IV to each tube. Mix and then add 2 ml of water to each tube. If fats or oils are present, these will appear as floating red droplets or as a floating red layer colored by Sudan IV. In Table 5B-1, record the reactions that occur in each of the test tubes.

   b. Why do the droplets float, rather than mix with water? ______________________

   ____

Table 5B-1 Data Table for the Sudan IV Solubility Test

Substance	Sudan IV Solubility Reaction
1.	
2.	
3.	
4.	
5.	

c. Which substances reacted with Sudan IV? ______________________________

____

d. Which substances did not react with Sudan IV? ______________________________

Why not? ______________________________

Did the results for each test support your hypotheses? ____________________

Did your results support your null hypothesis? ____________________

Did the results for each test agree with your predictions? ____________________

Explain any discrepancies. ______________________________

EXERCISE C Testing for Proteins and Amino Acids

Proteins are made up of one or more polypeptides, which are linear polymers of smaller molecules called **amino acids** (Figure 5C-1a). Amino acids derive their name from the amino group and the carboxyl group (acidic) that each possesses. Polypeptides are formed when amino acids are joined together by **peptide bonds** between the amino group of one amino acid and the carboxyl group of a second amino acid (Figure 5C-1b).

The biuret reagent reacts with peptide bonds and therefore reacts with proteins, such as egg albumin, but not with free amino acids, such as glycine and alanine. On the other hand, the reagent ninhydrin reacts with the amino group of free amino acids, but not with polypeptides.

Figure 5C-1 *(a) Structure of an amino acid. Note the presence of an amino (—NH$_2$) group and a carboxyl (—COOH) group. (b) Two amino acids are joined by a peptide bond when a molecule of water is "split out" from their amino and carboxyl groups. A polypeptide is made up of many amino acids joined in this way. "R" represents a side group that is characteristic for each amino acid. (Here and in Figure 5C-2, amino acids are shown in their nonionized form.)*

H—N(H)—C(H)(R)—C(=O)—OH ↓ H—N(H)—C(H)(R)—C(=O)—OH

amino acid amino acid

(a)

H—N(H)—C(H)(R)—C(=O)—N(H)—C(H)(R)—C(=O)—OH + H_2O

dipeptide

(b)

Objectives

☐ Distinguish between free amino acids and proteins (polypeptides) on the basis of their ability to react with either biuret reagent or ninhydrin.

PART I Testing for Protein with Biuret Reagent

The biuret reagent is light blue, but in the presence of proteins it turns violet. Other types of molecules may cause other color changes, but only the violet color indicates the presence of polypeptides.

Formulate a **hypothesis** and **predict** what you might expect to find for each of the substances to be tested with biuret reagent (Table 5C-1).

Hypothesis **Prediction**

1. Distilled water ______________________________
2. Egg albumin ______________________________
3. Potato starch ______________________________
4. Glucose ______________________________
5. Amino acid ______________________________

What is your **null hypothesis** for this group of tests?

What is the **independent variable**?

What is the **dependent variable**?

Procedure

1. Obtain five clean test tubes and use a wax pencil to number them from 1 through 5.
2. To the test tubes, add 2 ml of the solutions listed in Table 5C-1, matching each number to the number on the tube.
3. Add one dropperful (approximately 2 ml) of biuret reagent to each tube.
4. After an incubation period of 2 minutes, record your results in Table 5C-1 and determine whether the solution treated contains protein. Base your conclusions only on the presence or absence of the violet color.

Table 5C-1 Data Table for the Biuret Test

Substance	Color with Biuret Reagent after 2 Minutes	Protein Present (+) or Absent (−)
1. Distilled water		
2. Egg albumin		
3. Potato starch		
4. Glucose		
5. Amino acid		

a. What does this test tell you about the biochemical composition of starch or glucose?

____

b. Why has water been included as one of the test substances? ______________________________

c. Which substances reacted with biuret reagent? ______________________________

Why? ______________________________

d. Which substances did not react with biuret reagent? ______________________________

Why not? ______________________________

____

Did the results for each test support your hypotheses? ______________________________

Did the results support your null hypothesis? ______________________________

Did the results for each test agree with your predictions? ______________________________

Explain any discrepancies. ______________________________

✔ PART 2 Testing for Amino Acids with Ninhydrin

Ninhydrin reagent turns purple or violet in the presence of the free amino groups in amino acids. In the presence of proline, however, it turns yellow. Proline reacts differently because its amino group is not free but is, instead, part of the ring structure of the molecule (Figure 5C-2a, b).

Figure 5C-2 *(a) The amino acid leucine, with free amino group. (b) Proline, with amino group incorporated into a ring.*

leucine (a): CH_3, CH_3 — CH — CH_2 — H_2N—C(H)—C(=O)—OH

proline (b): CH_2—CH_2, CH_2, CH—C(=O)—OH, N—H

Procedure

1. Obtain a piece of filter paper and divide it into four quadrants with a pencil. Letter the quadrants A, B, C, and D.
2. Place one drop of each solution (labeled A, B, C, D) onto the filter paper in the quadrant with the corresponding letter. Allow the spots to dry.
3. Apply one drop of ninhydrin to each spot. Caution: Ninhydrin is poisonous; avoid contact with your skin. Allow the paper to dry at room temperature for 20 to 30 minutes. (The reaction will occur more quickly if the paper is lightly passed over a warm hotplate.)
4. One of the solutions contains proline, two of the solutions contain amino acids other than proline, and one is distilled water. In Table 5C-2, indicate the content of each solution.

Table 5C-2 Data Table for the Ninhydrin Test

Solution	Final Color with Ninhydrin	Type of Molecule in Solution
A		
B		
C		
D		

EXERCISE D Chromatography of Amino Acids

Amino acids can be classified as **nonpolar, polar uncharged,** or **polar charged** (acidic or basic) molecules, based on the structure of their R groups (Figure 5D-1). Depending on the nature of these R groups, different amino acids will be more or less soluble in different types of solvents. For instance, nonpolar amino acids will be more soluble in nonpolar solvents such as chloroform, whereas polar amino acids will be more soluble in polar solvents such as water. These differences in solubility allow mixtures of amino acids to be separated by **chromatography.**

Figure 5D-1 *Three types of amino acids, classified by R groups. (a) Nonpolar amino acids: R group composed of an unsubstituted hydrocarbon chain, often with branches or rings. (b) Polar uncharged amino acids: R group composed of substituted group, usually nonionized. (c) Polar charged amino acids: R group containing acidic (—COOH) or basic (—NH_2) groups. Here, all amino acids are shown in their fully ionized form at pH 7.*

lysine (basic)

leucine (a) — serine (b) — aspartic acid (acidic) (c)

Chromatography, a method for separating and identifying biological molecules, makes use of the tendency for molecules to show selective attraction to various substances, depending on their polarity (having charged or uncharged polar groups). Since molecules show characteristic differences in attraction, these differences can be used to identify unknown molecules and to separate and identify mixtures of molecules in solution. There are many chromatographic techniques, but most use the same basic method. The mixture to be tested is applied to a solid, stationary matrix material (called the *stationary phase*), which selectively absorbs the molecular mixture. It is then exposed to a motile substance (the *mobile phase*). The solid matrix can be a powder of fine "beads" packed in a vertical column (column chromatography) or spread in a thin layer on a glass plate (thin-layer chromatography). High-quality filter paper can also serve as a solid matrix (paper chromatography). The mobile phase can be a liquid (or a combination of several different liquids) or a gas. As the mobile phase moves through the stationary phase, different molecules within the mixture are separated or *partitioned* according to their relative attraction for each of the two phases. The tendency for different types of molecules in a mixture to bind to the stationary phase or to dissolve in the mobile phase will determine how far they will be moved by the mobile phase.

In a column, changing the pH or type of solvent (polar vs. nonpolar) causes different molecules to move down and finally out of the column, where they can be collected in tubes. In paper or thin-layer chromatography, after the mobile phase has moved across the stationary phase, each type of separated molecule appears as a distinct spot on the stationary phase (the plate or the paper). The stationary phase on which the molecules have partitioned themselves is called a **chromatogram.**

Paper chromatography can be carried out in either an ascending or a descending mode. In ascending chromatography, one end of the paper matrix is placed in the bottom of a chamber containing a solvent. As the solvent moves up the paper, its progress can be noted by observing the "solvent front." In descending chromatography, the solvent is contained in a trough at the top of the chamber.

It is possible to identify separated compounds by the *ratio* of the distance traveled by a compound in a particular solvent to the distance traveled by the solvent. This ratio is known as a compound's R_f value.

$$R_f = \frac{\text{distance of spot from origin}}{\text{distance of solvent (solvent front) from origin}}$$

For precise analysis, it is best to chromatograph unknown compounds together with known compounds, then compare the distances traveled.

Locating substances on chromatograms can be accomplished in various ways—by using fluorescent or other dyes (usually applied to the chromatogram in spray form) or by autoradiography (visualization of spots containing radioactivity).

Objectives

- ☐ Distinguish among nonpolar, polar uncharged, and polar charged amino acids based on their solubility properties.
- ☐ Describe the process of chromatography and how it can be used to separate different types of molecules.

Procedure

1. Place a clean paper towel on a clean counter. Cut a piece of Whatman filter paper into an 8-inch × 11-inch rectangle and place it on the paper towel (the paper towel helps to keep the filter paper clean.)
2. Orient the paper so that the 11-inch-long side is facing you.
3. Using a *pencil* and a ruler, draw a light line 1 inch from the bottom across the full width of the paper. Beginning 1½ inches from the left end of the line, make a series of six small cross marks on this line every 1½ inches (Figure 5D-2a).

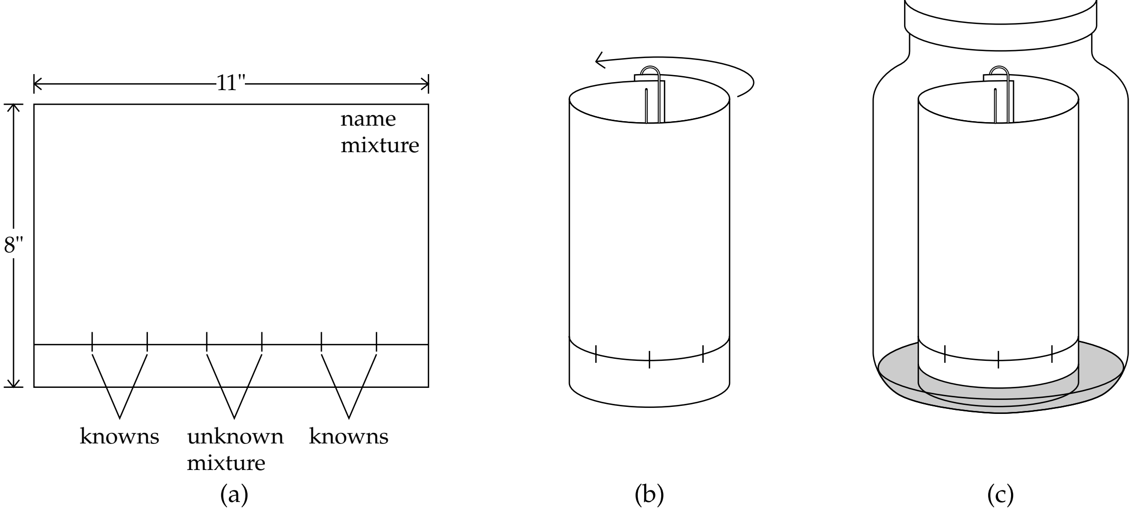


Figure 5D-2 *Preparation of a paper chromatogram. (a) Use a pencil to lightly mark the placement of amino acid samples. Use duplicate spots for the unknown mixture. (b) Roll the paper into a cylinder and hold it together at the top and bottom with plastic paper clips. (c) Place the paper cylinder into a gallon jar containing ¼ to ½ inch of chromatography solvent.*

4. Your instructor will provide you with an "unknown" sample containing a mixture of three amino acids. If several unknown solutions are being used in your laboratory, be sure to record the letter or number that your instructor has used to identify the unknown you selected. Code for unknown solution ______. You will also receive four amino acid "knowns." Record the names of the known samples:

Amino acid 1 ____________________

Amino acid 2 ____________________

Amino acid 3 ____________________

Amino acid 4 ____________________

Based on the structures of the known amino acids, formulate a hypothesis about the differences in their migration tendencies as measured by chromatography using a nonpolar solvent.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** about the migration patterns of amino acids 1, 2, and 3?

What is the **independent variable**?

What is the **dependent variable**?

5. Using a wooden applicator stick, apply a small spot of unknown solution (2 mm in diameter) to the filter paper on the line at one of the cross marks located at the center of the paper. Allow the spot to dry (you may use a hair dryer if available). Repeat this process at the same mark two additional times. *Do not* press the applicator stick to the paper hard enough to make a dent in the paper. On a separate sheet of paper, draw and label the chromatogram to indicate the mixture you have applied to each cross mark.
6. Apply another sample of the same unknown to a second mark using the same procedure as in step 5.
7. On the other cross marks, apply spots of the known amino acids—one amino acid per cross mark. Apply each amino acid three times, allowing spots to dry between applications. On the drawing of your chromatogram, be sure to indicate which amino acid was applied to each mark.

   Mark 1 ____________________ Mark 4 ____________________

   Mark 2 ____________________ Mark 5 ____________________

   Mark 3 ____________________ Mark 6 ____________________

8. Roll the chromatography paper into a cylinder and hold it together at the top and bottom with *plastic* paper clips (Figure 5D-2b).
9. Cover the bottom of a gallon glass jar with chromatography solvent (0.25 to 0.5 inch deep).
10. Place the cylinder of chromatography paper into the jar (Figure 5D-2c) and allow it to run for at least 4, preferably 5 to 6, hours. The instructor will remove your chromatograms and mark the solvent front. The chromatograms will then be allowed to air dry.
11. **Next Day:** Spray your chromatogram with ninhydrin or, alternatively, dip the chromatogram into a solution of ninhydrin. Caution: Wear gloves; if you use the spray, work under a hood. Heat the chromatogram according to directions from you instructor. Amino acids will turn purple in the presence of ninhydrin, except for proline, which will turn yellow.
12. Circle spots and record their colors in the table that follows. Determine the R_f values for each spot of unknown amino acid on your chromatogram, then determine the R_f value for each spot of known amino acid. To find the R_f value, estimate the center of each amino acid spot,

Unknown (Code ______)		Color	R_f	Knowns	Color	R_f
Sample 1:	Spot 1	______	______	Amino acid 1	______	______
	Spot 2	______	______	Amino acid 2	______	______
	Spot 3	______	______	Amino acid 3	______	______
				Amino acid 4	______	______
Sample 2:	Spot 1	______	______			
	Spot 2	______	______			
	Spot 3	______	______			

measure the distance it has traveled from the origin (cross mark), then measure the distance the solvent front has traveled from the origin. Find the ratio of these distances and record this information in the table.

13. Compare R_f values for the knowns and your unknown mixture. Identify and record the amino acids in your mixture: ______________________, ______________________, ______________________

Do the R_f values for the known amino acids support your hypothesis? ______ *Your null hypothesis?* ______

Was your prediction correct? ______________________

If there are discrepancies between your hypothesis and your results, how might these be explained?

____

____

EXERCISE E Analyzing Unknowns Qualitatively

Objectives

- ☐ Use knowledge gained in the previous exercises to identify the organic molecules present in unknown solutions.

Procedure

A number of commercial products and food samples containing a variety of compounds are available for testing. Using reagents and methods from the previous exercises, identify the kinds of molecules contained in these solutions.

Formulate a **hypothesis** and **predict** what you might expect to find for each test (positive or negative result) based on your knowledge of the substance and the tests performed during this laboratory. (Your instructor will tell you which substances are to be tested.)

Substance	Hypothesis	Prediction
______	______	______
______	______	______
______	______	______
______	______	______

_______________ ____

_______________ ____

_______________ ____

_______________ ____

_______________ ____

_______________ ____

Conduct all tests according to directions in Exercises A through C. Record the results in Table 5E-1. Report your results according to your instructor's directions and identify each numbered unknown.

The following hints will help you conduct successful analyses of the unknowns:

- *Never* insert your pipette into the stock bottle of solutions. Instead, pour a small amount into a beaker and pipette from this. Empty and rinse out the beakers at the end of the period.
- The success of this lab relies on using clean glassware and avoiding cross-contamination between solutions. Therefore, wash all glassware with warm soapy water; rinse thoroughly several times with tap water and once with distilled water before using in the next set of tests.
- Make sure you are able to differentiate between a positive reaction (the color change specific for the test being done) and a negative reaction (no color change or a change to a color other than the one specific to the test).

Table 5E-1 Data Table for Analyzing Unknowns Qualitatively

Unknown Tested	Positive (+) or Negative (−) Results				
	Benedict's Test	Lugol's Test	Biuret Test	Ninhydrin Test	Sudan IV Test
1.					
2.					
3.					
4.					
5.					
6.					
7.					
8.					
9.					
10.					

Did you predict the contents of each unknown accurately? (Your instructor will provide a list of expected test results for the unknowns.) ______________________________

If not, investigate the contents of the unknowns by examining the product labels, and explain your results.

____

____

Laboratory Review Questions and Problems

1. Explain the limitations of Benedict's test in determining whether or not sugar is present in a certain food product. Why do all monosaccharides, but only some disaccharides, react with Benedict's reagent?

2. What did you learn about the specificity of the biuret reagent?

3. In this lab, you used a biuret reagent to determine the presence of albumin (egg white) in solution. Why didn't you use ninhydrin as the colorimetric reagent?

4. The leaves of many plants are coated with a waxy substance that causes them to shed water. How would you expect this substance to react in the Sudan IV test?

5. Ninhydrin reacts with a mixture of amino acids and turns purple. Could proline be one of the amino acids? ______ How could you find out if the mixture contained proline?

6. Several individual unknowns are tested to determine the type of molecule present. Given the completed table below, indicate whether unknowns 1 through 5 are protein, reducing sugar, starch, lipid, or free amino acids (+ = positive result).

Unknown	Benedict's Test	Lugol's Test	Biuret Test	Ninhydrin Test	Sudan IV Test	Answer
1	–	–	+	–	–	______
2	+	–	–	–	–	______
3	–	+	–	–	–	______
4	–	–	–	+	–	______
5	–	–	–	–	+	______

7. Mixtures of unknowns are tested with several colorimetric reagents. Given the results in the following table, determine which of the four choices below best describes the contents of each tube (+ = positive result).

Test Tube	Benedict's Test	Lugol's Test	Biuret Test	Ninhydrin Test	Sudan IV Test
1	−	−	+	+	−
2	+	−	+	−	+
3	+	+	−	−	+

a. Tube 1: reducing sugar and protein
   Tube 2: lipid, free amino acids, and protein
   Tube 3: starch, reducing sugar, and lipid
b. Tube 1: protein and free amino acids
   Tube 2: starch, protein, and lipid
   Tube 3: free amino acids, starch, and protein
c. Tube 1: protein and free amino acids
   Tube 2: lipid, reducing sugar, and protein
   Tube 3: lipid, reducing sugar, and starch
d. Tube 1: free amino acids and lipids
   Tube 2: lipid, starch, and free amino acids
   Tube 3: starch, free amino acids, and reducing sugar

8. You test several unknowns and obtain the following results:

Solution	Results of Lugol's Test	Results of Benedict's Test	Results of Ninhydrin Test
I	Yellow	Blue	Violet
II	Yellow	Orange	Clear
III	Black	Blue	Clear
IV	Brown	Cloudy blue	Yellow
V	Yellow	Blue	Clear

a. Which solution contains starch?
b. Which solution is most likely glucose?
c. Which solution contains an amino acid other than proline?

9. What are some good sources of carbohydrates, proteins, and fats in a typical breakfast, lunch, and dinner?

10. What nutrients (biological molecules) would you obtain from red meat? Do nutritionists recommend that some fats be present in your diet? What would you use these for?

11. Some vitamins should not be taken in excess. Which ones? Why?

12. Four known amino acids (*AA*) produce a chromatograph with R_f valves as follows: $AA_1 = 0.25$, $AA_2 = 0.66$, $AA_3 = 0.50$, $AA_4 = 0.80$. A mixture of three amino acids yields spots that are 2 cm, 5.2 cm, and 6.4 cm from the origin. The solvent front is 8.0 cm from the origin. Which three of the four amino acids are contained in the mixture?

13. Some amino acids are called essential amino acids. What does this mean? Fatty acids with more than one double bond are considered as essential fatty acids. Animals cannot make fatty acids with more than one double bond. What are the sources of essential fatty acids?

14. In winter, plants exchange the saturated lipids in their membranes for unsaturated lipids. Unsaturated lipids are "bent" and keep the membranes more fluid because they cannot be stacked closely together. Of what advantage would this be for herbaceous plants that live through the winter? (Hint: what happens to bacon grease or the grease on top of soup when you put it in the refrigerator?)

LABORATORY

Prokaryotic Cells

OVERVIEW

Understanding the nature of cell structure and function is important to an understanding of organisms. All organisms are composed of cells, whether they exist as single cells, colonies of cells, or in multicellular form. Cells are usually very small, and for this reason, a thorough understanding of subcellular structure and function has been possible only through advances in electron microscopy and molecular biology.

There are two general types of cells: **prokaryotic** and **eukaryotic.** These two words have their root in the Greek word *karyon* (nut), which refers to a cell's nucleus. The prefix *pro-* means "before" or "prior to." Thus *prokaryotic* means "before having a nucleus." Prokaryotic cells do not have a membrane-bound nucleus and their genetic material (DNA) is only loosely confined to a nuclear area within the cell. Bacteria, including the cyanobacteria (formerly known as blue-green algae), are prokaryotes. All other organisms are eukaryotes. The prefix *eu-* means "true." The cells of eukaryotes have true, membrane-bound nuclei containing their genetic material.

Prokaryotic and eukaryotic cells also differ in several other ways. Eukaryotic cells are generally larger and contain additional specialized compartments (**membrane-bound organelles**) in which cell functions such as energy production may occur. Prokaryotic cells lack membrane-bound organelles; their cell functions are carried out in the cytoplasm.

During this laboratory you will investigate some of the structural and biochemical properties of prokaryotic cells. During Laboratory 7 you will study eukaryotic cells.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

EXERCISE A Producing Protobionts

Evidence suggests that the first cells were prokaryotic. They were probably anaerobic, living in an atmosphere with little or no oxygen, and heterotrophic, consuming organic materials as a carbon source. But how did these first cells come to be? In the 1930s, the Russian scientist A. I. Oparin suggested that molecular aggregates, called **coacervates,** which can be prepared in the laboratory, may resemble the precursors to the first cells. These aggregates are composed of high-molecular-weight substances that, when dissolved in water, aggregate to form viscous droplets.

Although these droplets are not living cells, coacervates and cells have several characteristics in common. They are able to maintain an internal environment that differs from their surroundings and they

can selectively absorb substances from their surroundings. Enzymes are included among the high-molecular-weight molecules—usually a combination of lipids, polypeptides, polysaccharides, and nucleic acids—used to form coacervates. Enzymes can catalyze reactions, including syntheses, hydrolyses, and electron transfer.

Coacervates are not alive, hence they are called **protobionts** (*proto-*, "before", plus *bionts,* "life"). However, the formation of coacervates may have allowed organic molecules to interact with one another to form new structural entities—the forerunners of cellular organelles.

Objectives

- ☐ Demonstrate how molecules of various molecular weights can be combined to form coacervates.
- ☐ Relate the biochemical properties of molecules to their role in coacervate formation.

Procedure

1. In a test tube, mix together 5 ml of 1% gelatin (protein) and 3 ml of 1% gum arabic (carbohydrate). Cover the tube with a piece of Parafilm and shake well.
2. Measure the pH by transferring a drop of the solution to a small section of pH paper using a glass stirring rod. Rinse the stirring rod. pH = ______
3. Prepare a wet mount of the mixture and observe it under the low power of the microscope (10×). Save the slide.
4. Add acid (1% HCl) to the test tube containing the gelatin/gum arabic mixture, one drop at a time, shaking the mixture after each addition. After each addition wait to see whether the solution becomes cloudy. If it remains clear, add another drop. When the solution turns cloudy, shake well. If it turns clear, add another drop. *Caution:* Excessive acid will cause the cloudiness to disappear. At that point, the acidity required for the formation of coacervates has been exceeded and the procedure must be repeated.
5. Once the solution is permanently cloudy, make another pH reading. pH = ______ Prepare another wet-mount slide of the solution. At this time, coacervates should be visible. If you have difficulty observing the coacervate spheres, adjust the light or use a higher power.
6. Observe the original slide to assure yourself that its solution has no coacervates.

   a. How do you think the pH may be affecting the protein or carbohydrate molecules?

   ____

7. Use congo red, neutral red, or methylene blue to stain the coacervates. Place one drop of coacervate solution on a glass slide and mix with one drop of a stain. Place a coverslip over the preparation and observe under the compound microscope at high power. Use a fresh drop of coacervate solution for each stain.

   b. Do all of the dyes stain the coacervates? ______ *Congo red?* ______ *Neutral red?* ______ *Methylene blue?* ______

   Congo red, in the presence of weak acids, changes color from red to blue. It is often used as an indicator but can also be used to stain carbohydrates. *Neutral red* is yellowish at higher pH values but turns red to pink at a slightly acidic pH and blue in strong acids. Often used as an indicator, neutral red stains cell cytoplasm containing carbohydrates and proteins. *Methylene blue* is also used as a general stain for cytoplasm and nuclei.

   c. How would you explain your results from staining the coacervates based on the properties of the stains described here? ______________________________

   ____

   ____

8. Add a drop of 5% NaCl to the edge of the coverslip of each of your stained slides. Observe the coacervates as the NaCl diffuses into the solution.

   d. What happens to the coacervates? ______

   Why? ______

   ______

9. When you have a finished observing the coacervates, add more acid to the mixture in the test tube, a drop at a time. Measure the pH at three-drop intervals. Continue to add acid until the solution clears.

   e. How do changes in pH affect the production of coacervates? ______

   f. Which molecules do you think are responsible for forming the membrane-like barrier between the coacervate and its environment? ______

   Why? ______

   ______

✓ EXERCISE B Examining Bacterial Cells

Present-day bacteria are extremely small (approximately 1 to 2 μm in diameter). Most are heterotrophic, depending on preformed food, but some are photosynthetic and make their own food. Morphologically, bacteria are either round (**cocci**), rod-shaped (**bacilli**), or spiral-shaped (**spirilla**). To view them with the light microscope, you must use an oil-immersion lens (100×). Even then, not much more than their basic shapes will be visible. With the aid of the electron microscope, however, you can study these prokaryotic cells more closely. You can even use special staining techniques to learn about their structure.

Objectives

- ☐ Give two general characteristics of prokaryotic cells.
- ☐ Distinguish among the three morphological types of bacteria.
- ☐ Describe the subcellular structure of a typical bacterium.
- ☐ Compare the structures of gram-positive and gram-negative bacteria.

✓ PART I Observing Bacteria Using the Light Microscope

You can use the light microscope to study bacteria, but only their external features will be distinguishable. It is possible to identify the three morphological types of bacteria (coccus, spirillum, and bacillus) by observing their shape (Figure 6B-1). You will also note that bacteria are often found in clusters or in chains. Some have one or more flagella (Figure 6B-2).

Procedure

1. Observe a prepared slide of *Escherichia coli* on demonstration. Describe its shape.

   ______

2. Observe a prepared slide of *Staphylococcus aureus* on demonstration. Describe its shape.

   ______

3. Observe a prepared slide of *Spirillum volutans* on demonstration. Describe its shape.

   ______

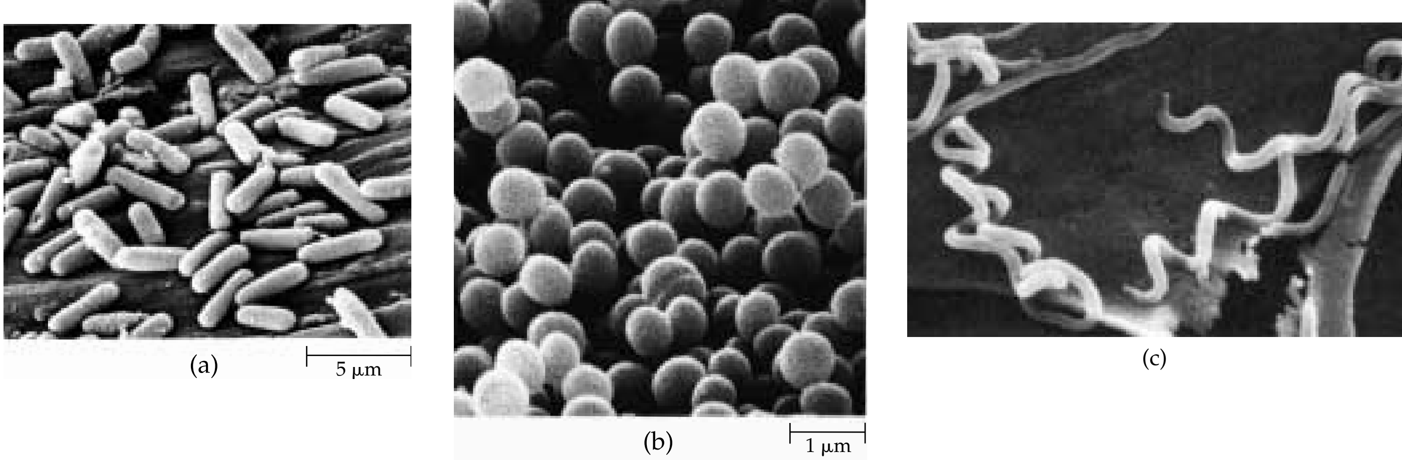


Figure 6B-1 *The cells of many familiar genera of bacteria include the (a) rod-shaped bacillus, (b) spherical coccus, and (c) helical spirillum.*

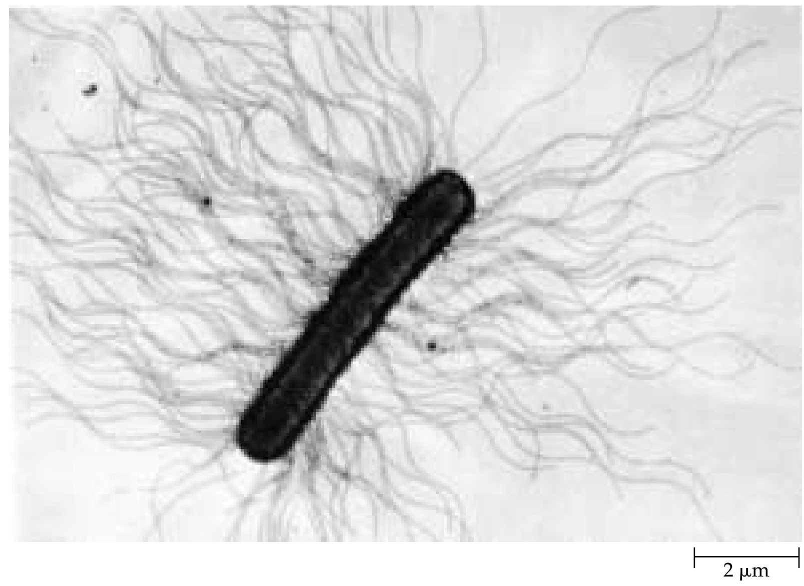


Figure 6B-2 *A bacterial cell,* Proteus mirabilis, *showing numerous flagella.*

4. Some bacteria have **flagella,** threadlike organelles used in locomotion. Bacterial flagella are composed of the protein flagellin. Observe the flagellated bacteria on demonstration. Do they have one or more than one flagellum? ___________________

✔ **PART 2 Observing Bacteria Using the Transmission Electron Microscope (TEM)**

The resolving power of an electron microscope is approximately 1,000 times that of a light microscope. This allows us to observe the subcellular structure of bacteria. The most prominent feature within the bacterial cell is the **nucleoid** region, which may appear as a lighter, fibrous central area. The genetic material of the cell is dispersed throughout this region. The cytoplasm is filled with granular-looking bodies called **ribosomes.** Both a **cell membrane** and a thicker **cell wall** (composed of peptidoglycan; a polymer of amino sugars, and other polymers in some species) surround the bacterium. Outside the cell wall there is often a layer of "slime" that forms a polysaccharide **capsule.** The capsule may help to protect bacteria against attack by the immune system of a host organism and from dehydration. Many bacteria do not produce capsules, and even those with capsules will not die if the capsule is destroyed (Figure 6B-3).

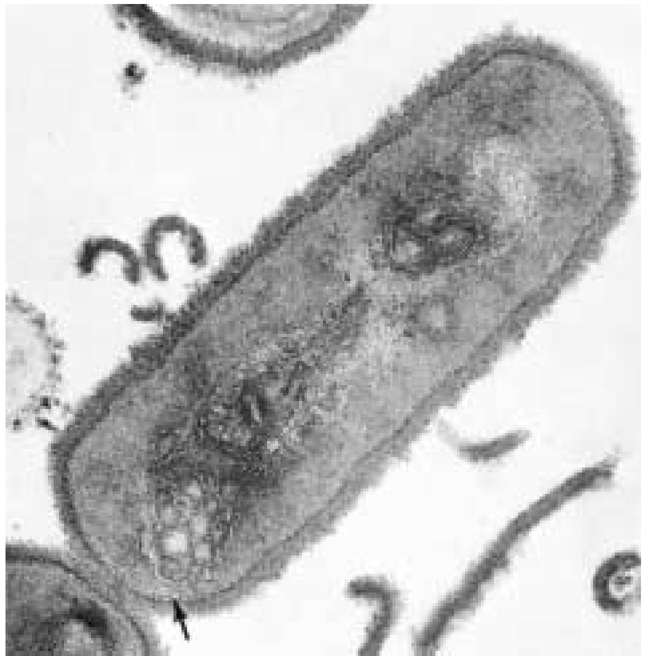


Figure 6B-3 *Convoluted membranes of a large mesosome* (arrow) *extend throughout this bacterium. The mesosome is continuous with the cell membrane. A thick, slimy capsule surrounds the cell.*

Some bacteria possess **mesosomes,** whorls of membranous material that extend inward from the cell membrane. The function of mesosomes is not yet certain, but they may play a role in cell division or in various energy-releasing reactions. In photosynthetic bacteria (see Part 4 of this exercise), the cell membrane often folds extensively to form an internal membrane system containing chlorophyll and other pigments.

Procedure

Figure 6B-4 is a transmission electron micrograph of an *Escherichia coli* cell that is in the process of dividing. The two new cells have not yet separated completely. The cell wall of *E. coli* is defined on its inner surface by the cell membrane and on its outer surface by a second, outer membrane. The cell wall exists in the light-colored area between the two membranes; this area is known as the **periplasmic space.** Identify the labeled structures in Figure 6B-4.

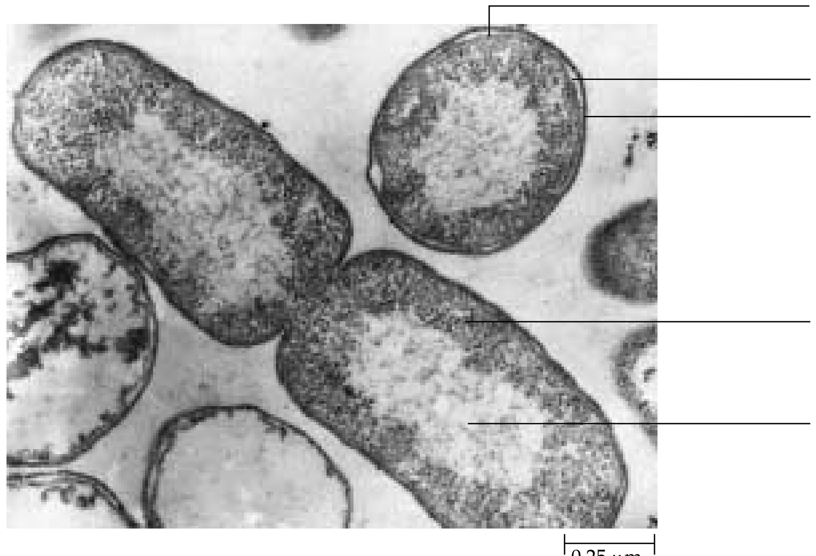


Figure 6B-4 Escherichia coli *cell in the process of dividing.*

PART 3 Using Gram Staining to Study Bacterial Cell Walls

The chemical nature and physical structure of bacterial cell walls differ among species. This difference is the basis for classifying bacteria into two major groups, **gram-positive**, and **gram-negative,** based on their staining properties with **Gram's stain,** a mixture of iodine and the dye crystal violet. When stained with Gram's stain and then treated with a decolorizing solution (alcohol or acetone), gram-positive cells retain the crystal violet color and appear deep purple-blue. Gram-negative cells lose the crystal violet color when washed with alcohol, but are stained red when treated with a second stain, safranin.

The key differences in the nature of the cell wall are shown in Figure 6B-5. Gram-positive bacteria (Figure 6B-5a) have a multilayered, cross-linked network of amino sugars (peptidoglycan) forming a cell wall outside the cell membrane. As many as 20 to 40 layers of peptidoglycan may be present, making the cell wall about 50 nm thick. Polymers of glycerol or ribitol usually extend from the cell membrane into the cell wall. Gram-negative bacteria (Figure 6B-5b) have a much thinner wall of peptidoglycan, usually only several layers thick. The cell wall lies outside the cell membrane but is covered by an additional outer membrane of lipid and protein. Under the electron microscope, the cell wall appears as the lighter layer between the two cell membranes, partly filling the periplasmic space.

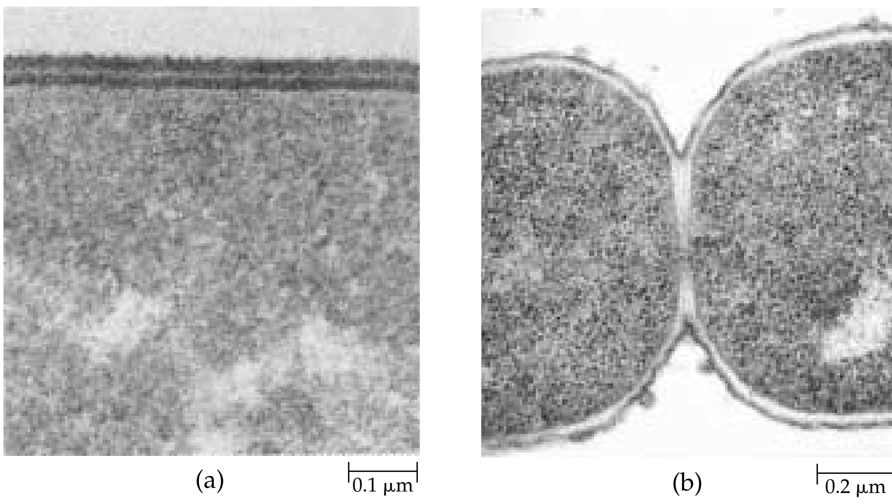


Figure 6B-5 *Electron micrographs of sections through the cell walls of (a) gram-positive* Bacillus polymyxa *and (b) gram-negative* Escherichia coli. *The wall of a gram-positive bacteria consists of a homogeneous layer of peptidoglycans and polysaccharides seen here as the lower dark band. The upper dark band is a layer of surface proteins. In gram-negative bacteria, a layer of peptidoglycan (cell wall) is sandwiched between inner and outer cell membranes.*

Procedure

When you go to the doctor's office and a bacterial infection is suspected, a Gram stain test might be performed to determine whether you are infected with gram-positive or gram-negative bacteria. For example, strep infections (from *Streptococcus* species) can readily be identified in this manner.

On the laboratory bench you will find two agar-slant cultures of bacteria. The bacteria are growing on the surface of the nutrient agar; if you look carefully, you can see a "slimy" coating of bacteria growing on the agar. Your challenge is to determine whether the two strains of bacteria, *Escherichia coli* and *Staphylococcus aureus,* are gram-positive or gram-negative.

Using what you know from your reading about the cell wall structure of the two genera of bacteria, form a hypothesis about the Gram staining properties of these two species.

HYPOTHESIS:

Null hypothesis:

*a. What do you **predict** will be the outcome of the Gram stain test?* ______________________

*b. What is the **independent variable** in this investigation?* ______________________

*c. What is the **dependent variable** in this investigation?* ______________________

1. Work in pairs. Use a clean glass slide. Do not touch the surface of the slide with your fingers. Label one end of the slide "E" and the other "S." One partner should carry out the following steps.
2. Sterilize a wire inoculating loop by flaming it until it is red hot.
3. Using the sterile loop, place two small drops of tap water on the slide approximately 2 cm apart between the "E" and "S" labels.
4. Resterilize the loop. Cool it for a few seconds. With the tip of the loop, remove a small amount of growth from the surface of the *E. coli* agar-slant culture. To maintain sterile conditions, be sure to flame the neck of the test tube after opening it and before closing it.
5. Mix the bacterial cells on the tip of the inoculating loop with the drop of water nearest the "E" label. With a circular motion, spread the spot out to the size of a dime or until it has a pale, milky appearance.
6. Immediately after making the smear, hold the loop *above* the flame to dry the inoculum, *then* flame the loop. (If you plunge the loop into the flame before it is dry, you will splatter bacteria all over yourself and your lab bench.)
7. The second partner should now repeat the above steps using the agar-slant culture of *S. aureus.* Place the *S. aureus* bacterial cells in the drop of water nearest the "S" label.
8. Allow the smears to *air dry.* Hold the slide by the end using forceps and "heat fix" it by passing the slide through the Bunsen burner flame (*right side up*) two or three times. (After heat fixing, the slide should not be too hot to handle. If it is, you have heated it too much.)
9. Place your slide across the edges of a small Petri dish. Place this dish inside a larger Petri dish.
10. Flood the slide drop by drop with **Gram's crystal violet** solution using a Pasteur pipette. Add two drops of 5% sodium bicarbonate buffer to the slide. Stain for 1 minute.
11. Pour off excess stain into the dish provided and *gently* wash the slide with water using a dropper or squirt bottle. Do not allow the dropper to touch the smear.
12. Flood the smears with **Gram's iodine** solution and allow it to react for 1 minute. Pour off the excess and let stand for 1 minute.
13. Wash off the iodine solution as in step 11 and gently blot the slide with bibulous paper or a paper towel.
14. Hold the slide at an angle and apply **Gram's alcohol** drop by drop until the violet color no longer appears in the washes from the smears. This should take only 10 to 15 seconds. *Do not overdo this step.*
15. Quickly rinse off the alcohol with water and blot.
16. Flood the slide with **safranin** and allow it to stain for 1 minute.
17. Wash the slide gently with water. Drain the excess water onto a paper towel and allow the slide to air dry.

18. When the slide is dry, observe it using an oil-immersion objective. Ask your instructor for assistance. You will use one of the oil-immersion microscopes on the demonstration table.*

RESULTS

d. Which bacterial species showed a positive test with Gram's stain? ______________________________

e. Which bacterial species showed a negative test with Gram's stain? ______________________________

CONCLUSION

19. Determine whether *Escherichia coli* and *Staphylococcus aureus* are gram-negative or gram-positive bacteria. *E. coli* ______________________________ *S. aureus* ______________________________

f. Do your results support your hypothesis? ________

g. Explain any discrepancies. ____

____

PART 4 Examining Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes. However, unlike other photosynthetic bacteria, which contain bacteriochlorophyll, cyanobacteria contain **chlorophyll *a***—the same type of chlorophyll found in eukaryotic green algae and plants. The chlorophyll molecules are not located within chloroplasts, as in higher plants, but are found, instead, within photosynthetic **thylakoid membranes** dispersed throughout the cytoplasm (Figure 6B-6). In addition to chlorophyll *a,* cyanobacteria contain other accessory pigments including the yellow and orange carotenoids and the phycobilins (reddish phycoerythrins and bluish allophycocyanins).

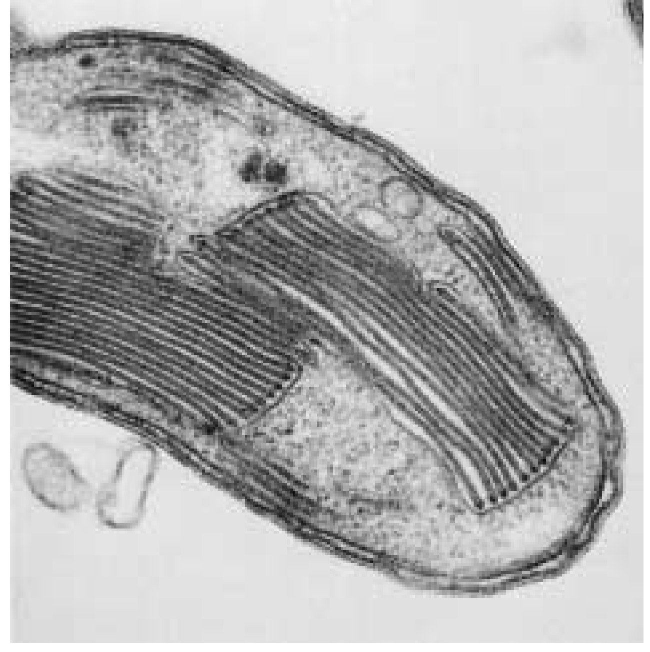


Figure 6B-6 *Photosynthetic membranes fold into stacks inside a bacterial cell.*

**Oil has a greater refractive index than air and thus better resolution can be obtained by replacing the layer of air between the end of the objective and the specimen with a drop of oil into which the objective is carefully immersed. This eliminates much of the refraction and loss of light that occurs when light passes through air before reaching the dry objective. The working distance of an oil-immersion objective (90× to 100×) is less than 1 mm and the objective must almost touch the slide to be in focus. The field of view is much smaller. Light intensity usually needs to be increased when working with oil. Review these principles in Laboratory 1.*

Procedure

1. Make a wet mount of one of the cyanobacterial species available in the laboratory. Observe the specimen using the 40× objective.
2. Draw a representative cell in the space below Figure 6B-7. Use this electron micrograph to assist you in labeling the parts of the cell you have drawn.

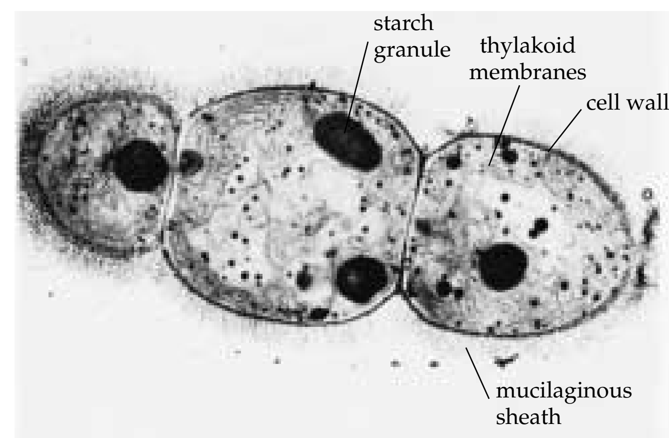


Figure 6B-7 *Electron micrograph of the cyanobacterium* Anabaena *sp. The large, dark bodies are composed of cyanophycean starch, the chief carbohydrate storage product of cyanobacteria.*

EXERCISE C Working with Bacteria

Microbiologists maintain specific types of bacteria in the laboratory as **cultures;** a culture containing only a single species of bacteria is called a **pure culture.** Since bacteria are found everywhere—in the air, water, soil, and food, on the body surface, and inside other organisms—it is necessary to follow strict procedures to protect laboratory cultures from contamination by unwanted strains of bacteria.

In the laboratory, bacteria are grown on culture media containing the nutrients necessary for their growth. The medium can be liquid (broth medium) or solid if **agar** (a complex carbohydrate product of seaweed) is added to the broth.

Bacteria are transferred from one medium to another or to a new stock of the same medium by **subculturing.** Pure cultures of bacteria, containing only one type of organism, are produced by preparing **streak plates** or **spread plates** that allow discrete **colonies** (small masses of bacteria resulting from multiplication of a single bacterium) to be isolated. These techniques are essential tools for the study of bacterial cells.

Objectives

- ☐ Subculture bacteria from a broth culture to an agar slant or to a second broth culture.
- ☐ Prepare a streak-plate culture from a mixture of bacteria to isolate single colonies.
- ☐ Prepare a spread-plate culture for isolating and culturing single colonies of bacteria.

Procedure

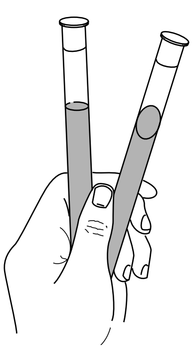
All steps should be carried out aseptically (under *sterile* conditions). A few precautions should also be taken: be careful never to lay contaminated or used equipment on laboratory benchtops; if a spill occurs, let your instructor know; dispose of all waste materials in special containers.

PART 1 Techniques for Transferring Cultures

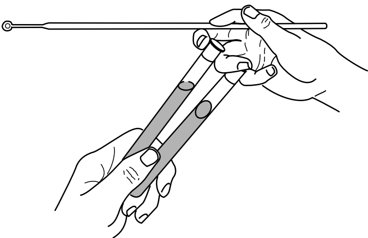
It is often necessary to begin new cultures by transferring bacterial cells from an existing culture to a new growth medium so as to maintain cultures that have become overgrown and have used up all the available nutrients.

Steps for Subculturing Bacterial Cells

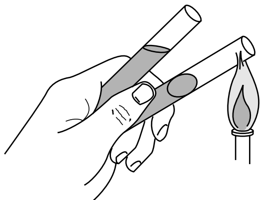
1. Obtain a 24-hour nutrient-broth stock culture of *Escherichia coli* (a gram-negative bacillus) or *Serratia marcescens* (a gram-negative, motile bacillus with red pigment) and an agar-slant tube. Label the tube to be inoculated with the name of the bacterium and your initials.
2. Hold both the stock tube and agar-slant tube in one hand using your thumb to separate them into a **V.**



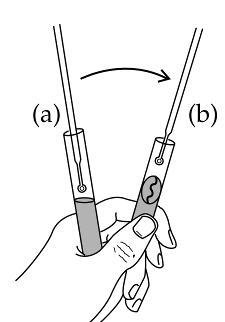
3. Obtain a wire inoculating loop from your instructor. Sterilize the loop by holding it in the hottest (blue) part of a Bunsen burner flame until the loop glows red. Then, continue to pass the lower two-thirds of the shaft through the flame. Once you have "flamed" the loop, never put it down; hold it for 10 to 20 seconds to allow it to cool. (Alternatively, a sterile, disposable plastic inoculating loop can be used—*do not* place it in the flame.)
4. Uncap both tubes by grasping the caps with the second, third, and fourth fingers of the hand holding the inoculating loop. Once removed, *do not* put the caps down or sterility will not be maintained.



5. Flame the necks of the two tubes briefly by passing them through the Bunsen burner flame.



6. Cool the transfer loop by touching it to the sterile inside surface of the culture tube and then (a) insert the loop into the broth culture and shake slightly. (b) Remove the bacteria-laden loop and insert it into the agar slant until you lightly touch the surface of the agar. Drag the loop across the surface of the agar in a zigzag line and remove. (To transfer a culture from a broth to a broth, see steps 8 to 10; to transfer from a broth to an agar plate, see Part 2 of this exercise.)



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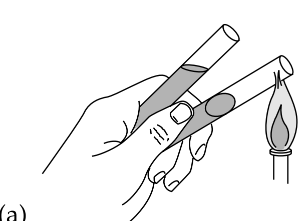
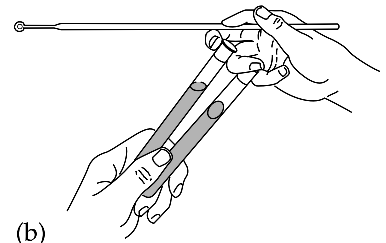
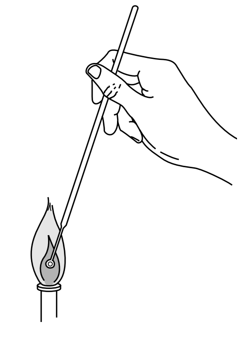
7. After inoculating, (a) reflame the necks of the tubes and (b) replace the caps on the correct culture tubes. (c) Always reflame the inoculating loop after use to destroy any remaining bacteria.


(a)


(b)


(c)

8. Resterilize the inoculating loop as in step 3.

9. Obtain a tube of sterile nutrient broth and, using the same stock culture tube as before, label the tube to be inoculated, uncap the two tubes, flame the necks of the tubes, and transfer a sample of bacteria from the culture tube to the nutrient broth with the inoculating loop. After inserting the loop into the sterile nutrient broth, shake the loop slightly to dislodge the bacteria.

10. After inoculating, reflame the necks of the tubes, replace the caps, and reflame the inoculating loop.

11. Incubate the tubes containing the inoculated broth and agar at 25°C for 24 to 48 hours. Examine your cultures for the presence of bacterial growth. You can recognize growth as turbidity in the broth culture and the appearance of a whitish (*E. coli*) or orange-red (*S. marcescens*) growth on the surface of the agar slant.

a. Why is the inoculating loop flamed before and after each transfer? ______________________

____

b. Why is it important NOT to place the caps to the tubes on your laboratory table during the transfer procedure? ____

____

c. Why is it important to cool the inoculating loop before transferring cells? ______________________

____

PART 2 Isolating Pure Cultures

The isolation of discrete colonies of bacteria is important in the study of the morphological and biochemical characteristics of bacteria and in recombinant DNA studies.

1. Obtain a nutrient broth culture of a mixture of *S. marcescens* and *E. coli* and a Petri dish of nutrient agar. Label the Petri dish with the names of the bacteria you are using and with your initials.

2. To prepare a **streak plate,** obtain a loopful of culture (follow sterile procedures as in Part 1) and, after carefully lifting one side of the lid of the Petri dish, deposit the bacteria (Figure 6C-1) onto the surface of the agar toward one side of the dish. When performing this type of transfer, always keep the lid of the Petri dish above the agar surface—*never* remove the lid completely and lay it down on the laboratory bench and *never* completely uncover the agar surface. By lifting only one side of the lid, you will prevent dust and other contaminants

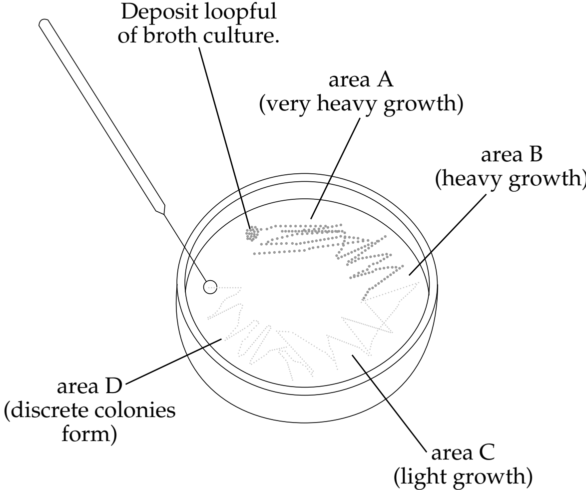


Figure 6C-1 *Preparation of a streak plate.*

from coming into contact with the surface of the agar and the inner surface of the lid of the Petri dish.

3. Reflame and cool the loop (you may stab it into the agar at the side of the plate several times or touch it to the surface of the agar until it no longer sizzles). Drag the loop through the bacteria rapidly, moving back and forth in a zigzag pattern (Figure 6C-1, area A).
4. Reflame and cool the loop. (In preparation of a streak plate, the loop is reflamed to help obtain a more dilute solution of bacteria.) Turn the Petri dish 90° and draw the loop through the first streak and continue streaking in a zigzag manner (Figure 6C-1, area B).
5. Again turn the agar plate 90° and repeat step 4 (Figure 6C-1, area C).
6. Once again, turn the plate 90°, and touching the loop to a corner of the streaks in area C, drag the loop across the surface in a zigzag motion. Be careful not to touch any previously streaked areas. Reflame the loop at the end of this first inoculation procedure.
7. Incubate plates for 48 to 72 hours at 25°C in an *inverted* position. (This precaution prevents the condensation that forms on the lid of the Petri dish from dropping onto the culture.)
8. To prepare a **spread plate,** obtain a nutrient broth culture of a mixture of *E. coli* and *Micrococcus luteus* (a gram-positive coccus bacterium with yellow pigment) and a sterile Petri dish of nutrient agar. Label the agar plate with the names of the organisms you are using and with your initials.
9. Obtain a glass spreading rod (this may be a glass rod bent into the shape of an **L** or a rod with a triangular bend on the end). Place the spreading rod into a beaker of 95% ethyl alcohol. Be sure that the beaker contains enough alcohol to cover the lower bent portion of the rod.
10. With a sterile loop, place a sample of the bacterial culture from the broth tube onto the center of the agar. (Remember to hold the lid face down above the culture plate to help prevent contamination.) Replace the cover and reflame the inoculating loop before putting it aside.
11. Remove the spreading rod from the alcohol. Keep the bent portion pointing downward (to prevent alcohol from running up the handle and onto your fingers). Pass the rod through the flame of the Bunsen burner and allow the alcohol to burn off completely. Allow the rod to cool for 10 to 15 seconds.
12. Lift the lid of the Petri dish and touch the rod to the clean surface of the agar to make sure it is cool before touching it to the bacteria in the middle of the plate.

13. Lightly move the spreading rod back and forth across the surface of the agar while spinning the plate around. (You may use a turntable to spin the plate if available, or you can do this by hand using the fingers of the hand holding the lid—you may wish to practice this technique on an empty dish first.)

14. Recover the plate and incubate in an *inverted* position at 25°C for 48 to 72 hours.

a. Observe the growth on both the streak plate and spread plate. Describe and make a drawing of each.

Streak plate

Spread plate

b. On each of the plates, locate two colonies that are morphologically different (I and II) and record your observations in Table 6C-1. Colonies may differ in the following ways: (1) color; (2) form: circular, irregular, or spreading; (3) elevation: flat or raised; and (4) size.

c. Which organism do you think is present in each of the colonies you observe? Record this information in Table 6C-1.

Table 6C-1 Isolating Pure Colonies of Bacteria

	Description	Organism
Streak plate Colony I		
Colony II		
Spread plate Colony I		
Colony II		

d. How might you prepare a pure culture of E. coli, S. marcescens, *or* M. luteus *from the streak or spread plates?* ______________________________

____

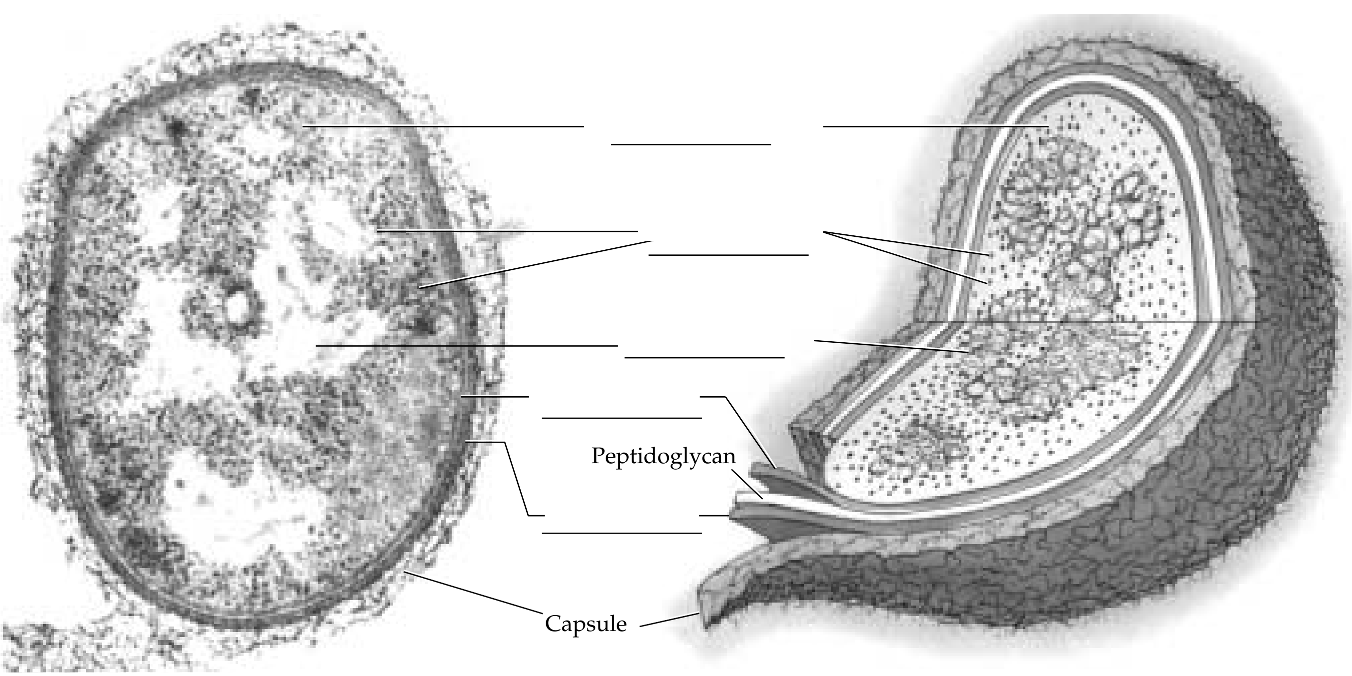
____

15. Compare the bacterial colonies you have observed with the viral plaques on demonstration. Viral plaques are formed by bacteriophages (viruses that attack bacteria). They are prepared as follows: A layer of harder agar is overlaid with a layer of soft agar containing a mixture of bacterial cells and bacteriophages. The bacteria reproduce and spread throughout the agar to

form a confluent layer, or "lawn," across the surface of the plate, except where a bacteriophage infection occurs. When the bacteriophage replicates inside the host cell, it causes the bacterial cell to *lyse* (dissolve); new virus particles then infect nearby cells. The process of viral replication and cell lysis continues, producing a clear, circular area called a **plaque** in the bacterial lawn where the bacterial cells were destroyed.

Laboratory Review Questions and Problems

1. Label the parts of the typical gram-negative bacterial cell shown below.



2. Coacervates are called protobionts. Why are they not called cells? What characteristics do they share with cells?

3. All cells are surrounded by a membrane that separates the external environment from the internal environment. A typical cell membrane is formed by a lipid bilayer in association with proteins. The protobionts made during this laboratory were composed of carbohydrate and protein. How is a barrier formed in these protobionts? Which molecules could form a membrane-like barrier?

4. Describe the structure of a bacterial chromosome. How does it differ from a eukaryotic chromosome?

5. Bacterial flagella are different in composition and structure from the flagella of eukaryotes. What types of differences exist? Do the flagella of prokaryotes move in the same way as the flagella of eukaryotes?

6. Cyanobacteria can carry out photosynthesis, yet they do not contain chloroplasts. Where is chlorophyll found in these organisms?

7. Your doctor tells you that you have a bacterial infection in your salivary glands. You are given the opportunity to view the slide made from a sample of your saliva. It is labeled "gram-negative, coccus." Describe what you will see on the slide.

8. Explain how the structure of the bacterial cell wall affects the results of the Gram staining procedure.

9. Your laboratory instructor is going to observe and grade your technique while you inoculate a broth culture and make a streak plate from an agar slant of *E. coli.* List four things that you should be careful to do (or not do) to receive an "A" for your aseptic technique.

10. You are given a broth culture of *E. coli* mixed with several other species of bacteria. Explain how you would establish a pure culture of *E. coli* for further laboratory investigations.

11. You are in the microbiology laboratory and are given the task of cleaning up after the last three lab sessions. You decide to sort the plates into "like" types. You find several (A) with small white circular areas of growth and others (B) that appears fairly "slimy" all over but with small circular clear areas dotted at various locations around the plate. You have others (C) that have irregular areas of red-colored growth and several (D) with a yellow-colored growth. You tentatively try to identify what is in the plates so you will know how to handle them. What do you decide about each?

LABORATORY

7

Eukaryotic Cells

OVERVIEW

All eukaryotic organisms are composed of cells, whether they exist as single cells, colonies of cells, or in multicellular form. Your body is composed of 50 to 100 trillion cells, most of which are very small, with specialized structures that allow for a diversity of functions.

All eukaryotic cells have their genetic material enclosed by a nuclear membrane, the nuclear envelope. In addition, a variety of subcellular membrane-bound organelles are present. These include plastids, mitochondria, lysosomes, microbodies, and Golgi complexes. Internal membrane systems, or endoplasmic reticula, divide the cell into specialized compartments. Non-membrane-bound organelles, such as ribosomes, centrioles, microtubules, and microfilaments, are also present in eukaryotic cells.

During this laboratory you will investigate the structure of plant and animal cells and will learn how biochemical analysis, cell fractionation, and transmission electron microscopy have allowed scientists to unlock the secrets of eukaryotic cells.

STUDENT PREPARATION

Prepare for this laboratory by reading the pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

EXERCISE A Examining Plant Cells

The cells of plants are eukaryotic, containing both a membrane-bound nucleus and membrane-bound organelles. A large central **vacuole** surrounded by a membrane (the **tonoplast**) is used for storing water, pigments, and wastes. Within the cytoplasm are various types of **plastids** responsible for photosynthesis and for storing starch or pigments. A **cell wall** composed of cellulose surrounds the plant cell.

Objectives

☐ Identify the structures of a typical plant cell.

Procedure

1. Prepare a wet-mount slide of an *Elodea* leaf. Observe the thick cell wall, thinner cell membrane, cytoplasm, nucleus, and chloroplasts. A large central vacuole should be apparent. These structures characterize a generalized plant cell.
2. On the top half of a separate sheet of paper, draw a representative *Elodea* cell and label its parts.

3. Prepare a fresh wet-mount slide of *Elodea.* Observe your slide using high power (40×).

Do the chloroplasts appear to move? ______ Describe their movement.

4. Prepare a wet-mount slide of onion tissue. Onions (*Allium*) have layers of modified leaves (scales) that can easily be separated from one another. Peel off a portion of one layer and examine the concave side of the piece you have obtained. The surface is covered by a thin layer of cells, the epidermis.

5. Remove a small piece of the epidermis (approximately 3 × 8 mm) by breaking the scale gently, leaving the epidermis intact. Peel the epidermis from one of the halves of the scale. Prepare a wet-mount slide of the isolated epidermis.

6. Observe the onion cells using low power (10× objective) and then high power (40× objective).

7. If it is difficult to see the cells, add a drop of Lugol's solution (I_2KI) at the edge of the coverslip. Does this solution stain the cells as it reaches them? ______

8. Draw a representative onion cell on the lower half of the sheet of paper you used for your drawing of *Elodea.* Compare the onion cell with the *Elodea* cell. Since they are both plant cells, they should be similar. You will note that onion cells lack one structure that is very conspicuous in *Elodea* cells. What is this structure? ______________________

a. List the similarities and differences between Elodea *cells and onion cells.*

Similarities	*Differences*
______________________	______________________
______________________	______________________
______________________	______________________
______________________	______________________

9. Use a razor blade to slide a piece of tissue, as thin as possible, from a potato. Be careful not to cut your fingers. Prepare a wet-mount slide; use a drop of water.

10. Study the slide at low power (10× objective) and then at high power (40× objective). Add a drop of Lugol's solution (I_2KI) to the side of the coverslip and observe the cells as the iodine solution makes contact with them.

b. How does the reaction of iodine with the potato cells compare with what you observed in your onion epidermis preparation (step 7)? ______________________

____

____

c. What does this tell you about the differences between the storage products in onions and potatoes?

____

____

d. Do you see any chloroplasts? ______ *Why or why not?* ______________________

e. You will probably see some small oval-shaped blue-black structures. These ***leucoplasts*** *store starch. Why did they turn blue?* ______________________

____

11. If a banana is available, scrape a small amount of tissue from its surface and spread it onto a slide. Add a drop of Lugol's solution and a coverslip. Observe the preparation using high power (40× objective).

f. How do these observations compare with what you saw in the potato?

____

Plastids are membrane-bound organelles unique to plants. You have already been introduced to two types—chloroplasts (containing chlorophyll) and leucoplasts (containing starch). **Chromoplasts** contain several types of pigment including carotenoids, which give plants an orange or yellow color.

12. Use a razor blade to slice a piece of tissue, as thin as possible, from the outer portion of a peeled carrot. Prepare a wet-mount slide.

g. Can you see the chromoplasts? Describe them. _______________________

____

EXTENDING YOUR INVESTIGATION: CYTOPLASMIC STREAMING

Many environmental conditions can affect the natural functions of cells, including the movement of chloroplasts. Microfilaments, composed of the protein actin, direct the movement of chloroplasts within the cell cytoplasm. What changes in the cellular environment do you think might disrupt, increase, or decrease the rate of chloroplast movement? Formulate a hypothesis that predicts how alterations in the environment might affect chloroplast movement in *Elodea.*

HYPOTHESIS:

NULL HYPOTHESIS:

Can you **predict** how changing the environment might affect chloroplast movement?

What is the **independent variable?** _______________________

What is the **dependent variable?** _______________________

Design an experimental procedure to test your hypothesis.

PROCEDURE:

Describe what you observed during your experiment.

RESULTS:

Do your results support your hypothesis?

Your null hypothesis?

What do you **conclude** about how the environment affects chloroplast movement?

✔ EXERCISE B | Examining Animal Cells

The cells of animals, like those of plants, are eukaryotic with a membrane-bound nucleus and membrane-bound organelles. Unlike plant cells, however, they have no central vacuole (although they may have small vacuoles) and no plastids. A plasma membrane surrounds the cell, but there is no cell wall.

✔ PART 1 Studying Animal Cells Using Light Microscopy

Animal cells can be studied using the light microscope, but most of the cellular organelles within the cytoplasm are not visible without the use of special staining techniques. The nucleus and **nucleolus,** where ribosomes are manufactured, are usually apparent in most cells.

To study the structure of animal cells you will use prepared slides of animal **tissues.** These are collections of cells that have a similar function. The cells are usually organized into sheets.

Objectives

☐ Identify the structure of a typical animal cell.

Procedure

1. Observe a prepared slide of frog epithelium (simple squamous or cuboidal). Examine the slide using the 10× and the 40× objectives.
2. Sketch one or more of the cells in the space below. Label the nucleus, cytoplasm, and plasma membrane.
3. If available, observe a slide of salamander epithelium that has been stained to demonstrate mitochondria—membrane-bound organelles used for cellular energy production. Use the 40× objective to examine the cells.

   a. Do the cells you observe have a cell wall? ______ *Plastids?* ______ *Chlorophyll?* ______

   b. List the similarities and differences between the plant cells and the animal cells you have observed.

Similarities	*Differences*
____________	____________
____________	____________
____________	____________
____________	____________

✔ PART 2 Studying Animal Cells Using Cytochemical Stains

Staining very thin sections of tissue highlights the internal structures of cells, enabling us to examine them in more detail. Stains may be general, staining many parts of the cell, or specific, reacting only with particular biochemical macromolecules. If a certain type of macromolecule happens to be located within a certain organelle, specific cytochemical stains allow us to determine the chemical composition of that organelle.

Objectives

- ☐ Describe how scientists can use the microscope to study the biochemistry of cells.
- ☐ Describe how the biochemical composition of cellular organelles can be determined and give specific examples.

Procedure

On demonstration you will find several examples of slides stained with special cytochemical stains specific for biochemical compounds such as DNA, RNA, protein, starch, and lipid. In each pair of slides you will study, one tissue sample has been treated with enzymes to remove the substance being studied. Examine the pairs of demonstration slides listed below and record your observations.

1. DNA in mammalian liver (Feulgen stain):

Color of nuclei ____________________

Color of cytoplasm ____________________

Other characteristics ____________________

____

Mammalian liver treated with DNase to remove DNA (Feulgen stain):

Color of nuclei ____________________

Color of cytoplasm ____________________

Other characteristics ____________________

____

a. Based on your observations, what does Feulgen reagent stain? ____________________

2. RNA in mammalian liver (methyl-green-pyronin stain):

Color of nuclei ____________________

Color of cytoplasm ____________________

Other characteristics ____________________

____

Mammalian liver treated with RNase to remove RNA (methyl-green-pyronin stain):

Color of nuclei ____________________

Color of cytoplasm ____________________

Other characteristics ____________________

____

b. Based on your observations, what does methyl-green-pyronin stain? ____________________

3. Glycogen in mammalian liver (carmine stain):

Color of nuclei ____________________

Color of cytoplasm ____________________

Other characteristics ____________________

____

Mammalian liver treated with amylase to remove glycogen (carmine stain):

Color of nuclei ____________________

Color of cytoplasm ____________________

Other characteristics ____________________

____

c. Based on your observations, what does carmine stain indicate is present in liver cells?

____

4. Fat tissue (osmium tetroxide):

Identifying characteristics ____________________

____

Fat tissue treated with lipase to remove lipid (osmium tetroxide):

Identifying characteristics ____________________

____

d. Based on your observations, how does osmium tetroxide indicate the presence of fat?

____

5. Based on the observations made above, describe what you would observe in the following slides.

e. Carmine stain used on a liver sample from a starved rat (with no stored glycogen).

____

f. Feulgen stain used on a chromosome preparation from Drosophila.

____

g. Osmium tetroxide used to stain a section of adipose (fat) tissue from an obese mouse.

____

✔ EXERCISE C | The Strange Shapes of Cells

You have just studied some representative plant and animal cells, all of which have a fairly simple shape and are of an approximately similar size. You should realize, however, that cells come in all sizes and shapes with a multitude of specializations for function or for interaction with other cells.

The smallest bacterial cells (mycoplasmas) are about 0.1 μm in diameter. Most other bacteria are on the order of 1 to 2 μm in diameter (Laboratory 6). A human liver cell has a diameter of 20 μm. A human ovum is approximately 100 μm in diameter—1,000 times larger than the smallest cells. Then, of course, there are the unusual cases to consider: for example, the ostrich's ovum, a single cell approximately 1,000 times the size of a human ovum.

Objectives

☐ Recognize diverse forms of specialization in cell structure.

Procedure

Observe examples of some of the diverse cell types that may be available in the laboratory. Sketch and label these on a separate of paper and insert your drawings into your laboratory manual.

Nerve cell A highly specialized cell consisting of a cell body (containing the nucleus) and long cytoplasmic extensions for transmission of nerve impulses.

Stentor A ciliated, single-celled eukaryote belonging to the kingdom Protista.

Acetabularia An unusually large, single-celled green alga (2 to 5 cm) possessing a foot (containing the nucleus), a stalk, and a cap.

Volvox A colony of biflagellated green algal cells.

Human sperm cell A highly differentiated sex cell consisting of three different regions: the head (containing the nucleus), the midpiece (with a large mitochondrion), and the tail composed of a flagellum.

Starfish egg A large, highly differentiated sex cell containing an extremely large nucleus called a germinal vesicle. The **nucleolus** (site of ribosome synthesis) is visible within the nucleus as a dark dot.

EXERCISE D | Cell Fractionation: A Study of Eukaryotic Cells

Cells can be broken apart by several means such as electric shock, sonication (vibration), or grinding. The cell membranes and endoplasmic reticulum are broken open, but the membrane fragments quickly "reseal" to form smaller vesicles. If gentle techniques are used, the cell organelles, including nuclei, mitochondria, and chloroplasts, remain intact and retain most of their original biochemical function.

A suspension of cell parts and organelles is called a **homogenate.** The many components of a cell homogenate can be separated by **differential centrifugation:** the use of different amounts of centrifugal force (generated at different speeds) to sediment cell parts of various sizes and densities. At low speeds, large nuclei and unbroken cells sediment to form a **pellet** at the bottom of the centrifuge tube. The remaining cellular organelles are found in the **supernatant** above the pellet. At slightly higher speeds,

chloroplasts and mitochondria can be separated in the pellet. The material still remaining in the supernatant is the **microsomal fraction** containing small cellular organelles (lysosomes, microbodies, ribosomes) and membrane vesicles.

If this exercise, you will separate the organelles from the cells of pea seeds using differential centrifugation. You will identify the types of organelles in the many cell fractions by using specific colorimetric staining reactions.

Objectives

- ☐ Describe how cell structure can be studied by fractionating cells.
- ☐ Determine the presence of cellular organelles within a cell fraction.

Procedure

Your instructor will homogenize a sample of pea seeds that have been soaked overnight to soften. Stir the homogenate before using. See Figure 7D-1 for a flow chart of this experiment.

1. Work in pairs. Fill a 15-ml centrifuge tube with homogenate until it is three-fourths full. Have your laboratory partner do the same so that there is an identical amount of material in the two tubes. Write your initials on the side of the tube.
2. Place your tube in a clinical centrifuge *opposite* your lab partner's tube. (The rotor must always be balanced with equal weights or volumes opposite each other.)
3. Close the top of the centrifuge and slowly increase the speed to 200 × gravity (200 × g; setting 4 on a clinical, table-top centrifuge). Centrifuge for 3 minutes.
4. While your material is in the centrifuge, obtain some of the cellular debris left on the cheesecloth when your instructor prepared the "pea soup." Place a small amount of this material on a slide and add a drop of water. Cover with a coverslip and observe the material using the light microscope (magnification 40×)

   a. Describe what you observe. ______________________________

   ____

   b. Do you see cell wall fragments? ________ *If yes, describe them.*

   ____

   c. Do you see intact cells? ________

   d. Add a drop of I_2KI solution at the edge of the coverslip. What does this stain?

   ____

   e. Describe what you see. ______________________________

5. After centrifugation is complete, use a Pasteur pipette to remove a small amount of sediment from the bottom of each tube and save it in a clean test tube. Return the original tubes to the centrifuge (a balanced pair) and slowly increase the speed to 1,300 × g (setting 7 on the clinical centrifuge). Centrifuge for 10 minutes.
6. While your material is in the centrifuge, investigate the contents of the sediment. Place a drop of the sediment on a slide and mix it with a drop of I_2KI. Cover with a coverslip and observe at 40×. Pea seedlings store food reserves as starch grains. Starch grains have characteristic shapes and are sometimes used for classifying plants.

   f. Describe the structure of the starch grains you observe. ______________________________

   ____

   g. Why are they stained blue? ______________________________

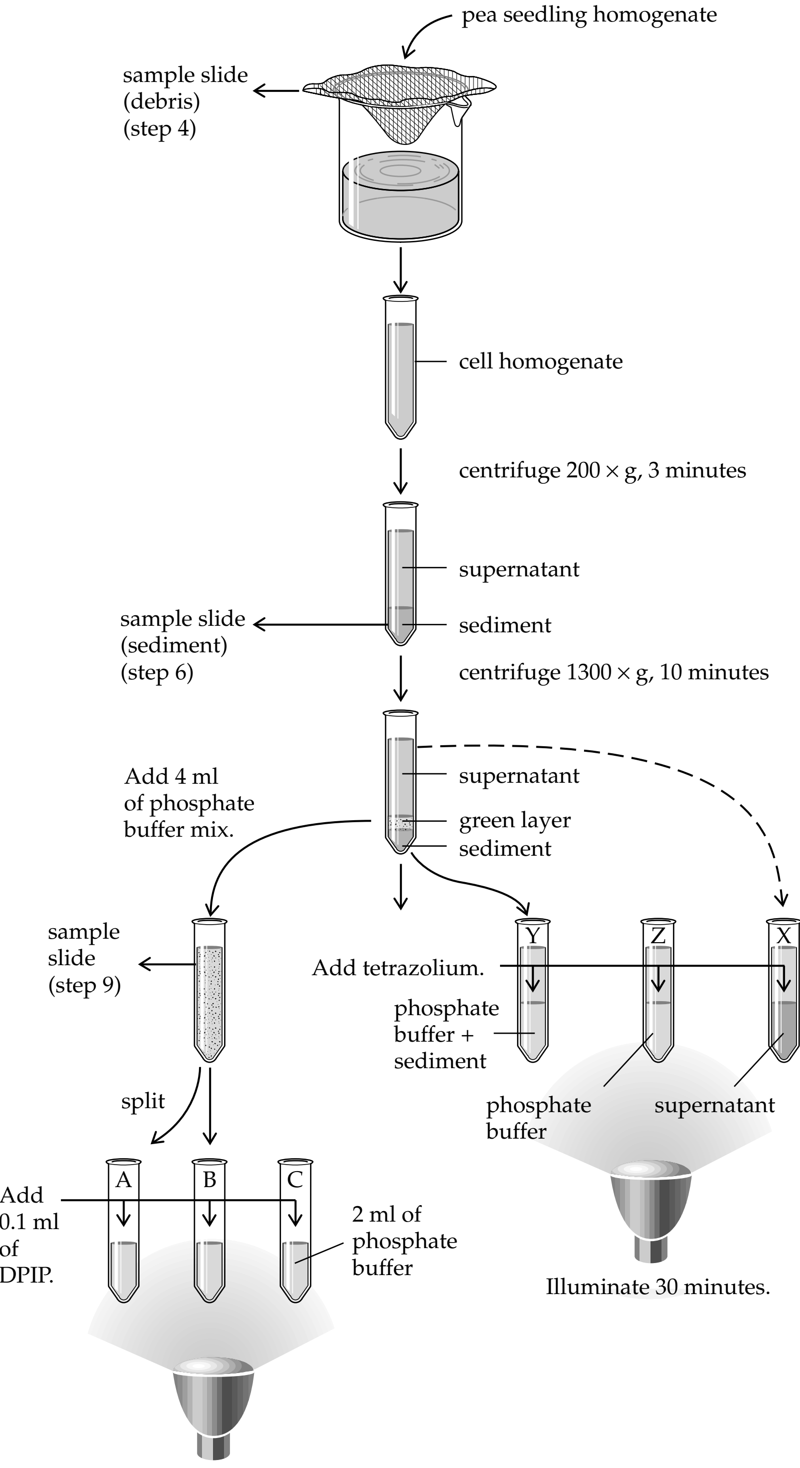


Figure 7D-1 *Cell fractionation procedure.*

h. Why are there so many starch grains in pea seeds? ______________________

____

7. After centrifugation is complete, carefully remove your sample tubes from the centrifuge.

i. Describe what the contents of the tube look like. ______________________

8. Nuclei and chloroplasts will be in a green layer above the sediment. Do you see this layer? Hold the tube in front of a light source and, using a Pasteur pipette, *carefully* remove material from this layer. (You will have to place the tip of the Pasteur pipette next to the side of the tube. You may wish to mark the pipette tip with a waterproof black marker to make it easier to see.) After removing this material, store the centrifuge tube on ice.

9. Place the material you remove into a small test tube on crushed ice. Add 4 ml of phosphate buffer and resuspend the contents. Use one drop of the material to make a wet-mount slide. Observe using high power (greater than 40× if possible).

j. Describe what you see. ______________________

10. Mark two test tubes "A" and "B," and put 2 ml of the sample into each tube. Cover tube with aluminum foil. Keep both tubes on ice. Place 2 ml of phosphate buffer into a third tube and label this tube "C." Add 0.1 ml of DPIP to each tube. Cover with parafilm and mix by inverting. Use the spectrophotometer to read the absorbance at 600 nm for each of the three tubes and record your data in the "before" column of Table 7D-1. If a spectrophotometer is not available, compare the colors of the three tubes and record this information as qualitative observational data. Tube C is your blank.

Table 7D-1

	Color		Absorbance (600 nm)		
Tube	Before	After	Before	After	Difference
A					
B (dark)					
C					

DPIP (2,6-dichlorophenol-indophenol) is a blue dye that can act as a hydrogen and electron acceptor. During the light-dependent reactions of photosynthesis, DPIP can substitute for $NADP^+$ and is reduced by the addition of hydrogen and electrons. When DPIP is reduced it becomes colorless.

k. If DPIP, when mixed with the chloroplast sample, loses its blue color, what is probably happening in the sample? ______________________

11. Form a hypothesis that predicts what changes you might expect in the three tubes and why this is so.

HYPOTHESIS:

NULL HYPOTHESIS:

12. Illuminate the three tubes with a 100-watt light. After 30 to 45 minutes, record your results in Table 7D-1 by observing the color in the three tubes or, if you are using a spectrophotometer, by reading the absorbance at 600 nm.

l. Do your results support your hypothesis? ______ *How do tubes A and B compare?*

m. What do you conclude about the contents of the green layer formed during the cell fractionation?

____

13. Locate the centrifuge tube on ice (end of step 8). Into a clean test tube add enough of the yellow-green supernatant layer to fill the tube approximately one-third to one-half full, and label this tube "X." Carefully pour off the remaining supernatant from the centrifuge tube. Suspend a sample of the sediment in an amount of phosphate buffer equal to the contents of tube X. This should produce a slightly cloudy, translucent mixture. Label the tube "Y." Into a third tube add an amount of phosphate buffer equal to that in tubes X or Y, and label this "Z."

14. You will use the dye **tetrazolium*** to test for mitochondrial activity. Tetrazolium will indicate whether the mitochondrial electron transport system is present and working. Tetrazolium can act as an electron acceptor (in place of cytochrome in the electron transport system) and will turn red when reduced by addition of electrons.

n. The yellow-green supernatant contains chlorophyll pigments from broken chloroplasts, but what else might it contain? ______________________________

o. How does the size of mitochondria compare with that of chloroplasts? ______________

15. Form a hypothesis that predicts which cellular fraction (X or Y) contains mitochondria and why this is so.

HYPOTHESIS:

NULL HYPOTHESIS:

16. Add tetrazolium solution to tubes X, Y, and Z to fill each tube approximately two-thirds full (the amount of tetrazolium should be equal to the amount of homogenate in the tube).

17. Place all three tubes in a beaker of warm water (35–40°C) in front of a 100-watt light for 30 minutes (check the water temperature after 15 minutes and replace with warm water if necessary).

18. RESULTS: After 30 minutes, record your observations in the following table.

p. Do your results support your hypothesis? ______ *Your null hypothesis?* ______

q. What can you conclude about the size of mitochondria relative to the size of chloroplasts in pea seeds? ______

Tube	Color
X	
Y	
Z	

✔ EXERCISE E | A Closer Look at Eukaryotic Cells

Figure 7E-1 shows two pictures, or *micrographs*, of an onion cell nucleus. (A micrograph is a photograph taken using a microscope.) One is a photomicrograph taken under the light microscope. The other is an

**Tetrazolium is a poison. If desired, see alternate directions in Preparator's Guide.*

electron micrograph. Note that the overall magnification of the two onion cells is approximately equal. Why, then, do the cells look so different? The reason is that the light microscope and electron microscope differ in their ability to resolve, or distinguish, fine detail. An electron microscope is able to resolve two structures as separate when they are only 0.0001 μm apart, whereas the light microscope can resolve objects only when they are approximately 0.5 μm apart (Laboratory 1).

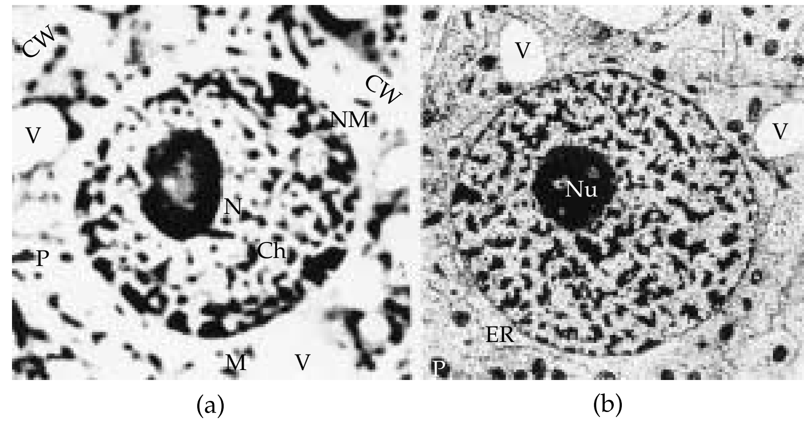


Figure 7E-1 *Photograph of an onion cell nucleus using (a) a light microscope and (b) an electron microscope.*

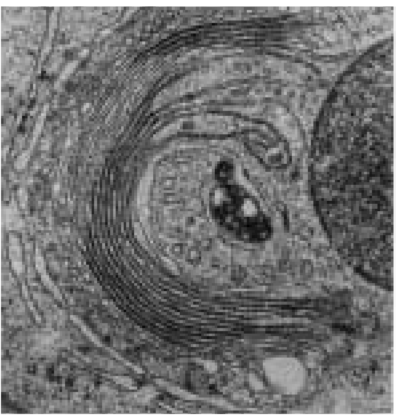
Objectives

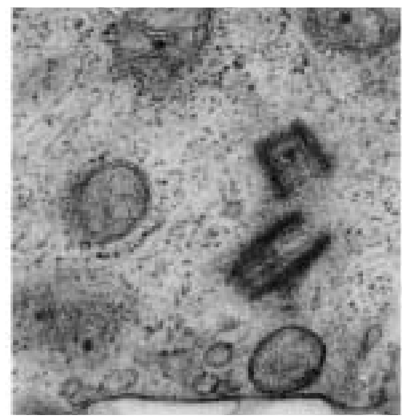
☐ Recognize membrane-bound and non-membrane-bound organelles in transmission electron micrographs.

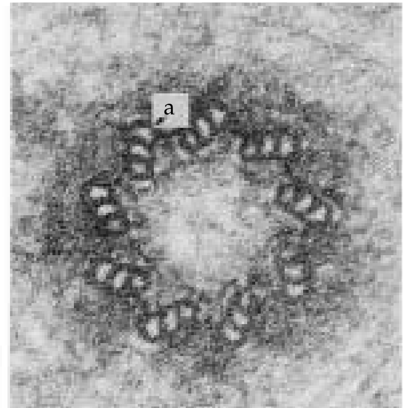
Procedure

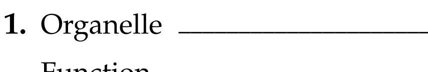
Eukaryotic cells are compartmentalized into many subcellular membrane-bound organelles. Non-membrane-bound particles and organelles are also present.

1. In the following transmission and scanning electron micrographs, indicate the type of organelle that is the main subject of the micrograph and describe its function. (The organelle in micrograph 6 is named for you.)
2. Use your knowledge gained from lectures and from reading your textbook to identify the lettered structures and list their functions (where indicated). If you are not sure of the identity of a particular structure, look for examples in labeled micrographs available in the laboratory or in your text.





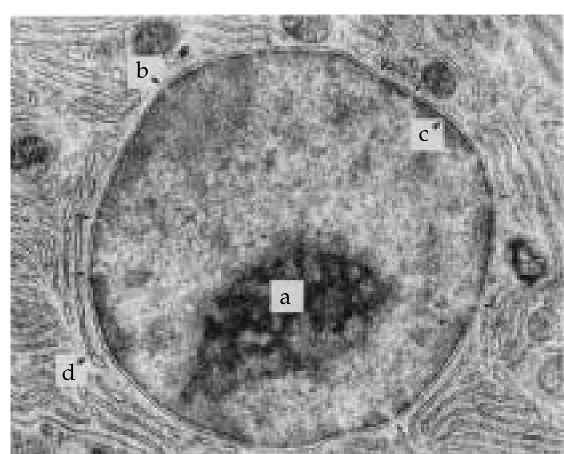




1. Organelle ____________________
Function ____________________

2. Organelle ____________________
Function ____________________

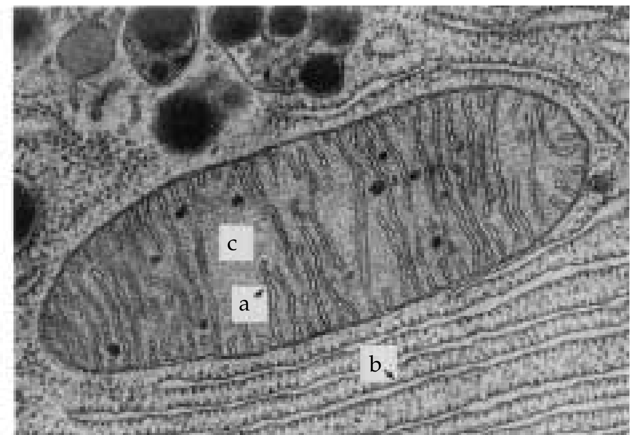
3. Organelle ____________________
Function ____________________
a ____________________



4. Organelle ____________________

Function ____________________

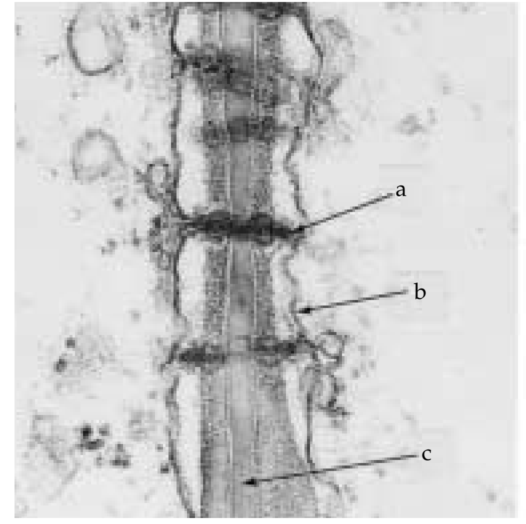
Part | Function
a __________ __________
b __________ __________
c __________ __________
d __________ __________



5. Organelle ____________________

Function ____________________

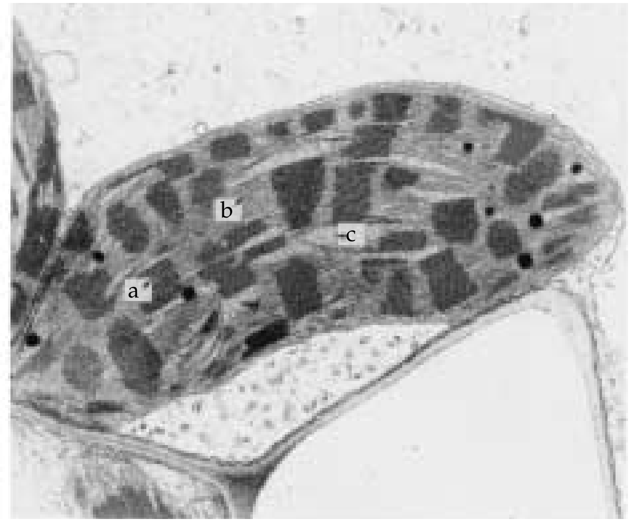
Part | Function
a __________ __________
b __________ __________
c __________ __________



6. Cell junction

Function ____________________

Part
a __________
b __________
c __________



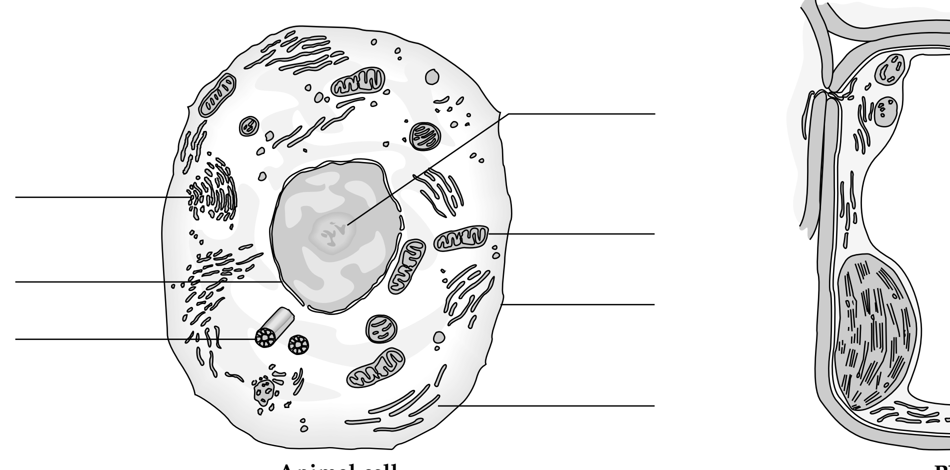
7. Organelle ____________________

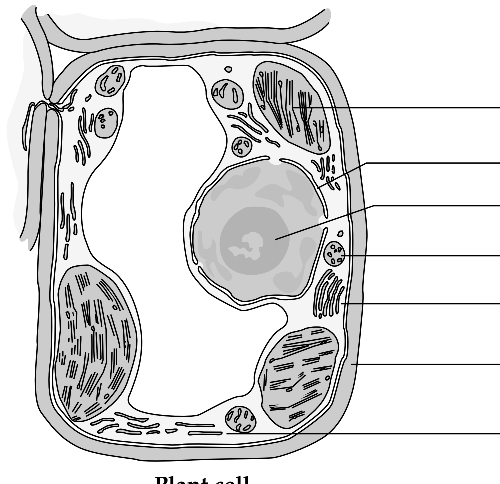
Function ____________________

Part | Function
a __________ __________
b __________ __________
c __________ __________

Laboratory Review Questions and Problems

1. Label the parts of the generalized animal cell and plant cell shown below.


Animal cell


Plant cell

2. Complete the following table. If possible, add extra information on the structure of each organelle.

Organelle(s) or Other Structure(s)	In Prokaryotes?	In Eukaryotes?	Function
Nucleus with nuclear membrane			
Nucleolus			
Chromosomes			
Cytoplasm			
Mitochondria			
Golgi complexes			
Endoplasmic reticulum			
Ribosomes			
Centrioles			
Chloroplasts			
Cell wall			
Flagellum			

3. A pathologist is studying a set of slides made from a patient's tissues, which have been stained with a series of cytochemical stains. The pathologist observes that the cell nuclei on all of the slides are red. The physician has informed the pathologist that all of the slides have been stained with Feulgen stain. Why are the nuclei red?

   Two sections of tissue treated with osmium tetroxide appear to contain large dark bodies within the cells. The slides were not labeled to indicate the organ from which the tissue was taken. The pathologist's apprentice wants to label them as liver. Is this label likely to be correct? Why or why not?

4. What are plastids? How many types of plastids did you observe during this laboratory period? What is the function of each type?

5. In *Elodea,* the chloroplasts appear to move within the cytoplasm. When you observed this movement, did they all move in the same direction? What cellular structures guide this movement?

6. Why are central vacuoles important to plant cells? For growth? Waste disposal? Storage? For improving the surface-to-volume ratio?

7. Describe the structure of a plant cell wall. Distinguish between the primary cell wall, the secondary cell wall, and the middle lamella.

8. What is the cytoskeleton of a cell? What are its parts? Can it be observed using the light microscope? Why or why not?

9. What is the largest cell that you observed during this laboratory period? ______________

   The smallest? ______________ Why are cells (even fairly "large" ones) usually small?

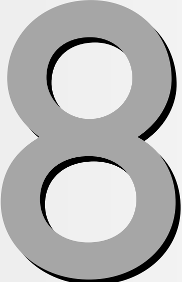
10. To study subcellular organization it may be necessary to homogenize the cells. What is the purpose of homogenization?

11. What physical properties of cell organelles determine their behavior during differential centrifugation? Why are different speeds of centrifugation used? Rank the cell parts that you have studied by their size (smallest to largest).

12. Organelles are often studied by separating them on a *sucrose gradient*. A centrifuge tube is filled with a sucrose solution of increasing concentration from the top of the tube to the bottom (5% at the top to 20% at the bottom). What would you expect to observe if you put a layer of cell homogenate on top of the sucrose and then subjected the sample to high-speed centrifugation?

13. Both 2,6-dichlorophenol-indophenol (DPIP) and tetrazolium are used as colorimetric indicators of metabolic functions in cells. For what functions were they used as tests in this laboratory? What do DPIP and tetrazolium have in common in their mechanisms of action? What is reduction? How can you tell that DPIP and tetrazolium have been reduced? When does this reduction take place during (a) photosynthesis and (b) cellular respiration?

LABORATORY



Osmosis and Diffusion

OVERVIEW

Cells consist of highly complex organizations of molecules, whose behavior can be explained by the laws of physics and chemistry. One of these laws, the **law of diffusion,** is of particular importance to our understanding of the movement of molecules into and out of cells. The law of diffusion states that molecules tend to move from areas of higher chemical potential to areas of lower chemical potential.

Chemical potential (μ) is a measure of free energy available to do the work of moving a mole of molecules from one location to another and, in some cases, through a barrier such as a cell membrane. In a solution, the greater the concentration of molecules of a dissolved substance (**solute**), the higher is the chemical potential (free energy per mole) of that substance. Thus, we can also say that molecules tend to move from areas of higher concentration (higher chemical potential) to areas of lower concentration (lower chemical potential) (Figure 8-Ia).

Osmosis, a special case of diffusion with special relevance for cells, is the movement of water molecules from regions of higher water potential to regions of lower water potential across a semipermeable or selectively permeable membrane. **Water potential (ψ)** is a measure of the chemical potential, or free energy per mole, of water molecules. Water potential is affected by the amount of other substances (solutes) dissolved in the

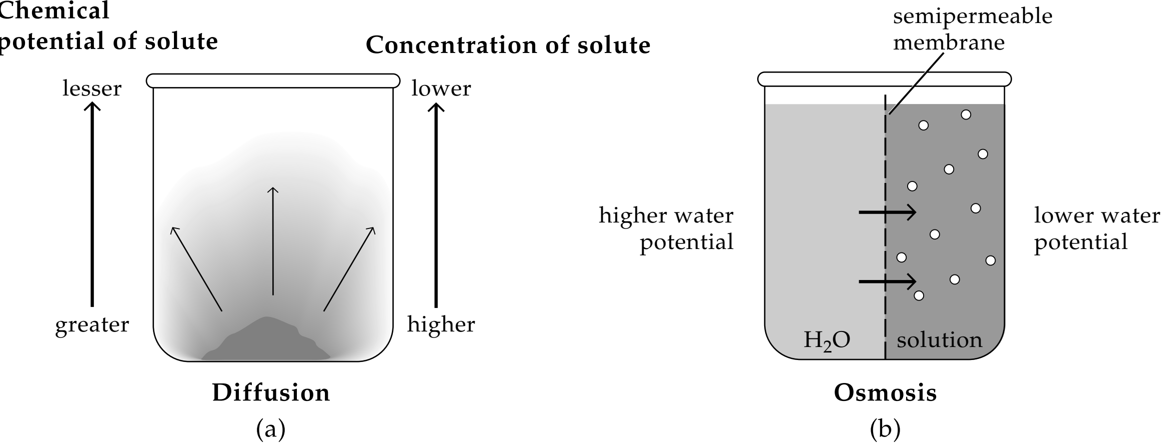


Figure 8-I *Diffusion and osmosis.*

water. The addition of solute to water lowers its water potential (the chemical potential of water molecules in the solution). Pure water, not under pressure (that is, at atmospheric pressure), has a water potential of zero, but as solute is added to pure water, the water potential becomes negative. The more solute is dissolved in water, the lower (more negative) is the water potential of the solution.

If pure water is separated from a solution by a semipermeable membrane, water molecules will move across the membrane from an area of higher water potential to an area of lower water potential. In other words, water tends to move across membranes toward areas with a higher concentration of solutes, where the water potential is lower (Figure 8-Ib).

Both diffusion and osmosis are examples of **passive transport** because molecules, whether water or solute, move "down" concentration or free-energy gradients. To move molecules *against* a gradient requires an energy input: this type of transport is called **active transport.**

During this laboratory period, you will examine the principles of solute and water movement in both artificial and living systems.

STUDENT PREPARATION

Prepare for this laboratory by reading the pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

✔ EXERCISE A | Brownian Movement

In order to understand how substances pass through a membrane, it is important to realize that molecules, when at temperatures above absolute zero, are in constant motion. Molecular motion is a form of energy: the translational, vibrational, and rotational kinetic energies of molecules. Although individual molecules are impossible to see, their existence is revealed by the jiggling—called **Brownian movement**—of minute particles suspended in water.

Objectives

- ☐ Define Brownian movement.
- ☐ Describe the effects of increased temperature on the movement of water molecules.

Procedure

Place a drop of powdered carmine suspension (carmine is not soluble in water) on a slide and cover with a coverslip. Examine the slide at high power (40×).

a. Do the carmine particles move randomly or in a definite path? ________

b. Can you see the water molecules? ________ *Are the water molecules moving?* ________

c. Is the movement of a carmine particle due to the movement of its own molecules or to bombardment by water molecules? ______________________________ *Explain.* ____

____

d. Would an increase in temperature increase or decrease the rate of Brownian movement? _______________

Why? ____

EXERCISE B Diffusion

The molecules in solids, liquids, and gases are in constant motion. In liquids and gases, the molecules are free to move and tend to migrate from regions of higher chemical potential (μ) and higher concentration to regions of lower chemical potential and lower concentration. They will move until they are uniformly distributed throughout the medium.

How fast does diffusion occur? The rate is dependent on the particle size and on the difference in chemical potentials of the molecules in the two regions: the larger the difference, the faster the rate of diffusion.

Objectives

- ☐ Define diffusion.
- ☐ Describe how diffusion of a gas in a gas or of a liquid in a liquid can be observed.
- ☐ Describe the effect of the molecular weight of a substance on its rate of diffusion.

PART 1 Diffusion of a Gas in a Gas

Procedure

We can study the process of diffusion of a gas in a gas by observing the movement of ammonium hydroxide vapors in air.

1. Wet a 4- to 6-inch strip of filter or chromatography paper with a solution of phenolphthalein (phenolphthalein is an indicator of pH changes).
2. Add 10 to 20 ml of ammonium hydroxide solution to a 250-ml graduated cylinder. As you pour, avoid wetting the sides of the container.
3. Suspend the filter paper from the hook in the bottom of the cork provided by your instructor. Immediately lower the phenolphthalein-soaked strip into the vessel and secure the cork. The bottom of the strip should *not* touch the ammonium hydroxide.

   a. *Describe the changes that take place in the filter paper over the next several minutes.*

   ____

   b. *Does the filter paper change color all at once?* ________

   c. *Explain the results of your experiment by relating the chemical potential of the ammonium hydroxide molecules to the process of diffusion.* ______________________________

   ____

PART 2 Diffusion of a Liquid in a Liquid

We can make some observations about the rate of diffusion and changes in concentration of a diffusing substance by observing the diffusion of a colored liquid in water. The rate of diffusion is determined by the magnitude of difference between the concentrations of a diffusing substance in two different locations, the area of origin and the area of destination.

Procedure

1. Place a clear plastic Petri dish on a white piece of paper. Center a thin plastic ruler (white or clear) under the bottom of the Petri dish.
2. Pour 30 ml of distilled water into the Petri dish. Allow it to stand undisturbed for a few minutes to minimize water convection currents.

3. Gently add one drop of blue food coloring or India ink to the center of the dish. Cover the Petri dish with its lid to prevent disturbance from air currents.
4. Find the rate of diffusion of the dye or ink into the water by measuring the diameter (D) in millimeters of the pigmented spot at 1-minute intervals for 15 minutes. Record your results in Table 8B-1.

Table 8B-1 Diffusion of a Liquid in a Liquid

Minute	Diameter (D)	Radius ($D/2$)
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		

5. Graph the radius ($D/2$) of the pigmented spot against time, in 2-minute intervals (use Figure 8B-1).

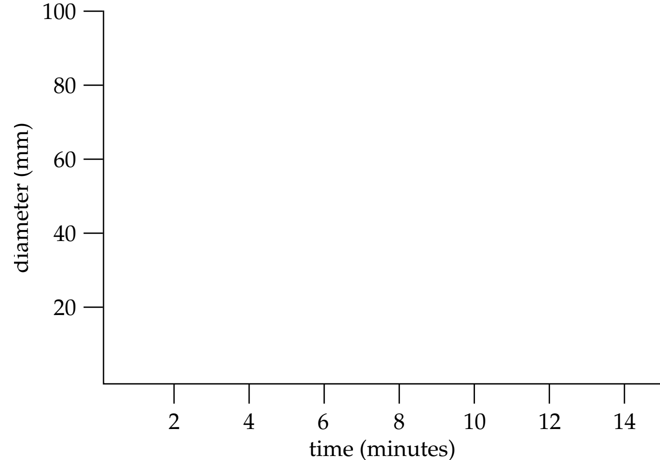


Figure 8B-I *Graph the radius of the pigmented spot against time.*

6. Choose three 2-minute intervals and calculate the rate of diffusion from the data in your graph. Use the following equation and record your results in Table 8B-2.

$$\text{Rate of diffusion} = \frac{(\text{radius at start of 2-min interval}) - (\text{radius at end of 2-min interval})}{2 \text{ min}}$$

$$= ______ \text{ mm/min}$$

Table 8B-2 Rate of Diffusion

Time Interval	Rate of Diffusion (mm/min)
Minute ______ to ______	____________
Minute ______ to ______	____________
Minute ______ to ______	____________

In diffusion, the random movements of individual molecules produce a net movement from an area of greater concentration to an area of lesser concentration. Eventually, both types of molecules (in this case, water molecules and pigment molecules) will be evenly distributed.

a. *Does the net movement of molecules slow down as equilibrium is reached?* ______ *Why?*

____

b. *What can you say about the chemical potential of the pigment molecules at different distances from the center of the spreading pigment spot?* ____

c. *How is the chemical potential of the pigment molecules related to their rate of movement?*

____

d. *Does net diffusion eventually come to an end?* ______ *Why?* ______________________________

____

EXTENDING YOUR INVESTIGATION: DOES TEMPERATURE AFFECT THE RATE OF DIFFUSION?

Would using hot water or cold water (refrigerated overnight) in the experiment in Exercise B, Part 2, have an effect on diffusion rates? What do you think would happen? Formulate a hypothesis.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will happen during your experiment?

What is the **independent variable** in this investigation?

What is the **dependent variable** in its investigation?

PROCEDURE: Repeat the experimental procedure in Exercise B, Part 2, using hot water and cold water (refrigerated overnight).

RESULTS: Record your data in the tables below.

Diffusion of a Liquid in Hot Water

Minute	Diameter (*D*)	Radius (*D*/2)
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		

Diffusion of a Liquid in Cold Water

Minute	Diameter (*D*)	Radius (*D*/2)
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		

What did you observe during your experiment?

Determine the rates of diffusion of the colored liquid in both hot and cold water and record them below.

Time Interval	Rate (mm/min) HOT	Rate (mm/min) COLD
Minute ______ to ______	______	______
Minute ______ to ______	______	______
Minute ______ to ______	______	______

Do your results support your hypothesis?

Your null hypothesis?

Was your prediction correct?

What do you **conclude** about the effects of temperature on the rate of diffusion?

PART 3 Effect of Molecular Weight on the Rate of Diffusion

In this experiment, two different gases are used. Ammonia (NH_3) and hydrogen chloride (HCl) react chemically to form a white salt, ammonium chloride (NH_4Cl):

$$NH_3 + HC \longrightarrow NH_4Cl$$

The distance that substances (in this case, two gases) travel per unit of time (the rate of diffusion) is related to the molecular weights of the diffusing substances:

Procedure

1. Your instructor will soak a cotton plug with HCl and another with NH_4OH. Both plugs will be inserted simultaneously into opposite ends of a large glass tube and the ends will be capped with rubber stoppers (Figure 8B-2). *Note the time of insertion*. Time ______

Ammonium hydroxide (NH_4OH) dissociates into ammonia (NH_3, a gas) and water. Ammonia diffuses toward the opposite end of the tube. At the same time, hydrochloric acid vapors (HCl) diffuse toward the ammonia. The overall reaction is

$$NH_4OH + HCl \longrightarrow NH_4Cl + H_2O$$

2. When the two gases meet, they react to form a white ring (ammonium chloride) around the glass tubing. Record the time at which the white ring is first seen. Time ______

The diffusion rate (r) of a molecule is inversely related to the square root of its molecular weight (MW),

$$r = \frac{1}{\sqrt{MW}}$$

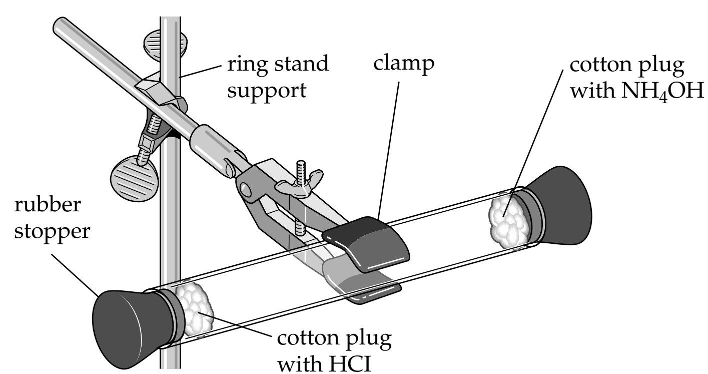


Figure 8B-2 *Experimental apparatus for determining the effect of molecular weight on the rate of diffusion.* ***Caution:*** *Both HCl and NH_4OH are poisonous. They can cause burns and can be fatal if swallowed. Carry out this experiment in a fume hood.*

and the ratio of the distances traveled by NH_3 (d_1) and HCl (d_2) should be close to the ratio of the diffusion rates:

$$\frac{r_1 = 1/\sqrt{MW_1}}{r_2 = 1/\sqrt{MW_2}} = \frac{r_1}{r_2} \propto \frac{d_1}{d_2}$$

The molecular weight of ammonia gas (NH_3) is 17; the molecular weight of hydrogen chloride gas (HCl) is 36.

3. Measure the distance to the white ring from the front edge of each of the cotton plugs.

- Distance of NH_4Cl precipitate from NH_4OH plug, d_1 = _______ mm
- Distance of NH_4Cl precipitate from HCl plug, d_2 = _______ mm
- Ratio d_1/d_2 = _______

4. Now calculate the ratio of diffusion rates from the molecular weights.

- Diffusion rate for NH_3, $r_1 = \frac{1}{\sqrt{MW_1}}$ = _______
- Diffusion rate for HCl, $r_2 = \frac{1}{\sqrt{MW_2}}$ = _______
- Ratio r_1/r_2 = _______

a. Is the ratio of the distances approximately equal to the ratio of the rates? _______ *Why?*

____

b. Was the white precipitate in the center of the tube? _______ *Why or why not?*

____

c. Which gas traveled faster? _______________________

Why was the white ring of NH_4Cl not seen immediately after the plugs were inserted?

____

d. What is the relationship between molecular weight and the rate of diffusion of a gas?

____

EXERCISE C | Diffusion Across a Selectively Permeable Membrane

The cell membrane is a **selectively permeable** membrane. Small hydrophobic solute molecules, water, and other very small polar, uncharged molecules can move freely through the cell membrane. But larger molecules, and even small charged ions, may pass more slowly or sometimes not at all. All cell membranes are dynamic systems that can alter their lipid and protein contents to change their pore size. By regulating membrane permeability in this way, cells allow specific molecules or ions to move into or out of the cell as needed.

Solutes that diffuse through a selectively permeable membrane always move from the solution that contains more of the solute to the solution containing less solute (Figure 8C-1). The solutes diffuse from an area of higher chemical potential to an area of lower chemical potential. If the two solutions contain equal concentrations of a solute, the chemical potentials are equal and no net movement of particles occurs.

In the laboratory, molecules of different sizes can be separated by **dialysis** using artificial membranes made into tube-shaped bags (dialysis bags). These membranes are **semipermeable.** (Since the membranes are nonliving, the pore sizes cannot be changed to "select" molecules of differing sizes.) The size of the pores in the dialysis tubing determines which substances can pass through. Molecules larger than the pore size remain inside or outside the bag, while smaller molecules and ions diffuse through the membrane. In

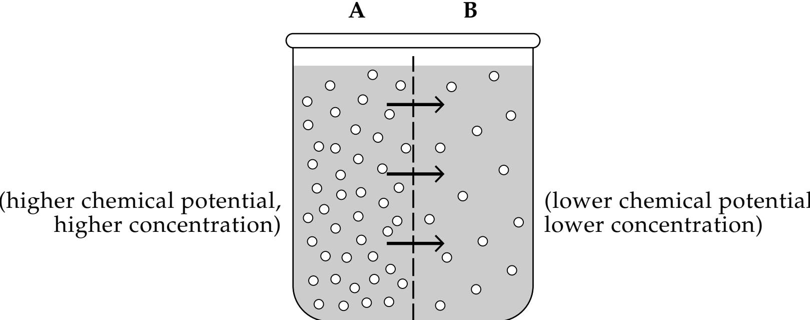


Figure 8C-1 *When two solutions are separated by a selectively permeable membrane, solute will move from an area of higher solute concentration and higher chemical potential to an area of lower solute concentration and lower chemical potential.*

all cases, molecules move from an area of higher concentration or higher chemical potential to an area of lower concentration or lower chemical potential.

In this experiment, solutes dissolved in water are separated by a semipermeable membrane. A solution of starch and glucose is placed inside a dialysis bag, and I_2KI (Lugol's solution) is added to the water outside the bag. Molecules can move both ways across the dialysis bag membrane *if* they are small enough.

a. Which molecule(s) do you think will move through the dialysis bag? ______________________________

Why? ____

b. Which molecule is least likely to move through the dialysis bag? ______________________________

Why? ____

Formulate a hypothesis that offers a tentative explanation for how molecular size might be related to the movement of glucose, starch, and I_2KI molecules in this experiment.

HYPOTHESIS:

NULL HYPOTHESIS:

Which way do you **predict** that the solute molecules used in this experiment might flow?

What is the **independent variable** in this investigation?

What is the **dependent variable** in this investigation?

Use the following procedure to test your hypothesis.

Procedure

1. Work in pairs. Obtain a piece of dialysis tubing and tie a knot in one end of it.
2. Add 4 Pasteur pipettefuls of 15% glucose solution to the bag, then use a different Pasteur pipette to add 4 pipettefuls of 1% starch solution to the glucose in the bag.
3. Hold the bag closed and mix its contents. Record its color in Table 8C-1. Carefully rinse off the outside of the bag in tap water.
4. Fill a beaker two-thirds full with water. Add about 4 dropperfuls of Lugol's reagent (I_2KI) to the water in the beaker. In Table 8C-1, record the color of the solution in the beaker.

Figure 8C-2 *Setup for dialysis experiment.*

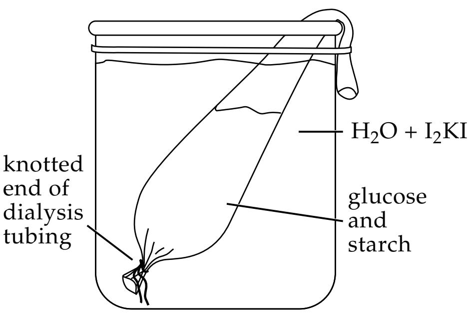


Table 8C-1 Data for Dialysis Experiment

	Original Contents	Original Color	Final Color	Color After Benedict's Test
Bag	Glucose and starch			
Beaker	$H_2O + I_2KI$			

5. Place the bag in the beaker so that the untied end of the bag hangs over the edge of the beaker. Do *not* allow the liquid to spill over the top of the bag. If the bag is too full, remove some of the liquid and rinse the bag again. Place a rubber band around the beaker so that the top of the bag is held securely in place (Figure 8C-2).
6. Allow the setup to stand until you see a distinct color change in the bag or in the beaker. (You may wish to go on to another exercise and then return to check your setup after 30 minutes or more.) In Table 8C-1, record the final color of the solution in the bag and the solution in the beaker.
7. After you have observed the color change, stir the contents of the beaker, remove 3 ml of the solution from the beaker, and test with Benedict's reagent according to the directions in Laboratory 5. (You may use TesTape if available.) Record your results in Table 8C-1.
8. Now use Benedict's reagent (or TesTape) to test 3 ml of the solution from the bag. Record your results in Table 8C-1.

c. I_2KI reagent is used to test for the presence of which type of molecule? ______________

What color change occurs in the presence of this molecule? ______________

d. TesTape or Benedict's reagent is used to test for the presence of which type of molecule? ______________

What color change occurs in the presence of this molecule? ______________

e. Which substance or substances entered the bag? Which one(s) left the bag? Use Table 8C-2 to give evidence for your answer in terms of the color changes that occurred.

Do your results support your hypothesis? _______ *Your null hypothesis?* _______

What do you ***conclude*** *about the effects of molecular size on the diffusion of molecules across a semipermeable membrane?* ______________________________

Plasma membranes remain selectively permeable as long as they are alive and normal. A membrane may become temporarily more permeable if the cell is in a state of shock; however, upon cell death, the membrane becomes completely permeable. Anesthetics, certain toxins, and low and high temperatures change the permeability of cell membranes.

Table 8C-2 Evidence: Substances Leaving and Entering

	Outside	Inside
I_2KI		
Starch		
Glucose		

9. Examine the boiled red cabbage and boiled beet cubes on demonstration. A pigment located in the cell vacuoles of these plants is responsible for their color.

f. Where do you find the red pigment after boiling? ______________________________

g. What does boiling do to the structure of a membrane? ______________________________

h. How has this affected the differential permeability of the vacuolar membrane?

____

EXERCISE D A Look at Osmosis

The movement of water molecules across a semipermeable or selectively permeable membrane is a special case of diffusion known as **osmosis.** Water molecules move from an area where the chemical potential is higher to an area where the chemical potential is lower. Recall that the chemical potential of water molecules is called water potential. Thus, as a rule, water molecules will always diffuse from an area of higher water potential to an area of lower water potential.

Water potential (ψ) results from the combined actions of **osmotic potential** (ψ_π), which is dependent on solute concentration in a solution, and **pressure potential** (ψ_p), which results from the exertion of positive pressure or negative pressure (tension) on a solution. We can express this relationship as

$$\underset{\text{water potential}}{\psi} = \underset{\text{osmotic potential}}{\psi_\pi} + \underset{\text{pressure potential}}{\psi_p}$$

In general, added positive pressure causes the water potential of a solution to become more positive (higher). Addition of solute causes the water potential of a solution to become more negative (lower) if

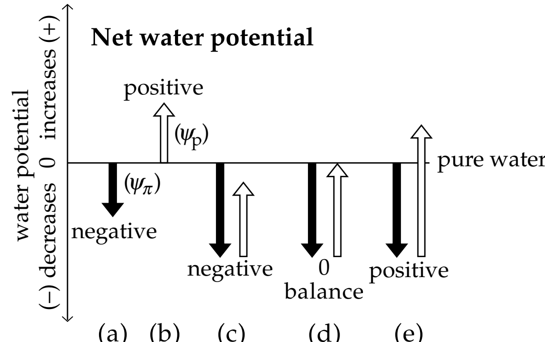


Figure 8D-1 *Pure water has a water potential (ψ) of 0 (if the water is not under pressure). The addition of solute (indicated by the solid black arrow) lowers water potential. In (a), solute has been added to pure water and water potential declines. Pressure (indicated by the open arrow) increases water potential. In (b), pressure has been applied to pure water. As you can see in (c) through (e), the net water potential (ψ) depends on the effects of both solute concentration (ψ_π) and pressure (ψ_p).*

there is no pressure to offset the effects of adding solute (Figure 8D-1). As a result, water potential (ψ) can be negative as in (c) and zero as in (d) or positive as in (e) (Figure 8D-1).

a. The water potential of solutions not under positive pressure is always ______________.

b. Can the water potential of a solution ever be positive? _______ *How?* ____________________________________

c. How would negative pressure (tension) affect water potential? ____________________________

Differences in water potential result in a tendency of water to leave one area in favor of another. The addition of solute to water *lowers* the **osmotic potential** of a solution (makes ψ_π more negative) and, therefore, lowers the water potential of that solution. Thus, in the absence of other factors (such as pressure; that is, $\psi_p = 0$), osmosis results in the net movement of water across a semipermeable membrane from an area of lower solute concentration (higher water potential) to an area of higher solute concentration (lower water potential).

Note: If two solutions, separated by a semipermeable membrane, have identical osmotic potentials, they are **isotonic** (they contain the same amounts of non-penetrating solutes, even if the chemical composition of the solutes is different). If they are not isotonic, then the solution with the greater concentration of solute (and more negative osmotic potential) is **hypertonic** to the other solution. A solution that is **hypotonic** has fewer solute particles and a less negative osmotic potential than the solution to which it is compared. During osmosis, water will move from a hypotonic solution into a hypertonic solution.

Sucrose is a disaccharide sugar. Suppose pure water is separated from a sucrose solution by a semipermeable membrane that is not permeable to the sugar; sugar is a non-penetrating solute (Figure 8D-2).

Which way will water molecules move? ____________________________

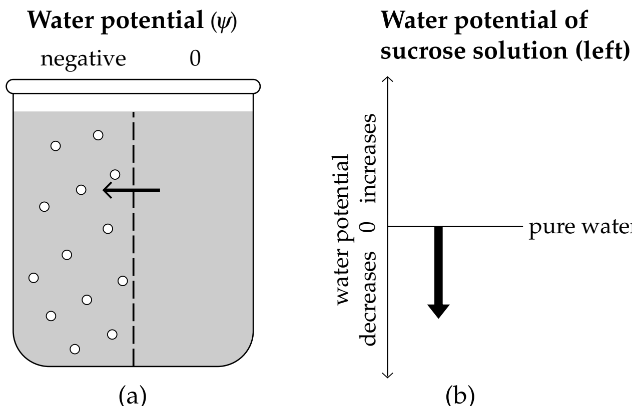
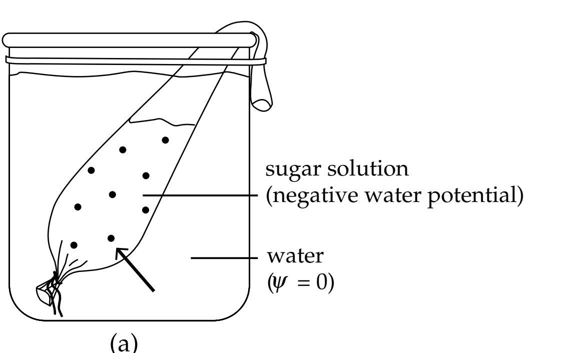


Figure 8D-2 *When non-penetrating solute, in this case sucrose, is added to pure water (left-hand side of beaker), water potential declines from zero to a negative value. Water always moves from an area of higher water potential to an area of lower water potential.*

Now, suppose you put the sucrose solution into a dialysis bag, permeable only to water, tie both ends of the bag, and place the bag in a beaker of pure water (Figure 8D-3).

d. Where is the water potential higher—inside or outside the bag? ____________________________ *Why?*



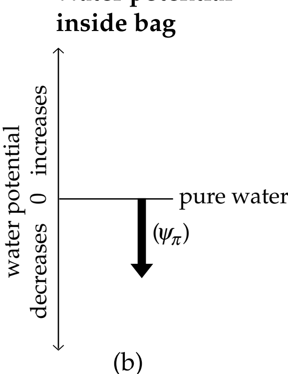


Figure 8D-3 *If a sucrose solution is placed in a dialysis bag and the bag is submerged in pure water, osmosis will move water from an area of higher water potential (pure water, $\psi = 0$) to an area of lower water potential (sucrose solution with a negative ψ).*

If solute is added to the water outside the bag, water will continue to flow from the beaker into the bag as long as ψ of the solution in the bag is more negative (lower) than ψ of the solution in the beaker (Figure 8D-3). The **rate** of diffusion depends on the size of the difference between the two water potentials (the larger the difference, the faster the flow).

Don't forget that **osmotic potential** (ψ_π) is a measure of the tendency for water to move into a solution. The more solute is added, the greater the osmotic potential (ψ_π) becomes (a *larger* negative value) and this large negative value lowers the overall water potential. Look at the size of the solid arrows (ψ_π) in Figure 8D-4. The arrow is smaller (a *smaller* negative value) for the solution in the beaker than for the solution in the bag (a *greater* negative value). You might think of osmotic potential as the "potential" for one solution (the one with the greater negative value of ψ_π) to "suck" water in from the other solution.

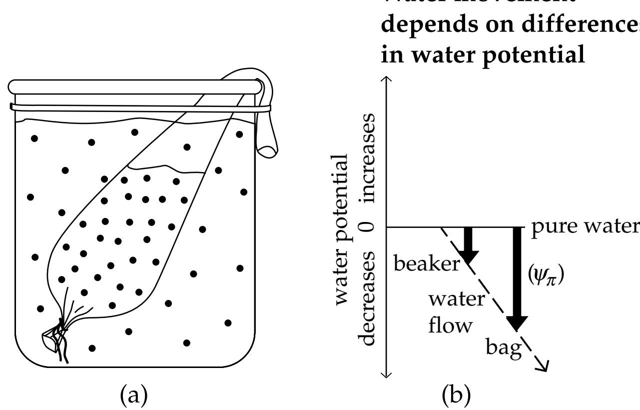


Figure 8D-4 *The more solute in a solution, the greater its osmotic potential (ψ_π) and the lower its water potential. Water tends to move toward regions of more negative water potential, so water moves from the beaker into the bag (dashed line).*

e. In Figure 8D-4, where is osmotic potential greater—inside or outside the bag? ____________________ *Why?*

f. Which way will water move? __________________________ *Why?* ___________________________________

If water potentials on the two sides of a selectively permeable membrane (such as a dialysis bag or cell membrane) eventually become equal, the *net* water flow will stop. The solutions will be at equilibrium. Individual water molecules, however, will continue to move back and forth across the membrane while the solution is at equilibrium (water movement does not stop; just *net* water movement ceases).

But, how can water potentials become equal if the bag always contains sucrose and the liquid outside the bag is pure? As water moves into the dialysis bag, the bag will swell. The same thing would happen to a cell (which contains solute in its cytoplasm or, in plant cells, in its vacuole). But, if a barrier (such as the wall of a tied dialysis bag or a cell wall) prevents indefinite expansion, then pressure will build up inside the compartment (bag or cell) into which the water is moving. An increase in positive pressure raises the pressure potential (ψ_p) and, because of this, the water potential inside the compartment becomes more positive (less negative). The pressure will "push" on the walls of the bag. The pressure that builds up (due to osmosis) is hydrostatic pressure, and it will build up until it equals the **osmotic pressure** (the amount of pressure necessary to stop water movement into the cell). See Figure 8D-5. When osmotic pressure is exerted against the walls of a cell, it is called **turgor pressure.** Turgor pressure is what makes the celery in your salad firm and keeps green plants standing up straight. Loss of turgor results in wilting.

As pressure builds inside a dialysis bag (or cell), the water potential gradient (difference) between the solution outside the bag and the solution inside the bag decreases. Water continues to flow into the bag until enough pressure builds up within the bag to offset the tendency for water to be drawn inward by the solute. Net water movement into the bag stops. At this point, the water potential inside the bag is equal to the water potential outside the bag (Figure 8D-5).

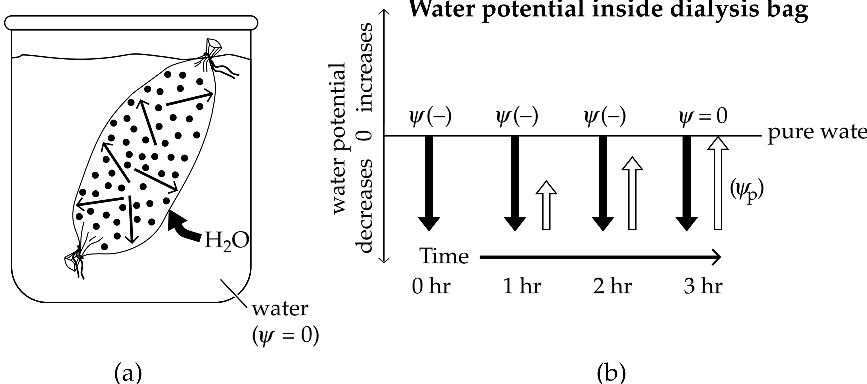


Figure 8D-5 *(a) As water moves into a dialysis bag containing a sucrose solution (assume the bag is not permeable to sucrose), pressure builds up over time (b) until the positive pressure exerted on the walls of the bag (ψ_p) balances the inflow of water.*

Objectives

- ☐ Define osmotic potential and osmotic pressure.
- ☐ Describe how to measure osmotic potential.
- ☐ Define turgor.
- ☐ Describe what causes plasmolysis in living plants.
- ☐ Relate osmotic potential to solute concentration and water potential.

PART I Measuring Osmotic Potential

In this experiment, you will investigate the relationship between osmotic potential and the movement of water through a selectively permeable membrane by the process of osmosis. You will place several dialysis bags containing solutions of different sucrose concentrations into beakers containing distilled water. The direction of water movement can be determined by finding the mass of (weighing) the bags before and after placing them in distilled water: the dialysis bags are permeable to water, but not to sucrose, and can gain or lose water.

At atmospheric pressure, the water potential of the pure water in the beakers can be assumed to be zero ($\psi = 0$); no solute is present, thus osmotic potential is zero ($\psi_\pi = 0$) and pressure potential is also zero ($\psi_p = 0$).

The water potential of the sucrose solutions in the dialysis bags will be negative (recall that in the absence of positive pressure, the addition of solute to pure water decreases osmotic potential), thus the water potential of the solutions inside the bags will be lower (ψ is negative) than the water potential of the water outside the bags, where ψ is zero. Can you predict which way water will move?

Procedure

1. Obtain six 25-cm strips of presoaked dialysis tubing and keep them in fresh distilled water.
2. Tie a knot at one end of each piece of dialysis tubing to form six bags. Use a pipette to put 15 ml of each of the solutions listed in Table 8D-1 into separate bags. After adding the solution, remove most of the air from the bag by drawing the unfilled portion between two fingers. Tie a knot near the open end to seal the solution within the tube. You should have 1.5 to 2 times as much empty space in the tube as that taken up by the volume of the solution. This will leave enough unfilled space within the tube to accommodate the possible accumulation of water. Be sure to keep track of which tube contains which solution.
3. Carefully blot the outside of each bag. Determine the initial mass of each bag and record it in Table 8D-1.
4. Fill six 250-ml beakers (or plastic cups) three-quarters full with distilled water.

Table 8D-1 Data for Measuring Osmotic Potential

Contents of Dialysis Tube	Initial Mass	Final Mass	Percent Change in Mass
Distilled water			
0.2 M sucrose			
0.4 M sucrose			
0.6 M sucrose			
0.8 M sucrose			
1.0 M sucrose			

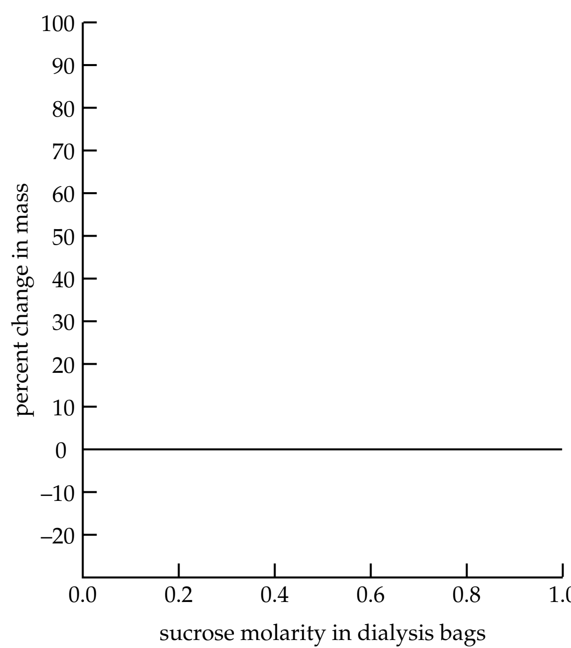


Figure 8D-6 *Percent change in mass of dialysis bags containing different molarities of sucrose.*

5. Place each bag in one of the beakers of distilled water and label the container to indicate the molarity of the solution in the dialysis bag. Make sure that all parts of the bag are completely covered by water.
6. Let stand for 1 hour.
7. At the end of the required time, remove the bags from the water and carefully blot them. Determine the mass of each bag and record your data in Table 8D-1.
8. Calculate the percent change in mass for each bag.

$$\% \text{ Change} = \frac{\text{final mass} - \text{initial mass}}{\text{initial mass}} \times 100$$

Graph your data in Figure 8D-6.

a. What is the relationship between the increase in mass and the molarity of sucrose in the dialysis bags? ______________________________

____

b. *How does the increase in sucrose concentration affect the water potentials of the various solutions inside the dialysis bags?* ______

c. *Does it affect the water potential of the solution outside the bags?* ______

d. *A solution (A) that contains more non-penetrating solute than another solution (B) is often said to be hypertonic (hyper- = more than; solution A is hypertonic to solution B). Likewise, solution B can be described as being hypotonic (hypo- = less than) to solution A. Use the terms* hypertonic *and* hypotonic *to describe the difference in water potential between solutions A and B.* ______

______

e. *If two solutions are* isotonic *to one another, how do their solute concentrations compare?*

______

f. *Predict what would happen in your experiment if all the bags were placed in a 0.4-M sucrose solution instead of in distilled water.* ______

______

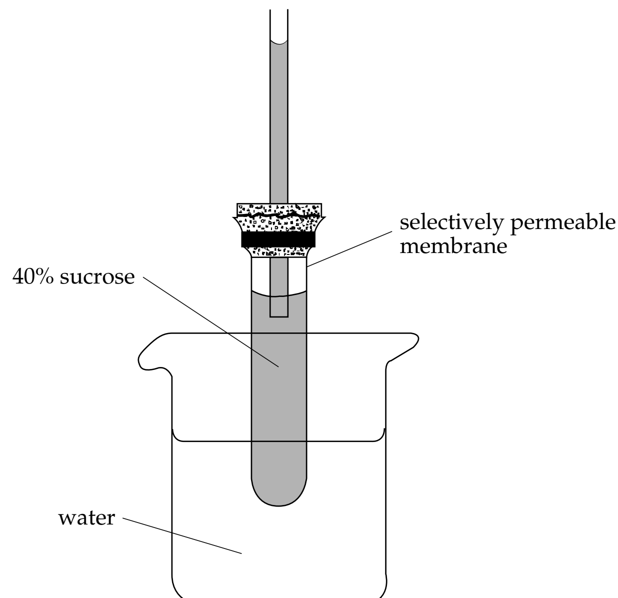
g. *What would a graph of percent change in mass look like for this experiment? Draw it on Figure 8D-6.*

PART 2 Measuring Pressure Potential: The Osmometer

The osmometer provides a way to demonstrate how changes in pressure potential can affect the water potential of a solution. In the osmometer on demonstration (Figure 8D-7), a dialysis bag containing a 40% sucrose solution has been suspended in a beaker of water. The bag is semipermeable—allowing passage of solvent (water) but no passage of solute (sucrose).

Water will move into the bag (where water potential is negative) and the sucrose solution will rise in the glass tubing of the osmometer until the water potential on both sides of the membrane is equal. At this point, the solution in the tube exerts enough positive hydrostatic pressure (pressure resulting from the movement of water or "osmotic pressure") on the contents of the dialysis bag to equal, or offset, the negative osmotic potential of the sucrose solution. The water potential of the solution in the bag will be zero ($\psi = 0$), like that of the water surrounding the bag, and *net* movement of water into the bag stops.

Figure 8D-7 *A simple osmometer.*



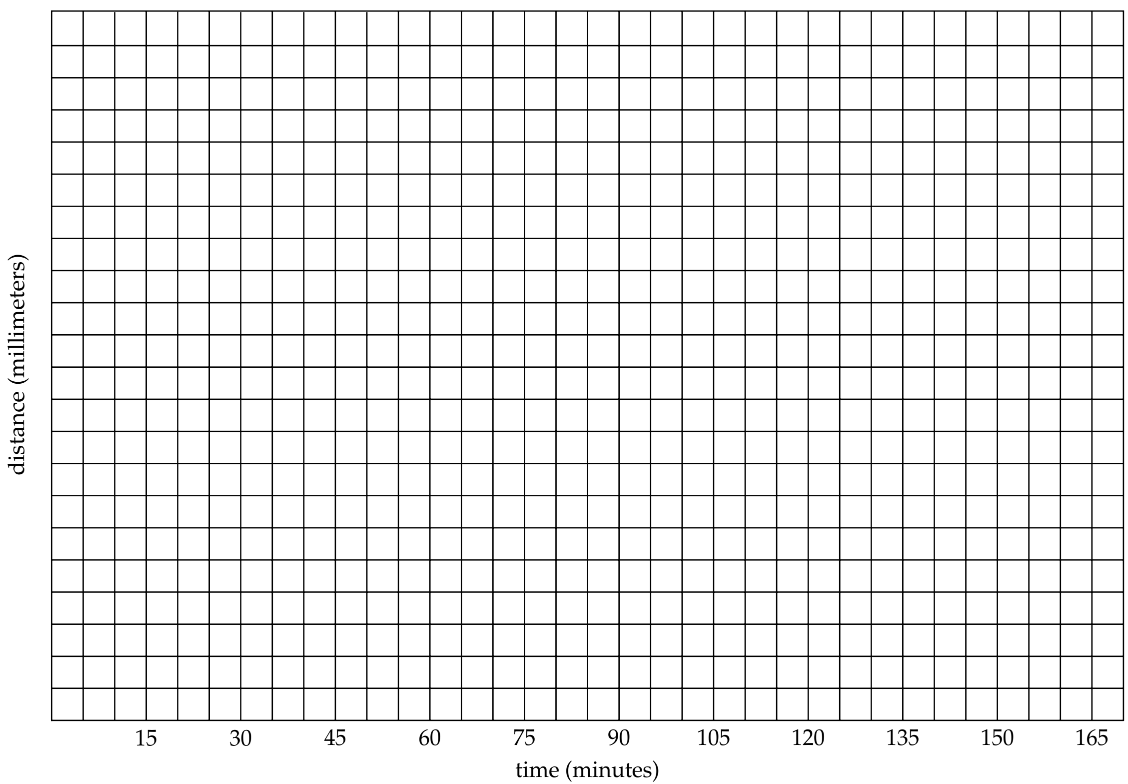


Figure 8D-8 *Graph osmotic pressure in millimeters of solution traveled up the osmometer tube.*

Procedure

1. At 15-minute intervals during the laboratory period, observe the changes in the osmometer on demonstration. On a separate sheet of paper, record the time and the height, in millimeters, of the solution in the tube.
2. Plot your data in Figure 8D-8.

   a. Why does water move into the bag? ______________________________

   ____

   b. In which solution (inside the bag or outside) is the water potential higher? ______________

   In which is solute concentration higher? ______________

   c. Which solution (inside the bag or outside) is hypertonic? ______________ *Hypotonic?*

   ____

   d. How is osmotic pressure (the tendency to resist further net water movement) exerted within the dialysis bag? ______________________________

   e. What does this pressure do to the water potential of the solution in the osmometer?

   ____

   f. What is the water potential inside the bag when the fluid stops rising in the glass tube?

   ____

How do you know? ____

____

g. Does osmosis stop after a certain period of time? (Be specific.) ____________________

PART 3 Measuring the Water Potential of Living Plant Cells

When a solution, such as that inside a potato cell, is separated from pure water by a selectively permeable membrane, water will move (by osmosis) from the surrounding area where water potential is higher (ψ = 0) into the cell where water potential is lower due to the presence of solutes (ψ is negative). (We will assume, for purposes of explanation, that solute is not diffusing.) The movement of water into the cell causes the cell to swell, and the cell membrane pushes against the cell wall to produce an increase in pressure (**turgor**).

Positive turgor pressure inside the cell continues to build up until the water potential of the cell equals the water potential of the pure water outside the cell ($\psi_{\text{cell}} = \psi_{\text{outside}} = 0$). At this point, a dynamic equilibrium exists and net water movement ceases (Figure 8D-9).

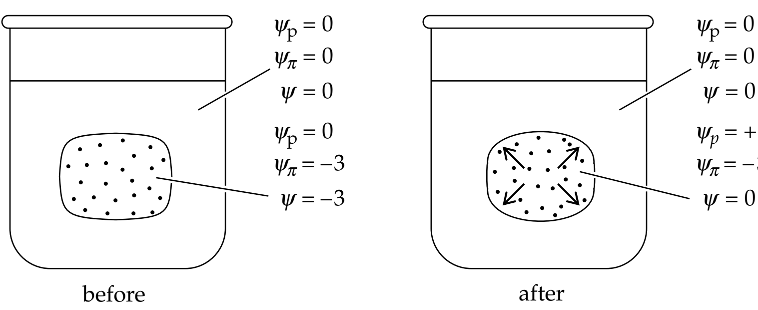


Figure 8D-9 *Water moves into a cell from an area of higher water potential outside the cell ($\psi = 0$) to an area of lower water potential inside the cell ($\psi = -3$). Net water movement ceases when enough pressure builds up in the cell so that the water potential inside the cell equals the water potential outside the cell ($\psi = 0$ for both). Recall that $\psi = \psi_\pi + \psi_p$.*

If solute is added to the water outside the potato cells, the water potential outside the cells is decreased, and, eventually, if enough non-penetrating solute is added to the water, the water potential outside the cell will end up being the same as the water potential inside the cell; then no net movement of water will occur. This does not mean, however, that the solute concentrations inside and outside the cell are equal, because water potential inside the cell results from a combination of both pressure potential (ψ_p) and osmotic potential (ψ_π) (Figure 8D-10).

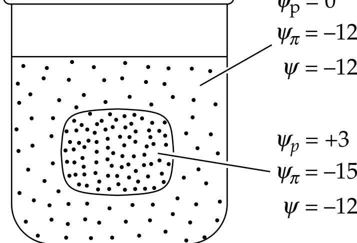


Figure 8D-10 *No net water movement occurs when the water potentials of the cell and its surroundings are equal ($\psi = -12$ for both), but ψ_π for the cell and solution are not the same. It is ψ_p inside the cell that makes it possible for $\psi_{cell} = \psi_{solution}$.*

In this experiment, you will calculate the water potential of potato-tissue cells soaked in different molarities of sucrose. The original turgor pressure of the cells cannot be measured, and thus the osmotic potential of the cells cannot be determined. However, you will be able to measure the water potential of the cells by determining at what molarity of sucrose net movement of water into the potato tissue stops.

Your instructor may ask you to compare different types of potatoes or even different types of vegetables. Does one type of potato or vegetable have a higher water potential than another? Does a potato stored in the refrigerator have a higher water potential than one stored on the shelf in the pantry? Consider the available materials and formulate a hypothesis.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** would occur in an experiment to test your hypothesis?

What is the **independent variable** in your investigation?

What is the **dependent variable** in your investigation?

Now use the following procedure to test your hypothesis.

Procedure

1. Work in pairs within groups of 4. Each pair of students in the group should obtain 100 ml of each of three sucrose solutions or distilled water, as listed in Table 8D-2. Pour each solution into a separate 250-ml beaker or plastic cup and label each container to indicate the molarity of sucrose it contains.
2. Each pair of students should use a cork borer (approximately 5-mm inner diameter) to cut 12 potato cylinders. Cut each cylinder to 3 cm in length. Do not include any skin on the cylinders. For the group's experiment, all cylinders should be cut from the same potato.
3. Keep your potato cylinders in a covered beaker or Petri dish until it is your turn to use the balance for finding the mass of the cylinders.
4. Quickly determine the mass of four cylinders together, and record it in the table below. Put all four cylinders into one of the three sucrose solutions.
5. Determine the masses of the other groups of four cylinders, and record them in the table. Put these cylinders into the other two solutions.
6. Cover all beakers to prevent evaporation.
7. Let the beakers stand for 2 hours or overnight, according to directions from your instructor.
8. Record the temperature of each solution after standing.
9. Remove the cores from one of the beakers, blot them gently by rolling lightly on a paper towel, and find their mass. Then do the same for the other groups of cylinders.
10. Record the final masses of your other two sets of cylinders.

Contents of Beaker	Initial Mass	Final Mass
1.		
2.		
3.		

11. Obtain the data for the remaining sucrose solutions from other members of your group. Record all data in Table 8D-2.

Table 8D-2 Group Data

Contents of Beaker	Initial Mass	Final Mass	Percent Change in Mass
Distilled water			
0.2 M sucrose			
0.4 M sucrose			
0.6 M sucrose			
0.8 M sucrose			
1.0 M sucrose			

12. Calculate percent change in mass as in Exercise D, Part 1, and graph your data in Figure 8D-11.

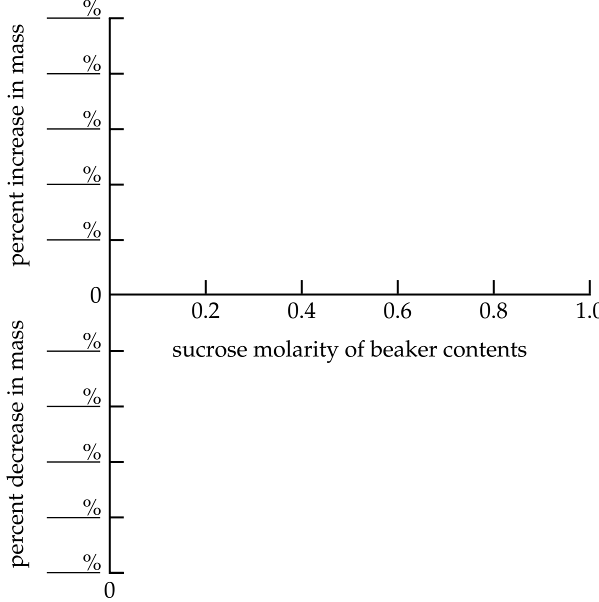


Figure 8D-11 *Percent change in weight of potato cores at different molarities of sucrose. Label the vertical axis in appropriate percent intervals.*

13. Determine the osmolarity of the sucrose solution in which the mass of the potato cores does not change. To do this, draw the straight line that best fits your data points (Figure 8D-11). The point at which this line crosses the *X*-axis represents the molar concentration of sucrose with a water potential equal to that of the potato tissue. At this concentration, there is no net gain or loss of water from the potato-tissue cells.

Molar concentration = ______________ *M*

14. The osmotic potential of a sucrose solution can be calculated from the molarity of the solution by using the formula

$\psi_\pi = -iCRT$

where

i = ionization constant (for sucrose, this is 1 because sucrose does not ionize in water; for NaCl, $i = 2$)

C = osmotic molar concentration

R = pressure constant (handbook value: $R = 0.0831$ liter bar/mol K)

T = temperature in degrees Kelvin (273 + °C of solution)

The units of measure will cancel as in the following example. For a 1.0-M sugar solution at 30°C under standard atmospheric conditions:

$$\psi_\pi = -1 \left(1.0 \frac{\cancel{\text{mol}}}{\cancel{\text{liter H}_2\text{O}}}\right) \left(0.0831 \frac{\cancel{\text{liter}}\ \text{bar}}{\cancel{\text{mol K}}}\right) (303\ \cancel{\text{K}}) = -25.18 \text{ bars*}$$

$$\psi_\pi = -i \quad \times \quad C \quad \times \quad R \quad \times \quad T$$

15. Knowing the osmotic potential of the sucrose solution (ψ_π) and knowing that the pressure potential of the solution is 0 ($\psi_p = 0$), since the solution is open to the atmosphere, allows you to calculate the water potential of the solution. It will be equal to the osmotic potential.

$$\psi = \psi_p + \psi_\pi$$
$$= 0 + \psi_\pi$$
$$= \psi_\pi$$

The water potential of the solution at equilibrium will be equal to the water potential of your potato cells. *a. Why?* ______________________________

16. Determine the water potential of your potato cells.

$\psi_{\text{potato cells}}$ = ____________

b. Is the osmotic potential of the potato cells the same as that of the sucrose solution when $\psi_{potato} = \psi_{solution}$? ______ *Why or why not?* ______________________________

____

Do your results support your hypothesis? ______ *Your null hypothesis?* ______

Was your prediction correct? ______

What do you ***conclude*** *about the water potential of your experimental material(s)?* ______________________________

____

c. If a potato is allowed to dehydrate by sitting in the open air, would the water potential of the potato cells become higher or lower? ______ *Why?* ______________________________

____

**A pressure of 1 bar is just slightly less than 1 atmosphere. [An atmosphere (atm) is the pressure exerted at sea level by an imaginary column of air—approximately 1 kg of pressure per cm^2.] Plant biologists usually measure water potential (ψ) in a unit of pressure called the megapascal (MPa): 1 atm = 0.1 MPa. We can also say that*

1 bar = 0.1 MPa

To express the above answer in MPa, simply move the decimal point one place to the left:

−25.18 bars = −2.518 MPa

EXTENDING YOUR INVESTIGATION: WATER POTENTIAL OF DIFFERENT TUBER TYPES

A study of various types of tubers was conducted to determine the percent carbohydrate and percent water content. The data are given below.*

Tuber	Percent Carbohydrate	Percent Water
Beets	9.6–9.9	87.6
Carrots	9.3	88.2
Parsnips	13.5–18.2	78.6
Sweet potato	27.9	68.5
Turnip	7.1–8.1	90.9
White potato	19.1	77.8

When students in an introductory biology class used some of these tubers for their water potential study (as in Exercise D, Part 3), they recorded the following results:

Sweet potato $\psi = -10.93$ bars

Turnip $\psi = -18.39$ bars

The students had hypothesized that sweet potatoes would have a lower water potential than turnips on the basis of carbohydrate and water content. Their hypothesis was *not* supported by their data. How might you explain this?

**Data from A. E. Leach and A. L. Winston,* Food Inspection and Analysis, *John Wiley & Sons, New York, 1920; and Morris J. Jacobs (ed.),* The Chemistry and Technology of Food and Food Products, *Interscience Publishers, New York, 1944.*

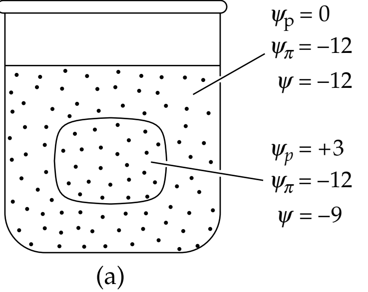
PART 4 Observing Osmosis in a Living System

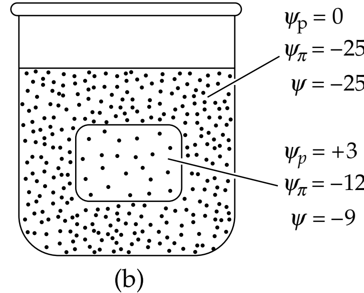
If a plant cell is immersed in a solution that has a higher solute concentration than that of the cell (Figure 8D-12), water will leave the cell, moving from an area of higher water potential to an area of lower water potential. The loss of water from the cell will cause the cell to lose turgor. Macroscopically, you can see the effects of loss of turgor in wilted house plants or limp lettuce. Microscopically, increased loss of water and loss of turgor become visible as a withdrawal of the protoplast from the cell wall (**plasmolysis**) and as a decrease in the size of the vacuole (Figure 8D-13).

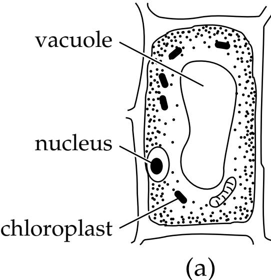
Procedure

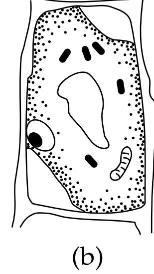
1. The various plant materials on display were placed into either pure water or a saltwater solution.

Figure 8D-12 *When a plant cell is placed in a solution of (a) equal solute concentration ($\psi_{\pi(cell)} = \psi_{\pi(surrounding)}$) or (b) greater solute concentration, water may leave the cell (why?), resulting in loss of turgor and, eventually, plasmolysis.*









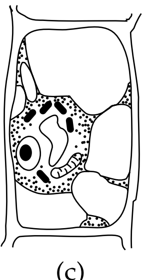


Figure 8D-13 *Plasmolysis in an epidermal cell of a leaf. (a) Under normal conditions, the plasma membrane is pressed against the cell walls. A large vacuole occupies the center of the cell, pushing the cytoplasm and nucleus to the periphery. (b) When the cell is placed in a solution with a higher concentration of solutes than that of the cell, water passes out of the cell, and the cell contents contract. (c) In an even more concentrated solution, the cell contents contract still further.*

a. *Compare the effects of water and salt solutions. What has happened?*

____

b. *Does cell turgor control the overall turgor of tissues?* ________ *The overall turgor of the plant part?* ________

c. *Can plant cells burst?* ________ *Explain.* ____

____

2. Obtain a filament of the green alga *Spirogyra* or a leaf from the tip of an *Elodea* plant. Place it in a drop of water on a slide, cover it with a coverslip, and examine the material first at low power (10×) and then at high power (40×). Locate a region of healthy cells and sketch the location of the chloroplasts in the left side of the space below.

3. While touching one corner of the coverslip with a torn piece of paper towel to draw off the water, add a drop of concentrated salt solution to the opposite corner of the coverslip. Be sure that the salt solution moves under the coverslip. Wait about 5 minutes, then examine as before. Record your observations in the space beside your drawing in step 2.

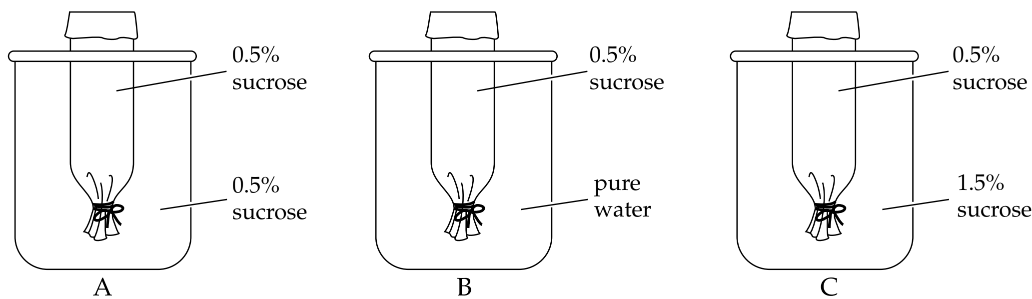
d. What happened when the water in which the cells were mounted was replaced by the salt solution?

____

e. Assuming that the cells have not been killed, what should happen if the salt solution were to be replaced by water? ______________________________

Laboratory Review Questions and Problems

1. Dialysis bags containing a 0.5% sucrose solution are placed in beakers containing the sucrose solutions indicated below. Dialysis bags are not permeable to sucrose.



Indicate the letter of the beaker in which the following events or situations occur.

a. The dialysis bag remains the same size. ______

b. The dialysis bag swells. ______

c. Water moves from the dialysis bag into the beaker. ______

d. The solution inside the bag is hypotonic to its surroundings. ______

e. Water potential inside the bag is higher than water potential outside the bag. ______

f. The solution inside the bag is isotonic to the solution outside the bag. ______

2. How does the method of salt or sugar curing (a process in which meat is packed in salt or sugar) help to preserve meat?

3. In some restaurants, sliced potatoes are soaked in water before they are fried. Can you provide an explanation for this practice?

4. Why is it important that solutions administered intravenously be isotonic to the recipient's blood? What would happen if an injected solution were hypertonic to your blood? What if it were hypotonic?

5. A gardener's favorite bush died several days after she applied twice the recommended amount of fertilizer. What probably happened?

6. A plant cell is placed in a beaker of pure water. What will happen to the cell?

7. An animal cell is placed in a beaker of pure water. What will happen to the cell? What is the reason for the difference between what happens to plant and animal cells placed in pure water?

8. A dialysis bag is attached to the end of a glass tube to make a simple osmometer. The dialysis bag contains a 1 M sucrose solution with a water potential of $\psi = -35$ bars. Since the osmometer is open to the air, ψ_p is initially 0. What is ψ_π of the sucrose solution in the bag? ______ If the osmometer is placed in a beaker of water and osmosis occurs, what will ψ, ψ_p, and ψ_π be at equilibrium for the solution in the bag and the water in the beaker?

	ψ	ψ_p	ψ_π
Bag			
Beaker			

9. A plant cell, when initially placed in pure water, has an osmotic potential of -4 bars and a pressure potential of $+2$ bars.

a. Which way will water diffuse?

b. When will net diffusion stop?

c. When equilibrium is reached, what has happened to the cell's osmotic potential and pressure potential values? ψ_π = ______ ψ_p = ______

10. A protozoan cell is placed in a 0.5 M sucrose solution at 27°C. Assume the cell has an initial osmotic potential of -2 bars. Because it lacks a cell wall, it cannot generate a turgor pressure and will always have a pressure potential of 0.

a. When the cell is placed in a sucrose solution, which way will water diffuse?

b. When will net diffusion stop?

c. What will be the appearance of the cell when equilibrium is reached?

d. What will be the cell's osmotic potential and pressure potential values at equilibrium? (Assume that the quantity of water lost by the cell will not appreciably change $\psi_{outside}$.)

ψ_π = ______ ψ_p = ______

11. A plant cell with a rigid cell wall is placed in a 0.2 M solution of NaCl at 27°C and is allowed to equilibrate. Assume the cell has an initial osmotic potential of −8 bars and an initial pressure potential of +2 bars. Based on this information:

a. In which direction will water diffusion occur?

b. What will be the cell's water potential at equilibrium?

c. As the cell becomes less turgid, what happens to ψ_p?

d. What will be the cell's osmotic potential and pressure potential at equilibrium? (Assume that the quantity of water lost by the cell will not appreciably change $\psi_{outside}$.)

ψ_π = ______ ψ_p = ______

e. Will the cell be more turgid or less turgid at equilibrium than when it was first introduced into the solution?

12. Assume an animal cell with a volume of 1 ml and an osmotic potential of −5 bars is placed into a 0.8 M solution of sucrose at 27°C and is allowed to equilibrate. (Remember that animal cells cannot build up pressures in excess of atmospheric pressure, therefore the pressure potential of animal cells is always 0.)

a. Which way will diffusion occur?

b. What is the water potential of the cell at equilibrium? (Assume $\psi_{outside}$ does not change.)

c. What is the volume of the cell at equilibrium? *Hint:* We can obtain a good estimate of volume changes by recognizing two facts: (1) as the cell shrinks, the cytoplasmic solute concentration increases; (2) the solute concentration is directly related to osmotic potential. Thus we can use van't Hoff's law, which expresses the classical chemical relationship between concentration (M) and volume (V) to determine volume changes: $M_1V_1 = M_2V_2$; however, here we substitute ψ_π for M:

M_1 (ψ_{π_1}) = −5 bars

V_1 = 100% initial volume

M_2 (ψ_{π_2}) = osmotic potential of cell at equilibrium

$$V_2 = \frac{M_1V_1}{M_2}$$

The answer will be expressed as a percent of the cell's initial volume.

LABORATORY

Mitosis

OVERVIEW

According to the cell theory, all cells come from preexisting cells. New cells are formed by the process of cell division, which involves both division of the cell's nucleus (**karyokinesis**) and division, of the cytoplasm (**cytokinesis**).

There are two types of nuclear division: **mitosis** and **meiosis.** Mitosis typically results in the production of two daughter nuclei that are genetically identical to each other and to the parent nucleus. Formation of an adult organism from a fertilized egg, asexual reproduction, regeneration, and maintenance or repair of body parts are all accomplished by mitotic cell division. Meiosis, on the other hand, reduces the chromosome number in daughter nuclei to half that of the parent cell. Gametes (sex cells) in animals and spores in plants are both produced by meiotic division.

During this laboratory period you will focus on mitosis; you will investigate meiosis in Laboratory 13.

STUDENT PREPARATION

Prepare for this laboratory by reviewing the text pages indicated by your instructor. Do Exercise A before coming to the laboratory and familiarize yourself with the contents of Exercises B through D.

✔ EXERCISE A The Cell Cycle—Interphase and "Getting Ready"

Objectives

☐ Name, identify, and describe the events occurring during the four phases of the cell cycle.

Procedure

The term **cell cycle** is used to describe the life history of actively dividing cells. Repeated cellular divisions are separated by periods of growth and preparation for the next division.

Even though it is a continuous process, the cell cycle can be separated into several stages (Figure 9A-1). After a cell divides, it enters **interphase,** which consists of three stages: $\mathbf{G_1}$**, S,** and $\mathbf{G_2}$**.** Interphase is often referred to as a "resting" stage, but cells in interphase are not really at rest. Indeed, they are actively preparing for the next division.

Figure 9A-1 *Stages of the cell cycle. G_1 and G_2 stand for the first and second "gaps" in the cell cycle. S stands for synthesis of DNA. Mitosis is also called "M phase."*

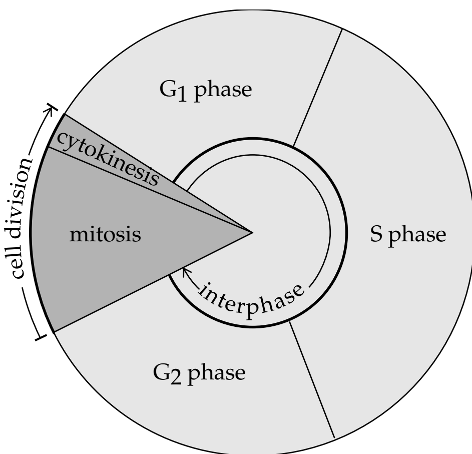
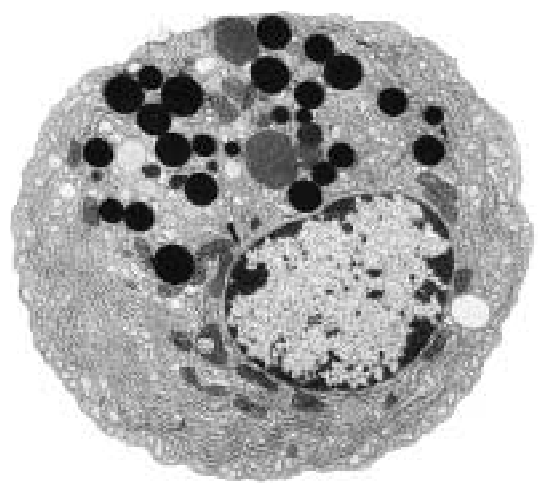


Figure 9A-2 *Electron micrograph of an isolated chief cell from mouse gastric mucosa. Note the darkly stained chromatin around the inner surface of the nuclear membrane.*



During interphase, DNA, with its chromosomal proteins, exists in a highly uncoiled state. Thus, when cell contents are stained, distinct chromosomal structures are not visible at this stage. Chromosomes appear, instead, as a granular material called **chromatin** within the nucleus (Figure 9A-2).

Events of G_1 During G_1 (first "gap"), the cell approximately doubles in size, and its enzymes and organelles double in number.

a. Why are the activities of G_1 important for a cell that is preparing to divide? ______________________________

____

____

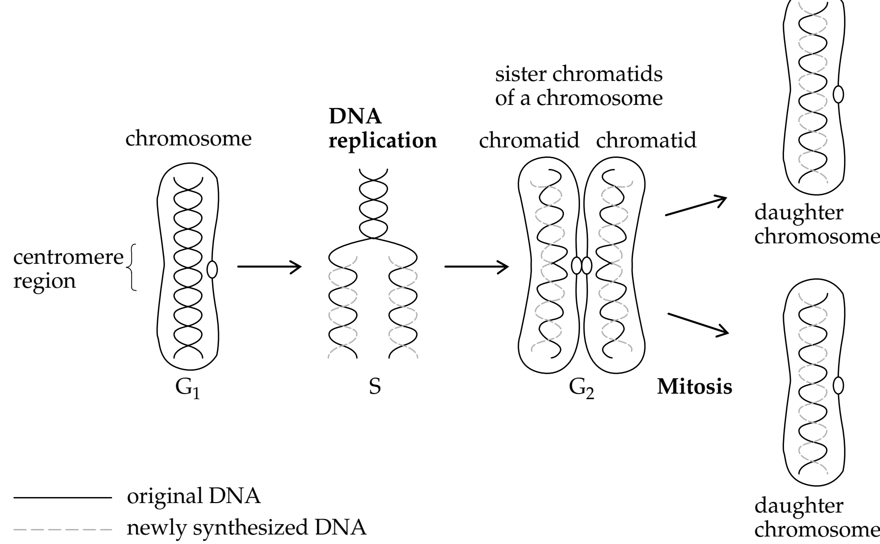
Late in the G_1 period, a critical **restriction point** is reached that determines whether or not the cell will continue to divide. If the cell does not proceed to the S phase, it exits the cell cycle and becomes "non-dividing." Nondividing cells are considered to be in the **G_0 phase.** Most cells of the body share this fate. Some, such as nerve cells, may never divide again; others can resume dividing if necessary. An example of the latter is the remarkable ability of the liver to regenerate after damage or transplant.

In order to pass the restriction point, a cell must reach a certain size. The ratio **volume of cytoplasm/genome size** appears to be of most importance. Other environmental factors such as cell density or adherence to a substrate may also play a part in regulation at this point in the cell cycle.

In addition, **protein kinase** enzymes fluctuate in concentration throughout the cell cycle. Kinase enzymes control cell cycle activity by phosphorylating specific proteins. Regulatory proteins, called **cyclins,** can in turn regulate protein kinases. Protein kinases controlled by cyclins are called **Cdk** proteins (cyclin-dependent kinases). A specific cyclin-Cdk enzyme complex is required for cells to pass the restriction point.

Events of S Before the S phase, each chromosome consists of a double-stranded helix of DNA. During the S phase, the two strands of the DNA helix unwind and separate, and each duplicates by a process called **replication.** By the end of the S phase, each chromosome is composed of two helices of DNA, called **chromatids,** joined at a region of the chromosome known as the **centromere** (Figure 9A-3).

Figure 9A-3 *Replication of chromosomal DNA. The two strands of a helix of DNA separate, and each directs the synthesis of its other half so that each chromatid will be composed of a double-stranded helical DNA molecule. The small oval shape located at the centromere region is a* kinetochore*—a small proteinaceous body to which spindle fibers attach during mitosis.*



Distinct chromosomes are not yet visible during this phase. The DNA molecules are structurally intact, but are largely uncoiled and dispersed as chromatin. Chromosomal proteins, also synthesized during the S phase, will eventually associate with DNA to help coil it into tightly packed chromosome structures prior to the onset of mitosis.

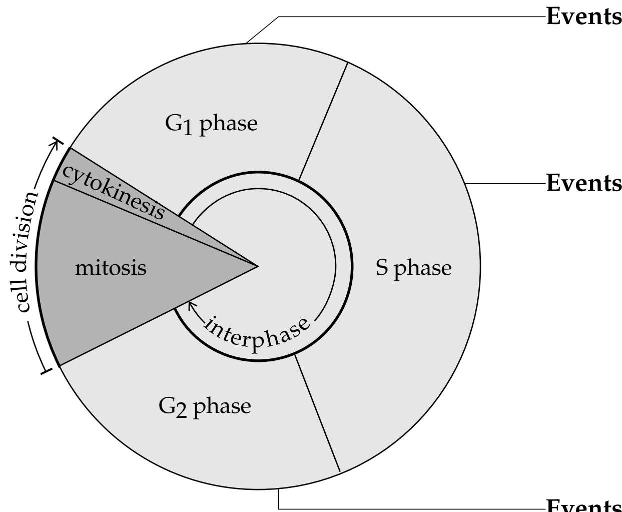
b. Why is it necessary for DNA to duplicate before cells divide? ____________________

____

Events of G_2 During the G_2 phase, structures involved directly in cell division are synthesized. **Spindle fibers** begin to assemble. These will become attached to chromosomes to guide their movement during cell division. In animal cells, a pair of **centrioles** completes division to form two pairs of centrioles. These will also play a role in the movement of chromosomes during the mitotic process. Cells of higher plants have spindle fibers but usually lack centrioles.

Regulatory proteins also serve an important role in the transition from the G_2 phase to the **M phase** (mitosis). This transition is regulated by a buildup of cyclins at the end of the G_2 phase and their regulation of a Cdk protein kinase as part of an enzyme complex known as **MPF** (M-phase promoting factor). At the end of the M phase, MPF activates an additional enzyme that destroys cyclin and turns off MPF activity.

c. Summarize the events of interphase in the following figure.



✔ EXERCISE B | Simulating the Events of Interphase, Mitosis, and Cytokinesis

Objectives

- ☐ Relate the process of DNA replication to the process of mitosis.
- ☐ Describe the structure of a chromosome in late prophase and identify its parts.
- ☐ Name, identify, and list the events occurring during the stages of mitosis.
- ☐ Describe the functions of centrioles, centromeres, and spindle fibers in mitosis.
- ☐ Compare the process of cytokinesis in animal and in plant cells.

Procedure

The purpose of this exercise is to simulate major features of mitotic cell division, *not* to duplicate it. The materials used will limit how closely you can approximate the actual process.

On your laboratory bench, you will find a chromosome simulation kit consisting of two strands of red beads and two strands of yellow beads, four hollow plastic cylinders, and four pieces of string. The strands of plastic beads represent chromosomes. The magnetic piece in each "chromosome" represents the centromere region. The four hollow plastic cylinders represent centrioles. The string will be used to simulate spindle fibers.

Figure 9B-1 shows a simulation of a chromosome as it would appear before the S phase of interphase, when DNA duplication occurs. It represents one double-stranded helical DNA molecule. Figure 9B-2 shows a simulated chromosome as it would appear after DNA replication. Each half of this duplicated chromosome is called a chromatid and contains its own helical DNA molecule. The two chromatids attached at the centromere region are identical and are called **sister chromatids.**

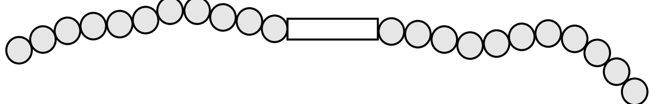


Figure 9B-1 *Early interphase. A chromosome, as it appears before DNA duplication in interphase, is composed of one double-stranded DNA molecule (see Figure 9A-3, G_1 phase).*

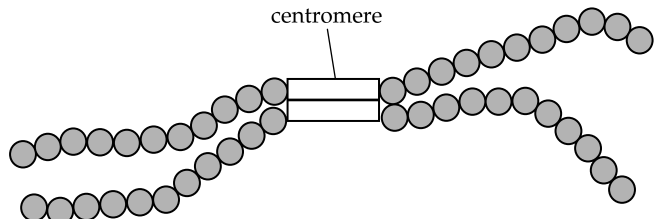


Figure 9B-2 *Mid-interphase. A chromosome as it appears after DNA duplication interphase. Each sister chromatid contains one double-stranded DNA molecule (see Figure 9A-3, G_2 phase).*

a. Next to each diagram (Figures 9B-1 and 9B-2), draw its representative DNA structure. Is the helix double-stranded or single-stranded in each chromatid? ______________________________

Interphase

Place one strand of red beads and one strand of yellow beads near the center of your work area. Together these two strands of beads represent a **homologous pair** of chromosomes.*

b. What is the significance of using two different colors for the two homologous chromosomes?

____

Could you have used two red strands, one short and the other long? ______ *Why or why not?*

____

Keep in mind that chromosomal DNA would not be seen in the form of chromosomes at this stage, but would exist as part of the diffuse chromatin contained within the nucleus. Position two of the hollow plastic cylinders "centrioles") at right angles to each other outside an imaginary nuclear membrane or envelope surrounding the chromosomes.

DNA synthesis occurs during interphase to produce chromosomes consisting of two chromatids. Simulate this process by bringing the magnetic centromere of the second red strand on the table. Do the same with the yellow strands. Two chromosomes, each consisting of two sister chromatids, are now before you (Figure 9B-3). Simulate centriole replication by placing two additional plastic cylinders next to the original centriolar bodies.

c. Why do you need to replicate the centrioles? ______________________________

____

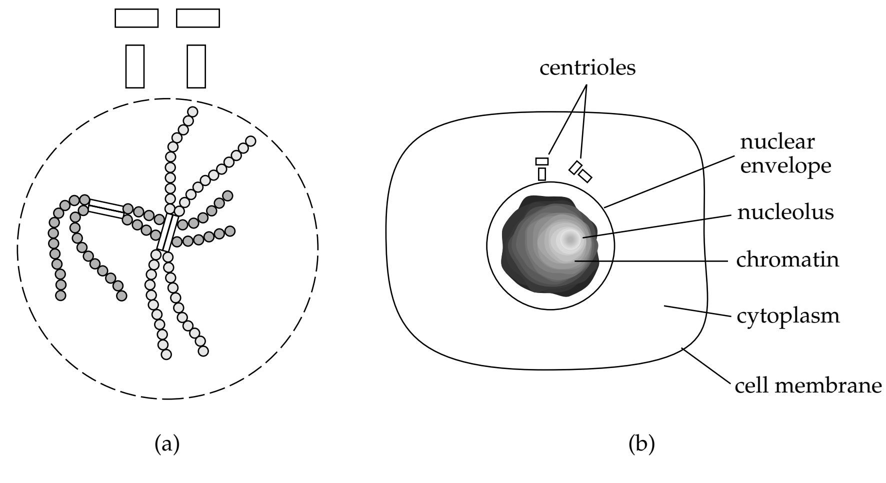


Figure 9B-3 *Late interphase. Chromosomal DNA has already duplicated and the centrioles have replicated by the end of interphase. The dashed line in (a) represents the nuclear envelope.*

**In diploid organisms, chromosomes of somatic cells occur in matched pairs that resemble each other in size, shape, and location of their centromeres. Matched pairs are called homologous chromosomes. One member of each pair is paternal and the other, maternal, in origin.*

Mitosis

Prophase

During prophase, chromatin condenses within the nucleus until the chromosomes and their individual chromatids are visible. The two pairs of centrioles, replicated during interphase, separate and migrate to opposite sides or **poles** of the nucleus. A dense material surrounding the centrioles is associated with microtubule formation and is often referred to as a *microtubule organizing center.* This material, in combination with the centriole pair at its center, is called a **centrosome.** Spindle fibers composed of microtubules begin to form. The assembly of microtubules is initiated by the centrosomes and requires the polymerization of globular **tubulin** protein molecules into fibers (similar to lengthening a string of beads). As the spindle fibers lengthen, the nuclear membrane and nucleoli disappear. The spindle fibers attach to a protein structure, the **kinetochore**, located at the centromere region of two sister chromatids. **Kinetochore microtubules** extend from the centrosomes to the kinetochores of chromosomes. Some fibers reach all the way across the cell from centrosome to centrosome, while others seem to stop short of attachment. Kinetochore microtubules shorten during anaphase by depolymerization of tubulin fibers at their kinetochore ends.

Begin your simulation of the events of prophase by separating the two pairs of cylindrical beads, moving one pair to the opposite side of the imaginary nuclear membrane that surrounds the chromosome bundle. Approximately 50 cm from each side of the chromosomes, tape down one centriole of each pair so that it is pointing toward the nucleus (Figure 9B-4).

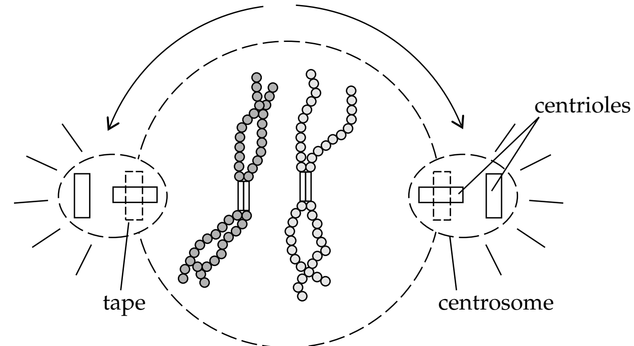


Figure 9B-4 *Early prophase. Centrioles have migrated to opposite sides of the nucleus and spindle fibers will soon become attached to the centromere regions of the chromosomes.*

Form a loop at one end of each piece of string in your kit. Draw the loop of one piece of string tightly around the centromere region of one of the sister chromatids (Figure 9B-5); repeat this process for the other sister chromatid and for the sister chromatids of the homologous chromosome. Extend the strings (spindle fibers) of sister chromatids toward opposite sides of the nucleus; thread each string through one of the cylinders (centrioles).

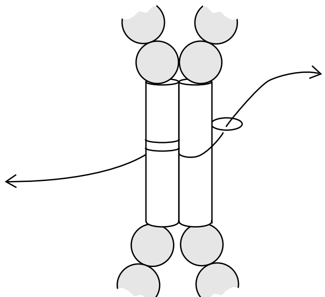


Figure 9B-5 *Attachment of spindle fibers to the centromere regions of sister chromatids.*

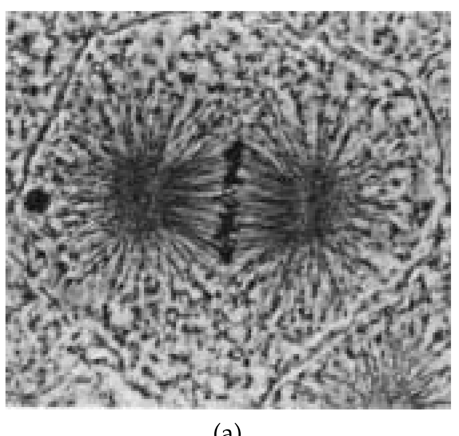
Metaphase

During metaphase, the centromere regions of sister chromatids are attached by spindle fibers to opposite centrioles and the chromosomes line up in single file at the middle, or equator, of the spindle apparatus (Figure 9B-6).

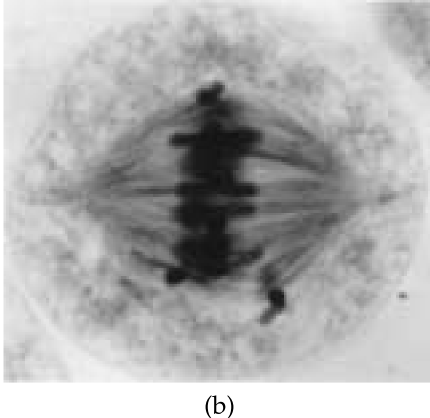
d. Why is it important that the chromosomes line up in single file during metaphase?

____

Figure 9B-6 *(a) The mitotic spindle apparatus of animal cells consists of astral fibers (the short fibers that surround the centrioles), spindle fibers that attach to chromosomes, and spindle fibers that extend between centrioles. (b) In cells of higher plants, the mitotic spindle apparatus has spindle fibers but lacks centrioles and astral fibers.*



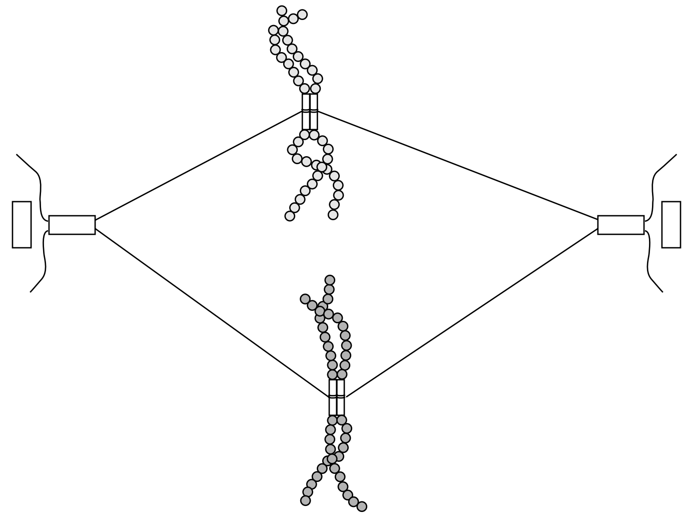
(a)



(b)

To simulate metaphase, center the chromosomes between the centrosomes (Figure 9B-7).

Figure 9B-7 *Metaphase. Chromosomes line up in single file on the mitotic spindle apparatus. Chromosomes appear to be perpendicular to the long axis of the spindle apparatus and are equidistant from each pole of the cell.*

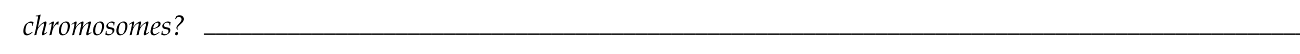


Anaphase

During anaphase, the sister chromatids of each chromosome separate at the region of their centromeres and move toward opposite poles. Recall that the spindle fibers shorten at their kinetochore ends and the chromosomes follow. Thus they appear to be pulled toward opposite sides of the cell. The separated chromatids are now called **daughter chromosomes.** The name of each of the two DNA strands, formerly held together at the kinetochore, has changed from chromatid to chromosome. When a strand of DNA has its own centromere region, it is called a chromosome.

To simulate anaphase, pull on the strings until the magnetic centromeres are separated. Continue pulling the daughter chromosomes toward the centrioles. Note that the arms of each chromosome follow the centromere toward the pole (Figure 9B-8).

e. During mitosis, when would you change the description of DNA strands from chromatids to chromosomes?



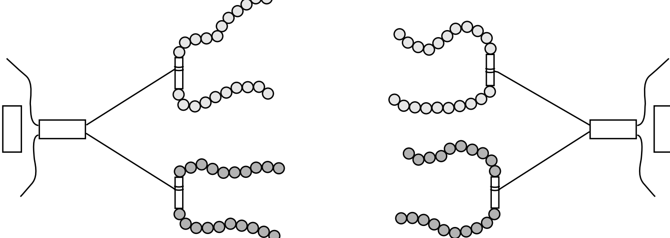


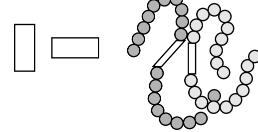
Figure 9B-8 *Anaphase. Centromere regions of sister chromatids separate, and daughter chromosomes move to opposite ends of the cell.*

Telophase

The spindle apparatus disappears during telophase and nuclei are re-formed as the nuclear membrane appears. Nucleoli also reappear. There are now two nuclei, one for each of the daughter cells. Chromosomes decondense and re-form diffuse chromatin.

f. How many helices of DNA are in each of the daughter chromosomes at this time? _______

To simulate telophase, remove the strings. Near each pair of centrioles, pile up the one red and one yellow chromosome drawn to that side of the cell during anaphase (Figure 9B-9). These represent the daughter nuclei and daughter cells. Note that you began cell division with two chromosomes: a red one and a yellow one.



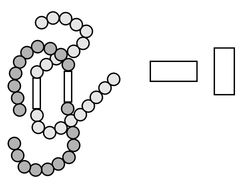


Figure 9B-9 *Telophase. Sister chromatids have separated, forming daughter chromosomes, and new nuclear membranes form around the separated chromosomal bundles.*

g. How many chromosomes do you have in each of your daughter nuclei? _______

Note: The number of chromosomes in a cell is determined by counting the number of visibly separate centromeres, regardless of whether the centromere region joins two sister chromatids in a chromosome or appears in an undivided chromosome composed of only one chromatid.

Repeat the procedure outlined above until you are thoroughly familiar with the major events of mitosis. Have your instructor check your work if you experience difficulty.

After you are comfortable with this simulation of the mitotic process, combine your chromosome kit with that of another student. Shorten the two yellow and two red strands from one of the kits so that there are only five beads on each side of the centromere region.

Now simulate mitosis in a nucleus with four chromosomes (two homologous pairs, one pair consisting of one short red and one short yellow chromosome, and the other of one long red and one long yellow chromosome). Draw each stage (prophase, metaphase, anaphase, and telophase) on a separate piece of paper and insert this into your laboratory manual. You should end up with two nuclei, each containing four chromosomes—two red and two yellow. Have your instructor check your work.

When you are finished, reattach the beads to the original chromosomes and separate the two kits in preparation for the next class.

Cytokinesis

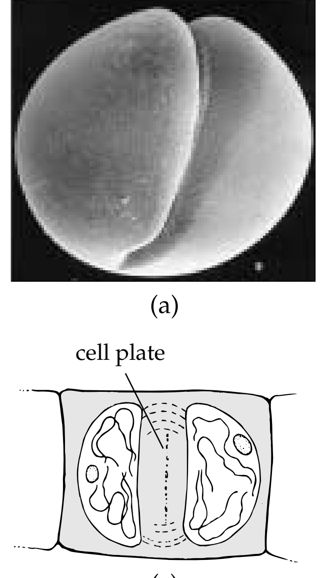
Division of the cytoplasm (cytokinesis) usually occurs as telophase of mitosis progresses. In animal cells, microtubules play a role in the formation of a furrow that constricts the cytoplasmic mass into two daughter cells (Figure 9B-10a).

In plant cells, a cell plate is formed along the midplane of the dividing cell as many vesicles (produced by Golgi bodies) become joined together. This creates a membrane-bound space, the cell plate. Membranes of either end of the cell plate fuse with the cell membrane to produce two separate daughter cells. The area between the cells becomes impregnated with pectin to form the middle lamella, and the two daughter cells synthesize cell walls (Figure 9B-10b).

h. How does the number of chromosomes in the two daughter cells that you formed in your simulations compare with the number of chromosomes in the parent cell? ____________________

i. How do the two cells that you formed in your simulation compare with the parent cell with regard to which chromosomes are present? ____

____



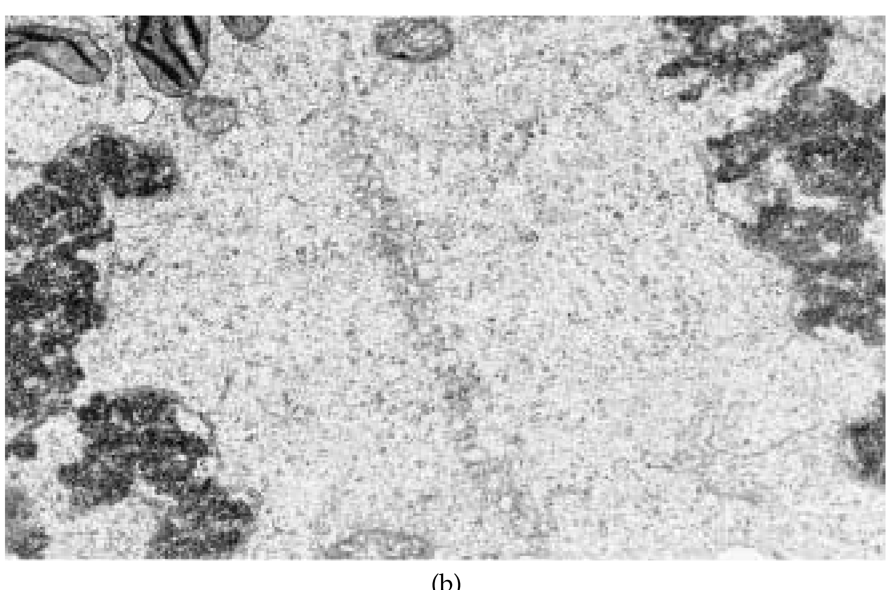


Figure 9B-10 *(a) Cytokinesis in an animal cell. (b) and (c) Cytokinesis in a plant cell.*

j. Would the constitution of the daughter cells be any different if you had lined up the metaphase chromosomes in a different order? ________

k. If the red chromosomes represent paternal chromosomes and the yellow chromosomes represent maternal chromosomes, what can be said about the genetic constitution of the daughter cells?

____

l. Keeping your answer to the previous question in mind, briefly describe how mitosis maintains a constant chromosome number and why daughter nuclei are always genetically identical. ______________________

____

✔ EXERCISE C | Mitosis in Living Tissues—Onion Root Tips

Objectives

- ☐ Identify the stages of mitosis in living tissues from the onion root tip.
- ☐ Relate the apparent frequency of mitotic figures to the growth pattern of root-tip tissue.

Procedure

Mitosis is easily studied using the root tips of actively growing plants. Roots of the common garden onion (*Allium cepa*) provide good material for such a study (Figure 9C-1). (*Allium cepa* has 16 chromosomes.)

1. Obtain a stained root tip for microscopic examination and place it on a slide in a drop of water. Add a coverslip, gently pressing downward with the eraser end of a pencil to spread the tissues apart.
2. Observe the preparation using a microscope (10×) and squash it further if necessary; do not be too heavy-handed initially.

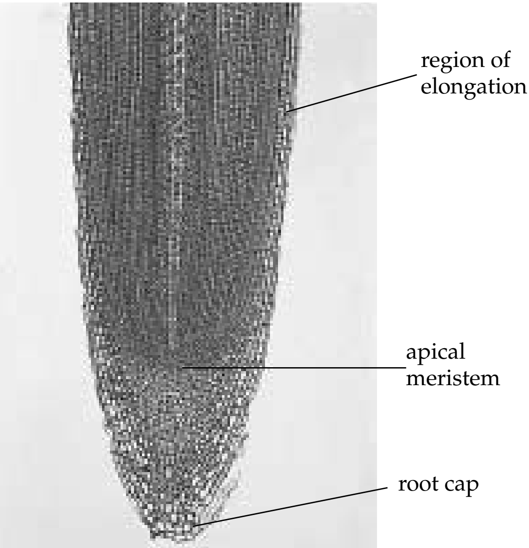


Figure 9C-1 *Median longitudinal section of an onion root tip.*

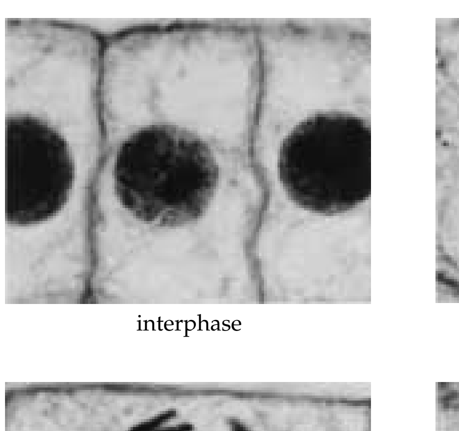
3. Use the microscope to examine the *Allium* root-tip slide. Scan the entire length of the slide to observe cells far from the tip and cells right at the tip. Are the cells in these areas dividing? Locate the area of cell division, the region where the greatest number of mitotic figures are represented, and draw a box around this area in Figure 9C-1. Label this area "region of cell division."

At the apex of the region of cell division, certain cells called "initials" give rise to new cells for root growth. When an initial cell divides, one daughter cell goes on to divide a few more times (in the region of cell division) before differentiating into specialized cells. The other daughter cell continues as an initial cell and remains in the apical region (called the *apical meristem;* **meristem** refers to undifferentiated plant tissue from which new cells are formed). Covering the apical meristem is the *root cap.* Behind the region of cell division, cells elongate and later differentiate as the root lengthens.

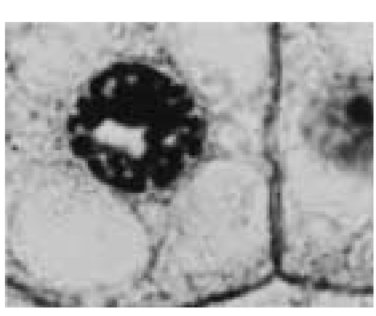
a. What might be the purpose of the root cap? ______________________________

4. Switch to high power (40×). Use Figure 9C-2 to identify cells in interphase, prophase, metaphase, anaphase, and telophase. Draw and label these cells on a separate sheet of paper. Insert the drawings into your notebook.

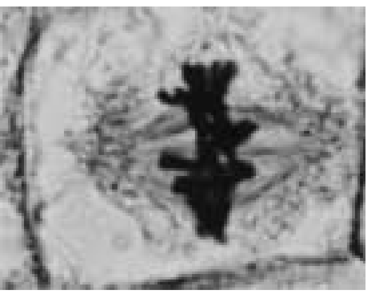
Figure 9C-2 *Stages of mitosis in the onion root tip.*



interphase

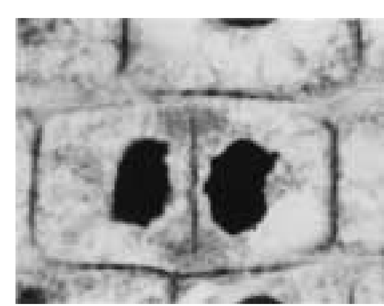


prophase



metaphase

anaphase



telophase

EXERCISE D Phases of the Cell Cycle in the Onion Root Tip

Now that you are familiar with the events of mitosis and the stages of the cell cycle, you can estimate the relative duration of each phase of the cell cycle by recording the frequency with which you find each phase in regions where cell division is actively taking place. The frequency of a phase is an indication of the relative length of that phase.

Objectives

☐ Use observations of cells in an onion root tip to describe the relative duration of the phases of the cell cycle.

Procedure

1. Obtain a prepared slide of onion root tip or use your fresh onion root-tip squash if the preparation is good and many mitotic figures are visible.

2. With the 40× objective in place, examine a single field of view (the area visible in the microscope with the slide stationary) in the apical meristem region and count the number of cells in the phases of the cell cycle listed in Table 9D-1. Make sure you are surveying the actively dividing area of the onion root tip. Repeat this count in at least two more nonoverlapping fields. Use Table 9D-1 to collect and calculate your results.

Table 9D-1 Percentage of Cells in Each Phase of the Cell Cycle

	Number of Cells				Percent of Grand Total (Total/Grand Total × 100)
	Field 1	Field 2	Field 3	Total	
Interphase					
Prophase					
Anaphase					
Telophase					
Grand total	X	X	X		

The duration of mitosis varies for different tissues in the onion. However, prophase is always the longest phase (1–2 hours), and anaphase is always the shortest (2–10 minutes). Metaphase (5–15 minutes) and telophase (10–30 minutes) are also of relatively short duration. Interphase may range from 12 to 30 hours.

Consider that it takes, on average, 16 hours (960 minutes) for onion root-tip cells to complete the cell cycle. You can calculate the amount of time spent in each phase of the cell cycle from the percentage of cells in that stage:

Percentage of cells in stage × 960 minutes (16 hours) = minutes of cell cycle spent in stage

3. Calculate the following (convert the times to hours and minutes):

Time spent in: prophase ______ hr ______ min

metaphase ______ hr ______ min

anaphase ______ hr ______ min

telophase ______ hr ______ min

Total time spent in mitosis ______ hr ______ min

Time spent in interphase ______ hr ______ min

a. *What percentage of the cell cycle is spent in mitosis?* ______ *In interphase?* ______

b. *How do your results compare with what is generally known about the* Allium cepa *cell cycle, as described above?* ______________________________

____

____

c. *If your data show little agreement with the information given on the* Allium cepa *cell cycle, would the cell cycle for the root-tip cells you observed have to be longer or shorter than the average of 16 hours in order to explain your results?* ______________________________

____

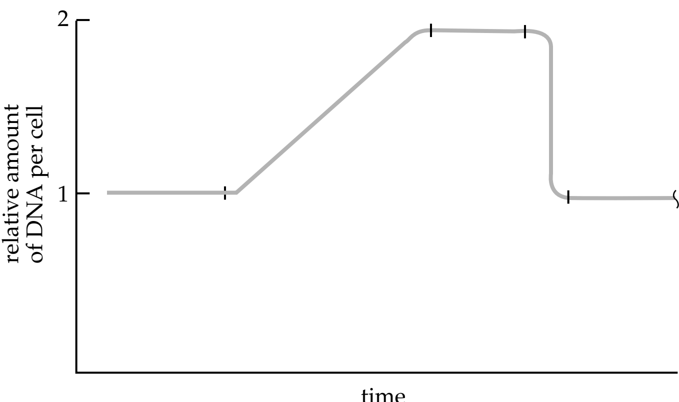
4. If you are using a prepared slide of an onion root tip, you will be observing a longitudinal section through the root tip.

d. How does a longitudinal section compare with a cross section? ______________________

e. Why do some of the cells appear to be empty? ______________________

Laboratory Review Questions and Problems

1. The diagram below shows the relative amount of DNA present in a cell progressing through the stages of the cell cycle. Label the line segments between the hatch marks to show G_1, S, G_2, and mitosis.



2. Complete the following table.

	Number of Chromatids per Chromosome
G_1	
S	
G_2	
Prophase	
Metaphase	
Anaphase	
Telophase	

3. How do protein kinases and cyclins regulate the cell cycle?

4. What role does cell division play in the growth of an organism?

5. What is the function of the centromere region of a chromosome?

6. What is a centrosome? How are centrioles and centrosomes related?

7. What is the function of spindle fibers? How do they lengthen and shorten?

8. When does a chromatid become a chromosome?

9. You have a diploid cell containing eight chromosomes ($2n = 8$). What would this cell look like at metaphase? How many chromosomes would each of the daughter cells have?

10. You are given two dishes of beads, one containing red beads and the other yellow. Describe how you would simulate the chromosomes of a diploid cell for which $2n = 4$.

11. Can a haploid cell undergo mitosis? Why or why not? If you had a haploid cell with four chromosomes ($n = 4$), what would it look like during metaphase?

12. Indicate the stage of mitosis (interphase, prophase, metaphase, anaphase, telophase) during which each of the following events occurs.

_______________ Nuclear membrane disappears.

_______________ Centrioles replicate.

_______________ Chromosomes are arranged in single file between the poles of the cell.

_______________ Chromatids separate.

_______________ Spindle fibers form.

_______________ Cell plate forms.

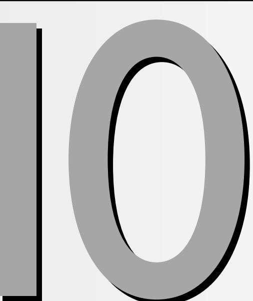
_______________ DNA replication occurs.

_______________ Chromosomes first become visible as long thin strands.

_______________ Chromosomes move to opposite poles of the cell.

LABORATORY

Enzymes



OVERVIEW

Without enzymes, most biochemical reactions would take place at a rate far too slow to keep pace with the metabolic needs and other life functions of organisms. **Enzymes** are catalysts that speed up chemical reactions but are not themselves consumed or changed by the reactions.

The cell's biological catalysts are proteins. These enzymes have a very complex three-dimensional structure consisting of one or more polypeptide chains folded to form an **active site**—a special area into which the **substrate** (material to be acted on by the enzyme) will fit.

Changes in temperature, alterations in pH, the addition of certain ions or molecules, and the presence of inhibitors all may affect the structure of an enzyme's active site and thus the activity of the enzyme and the rate of the reaction in which it participates. The rate of an enzymatic reaction can also be affected by the relative concentrations of enzyme and substrate in the reaction mixture.

During this laboratory period, you will investigate how changes in pH, temperature, substrate concentration, and enzyme concentration affect the enzymatic activity of catecholase. We will also observe the action of rennin during the process of cheesemaking.

STUDENT PREPARATION

Prepare for this laboratory by reviewing the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

EXERCISE A Investigating the Enzymatic Activity of Catecholase

During this exercise you will study the activity of the enzyme **catecholase** contained in some fruits and vegetables. Peeled potatoes and bruised fruits turn brown when exposed to air because catecholase facilitates a reaction between catechol and oxygen. In the presence of oxygen, the compound **catechol** is oxidized by the removal of two hydrogen atoms. Catechol is thus converted to benzoquinone, and oxygen is reduced by the addition of two hydrogen atoms to form water. Benzoquinone molecules then link together to form long, branched chains. These chains are the structural backbones of the red and brown melanoid pigments that cause darkening.

Throughout this exercise, work in pairs. Each pair of students should perform all four parts of the exercise. If you will be using the spectrophotometer to collect results, use the procedures labeled "quantitative." If you will be determining enzyme activity by observing color changes, use the procedures "qualitative."

catechol + $\frac{1}{2}O_2$ $\xleftrightarrow{\text{enzyme catecholase}}$ benzoquinone + H_2O

Note: Your instructor will dispense an extract of potato juice containing the enzyme catecholase to each group. The dropper bottle should be kept tightly covered at all times. Keep in mind that whenever you use potato juice in the following experiments, you are using an enzyme—catecholase—preparation.

Objectives

- ☐ Determine the effects of temperature on the enzymatic activity of catecholase.
- ☐ Describe the effects of changes in enzyme concentration on the rate of an enzyme-catalyzed reaction.
- ☐ Describe the relationship between substrate concentration and the maximum velocity of an enzyme-catalyzed reaction.

PART I The Effect of Temperature on Enzyme Activity

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as temperature increases, up to a point at which the rate is maximum. The rate then abruptly declines with a further increase in temperature (Figure 10A-1). Above 40°C, most enzymes active in living tissue become **denatured**—their secondary or tertiary protein structure breaks down.

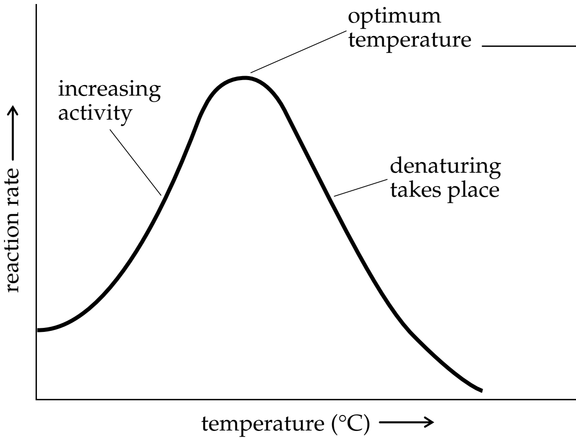


Figure 10A-1 *Effect of temperature on reaction rate.*

a. From what you have learned about proteins from reading your text or from lecture discussions, describe the significance of the secondary and tertiary structures of a protein.

____

____

b. Why would changing the secondary or tertiary structure of a protein affect its enzymatic activity?

____

____

Procedure (Quantitative)

1. Refer to Laboratory 4, Exercise A, for directions on how to use the spectrophotometer (Spectronic 20) and for an explanation of the theory behind its operation. Set the wavelength to 420 nm and adjust the machine to 100% transmittance and 0% absorbance. Prepare three

tubes, each containing 3 ml of pH 7 phosphate buffer, 10 drops of potato juice, and 10 drops of water, as blanks for the three temperatures to be used. Label the tubes "10B," "24B," and "50B."

2. Fill three additional test tubes with 3 ml of pH 7 phosphate buffer. Label the tubes "10," "24," and "50." These are your "experimental" tubes.
3. Place the first experimental tube and its corresponding blank in an ice-water bath, leave the second tube and its corresponding blank at room temperature, and place the third tube with its blank in a beaker of warm water (approximately 50°C). Allow 10 minutes for the buffer in the first pair of tubes to reach 10°C (or less), the buffer in the second pair of tubes to reach 24°C (room temperature), and the buffer in the third pair of tubes to reach 50°C. Meanwhile, continue to step 4.
4. Take two test tubes containing potato juice and put one on ice (10°C) and the other in a warm-water bath (50°C). Take two test tubes containing catechol and put one on ice and the other in the warm-water bath.
5. After 10 minutes, add 10 drops of catechol (from a tube at the same temperature, or for the 24°C sample, from the room temperature stock) to each experimental tube. Cover each tube with Parafilm and invert several times to mix.
6. Use the blank for 10°C (tube 10B) to adjust the Spectronic 20 to 0% absorbance. Wipe condensation off the tube before reading. Add 10 drops of potato juice (from the tube at 10°C) to your 10°C experimental tube (tube 10). (Water has been used to replace this in your blank.) Cover the tube with Parafilm and invert it several times to mix the contents. Quickly read absorbance and record your reading in Table 10A-1 as the "0 minutes" reading. Return both the experimental tube and the blank to the 10°C beaker.
7. Repeat the procedure in step 6 for your room temperature (24°C) and 50°C experimental tubes. Remember to use appropriate blanks at each temperature. (Note: You may want to complete all readings at the first temperature and then follow with the other two sets of tubes, but this will take more time.)
8. At 2-minute intervals, determine the absorbance for each of your three experimental tubes (always adjusting the Spectronic 20 with the appropriate blank). Record your data in Table 10A-1. Allow the experiment to run for 10 minutes.

Table 10A-1 Effect of Temperature on Enzyme Activity

	Absorbance (420 nm)		
Minutes	At 10°C	At 24°C	At 50°C
0			
2			
4			
6			
8			
10			

9. Graph your data for the 6-minute time (use graph paper). Label the *X*-axis "temperature" and the *Y*-axis "absorbance (420 nm)."

   c. At what temperature was the amount of product produced by the enzyme-catalyzed reaction greatest? _______

d. How would product produced per minute relate to the rate of an enzyme-catalyzed reaction?

____

10. To determine rate, graph the data for each of the three tubes. Label the *X*-axis "time" and the *Y*-axis "absorbance (420 nm)." Find the slope of the straightest portion of each curve and compare these rates (absorbance/min).

e. At what temperature was the rate of the enzyme-catalyzed reaction greatest? _______ *Write this value in the space provided in Figure 10A-1.*

f. Do your results support the explanation given at the beginning of this exercise? _______ *If not, what might be the reason for this?* ____

____

11. Skip to "Final Step" on p. 10-5.

Procedure (Qualitative)

1. Fill three test tubes with 3 ml of pH 7 phosphate buffer. Label the tubes "10," "24," and "50."

2. Place the first test tube in an ice-water bath, leave the second tube at room temperature, and place the third tube in a beaker of warm water (approximately 50°C). Allow 10 minutes for the buffer in the first tube to reach 10°C (or less), the buffer in the second tube to reach 24°C (room temperature), and the buffer in the third tube to reach 50°C. Meanwhile, continue to step 3.

3. Take two test tubes containing potato juice and put one on ice (10°C) and the other in a warm-water bath (50°C). Take two test tubes containing catechol and put one on ice and the other in the warm-water bath.

4. After 10 minutes, add 10 drops of catechol (from a tube at the matching temperature, or for the 24°C sample, from the room temperature stock) to each tube of buffer.

5. Add 10 drops of potato juice (again, at the matching temperature) to each tube.

6. Allow the tubes to stand for 5 minutes (shake the mixture several times during the 5 minutes). Record your results in Table 10A-2 by indicating the intensity of color in each tube after 5 minutes (0 indicates no color; +, ++, and +++ indicate increasing intensities of color).

Table 10A-2 Effect of Temperature on Enzyme Activity

	Intensity of Color		
Minutes	10°C	24°C	50°C
5			

c. At what temperature was the amount of product produced by the enzyme-catalyzed reaction greatest? _______

d. How would product produced per minute relate to the rate of an enzyme-catalyzed reaction?

____

e. What was the purpose of shaking the tubes? ____

____

f. At what temperature was the rate of the enzyme-catalyzed reaction greatest? ______ Write this value on the blank provided in Figure 10A-1.

Final Step Indicate the relationship between temperature and enzymatic activity by writing the temperatures used in this experiment at appropriate positions along the horizontal axis of Figure 10A-1. To assure accuracy, it may be helpful first to mark highest and lowest temperatures on the graph itself.

Cooking hint: Placing peeled potatoes into ice water before cooking keeps them from darkening in color. Why? ____

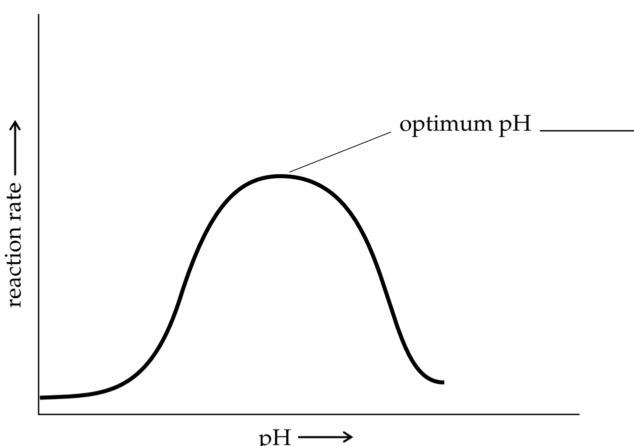
____

PART 2 The Effect of pH on Enzyme Action

The presence of various ions can interfere with the pattern of positive and negative charges within a protein molecule, thus changing the way the protein folds. In enzymes, the shape of the active site may be changed. We should expect, then, that changes in pH (reflecting the concentrations of hydrogen and hydroxide ions) would affect the action of enzymes.

The most favorable pH value—the point at which the enzyme is most active—is known as the optimum pH (Figure 10A-2). Extremely high or low pH values usually result in a complete loss of enzyme activity due to **denaturation,** the breakdown of the secondary and tertiary structure of a protein. Denaturation can also be caused by extremes in temperature, as explained in Part 1 of this exercise.

Figure 10A-2 *Effect of pH on reaction rate.*



a. How do you think extremes of pH might cause denaturation and loss of enzyme activity?

____

____

Procedure (Quantitative)

1. Prepare five tubes for blanks, each containing 3 ml of a different phosphate buffer (pH 4, pH 6, pH 7, pH 8, pH 10), 10 drops of potato juice, and 10 drops of water. Be sure to label blank tubes with a "B" and the pH value. These will also serve as controls.
2. Set the spectrophotometer wavelength to 420 nm and adjust the machine to 100% transmittance and 0% absorbance.
3. Label five additional test tubes with the following pH values: pH 4, pH 6, pH 7, pH 8, and pH 10. These will serve as experimentals.

4. Fill each experimental tube with 3 ml of the matching phosphate buffer.
5. Add 10 drops of potato juice to each experimental tube. Cover each tube with Parafilm and invert the tube several times to mix the contents.
6. Add 10 drops of catechol to each tube (in the blanks, water substitutes for catechol). Cover the tubes with Parafilm and invert each several times to mix the contents.
7. Allow the tubes to stand for 5 minutes (mix at 1-minute intervals). At the end of this time, record absorbance readings (at 420 nm). Be sure to use the proper blank to adjust the spectrophotometer before each reading. Record your data in Table 10A-3.

Table 10A-3 Effect of pH on Enzyme Activity

Absorbance (420 nm)				
pH 4	pH 6	pH 7	pH 8	pH 10

8. Graph your data on graph paper. Label the *X*-axis "pH" and the *Y*-axis "absorbance (420 nm)."

   b. At what pH was the amount of product in this enzyme-catalyzed reaction greatest? ________

   c. How does the amount of product produced per minute relate to the rate of the enzyme-catalyzed reaction? ________________________________

   d. At what pH was the rate greatest? ________

9. Skip to "Final Step" on p. 10-7.

Procedure (Qualitative)

1. Label five test tubes with the following pH values: pH 4, pH 6, pH 7, pH 8, and pH 10. These will serve as experimental tubes.
2. Fill each experimental tube with 3 ml of the matching phosphate buffer.
3. Add 10 drops of catechol to each tube. Cover the tube with Parafilm and invert several times to mix the contents.
4. Be prepared to observe color changes. Allow the tubes to stand for 3 to 5 minutes. Invert and mix each tube at 1-minute intervals. The rate of the enzyme-catalyzed reaction (measured by the amount of product produced during a given period of time) will be qualitatively proportional to the intensity of color developed in each reaction mixture. Record your results by indicating the intensity of color in each tube (0 = no color; +, ++, and +++ = increasing intensities of color). Record your data in Table 10A-4.

Table 10A-4 Effect of pH on Enzyme Activity

Intensity of Color				
pH 4	pH 6	pH 7	pH 8	pH 10

b. At what pH was the amount of product in this enzyme-catalyzed reaction greatest? ________

c. How does the amount of product produced per minute relate to the rate of the enzyme-catalyzed reaction? ____________________

d. At what pH was the rate greatest? ________

Final Step Indicate the relationship between pH and enzymatic activity by writing the pH values used in this experiment at appropriate positions along the horizontal axis of Figure 10A-2. To assure accuracy, it may be helpful to first mark pH values on the graph itself.

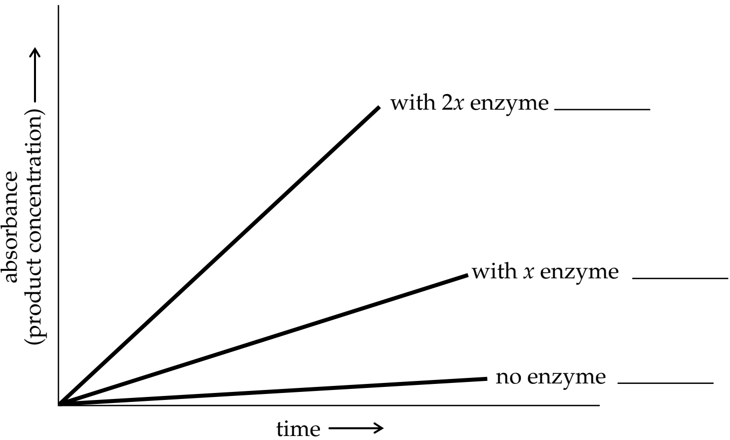
Cooking hint: Sprinkling lemon or orange juice over peeled fruit keeps the fruit from darkening. Citrus juice contains citric and ascorbic acids. *Why does citrus juice keep fruit from darkening?*

____

PART 3 The Effect of Enzyme Concentration on Enzyme Activity

When a substrate fits into the active site of an enzyme, an enzyme–substrate complex is formed. The enzyme assists with the chemical reaction and a product is formed. The reaction rate is usually directly proportional to the enzyme concentration (Figure 10A-3). The reaction rate will increase in proportion to an increasing enzyme concentration only if substrate is present in excess amounts so that the reaction is not limited by substrate availability.

Figure 10A-3 *Effect of enzyme concentration on reaction rate.*



a. If an adequate amount of an enzyme is present, how might availability of a substrate affect the production of a biochemical product in the cells of your body? ____________________

____

Procedure (Quantitative)

1. Label four test tubes as noted in the table below and add the required amounts of phosphate buffer, potato juice, and water to each tube. Cover each tube with Parafilm and invert the tube several times to mix. These tubes will serve as blanks.

Tube	pH 7 Phosphate Buffer	Potato Juice	Water
Blank A	3 ml + 20 drops	—	10 drops
Blank B	3 ml + 15 drops	5 drops	10 drops
Blank C	3 ml + 10 drops	10 drops	10 drops
Blank D	3 ml + 0 drops	20 drops	10 drop

2. Label four additional tubes as noted below. These tubes will serve as experimentals. Add the required amounts of phosphate buffer and potato juice. DO NOT ADD CATECHOL YET! Cover each tube with Parafilm and invert it several times to mix the contents.

Tube	pH 7 Phosphate Buffer	Potato Juice	Catechol (ADD LAST)
A	3 ml + 20 drops	—	10 drops
B	3 ml + 15 drops	5 drops	10 drops
C	3 ml + 10 drops	10 drops	10 drops
D	3 ml + 0 drops	20 drops	10 drops

3. Set the spectrophotometer wavelength to 420 nm and adjust the machine to 100% transmittance and 0% absorbance.
4. Adjust the spectrophotometer using blank A. Add catechol to experimental tube A. Cover the tube with Parafilm and invert it several times to mix. Immediately read absorbance and continue to take readings every 2 minutes for 6 minutes. Record your data in Table 10A-5.

Table 10A-5 Effect of Enzyme Concentration on Enzyme-Catalyzed Reactions

	Absorbance (420 nm)			
Minutes	A	B	C	D
0				
2				
4				
6				

5. Now repeat the procedure in step 4 using tubes B, C, and D, and the appropriate blanks. Record your data.
6. Graph your data on graph paper. Label the X-axis “time (minutes)” and the Y-axis “absorbance (420 nm).”
7. The absorbance is proportional to the amount of product produced by this enzyme-catalyzed reaction. The slope of the plotted line, expressed in absorbance units per unit of time, is an indication of the rate of the reaction. The slope of the line can be determined by taking any two points on a straight line plotted in step 6 and performing the following operation:

$$\text{Slope} = \frac{\text{absorbance}_2 - \text{absorbance}_1}{\text{time}_2 - \text{time}_1}$$

b. How do the rates of the reactions in tubes A, B, C, and D differ?

____

____

c. How does increasing enzyme concentration in the presence of unlimited substrate affect the rate of the enzyme-catalyzed reaction? ____

____

8. Skip to "Final Step" on p. 10-10.

Procedure (Qualitative)

1. Label four test tubes as noted in the table below. Add the indicated amounts of phosphate buffer and potato juice to each tube. DO NOT ADD CATECHOL YET! Cover each tube with Parafilm and invert it several times to mix the contents.

2. Add 10 drops of catechol to each tube. Cover each tube with Parafilm and invert it several times to mix the contents. (Do not use the same piece of Parafilm on all tubes!)

Tube	pH 7 Phosphate Buffer	Potato Juice	Catechol (ADD LAST)
A	3 ml + 20 drops	—	10 drops
B	3 ml + 15 drops	5 drops	10 drops
C	3 ml + 10 drops	10 drops	10 drops
D	3 ml + 0 drops	20 drops	10 drops

3. Allow the tubes to stand for 3 to 4 minutes; mix the contents of each tube at 1-minute intervals.

4. The rate of the enzyme-catalyzed reaction (measured as the amount of product produced during a given period of time) will be proportional to the intensity of color developed in each reaction mixture. In Table 10A-6, record your results by indicating the intensity of color in each tube after 3 or 4 minutes (0 = no color; +, ++, and +++ = increasing intensities of color).

b. What changes did you observe throughout the period during which the reaction was occurring?

____

____

Table 10A-6 Effect of Enzyme Concentration on Enzyme-Catalyzed Reactions

Intensity of Color			
A	B	C	D

c. At what enzyme concentration was the amount of product in this enzyme-catalyzed reaction greatest? ________

d. At what enzyme concentration was the rate greatest? ________

Final Step Record your observations of the relationship between enzyme concentration and the rate of reaction by labeling the lines in Figure 10A-3 with A, B, or C, representing the activity of catecholase in tubes A, B, and C. Add a line to represent the rate of the reaction observed in tube D.

e. Do your results support the concept that reaction rate is proportional to enzyme concentration? ____________

Explain. ____

____

PART 4 The Effect of Substrate Concentration on Enzyme Activity

If the amount of enzyme is kept constant but the amount of substrate is gradually increased, then the **velocity,** the rate of speed, at which the enzyme works (converts substrate to product) will increase until it reaches a maximum. At this point, increases in substrate concentration will not increase the velocity of the reaction because all of the available enzyme is participating in the enzyme–substrate complex. Thus, every enzyme has a "maximum" velocity at which it will work (Figure 10A-4).

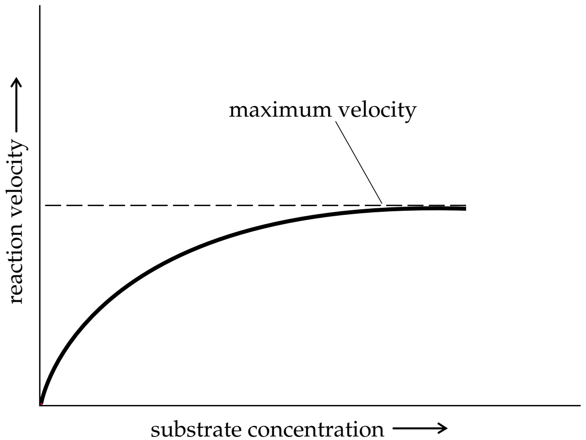


Figure 10A-4 *Effect of substrate concentration on reaction rate (velocity).*

a. What consequences would such maximum velocities have for cell metabolism?

____

Procedure (Quantitative)

1. Work in groups of four (two student pairs). Dilute the stock potato extract 1:1.
2. Label 10 tubes with the numbers indicated in the following table—make *two* blanks. One pair of students will work with the first four tubes and the other pair with the remaining four. Each pair of students will need a blank.

   Note: If there are enough students in your laboratory section, your instructor will assign two substrate concentrations to each pair (of course, you must make a blank also—therefore every student pair will need three tubes). Work with three other pairs of students in your laboratory to construct a team.

3. Add phosphate buffer and catechol to all tubes, as indicated.
4. Set the spectrophotometer to 420 nm and adjust it to 100% transmittance and 0% absorbance.
5. Add 30 drops of diluted potato juice to your blank. Cover the tube with Parafilm, and invert it several times to mix the contents. Use the blank to readjust the spectrophotometer to 0% absorbance.

Tube	pH 7 Phosphate Buffer	Catechol
Blank	5 ml + 48 drops	0 drops
1	5 ml + 47 drops	1 drop
2	5 ml + 46 drops	2 drops
4	5 ml + 44 drops	4 drops
8	5 ml + 40 drops	8 drops
16	5 ml + 32 drops	16 drops
24	5 ml + 24 drops	24 drops
32	5 ml + 16 drops	32 drops
48	5 ml + 0 drops	48 drops

6. Make sure that you know who is processing (reading absorbance for) which experimental tubes before you proceed with the experiment.

7. Now add 30 drops of diluted potato juice to one of your experimental tubes. Mix and immediately record the absorbance in the appropriate "0 minutes" column in Table 10A-7. Record the absorbance reading every 2 minutes for 6 minutes.

Caution: If two student pairs are using one spectrophotometer, be sure that one pair *follows* the other and uses the appropriate blank. "Zero time" is critical and must be read immediately!

Table 10A-7 Effect of Substrate Concentration on Enzyme-Catalyzed Reactions

	Absorbance (420 nm)			
Tube	0 minutes	2 minutes	4 minutes	6 minutes
1				
2				
4				
8				
16				
24				
32				
48				

8. Repeat step 7 with your other experimental tube(s). Remember to use your blank to adjust the spectrophotometer if others have used the machine.

9. Using graph paper, plot absorbance on the *Y*-axis and time on the *X*-axis. Your graph should contain a line for each experimental tube.

b. Does the amount of enzyme (30 drops of diluted potato juice) ever limit the reaction? ________

If so, when? ____

c. If you increased the amount of enzyme as you increased substrate so that neither enzyme nor substrate was limiting, what would happen to the reaction? ____________________

____

d. What would the curve look like? ____________________

10. Now, determine V_0 (initial velocity) for each of your enzyme concentrations. V_0 is the slope of the line tangent to the initial portion of your curve. Record V_0 in the appropriate space in the following table. (Your classmates will provide the missing values for this table.)

Tube	Substrate Concentration	V_0
1		
2		
4		
8		
16		
24		
32		
48		

11. Using graph paper, plot V_0 vs. substrate concentration for all tubes. This will plot as a single reaction curve. Place V_0 on the Y-axis and substrate concentration on the X-axis.

e. Do your observed results indicate that the enzyme has a maximum velocity, V_{max}, *at which it will work?* ________ *Explain.* ____________________

____

12. Every enzyme has a K_m value that is a measure of its activity. K_m is defined as the substrate concentration at which the reaction velocity is $\frac{1}{2}V_{max}$. You can find the K_m for catecholase by drawing a horizontal line on your graph at $\frac{1}{2}V_{max}$. Where it intersects your curve, drop a vertical line to the X-axis. Where this line crosses the X-axis, you can determine K_m (Figure 10A-5).

For catecholase V_{max} = ______________ K_m = ______________

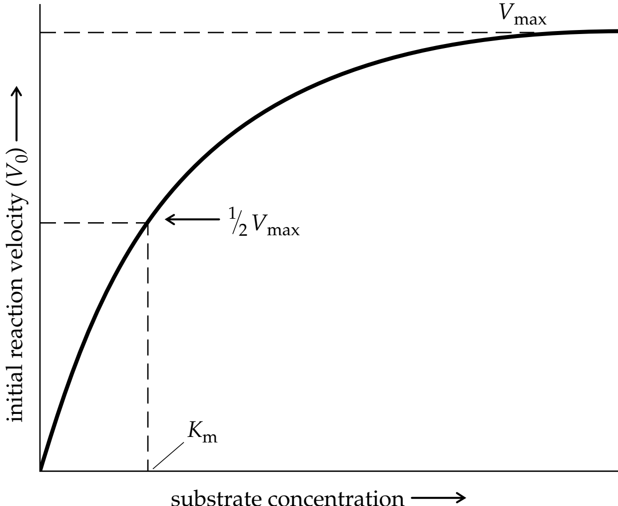
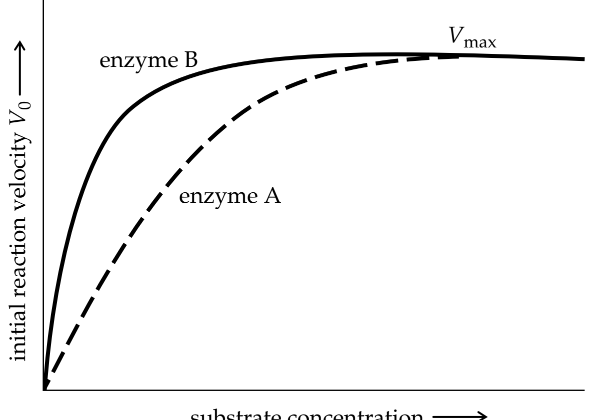


Figure 10A-5 *Determining* K_m *for an enzyme.*

Figure 10A-6



f. In Figure 10A-6, which enzyme (A or B) has the higher K_m? ________

g. Which enzyme has the fastest rate (steeper slope and higher V_0 *values at a given substrate concentration)?* ________ *Remember,* K_m *is a measure of affinity; does the enzyme with the fastest rate have the higher or lower* K_m*?* ________

h. From your answers, describe the relationship between the size of K_m *and the affinity of an enzyme for its substrate.* ____

13. Indicate the effects of substrate concentration on the rate of the catecholase reaction by writing the substrate values (1, 2, 4, 8, 16, and 24) at appropriate positions along the horizontal axis of Figure 10A-4.

Procedure (Qualitative)

1. Work in groups of four (two student pairs). Dilute the stock potato extract 1:1.

2. Label eight test tubes with the numbers indicated in the table below. Each student pair should fill four of the tubes with phosphate buffer and catechol, as indicated. Cover each tube with Parafilm and mix by inverting the tube several times.

Tube	pH 7 Phosphate Buffer	Catechol
1	5 ml + 47 drops	1 drop
2	5 ml + 46 drops	2 drops
4	5 ml + 44 drops	4 drops
8	5 ml + 40 drops	8 drops
16	5 ml + 32 drops	16 drops
24	5 ml + 24 drops	24 drops
32	5 ml + 16 drops	32 drops
48	5 ml + 0 drops	48 drops

3. Add 30 drops of diluted potato juice extract to each of the eight tubes. Cover each tube with Parafilm and mix by inverting the tube several times.

4. Incubate the mixtures for 5 minutes; mix each tube at 1-minute intervals.

5. The rate of the enzyme-catalyzed reaction (measured as the amount of product produced during a given period of time) will be proportional to the intensity of color developed in each reaction mixture. Record your results by indicating the intensity of color in each tube after 5 minutes (0 = no color; +, ++, and +++ = increasing intensity of color).

Intensity of Color							
1	2	4	9	16	24	32	48

b. Do your observed results support the idea that an enzyme has a maximum velocity at which it would work? ______ Explain. ______________________________

____

6. Indicate the effects of substrate concentration on the rate of the catecholase reaction by writing the substrate values (1, 2, 4, 8, 16, and 24) at appropriate positions along the horizontal axis of Figure 10A-4.

EXTENDING YOUR INVESTIGATION: MAKING JUICES JUICIER

Pectins are large polysaccharide molecules located primarily in the cell walls of plants. These molecules contribute to the sturdiness of plant cells. Pectin compounds are especially prevalent in fruits. When fruits are squeezed to make juices, pectins that remain in the cell walls help to keep particles of the fruit intact, as you may notice in the "lumpy" texture of orange or tomato juice. When pectin molecules are released into the juice during the process of squeezing, they help to hold the remaining particles in suspension and increase the juice's viscosity, a characteristic we recognize as the "thickness" or "body" of, for example, prune juice or fruit nectars. Pectins are added to fruit juices to make jellies and jams.

This thickening effect and the ability to keep particles in suspension are due, in part, to the size of the pectin molecules. They are large enough not to dissolve completely in water, but not so large and heavy that they exist as separate particles that separate easily from a liquid and sink, as would, say, particles of clay stirred into water.

Pectinases are enzymes that break down pectin molecules. In the commercial preparation of "clear" (as opposed to "homestyle") fruit juices, pectinases are often added to processed fruit to increase the amount of juice released and to allow particles of fruit to settle out of the liquid.

You will be given some applesauce and some pectinase. Formulate a hypothesis about the effect of pectinase on the applesauce.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will be the effect of pectinase on the applesauce?

Identify the **independent variable** in this investigation.

Identify the **dependent variable** in this investigation.

Now investigate the effects of pectinase on applesauce using the following procedure.

PROCEDURE:

1. Place approximately 25 ml of applesauce into each of two beakers, one labeled "no enzyme" and the other labeled "pectinase."
2. Add 0.5 ml of distilled water to the "no enzyme" beaker and 0.5 ml of pectinase to the "pectinase" beaker.
3. Use separate spatulas to stir the material in each beaker.
4. Let stand 10 minutes.
5. Place cheesecloth in a funnel and place the funnel into a graduated cylinder.
6. With the aid of the spatula, pour the contents of each beaker into a separate funnel and collect the filtrate.
7. Record the amount of juice collected in each cylinder after 5 minutes.

RESULTS:

Amount of juice collected without pectinase ______ ml

Amount of juice collected with pectinase ______ ml

Do your results support your hypothesis?

Your null hypothesis?

Was your prediction correct?

What do you **conclude** about the role of pectinase in making juices juicier?

EXERCISE B The Essentials of Cheesemaking

The enzyme **rennin** is used to coagulate the casein of milk during cheese production and serves as a good example of one of the many uses of enzymes. Although there is great variety among cheeses, the methods of production are basically very similar. Cheese is the solid portion (curd) of milk which has been separated from the liquid portion (whey). The sequence of procedures usually followed in cheesemaking is described below.

The Process of Cheesemaking

- Lactic acid-forming bacteria added to milk convert lactose to lactic acid, which sours the milk (25–30°C, 10–75 minutes). Amateur cheesemakers may use buttermilk as a starter (ripen for 4–12 hours).
- Coloring may be added for eye appeal. It has no effect on the quality of the cheese.
- Rennin (pH 5.5–6) coagulates ripened milk to form a precipitate (curd); liquid (whey) remains.
- Cut and stir—the smaller the curd, the harder and drier the cheese.
- Cook (38–39°C)

(continued on next page)

The Process of Cheesemaking (continued)

- Drain and salt.
- Press in cheesecloth-lined molds. Cheese may be inoculated with bacteria or mold to produce special cheeses.
- Seal in wax or treat periodically with brine or vegetable oil to keep contaminating organisms from growing on the surface.
- Cure and age to allow characteristic cheese flavor to develop.

Objectives

☐ Apply the principles of enzyme activity to cheesemaking.

Procedure

1. Work in pairs. Measure 250 ml of preripened milk (pH 5 to 5.6) into a beaker and *gently* warm it to 32°C.
2. Add 0.5 ml (10 drops) of Rennilase* to the milk while stirring continuously. Remove from the heat and leave *undisturbed* (without jarring) for 15 minutes or until the milk coagulates.
3. Using a glass rod, break up the curds and separate these from the whey. Filter the mixture through several layers of cheesecloth. Put the curds in a sterile Petri dish; add salt, pepper, or other seasoning to taste and mix. *Keep this preparation clean* if you wish to eat the cheese.

   a. At what pH and temperature is the action of rennin most effective in making cheese?

   ____

   b. Name two of your favorite types of cheese. What changes in the cheesemaking process do you think were required to produce each of these? ______________________________

   ____

   ____

   ____

   c. Dehydrated whey powder is a highly nutritious by-product of the cheese industry. Can you think of some uses for whey powder? ______________________________

   ____

Laboratory Review Questions and Problems

1. After discussing the results of all four experiments in Exercise A with your classmates, briefly summarize *your* conclusions on the effects of temperature, pH, enzyme concentration, and substrate concentration on the action of enzymes.

**Rennin is the enzyme that coagulates milk. It can be extracted in its crude form (rennet) from the lining of a portion of the cow's stomach (abomasum). Advances in genetic engineering, however, have allowed microbiologists to produce a bacterium having milk-coagulating properties. These bacteria can be grown in large numbers to produce greater amounts of the enzyme rennin. Rennilase is a trademark name for this product.*

2. What important consequences do these properties of enzymes (from question 1) have for the metabolism of cells and organisms?

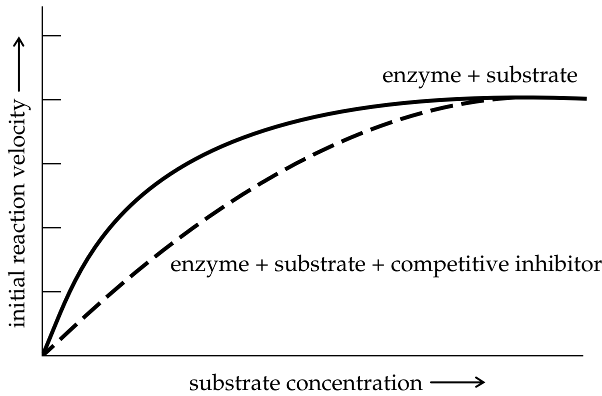
3. Define enzyme denaturation in terms of protein structure. What environmental factors can denature enzymes?

4. What does it mean to say that an enzyme-catalyzed reaction is either enzyme-limited or substrate-limited?

5. The K_m of an enzyme for a particular substrate is defined as the substrate concentration at which the reaction occurs at half its maximum rate. From Exercise A, Part 4, what would be the K_m (expressed here in drops) of catecholase? ____________________

6. The K_m usually, but not always, is inversely related to the strength of binding of a substrate to an enzyme. (In such cases $1/K_m$ is a measure of binding affinity.) Would an enzyme have lower or higher K_m values for more tightly bound substrates? ____________________

7. If a competitive inhibitor is introduced to a series of tubes in which enzyme and increasing amounts of substrate are mixed, the kinetics of the reaction would be as follows:



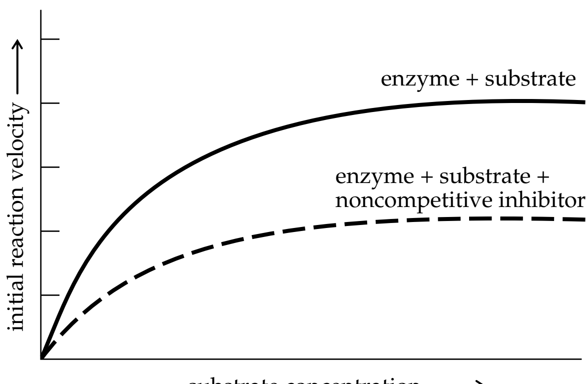
Competitive inhibitors bind to the active site of enzymes and compete with the substrate's ability to bind.

a. Is the K_m lower, higher, or the same for this reaction when a competitive inhibitor is added? ____________________

b. Explain why this is so. (*Hint:* Why is the maximum velocity the same for both reactions?)

c. How do the rates of the reactions compare?

8. If a noncompetitive inhibitor is added to tubes containing enzyme and substrate as above, the kinetics of the reaction would be as follows:



Noncompetitive inhibitors bind to areas on an enzyme other than the active site. They do not compete with the substrate, but affect reaction rate by changing the structure of the enzyme.

a. Is the K_m higher, lower, or the same for this reaction when a noncompetitive inhibitor is added? ____________________

b. Is the maximum velocity higher, lower, or the same? ____________________

c. Explain why this is so, based on what you know from your reading about the action of noncompetitive inhibitors.

Energetics, Fermentation, and Respiration

LABORATORY 11

OVERVIEW

Many metabolic reactions within the cell do not occur spontaneously but require a source of chemical energy in the form of ATP (adenosine triphosphate). The major source of ATP for most cells is the oxidation of glucose, a series of enzymatic reactions that results in the breakdown of carbon compounds into carbon dioxide, water, and energy.

The oxidation of glucose takes place in two major stages (Figure 11-1). The first is **glycolysis,** an **anaerobic** process (one that can proceed in the absence of oxygen).

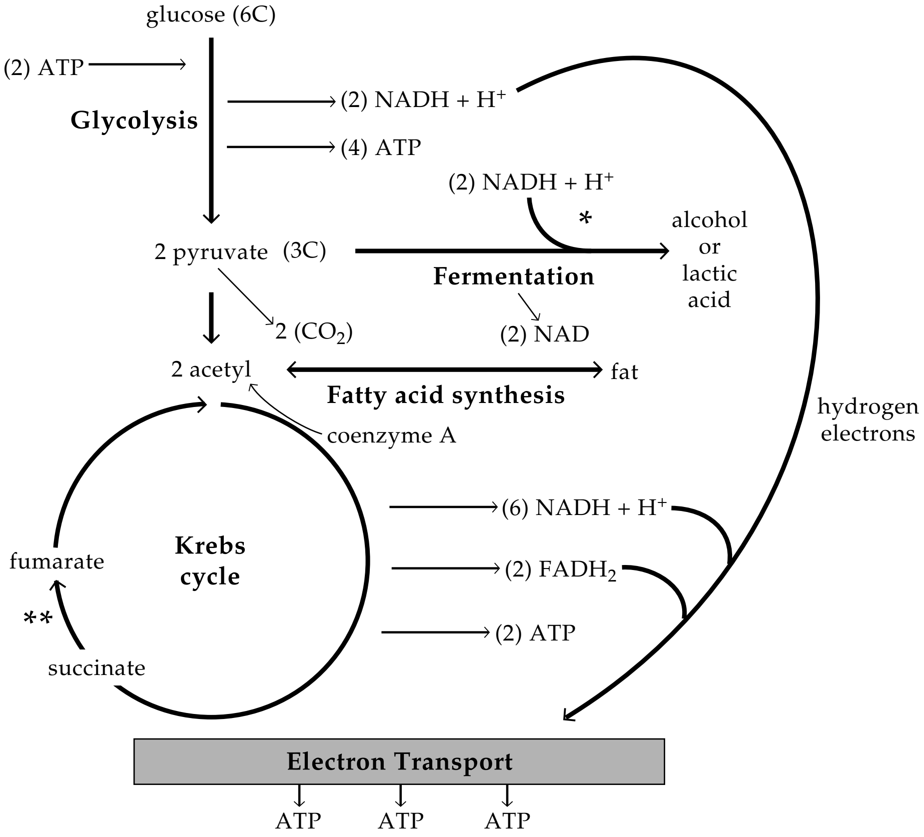


Figure 11-1 *Overview of cellular respiration.*

Glycolysis occurs in the cytoplasm of both **aerobic** (oxygen-requiring) and anaerobic organisms. The end product of glycolysis is pyruvic acid.

When oxygen is unavailable, the conversion of glucose to pyruvic acid is the major source of energy. In some organisms, such as yeasts, pyruvic acid can be further metabolized by a second series of reactions, the anaerobic process of **fermentation,** which results in the production of alcohol and carbon dioxide. Lactic acid can also be formed by the anaerobic metabolism of pyruvic acid. For example, when the oxygen supply available to muscle cells is depleted during strenuous exercise, lactic acid is produced. Eventually, as oxygen becomes available, lactic acid is used to resynthesize pyruvate.

When oxygen is available, the second stage in the oxidation of glucose is aerobic cellular respiration, which consists of the **Krebs cycle** (or *citric acid cycle*) and **electron transport.** These reactions, which take place in the mitochondria, greatly increase the energy harvest from the oxidation of glucose.

During this laboratory, you will study the processes of fermentation and respiration by making observations about the products of cellular reactions.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

✔ EXERCISE A Production of Carbon Dioxide and Ethanol by Fermentation

Yeasts are simple unicellular organisms related to mushrooms, molds, and mildews. They are called **heterotrophs** because they do not carry on photosynthesis, but obtain their food from outside sources such as grapes or grain. Yeasts are also classified as **facultative anaerobes**—they can live in aerobic or anaerobic environments. Under anaerobic conditions, yeasts carry out fermentation to produce alcohol and carbon dioxide. The alcohol in wine, beer, and other beverages is produced by the metabolic reactions of yeasts grown on grapes and grains such as barley.

$$\underset{\text{glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{ethanol}}{2C_2H_5OH} + \underset{\text{carbon dioxide}}{2CO_2} + \text{energy}$$

Very little net energy is produced during the process of fermentation—only two ATPs for every glucose molecule metabolized—but this is sufficient to sustain existing yeast cells.

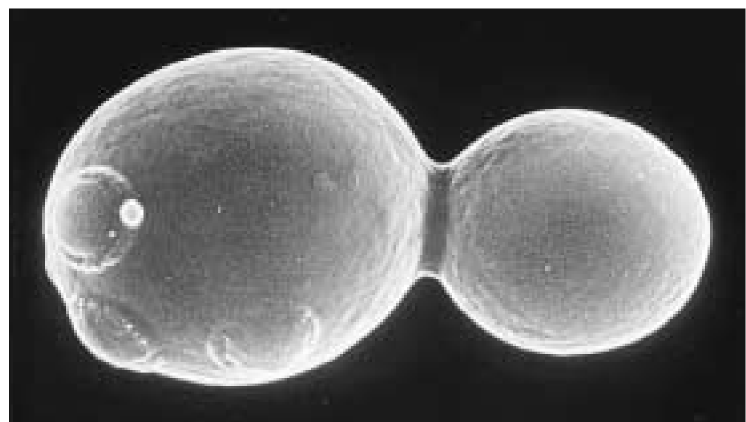
Objectives

- ☐ Describe the structure of a yeast cell with reference to the location of metabolic reactions involved in glycolysis and fermentation.
- ☐ Describe how to demonstrate carbon dioxide and ethanol production during fermentation.
- ☐ List the requirements for anaerobic fermentation

✔ PART I Examining Yeast Cells

Yeast cells are single-celled eukaryotes (Figure 11A-1). They can often be observed reproducing by budding.

Figure 11A-1 *Yeast cells. The cell on the right has almost completed the process of budding from the one on the left, which is in the process of producing three new yeast cells.*



Procedure

1. Using a Pasteur pipette, place several drops of the stock yeast solution onto the center of a microscope slide.
2. Add a drop of neutral red and apply a coverslip.
3. Use high power (40× objective) to observe the yeast.

   a. Why was the neutral red added to the yeast sample?

   ____

4. Under optimal conditions, yeast multiply by budding, a process involving an outpocketing of the cytoplasm and the subsequent pinching off of a new cell. Scan your slide slowly to see if you can detect cells with buds.

   b. How do you know that yeasts are eukaryotic, not prokaryotic? ____________________

   ____

   c. In what parts of the yeast cell does glycolysis take place? ____________________

PART 2 Production of Carbon Dioxide by Fermentation

Under anaerobic conditions, yeast cells break down sugars, releasing carbon dioxide gas. Evidence that fermentation is taking place in a yeast culture can be provided by bubbling the gas into an indicator solution.

Procedure

There are two flasks on demonstration: one contains a sugar and yeast solution and the other contains a 10% sucrose solution.

Each flask has a bent **U**-tube extending from the flask into a cylinder filled with a bromothymol blue indicator solution. If carbon dioxide passes from the **U**-tube into the cylinder, the solution in the cylinder will turn yellow.

a. In which flask is carbon dioxide being produced? ____________________

b. What is the source of the carbon dioxide? ____________________

c. As mixtures of grapes and yeast ferment, bubbles appear. What causes the formation of these bubbles?

____

PART 3 Requirements for Fermentation in Yeast

In this experiment, the class will study the role of yeasts as well as the food source requirements for fermentation. You will be assigned to one of four treatment groups by your laboratory instructor (Table 11A-1).

Table 11A-1

Group	Yeast	Boiled Yeast	Sugar	Water
I	10 ml	—	5% glucose	—
II	10 ml	—	5% sucrose	—
III	—	10 ml	5% sucrose	—
IV	10 ml	—	—	Distilled water

During the processes of glycolysis and fermentation, yeasts use sugars, but not all sugars are used at the same rate. Why do you think this might happen? Can you formulate a hypothesis about the requirements that must be met in order to maximize the rate of fermentation?

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will happen in the experiment (consider all treatments)?

What do you predict will happen in your treatment compared with other treatments?

What is the **independent variable** in this experiment?

What is the **dependent variable** in this experiment?

Now measure the fermentation rates for the four treatments by using the following procedure.

Procedure

1. In each assigned laboratory group, work in pairs. Obtain two plastic fermenting vials, one large and one small.
2. Add 10 ml of yeast suspension (boiled yeast suspension if you are assigned to Group III) to the smaller vial.
3. Finish filling the small vial to its brim with the sugar solution or with distilled water, as designated for your group.
4. Hold the small vial upright. Invert the large vial so that the bottom side is upward and lower it to cover the small vial.
5. Hold the two vials tightly together at their ends and invert the apparatus so that the small vial is now upside down within the larger vial (Figure 11A-2). As CO_2 is produced by the

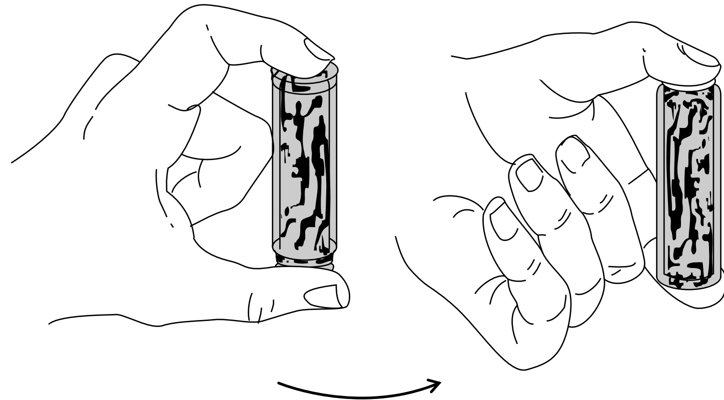


Figure 11A-2 *Fermentation apparatus.*

process of fermentation, it will collect in the upper portion of the small vial and the yeast mixture will be pushed downward and out into the larger vial.

6. If there is an air space at the top of the small vial, measure its length using a millimeter ruler and use this measurement as the zero point.
7. Measure the length of the gas column at 5-minute intervals for as long as possible (up to 45 minutes). Foaming within the large vial may obscure the length of the gas column. If you find it difficult to read, move the small vial until its side is pressed against the side of the outer vial.
8. Record your readings in Table 11A-2, and remember to subtract the value for the zero point from each reading.

Table 11A-2 Class Data for Yeast Fermentation Experiment, Recorded as Length of Gas Column (in millimeters)

	Group I					Group II					Group III					Group IV				
	Student Pair:					Student Pair:					Student Pair:					Student Pair:				
Minutes	1	2	3	4	Average	1	2	3	4	Average	1	2	3	4	Average	1	2	3	4	Average
0																				
5																				
10																				
15																				
20																				
25																				
30																				
35																				
40																				
45																				

9. Record the data for all teams (student pairs) in your group. Average the data for each time period and place all group data on the blackboard.

10. Copy the class data for other groups into Table 11A-2 and plot the data (using the averages) on graph paper. Always graph dependent variables along the vertical (*Y*) axis; in this case, "gas column (mm)"; "time (min)" should be plotted on the horizontal (*X*) axis. Be sure to label your graph. Use either colored pencils or different symbols to designate the four different groups.

11. Since the vials are uniform cylinders, the rate of increase in the length of the space filled by CO_2 is directly proportional to the rate of CO_2 production. To determine the actual rate, pick any two points on the straight-line portion of the curve (or a straight line approximated to the curve). Divide the difference in the gas column height (in millimeters) between the two points by the difference in time between the two points. The result will be the rate of CO_2 production in millimeters per minute.

    Millimeters can be converted to volume (milliliters) by using the formula for the volume (V) of a cylinder, $\pi r^2 h = V$, where r is the radius of the cylinder (in millimeters) and h is the height of the gas column (in millimeters). This will allow fermentation rates to be expressed as milliliters per minute. Record fermentation rates in Table 11A-3.

Table 11A-3

Treatment Group	Fermentation Rate (ml/min)
I	
II	
III	
IV	

a. What do the data from each group tell you about the process of fermentation?

Group I ______________________________

Group II ______________________________

Group III ______________________________

Group IV ______________________________

b. Why was Group IV included in this experiment? ______________________________

c. What is the effect of boiling the yeast (Group III)? ______________________________

d. Group III serves as a control for this experiment. What other setup could be included as a control for this experiment? ______________________________

Do your results support your hypothesis? ________ *Your null hypothesis?* ________

What do you ***conclude*** *about the role of yeasts in the process of fermentation?* ______________________________

____

____

What do you conclude about the importance of the type of sugar used as a food source for yeasts in the process of fermentation? ______________________________

____

✔ **PART 4** **Production of Ethanol by Yeast**

Some yeasts are more metabolically active than others and can produce more alcohol. Lugol's solution (I_2KI) can be used to test for metabolic activity in yeast. In this experiment, you will be looking at the **fermentation** reaction; see* in Figure 11-I.

Procedure

1. Obtain a yeast culture that has been incubating for 24 hours.
2. Using a 10-ml pipette, transfer a 5-ml sample of the clear solution from the yeast culture into a test tube.
3. Add 2 ml of 10% NaOH down the side of the tube and mix by gently shaking.
4. Slowly add 3 ml of Lugol's reagent (I_2KI), one drop at a time, while gently shaking the solution.
5. Allow the mixture to sit at room temperature for about 5 minutes.
6. Check for the presence of a yellow, flaky precipitate at the bottom of the vial. This layer contains iodoform, a compound produced when alcohol reacts with iodine.

   a. What is the source of the alcohol that reacts to form the iodoform layer?

   ____

EXERCISE B The Krebs Cycle Reactions in Bean Seeds

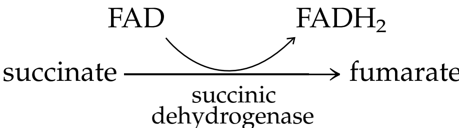
Within the mitochondria of aerobic organisms, pyruvic acid, the product of glycolysis, is further metabolized by a cyclic series of reactions called the **Krebs cycle** (or citric acid cycle). During the reactions of the Krebs cycle, special electron carriers (coenzymes FAD and NAD^+) are reduced (gain electrons) and a small amount of ATP is produced. Carbon dioxide is also produced.

It is the hydrogen electrons stripped from glucose and its breakdown products that are used to reduce the electron carriers FAD and NAD^+ to $FADH_2$ and $NADH + H^+$. When these reduced electron carriers are later oxidized by the electron transport chain, the released energy is used to phosphorylate ADP, thus regenerating ATP. The final electron acceptor of the electron transport chain is oxygen.

The Krebs cycle and electron transport reactions will occur only if oxygen is available to accept electrons. These pathways are part of the aerobic respiration process. The final products of respiration are carbon dioxide (CO_2), water (H_2O), and energy; all carbon-containing substrate molecules have been completely oxidized.

$$\underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{oxygen}}{6O_2} \longrightarrow \underset{\text{carbon dioxide}}{6CO_2} + \underset{\text{water}}{6H_2O} + \text{energy}$$

In this exercise, you will examine one of the reactions of the Krebs cycle—the conversion of **succinate** to **fumarate,** catalyzed by the enzyme succinic dehydrogenase, using the coenzyme FAD (flavin adenine dinucleotide) (see ** in Figure 11-I).



During this reaction, two hydrogen atoms are removed from succinate and are transferred to FAD, reducing it to $FADH_2$. For test purposes, certain dyes such as 2,6-dichloro-phenol-indophenol (DPIP) can function as hydrogen acceptors in place of FAD. DPIP is decolorized as it accepts hydrogen from succinate. The amount or rate of decolorization provides a means for observing and measuring the reaction.

Objectives

- ☐ List the requirements for the Krebs cycle reaction in which succinate is transformed to fumarate.
- ☐ Describe how DPIP can be used to study a reaction in which hydrogen is transferred from one compound to another.
- ☐ Describe how FAD functions as a coenzyme with succinic dehydrogenase.

Procedure

1. Work in pairs within groups of four. In this exercise, you will observe the decolorization of DPIP as it accepts hydrogen electrons from succinate (usually the function of FAD). As the DPIP becomes less blue, it will absorb less light (and consequently transmit more light). To observe the changes in absorbance and transmittance, you will use a spectrophotometer (the Spectronic 20).

Note: For a complete discussion of the operation of a spectrophotometer, review Laboratory 4. In this instrument, light is passed through a grating that, like a prism, separates it into bands of certain wavelengths. Colored indicator dyes such as DPIP absorb a maximum amount of light in the 600-nanometer (nm) wavelength range. If a sample is placed in the path of this light, a photocell on the other side of the sample will measure how much light has passed through (been transmitted). On separate scales, the instrument shows the percent of light transmitted and the percent of light absorbed.

2. Obtain 8 spectrophotometer tubes per group. Each pair of students will use 4 tubes.

3. Since you want to measure only decolorization of DPIP and not absorbance by other materials in your solution, a "blank" must be prepared. A blank contains all materials present in the sample except the one to be measured (DPIP). Each pair of students should prepare a blank as indicated in Table 11B-1. Make sure the contents are mixed by covering the tube with Parafilm and inverting it several times. With the machine empty, use the left-hand knob to set the spectrophotometer to 100% absorbance and 0% transmittance. Now insert the blank and use the right-hand knob to set the machine to 100% transmittance and 0% absorbance. Absorbance by DPIP alone can now be measured in the experimental tubes.

Table 11B-1

Tube	Lima Bean Juice	DPIP	Phosphate Buffer	Malonate	*Do not add until step 6:* Succinate
1	0.3 ml	0.3 ml	4.3 ml	—	0.1 ml
2	0.3 ml	0.3 ml	4.4 ml	—	—
3	0.3 ml (boiled)	0.3 ml	4.3 ml	—	0.1 ml
4	0.3 ml	0.3 ml	4.0 ml	0.3 ml	0.1 ml
5	0.3 ml	0.3 ml	4.1 ml	—	0.3 ml
6	0.3 ml	0.3 ml	3.8 ml	0.3 ml	0.3 ml
Blank	0.3 ml	—	4.6 ml	—	0.1 ml

4. Your instructor will homogenize and then centrifuge a solution containing 50 g of soaked lima bean seeds. The supernatant (the clear liquid remaining above the sediment) will contain isolated mitochondria. (One sample will be boiled for 5 minutes.)

5. To test tubes 1 through 6, add the reagents in the amounts indicated in Table 11B-1. *Do not* add succinate yet. Mix the contents of each tube by covering the tube with Parafilm and

inverting it several times. Use a *different* piece of Parafilm for each tube to avoid contamination. (After all ingredients have been added, all tubes should contain 5 ml of solution.)

Caution: If another group of students has used the Spectronic 20, use your blank to readjust the machine before making experimental readings. Remember, the blank tube contains all reagents except the dye (DPIP) and will allow you to zero the spectrophotometer to measure only those changes in color that result from changes in DPIP in the sample tubes.

6. Add succinate (see Table 11B-1) to one tube at a time (except tube 2). After adding the succinate, place a piece of Parafilm over the tube and invert the tube to mix in the succinate. Immediately read the absorbance at 600 nm. Do this for each of the three experimental tubes assigned to your student pair.
7. Read absorbances at 2-minute intervals and record your data in Table 11B-2. Take turns making absorbance readings. Remember to readjust the Spectronic 20 with your blank if necessary.

Table 11B-2 Absorbances of Bean Seedling Solutions

Minutes	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
0						
2						
4						
6						
8						
10						
12						
14						
16						

8. Graph your data on a sheet of graph paper. Place absorbance on the *Y*-axis and time (in minutes) on the *X*-axis. Use a different symbol or colored pencil to indicate each tube.
9. Discuss the results of your experiment. Describe what the data from each group show about the reaction succinate ⟶ fumarate.

Tube 1 ____________________

Tube 2 ____________________

Tube 3 ____________________

Tube 4 ____________________

Tube 5 ____________________

Tube 6 ____________________

a. How does this reaction indicate that the bean seedlings used in the test were alive?

____

b. How do the results in tube 3 support your answer to question a? ______________________

____

c. Which tube is used as a control? ____________

The malonate molecule is sufficiently similar in shape to the succinate molecule that it binds to the active site of the enzyme succinic dehydrogenase (Figure 11B-1). Succinic dehydrogenase cannot act on malonate. When malonate is bound to the enzyme, no $FADH_2$ or fumarate is produced. Thus, malonate is a competitive inhibitor of succinic dehydrogenase (Figure 11B-2).

Figure 11B-1 *Succinate and malonate are similar in shape, and both can bind to the enzyme succinic dehydrogenase.*

malonate: $^{-}O-C(=O)-CH_2-C(=O)-O^{-}$

succinate: $^{-}O-C(=O)-CH_2-CH_2-C(=O)-O^{-}$

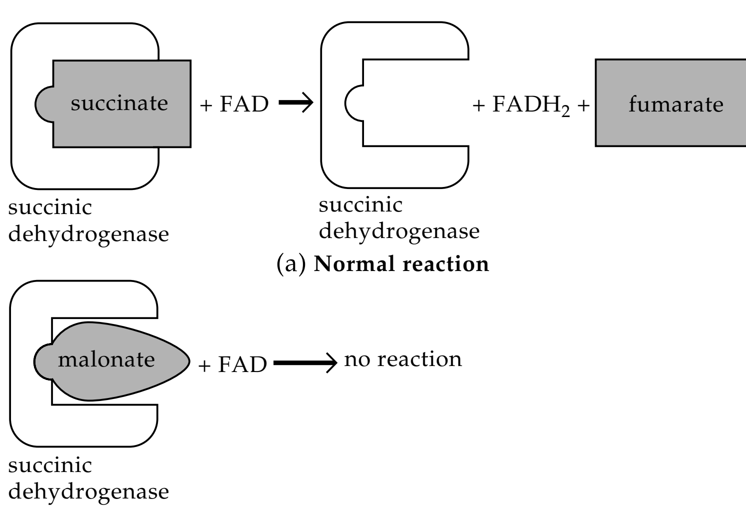


Figure 11B-2 *(a) The reaction of succinic dehydrogenase and its substrate, succinate. (b) When malonate occupies the active site for succinate in the succinic dehydrogenase enzyme, no reaction occurs.*

10. Now, describe the effects of malonate in your experiment. If succinate and malonate are both present in a mixture, they will compete for the enzyme's active site.

d. What did you observe in tube 4? ______________________

____

e. Based on what you know about competition, how do you explain your observations?

____

____

11. Compare your results for tube 4 with those for tube 6.

f. In which tube is more fumarate produced? ____________ *How do you know?*

____

g. *What is different about the contents of tubes 4 and 6?* ______________________

____

h. *How does increasing the amount of succinate affect malonate's ability to inhibit this reaction?*

____

12. From your textbook reading, explain the differences between the actions of competitive and noncompetitive inhibitors. Mercury (Hg) is a noncompetitive inhibitor of succinic dehydrogenase.

i. *How would Hg have affected the enzymatic breakdown of succinate by succinic dehydrogenase?*

____

____

j. *If you had included Hg (mercuric chloride, or $HgCl_2$) in any of the reaction mixtures, would you have expected to see DPIP decolorize?* ______ *Why or why not?* ______________________

____

k. *If you increased the amount of succinate 10-fold in the presence of $HgCl_2$, would you expect to see DPIP decolorize?* ______ *Why or why not?* ______________________

____

EXTENDING YOUR INVESTIGATION: STUDYING INHIBITION

How would changing the amounts of succinate and malonate change the activity of succinic dehydrogenase? If 10 times more succinate than malonate is available, what is the chance that succinate will combine with the enzyme before malonate? What would be the consequence of this? Do you think it would be possible to overcome competitive inhibition? Formulate a hypothesis to explain the interaction of malonate and succinate when combined in different ratios with succinic dehydrogenase.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** might happen as the succinate/malonate ratio increases?

What is the **independent variable** in this investigation?

What is the **dependent variable** in this investigation?

Design an experiment to test your hypothesis. Follow the same type of procedure as in Exercise B, combining lima bean juice, DPIP, phosphate buffer, succinate, and malonate.

PROCEDURE:

RESULTS: Record your results in the table below. What do the data collected from your experiment tell you about the competitive inhibitor, malonate?

Minutes	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
0						
2						
4						
6						
8						
10						
12						
14						
16						

Do your results support your hypothesis?

Your null hypothesis?

Was your prediction correct?

What do you **conclude** about the effect of competitive inhibitors on enzymatic reactions?

✔ EXERCISE C Heat Production During Respiration in Seedlings

When respiration occurs, energy is produced. Some of this energy is used to produce ATP and some is given off as heat.

Objectives

☐ Detect respiration in growing tissues by measuring heat generation.

Procedure (*Class Demonstration*)

1. Your instructor has placed some moist cotton into the bottom third of an insulated pint bottle (Thermos) (Figure 11C-1). The remainder of the bottle has been filled with germinating seeds presoaked in water.

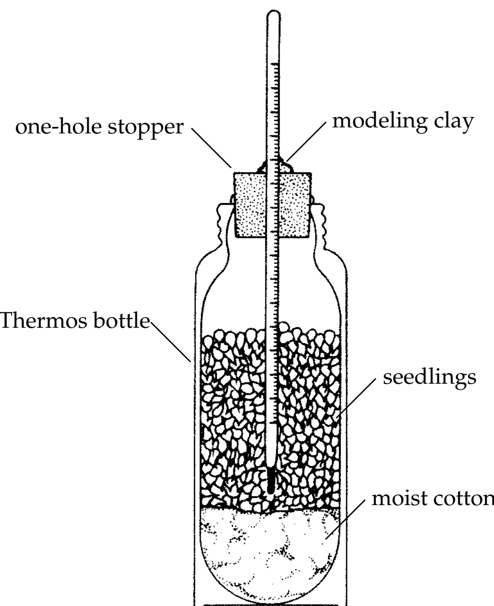


Figure 11C-1 *Setup for measuring the production of heat by seedlings in a Thermos bottle.*

2. A thermometer has been inserted so that its tip extends into the mass of seeds.
3. In the table that follows, record the time, the temperature inside the Thermos, and room temperature. Record these data several times during the laboratory period.

Time	Thermos Temperature	Room Temperature

a. Do your temperature readings indicate that the seeds are respiring? _______ *How do you know?*

____

b. What source of energy are the germinating seedlings using? _______________________

c. What influence might small increases in temperature have on respiration rates?

_______________________ *Large increases in temperature?* _______________________

d. Explain your answers to question c *in terms of your knowledge of enzyme structure and function.*

____

e. How is metabolic heat useful to plants and animals in their natural environments?

____

____

The heat production you have measured shows that respiration is not 100 percent efficient. When 1 mole of glucose (the molecular weight in grams) is oxidized in a respiring cell, approximately 434 kilocalories of useful energy are generated in the form of ATP. In contrast, the complete oxidation of glucose yields 687 kilocalories of energy measured as heat.

f. Using these figures, calculate the efficiency of respiration as a percent. ______

✔ EXERCISE D | Respiration in Plant Embryos

Several tests are used by the agricultural industry to check the viability of seeds before planting. One of these tests involves the use of the dye tetrazolium, which is colorless when oxidized but becomes reddish when reduced. The test relies on whether or not the seed's electron transport system is working. When tetrazolium is added to a living cell, it will interact with the electron transport system to accept hydrogen electrons as they are transferred from the cytochromes. When tetrazolium accepts these electrons, it is reduced and turns red or a deep pink. If the seed is dead, the electron transport system will not be functioning, no hydrogens will be available to reduce the tetrazolium, and the seed will remain colorless.

Objectives

- ☐ Explain how the indicator TTC (2,3,5-triphenyl tetrazolium chloride) can be used to determine whether seeds are viable.

Procedure

1. On the demonstration table you will find two groups (A and B) of seeds (beans or corn). One lot has been boiled and is dead. The other is alive. Test the two groups of seeds for viability. Cut three seeds from each group in half along their long axis with a sharp razor blade (Figure 11D-1).

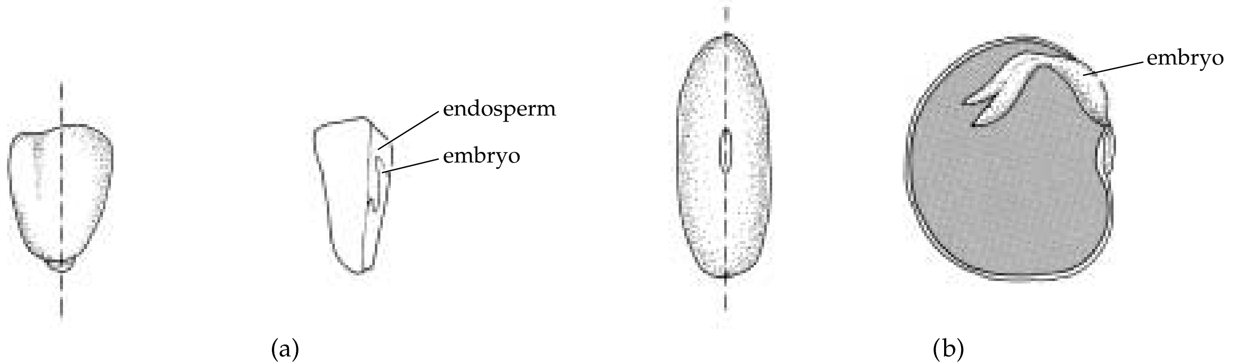


Figure 11D-1 *Seeds cut to show embryo. (a) Corn. (b) Bean.*

2. Compare your cross section of the seed with that shown in Figure 11D-1 to be sure that you have exposed the embryo. Locate the embryo, endosperm (food for the growing seed), and seed coat of your seeds.

   a. Which tissue would you expect to stain red after application of the TTC reagent?

   ____

3. Place several drops of TTC into two Petri dishes. Label one Petri dish for the A group of seeds and another for the B group. Place the seed halves with their cut side down in the Petri dishes; add enough TTC to completely cover the seeds.

   Caution: TTC is a poison; avoid contact with skin. Wash immediately if you come into direct contact with TTC.

4. At the end of the laboratory period, turn the seed halves over and examine them. Use forceps!

   b. Which group of seeds is alive and respiring? _______ *How do you know?* ____________________

   ____

Cyanide affects the electron transport system by binding to components of the system (the cytochromes), thereby inhibiting the transfer of electrons.

   c. If seeds were treated with cyanide, what results would they show in the tetrazolium test?

   ____________________________ *Explain.* ____

   ____

   ____

Laboratory Review Questions and Problems

1. Where in the cell do the following processes occur? glycolysis ______________

   fermentation ______________ Krebs cycle ______________ electron transport ______________

2. Why is fermentation considered to be an inefficient process?

3. Why is the process of fermentation called anaerobic?

4. Complete the following chart.

	Requirements	Products	Final Electron Acceptor
Alcoholic fermentation			
Lactic acid fermentation			
Aerobic respiration			

5. You have just finished running a 3-minute mile. Your legs are cramping badly and you are breathing very rapidly. How could heavy breathing be beneficial?

6. You have three unlabeled solutions—A, B, and C. You know that one is an enzyme solution, another is a solution of that enzyme's substrate, and the third contains an inhibitor of enzyme activity. You know that when the enzyme and the substrate react, the solution will turn red. You mix various amounts of the three solutions. From the results, determine the identities of solutions A, B, and C. Is the inhibitor competitive or noncompetitive?

Reaction	Buffer (ml)	Solution A (ml)	Solution B (ml)	Solution C (ml)	Color
1	0.4	0.1	0.1	0.1	Medium pink
2	0.5	—	0.1	0.1	Red
3	0.3	0.2	0.1	0.1	Light pink
4	0.4	0.2	—	0.1	Colorless
5	0.3	0.1	0.2	0.1	Dark pink
6	0.5	0.1	0.1	—	Colorless
7	—	0.1	0.4	0.2	Red

7. Ten glucose molecules are broken down during glycolysis. How many pyruvate molecules are produced? ______ Each of the 3-carbon pyruvate molecules can yield a 2-carbon acetyl fragment after CO_2 is removed. How many acetyl compounds can be produced from the 10 glucose molecules? ______ If these are used to produce a fatty acid, how many carbons would be in the fatty acid chain? ______ Draw the fatty acid in the space below (see Laboratory 5).

8. A farmer has a large bag of pea seeds left over from last year's planting. He would like to save some money by planting the seeds, but is not sure that a sufficient percentage of the seeds remains viable. How could he determine what percentage of the seeds would be expected to germinate?

LABORATORY

12

Photosynthesis

OVERVIEW

During the process of **photosynthesis,** plants capture a small fraction of the sun's energy and store it in the chemical bonds of carbohydrates. The carbon source for the organic compounds is the inorganic atmospheric gas carbon dioxide, which is reduced by the addition of electrons from H_2O to form carbohydrate ($C_6H_{12}O_6$). Oxygen (O_2) is released when water is "split" to provide hydrogen elections ($2H_2O \longrightarrow 4H + O_2$). The general formula for photosynthesis is:

$$\underset{\text{carbon dioxide}}{6CO_2} + \underset{\text{water}}{6H_2O} + \underset{\text{light energy}}{686 \text{ kilocalories}} \longrightarrow \underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{oxygen}}{6O_2}$$

Photosynthesis is the source of virtually all energy used by organisms (with the exception of a few chemosynthetic organisms); photosynthesis is the only method by which chemical energy is added to the ecosystem. As we examine the factors involved in energy capture and storage, remember that cells that are photosynthesizing are also carrying on cellular respiration, as are all animal organisms. Cellular respiration uses the O_2 produced by photosynthesis to break down glucose. Energy trapped in the chemical bonds of glucose is released for cellular work, and carbon is returned to the atmosphere in the form of CO_2. This CO_2 serves as a carbon source for further photosynthesis (Figure 12-1).

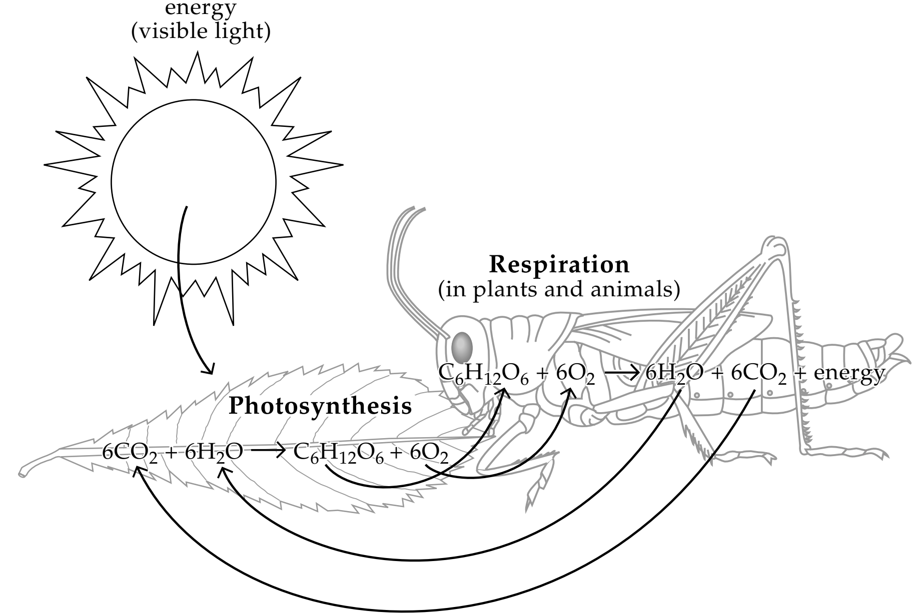


Figure 12-1 *Note that the product of photosynthesis, glucose, is the starting reactant for respiration. The photosynthetic reactions begin with water and carbon dioxide. Thus, with the addition of energy from sunlight, water and carbon dioxide are constantly recycled into higher-energy glucose molecules. The energy-requiring processes of life often obtain energy by breaking down glucose.*

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

EXERCISE A | The Energy-Capturing Reactions

Photosynthesis takes place in two stages. The first, the energy-capturing reactions, called **light-dependent reactions,** must take place in light. The light-dependent reactions are summarized by the equation:

$$H_2O + ADP + P_i + NADP^+ \xrightarrow[\text{chloroplasts}]{\text{light}} \tfrac{1}{2}O_2 + ATP + NADPH + H^+$$

Light energy is used to split water and energize the electrons of chlorophyll molecules. Some of this energy is then captured and used to form ATP and NADPH; O_2 is released as a by-product. You can determine the rate of this reaction by measuring the amount of product made, in this case O_2. Both light intensity and light quality (color or wavelength) affect the rate of the energy-capturing reaction.

Objectives

- ☐ Explain how to measure the rate of photosynthesis.
- ☐ Discuss the effect of light intensity on photosynthesis.
- ☐ Discuss the effect of light quality (wavelength) on photosynthesis.

PART I Determining the Effect of Light Intensity

The rate of photosynthesis can be affected by the amount of light available to plants. Since oxygen is a product of photosynthesis, measuring its production allows us to measure the rate of photosynthesis.

In this exercise, you will work with small pieces (disks) of spinach leaf tissue. The leaf tissue is riddled with gas-filled intercellular spaces. If leaf disks cut from spinach leaves are placed in a sodium bicarbonate ($NaHCO_3$) solution and subjected to a vacuum, sodium bicarbonate will replace the gases in the intercellular spaces and will serve as a carbon source for photosynthesis. Replacement of gas by liquid will cause the disks to sink to the bottom of the flask. As the light-dependent reactions of photosynthesis proceed, oxygen gas will be evolved and will diffuse into the intercellular spaces. When enough oxygen accumulates in the intercellular spaces, each leaf disk will regain its buoyancy and turn on edge or float to the surface. Thus, by observing the flotation of spinach leaf disks, you can indirectly measure oxygen production as an indicator of photosynthetic activity. An increase in the rate of photosynthesis should result in an increase in the number of floating disks.

Formulate a hypothesis about the effects of different intensities of light on photosynthesis.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will happen in an experiment used to test your hypothesis?

What is the **independent variable** in your experiment?

What is the **dependent variable in** your experiment?

Now, determine the effects of light intensity on photosynthesis by using the following procedure.

Procedure

1. Work in pairs. Each pair will be assigned a light bulb of a different wattage: 40, 60, 100, or 150 W. Several student pairs will use no light.
2. Place the bulb in the flood light assembly and adjust the light so that it is 27.5 cm above the base of the ring stand. The light intensities delivered from this height (W/m^2) will differ with the wattage of the bulb being used (see Table 12A-1).

Table 12A-1

Bulb (W)	Intensity (W/m^2)
None	0.000
40	0.036
60	0.064
100	0.100
150	0.154

3. Using a cork borer, cut 50 disks from spinach leaves. Avoid cutting through areas with large veins. Cut leaves on a paper towel or Styrofoam board. Immediately put the disks into your instructor's 500-ml flask (the flask contains $NaHCO_3$). All groups should place their disks in the same flask.
4. Your instructor will use a water aspirator to sink the disks in a vacuum, using the following procedure:
   - Attach a piece of vacuum tubing to the sidearm. Put a rubber stopper firmly in the mouth of the flask, and press a piece of tape securely over the hole of the stopper.
   - Turn the water to full force. When the solution begins to bubble, release the vacuum by peeling back the tape.
   - Swirl the flask, and apply the vacuum again.
   - Repeat this procedure three or four times until most of the disks fall to the bottom of the flask when the vacuum is released. As the solution infiltrates the disks, they will become a darker green.

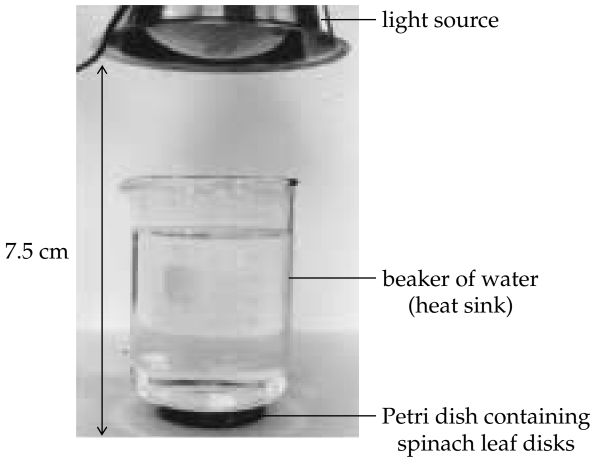


Figure 12A-1 *Setup for experiment demonstrating the effects of light intensity on photosynthesis. Adjust the space between the lamp and the Petri dish to 27.5 cm. (This setup, using different lamp-to-Petri-dish distances, will also be used for Exercise A, Part 3.)*

5. During this time, obtain a Petri dish with a clear lid (the dish is wrapped with black tape on the bottom to exclude excess light). If you are assigned to the "no light" treatment, you will use a Petri dish with both top and bottom covered with black tape. Fill the Petri dish approximately two-thirds full with fresh 0.2% $NaHCO_3$ solution.
6. Turn off the room lights and, using forceps, gently transfer 20 sunken spinach disks to your Petri dish and cover the dish—replace any floating disks until all 20 are sunken disks.
7. Center the Petri dish on the base of the ring stand under the light and cover the dish. If you are using no light, leave the dish on the laboratory table or place it in a dark drawer.
8. Place a 2-liter beaker, filled to the 1,600-ml mark with tap water, on top of the Petri dish to act as a heat filter (see Figure 12A-1).
9. Turn on the light and record the time. After 10 minutes, count the number of spinach disks that are floating. Record your results below.

Treatment	Number of Disks Floating (10 minutes)

10. When all groups have finished, record the class data and compute and record the average (arithmetic mean) for each light intensity in Table 12A-2.

Table 12A-2 Effects of Varying Light Intensities on Photosynthesis, Measured as Number of Floating Disks

	Group						
Intensity (W/m^2)	1	2	3	4	5	6	Mean
0.000							
0.036							
0.064							
0.100							
0.154							

11. Graph your data as percent of floating disks: (average/20) × 100. Make a bar graph using the grid in Figure 12A-2.
    a. *Why is it important to use mean values when making the graph:?*

    ____

    ____

Do your results support your hypothesis? _______ *Your null hypothesis?* _______

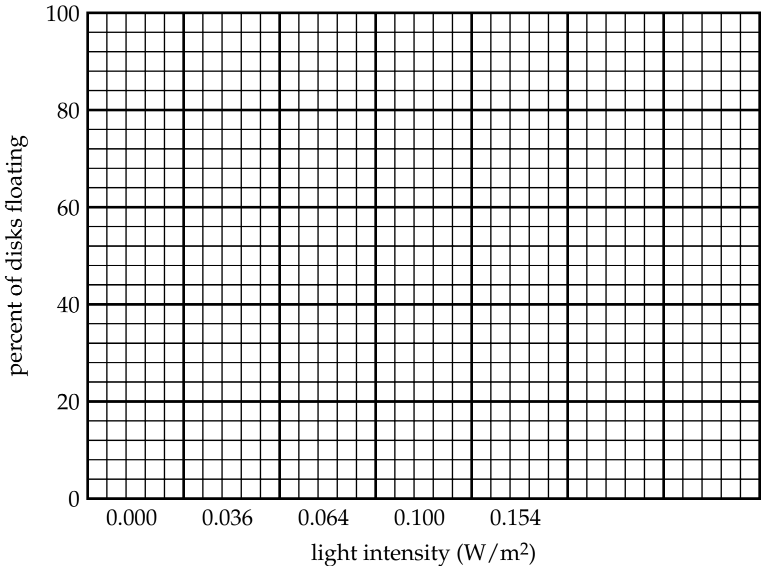
From your graph, what do you ***conclude*** *about the effects of light intensity on the rate of photosynthesis?*

____

____

Is the effect of increasing light intensity significant? To determine this, your instructor will collect data from all laboratory classes and will use these data to perform a chi-square median test. The results will assist you in determining whether to accept or reject your null hypothesis.

Figure 12A-2



✔ PART 2 The Spectrum of Visible Light

White light is composed of all the wavelengths (colors) in the spectrum of visible light (Figure 12A-3). These wavelengths are measured in nanometers. We see colors because objects contain **pigments** that selectively absorb some wavelengths of visible light and reflect or transmit others. What we recognize as an object's color is composed only of those wavelengths of light that are transmitted or reflected.

The various pigments found in chloroplasts—including chlorophylls *a* and *b,* xanthophylls, and carotenes—absorb different wavelengths of light, thus making use of a wide range of light energy for photosynthesis.

Procedure

1. Use the prism on the demonstration table to project a spectrum onto a piece of white paper.

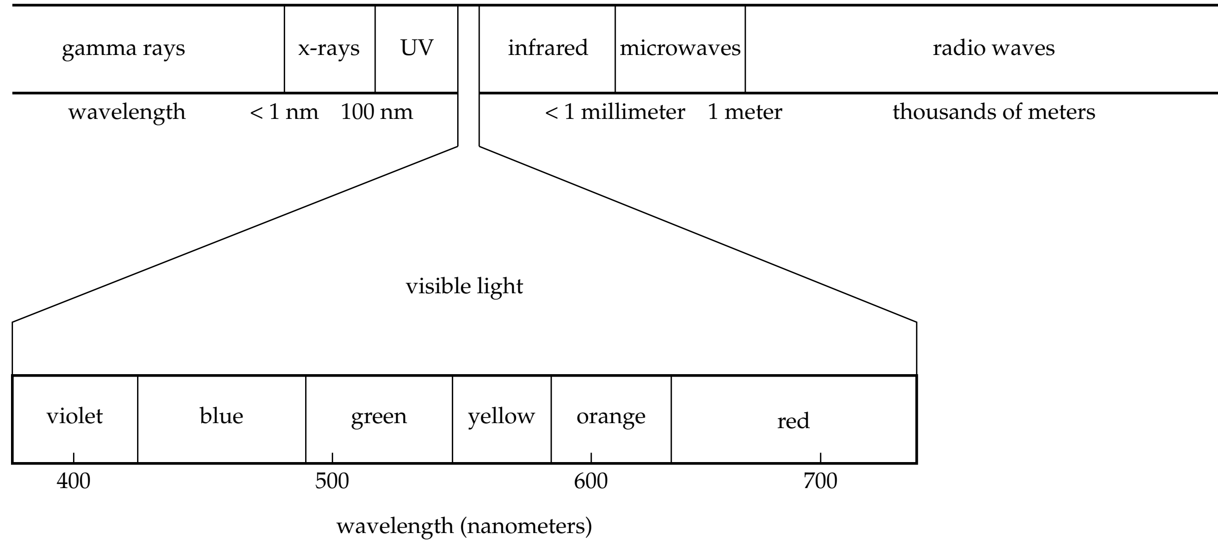


Figure 12A-3 *The spectrum of white light.*

2. Hold various colored filters between the light source and the prism. What do you predict will happen? Complete Table 12A-3.

Table 12A-3 Absorption and Transmission Properties of Colored Filters

Filter Pigment	Colors Absorbed	Colors Transmitted
Blue		
Green		
Red		

a. *Why do green plants appear green? (What colors of light would you expect plants to absorb or reflect?)* ______________________________

____

b. *Why does a black piece of material appear black?* ______________________________

____

PART 3 Determining the Effects of Light Quality—The Action Spectrum

An **action spectrum** defines the relative effectiveness of different wavelengths of light (colors) for light-dependent processes such as photosynthesis. In this experiment, you will test the effects of colors of light on photosynthesis in sections of spinach leaves.

As in Exercise A, Part 1, spinach leaf disks placed in sodium bicarbonate ($NaHCO_3$) and subjected to a vacuum will sink as the solution replaces the gases trapped in the intercellular spaces. In the presence of different colors of light, photosynthesis will produce varying amounts of oxygen. Accumulation of oxygen in the leaf disks will cause them to float.

You will test the effects of different wavelengths of light by placing the spinach leaf disks into Petri dishes, each covered by a filter that transmits only certain wavelengths of light. If some colors of light are more effective than others in promoting the reactions of photosynthesis, there should be a difference in the rate at which the leaf disks rise to the surface. These data can then be used to generate an action spectrum for photosynthesis.

Formulate a hypothesis that addresses how different wavelengths of light affect the process of photosynthesis in spinach leaf disks.

HYPOTHESIS:

NULL HYPOTHESIS:

What would you **predict** will occur in an experiment designed to test your hypothesis?

What is the **independent variable** in your experiment?

What is the **dependent variable** in your experiment?

Use the following to carry out your investigation.

Procedure

1. Work in pairs. Each pair should obtain one of five specially prepared Petri dishes. The bottoms of the Petri dishes are wrapped in black tape to eliminate extra light. The tops are covered with colored filters (clear or none, blue, green, or red), or with black tape if you are assigned to the dark treatment). Fill your dish two-thirds full with the 0.2% $NaHCO_3$ solution. Place your dish on the stand portion of a ring stand. (Students assigned to a "dark" treatment will place dishes in the dark in a drawer.)
2. Attach a 150-W photoflood lamp to your ring stand. Because filters absorb different amounts of light, adjust the lamp-to-Petri-dish distance to make all light intensities equal as follows: no filter (white light), 27.5 cm; blue, 23.5 cm; green, 21.0 cm; red, 26.5 cm (dark, in box or drawer) (see Figure 12A-1).

3–8. Follow steps 3–8 in Exercise A, Part 1, substituting (in step 5) Petri dish lids with colored filters for clear Petri dish lids, when assigned.

9. Turn on the light and record the time. After 10 minutes, count the number of spinach disks that are floating and record your results below.

Treatment	Number of Disks Floating (10 minutes)

10. When all groups have finished, record the class data and compute and record the average (mean) for each color (wavelength) of light in Table 12A-4.

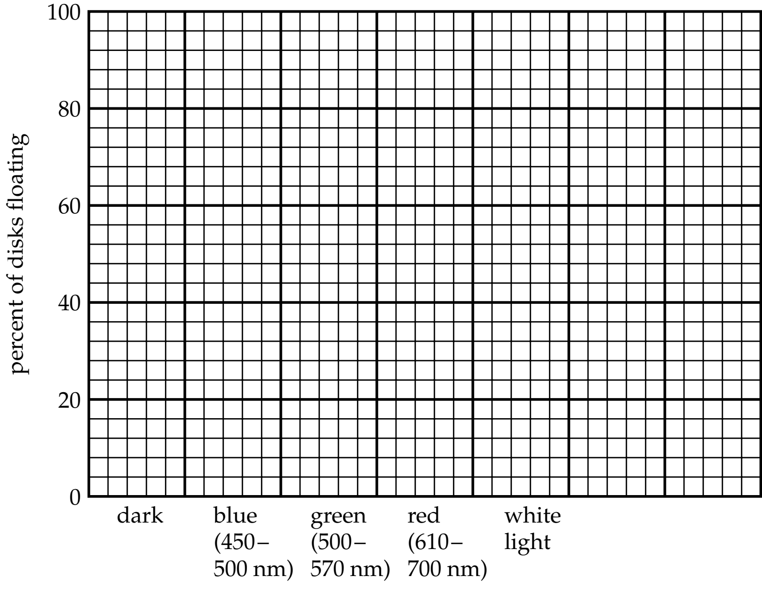
Table 12A-4 Effects of Different Wavelengths of Light on Photosynthesis, Measured as Number of Floating Disks

	Group						
Wavelength (λ)	1	2	3	4	5	6	Mean
White							
Blue							
Green							
Red							
Dark (no light)							

11. Graph your data as percent of floating disks: (average/20) × 100. Make a bar graph using the grid in Figure 12A-4, then superimpose an action spectrum curve along the tops of all the bars in your graph.

    a. *Why is this called an* action *spectrum?* ______________________

Figure 12A-4



Do your results support your hypothesis? ________ *Your null hypothesis?* ________

b. Which wavelengths of light are most effective in promoting photosynthesis? ____________________

c. Which are least effective? ____________________

From your graph, what do you ***conclude*** *about the effects of different colors of light on the rate of photosynthesis?*

____

Is the difference in photosynthetic rate at specific wavelengths significant? To determine this, your instructor will collect data from all laboratory classes and will use these data to perform a chi-square median test. The results will assist you in determining whether to accept or reject your null hypothesis.

EXERCISE B | The Pigments of Chloroplasts

Chlorophyll is the pigment responsible for the green color of plants. However, plants also contain pigments that absorb wavelengths of light other than those used by chlorophyll. *From your results in Exercise A, Part 3, why would this be necessary?* ____

Objectives

- ☐ Define "pigment." Explain the meaning of "absorption" and "transmission" of light by pigments.
- ☐ Describe the purpose and technique of chromatography.
- ☐ Name the pigments present in green plants.
- ☐ Explain how a spectrophotometer (Spectronic 20) works (*optional*).
- ☐ Using plant pigments as an example, explain what an absorption spectrum shows.
- ☐ Using photosynthesis and plant pigments as an example, discuss the relationship between absorption and action spectra.
- ☐ Give evidence that light is necessary for chlorophyll formation.

PART 1 How Plant Pigments Use the Light Spectrum

The process of **chromatography,** which separates complex mixtures into their component parts based on their solubility in different kinds of solvents, can help identify some of the pigments used in photosynthesis. Acetone has been used to extract pigments from spinach leaves.

Procedure

1. Obtain a piece of chromatograph paper precut to fit a large test tube and, using a *pencil,* draw a baseline 1.5 cm from the bottom or pointed end (Figure 12B-1a). (Try not to touch the paper because oil from your skin will interfere with the chromatography process.)
2. Use a capillary pipette to streak chlorophyll extract along the pencil line. The streak should be as thin as possible. (Dry with a hair dryer if available.) Reapply the chlorophyll extract at least 5 more times, keeping the pigment line as narrow as possible. (Alternatively, obtain a large spinach leaf or ivy leaf. Place the leaf with its lower surface down across the chromatography paper. Roll a dime or quarter across the leaf, pressing hard, to make a green pigment line across the paper as close to your pencil line as possible. Roll the coin back and forth to squeeze out as much pigment as possible.)

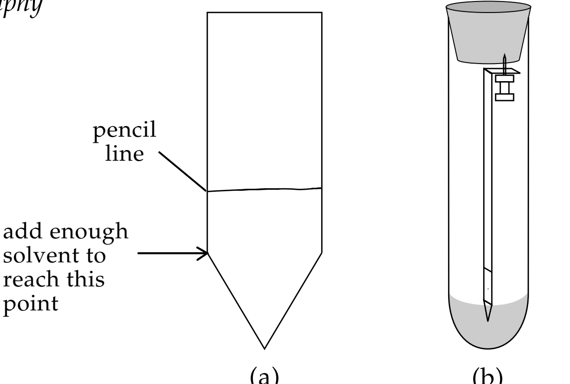
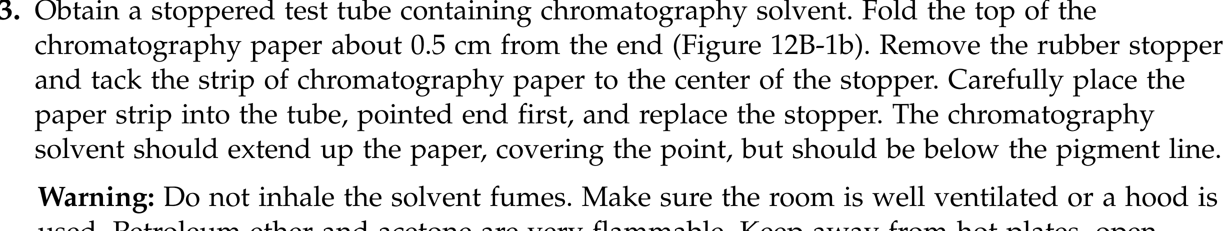


Figure 12B-1 *Setup for chromatography of plant pigments.*



3. Obtain a stoppered test tube containing chromatography solvent. Fold the top of the chromatography paper about 0.5 cm from the end (Figure 12B-1b). Remove the rubber stopper and tack the strip of chromatography paper to the center of the stopper. Carefully place the paper strip into the tube, pointed end first, and replace the stopper. The chromatography solvent should extend up the paper, covering the point, but should be below the pigment line.

   Warning: Do not inhale the solvent fumes. Make sure the room is well ventilated or a hood is used. Petroleum ether and acetone are very flammable. Keep away from hot plates, open flames, or other sources of high heat.

4. Place the tube into a test tube rack and do not disturb it while the chromatography procedure continues.
5. Allow the solvent to rise up the paper until it is approximately 1 cm from the top of the paper. Remove the paper and mark the location of the solvent front (Figure 12B-1c) using a pencil.
6. After the chromatogram has dried, use a pencil to circle each pigment spot. Record the color of each pigment spot and the distance the pigment has traveled from the origin. Use the center of the spot as your point of reference. The colors will fade quickly, so make your observations immediately.

Two kinds of green chlorophylls can be observed. Chlorophyll *a* is a bluer-green than chlorophyll *b,* which appears yellow-green. Chlorophyll *b* is found closer to the origin. Also present are two types of yellow-to-orange pigments called carotenoids; carotenes are orange-yellow and xanthophylls are yellow.

All four of the pigments you observe are found in the thylakoid membranes of chloroplasts. Both chlorophylls *a* and *b* consist of a large ring component (a tetrapyrrole ring, like that of hemoglobin), associated with a magnesium ion (Mg^{2+}). A long, nonpolar hydrocarbon chain attached to the ring anchors the charged ring portion of the pigment molecule to the nonpolar lipid layers of the thylakoid

membranes. The carotenes and xanthophylls are pure hydrocarbon chains, with alternating single and double bonds between carbons. Both xanthophylls and carotenes are very nonpolar and are embedded in the thylakoid membranes.

Recall that the chromatography solvent is nonpolar. Only nonpolar materials dissolve in nonpolar solvents. The more nonpolar a pigment is, the further it will travel with the solvent front (leading edge of the solvent traveling up the paper). The more polar a material is, the greater will be its tendency to stay in place, tightly bound to the water in the paper. In this way, the photosynthetic pigments become partitioned on the chromatography paper.

a. *Which pigment appears to be the most nonpolar?* ______________________

b. *Which pigment is the most polar?* ______________________

c. *What general structural feature of chlorophylls* a *and* b *causes them to remain close to the origin? (Hint: Charged ions are polar and hydrophilic.)* ____

d. *How many of the pigments present are chlorophyll?* ______

e. *How many of the pigments present are carotenoids?* ______

7. Draw a representation of your chromatogram below. Label the pigments.

PART 2 Absorption Spectra of Chloroplast Pigments

Having isolated and identified the pigments found in chloroplasts, you can now determine the wavelengths of light transmitted by each pigment and then by all the pigments in the chloroplast extract.

Using a spectrophotometer (Spectronic 20), white light will be passed through a grating, which, like a prism, separates light into bands of specific wavelengths. You select a wavelength—for example, in the green range—to pass through the chlorophyll extract. Green light shines on the sample, and a photocell on the other side of the sample measures how much light has passed through the sample (has been transmitted). The spectrophotometer then records, on separate scales, the percentage of light transmitted and the percentage of light absorbed (**absorbance value**). You can determine the absorbance for each pigment at different wavelengths and generate the absorption spectrum for each pigment. (See Laboratory 4, Exercise A.)

Procedure

1. Work in groups of four. Your instructor will assign each group a pigment isolated from chloroplasts.

If you are not already familiar with the use of the spectrophotometer from Laboratory 4, follow these instructions. Insert a spectrophotometer tube containing a white strip of paper into the sample holder. Rotate the tube until you see colored light reflected from the paper. Turn the wavelength knob. As you turn the knob, you will see the different colors of light in the spectrum. What color do you see at 450 nm? ____________ 550 nm? ____________

650 nm? __________ As you vary the wavelength of light, you vary the color of light that can be absorbed by a sample (in this case, a chlorophyll extract in the sample holder). The instructions for using the Spectronic 20 are beside the instrument on the demonstration table. Ask your instructor for assistance if necessary.

2. The chloroplast pigments (chlorophyll *a*, chlorophyll *b*, xanthophylls, carotenes) are dissolved in different solvents—methanol, petroleum ether, and ethyl ether. Since the solvents themselves absorb a small amount of light, this amount must be subtracted from the total absorption by the pigment (analogous to taring a balance). This is done by using a tube containing just solvent (a solvent *blank*). First, with no tube in the machine, use the left-hand knob to adjust to 100% absorbance and 0% transmittance. Select a pigment and correct for absorbance by the solvent; place a tube containing the solvent (blank) in the machine and use the right-hand knob to adjust to 100% transmittance and 0% absorbance. For each pigment, be sure that the blank is the same solvent that was used to separate the extract.

3. Place the sample of chlorophyll pigment extract into the proper Spectronic 20 tube. Read the absorbance value. Take a reading every 25 nanometers (nm) from 400 to 700 nm. (Since absorbance by the solvent blank changes with different wavelengths, you must rezero the machine, using the blank, every time you change the wavelength.)

4. Record your results in Figure 12B-2.

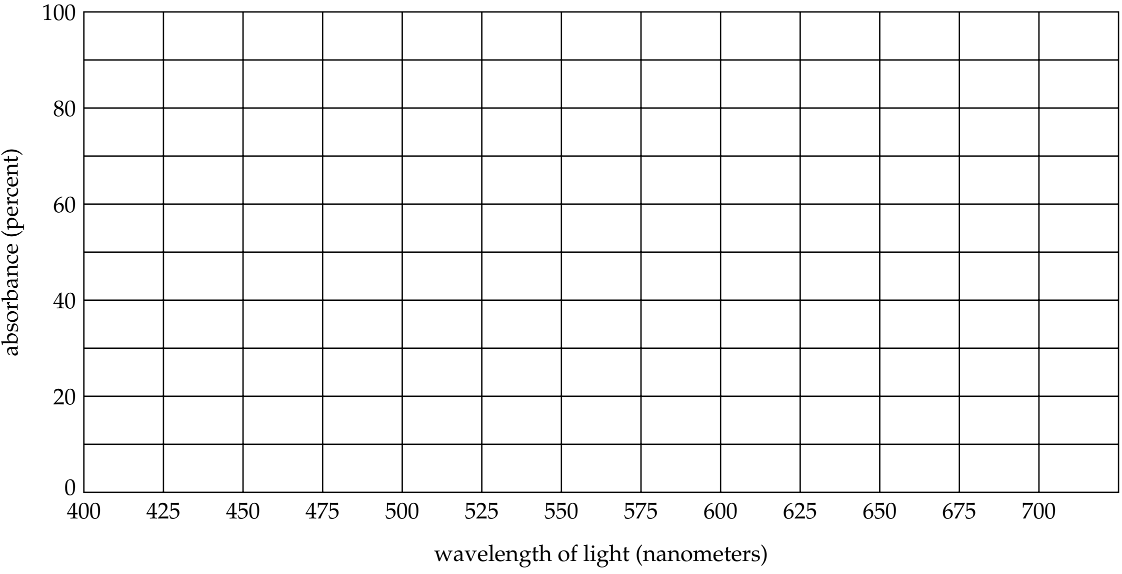


Figure 12B-2 *Graph the absorption spectrum of chloroplast pigments.*

5. Trace your graph onto your instructor's transparency. At the end of the period, examine all the graphs. Roughly sketch the curves for the other pigments on your own graph.

In Exercise A, you used spinach disks to investigate the wavelengths of light that are most effective in photosynthesis, and you generated an *action spectrum.* Now you have examined the wavelengths of light absorbed by individual chloroplast pigments and have generated an *absorption spectrum.*

a. In your own words, describe the difference between an action spectrum and an absorption spectrum.

____

6. Superimpose your results from your action spectrum (Exercise A, Part 3) onto Figure 12B-2 at the appropriate wavelengths.

b. What can you conclude about which pigments are responsible for photosynthesis?

____

✔ **PART 3** **The Role of Light in Chlorophyll Synthesis**

Procedure

Observe the two flats of wheat seedlings on demonstration. Record your observations below.

a. Appearance of light-grown seedlings: _______________

b. Appearance of dark-grown seedlings: _______________

c. What pigments are present in each set of plants? _______________

d. Is light necessary for the synthesis of chlorophyll? _______

e. Is light necessary for photosynthesis? (In Exercise A, what happened to the spinach disks placed in the dark?)

____

f. If chlorophyll is the pigment responsible for capturing the sun's rays, explain how the dark-grown seedlings managed to survive and grow. _______________

____

EXERCISE C The Light-Independent Reactions of Photosynthesis

Earlier, we indicated that photosynthesis takes place in two stages. In the first stage, energy from the sun is captured in ATP and NADPH. During the second stage, most of this energy is stored, by **carbon dioxide fixation,** in energy-rich carbohydrates. The six-carbon monosaccharide, glucose, is produced from CO_2 fixation.

$$6CO_2 + 18ATP + 12NADPH + 12H^+ \longrightarrow \underset{\text{glucose}}{C_6H_{12}O_6} + 18ADP + 18P_i + 12NADP^+ + 6H_2O$$

Again, this result is not accomplished in a single step, but requires many separate reactions.

Carbohydrate is packaged as a disaccharide, sucrose, for transport from the leaves to the nonphotosynthesizing parts of the plant, such as the roots or fruit. For longer-term storage, starch, consisting of long chains of glucose, is synthesized.

Must the light-dependent reactions occur before the light-independent reactions can occur? In some variegated plants, such as *Coleus,* parts of the leaves do not contain chlorophyll. Do they carry out light-independent reactions even though they cannot carry out the light-dependent reactions without chlorophyll?

Objectives

- ☐ Define carbon dioxide fixation.
- ☐ Discuss and give evidence for the role of plant pigments in carbon dioxide fixation.

Procedure

1. Obtain a leaf from a variegated *Coleus* plant.

2. Place a sheet of white paper over the leaf, hold it up to the window (or use a light table if available), and trace the outline of the pattern of colors. Label each section with its color.

3. Put the leaf into a boiling-water bath for 2 to 3 minutes. Notice that pink and purple pigments (anthocyanins) are removed by this treatment.
4. Transfer the leaf to hot alcohol (heated in a beaker placed in a water bath). Caution: Be very careful heating the alcohol—do not allow it to boil! Leave the leaf in the alcohol until all of the pigment has been leached out.
5. Place the leaf in a Petri dish and pour Lugol's solution (I_2KI) over it. Starch will stain dark blue-black.
6. Indicate the starch-containing areas on your leaf tracing, and complete Table 12C-1.

Table 12C-1 Relation of Plant Pigments to Starch Formation

Pigment	Starch Present?
None	
Anthocyanins	
Carotenes/xanthophylls	

a. In which parts of the plant did the carbon-fixing reactions result in the synthesis of starch? ________

b. Was chlorophyll present in these areas? ________________________________

c. Would you conclude that the energy-capturing reactions are a necessary preliminary to the carbon-fixing reactions? ________________________________

d. If you placed a plant that contained starch in the dark and tested a week later for starch, what would your results show? ________________________________

EXTENDING YOUR INVESTIGATION: DO ALL PLANTS STORE STARCH?

Plants store glucose molecules in different ways. Glucose molecules may be synthesized into the large starch molecules, amylose and amylopectin. Some plants use glucose to synthesize other storage compounds such as sucrose (glucose + fructose), and sometimes glucose is simply stored as glucose.

Pieces of potatoes and onions will be available to you. How do you think glucose is stored in each one? Formulate a hypothesis.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** you would find if you tested onions and potatoes for storage of the products of photosynthesis?

In this investigation you will use I_2KI and Benedict's reagent to test for the presence of starch and glucose in potatoes and onions.

What is the **independent variable** in this investigation?

What is the **dependent variable** in this investigation?

Use the procedure outlined below to conduct your investigation; or, using the materials available, design your own experiment.

PROCEDURE

1. Obtain a piece of potato and a piece of onion and place them in a plastic Petri dish.
2. Add several drops of Lugol's solution (I_2KI) to each.

   a. What do you observe? ____________________

   Recall that I_2KI turns blue-black in the presence of starch.

   b. Which material contains starch? ____________________

3. Obtain a new piece of potato and a new piece of onion. Use a pair of forceps or razor blade to crush the material. Add the onion pieces to one test tube and the potato pieces to another test tube. Label the tubes.
4. Add 2 ml of water to each tube. Add 2 ml of water to a third, empty tube.
5. Add 2 ml of Benedict's reagent to each of the three tubes.
6. Place the tubes in a beaker of water containing boiling chips and boil the material in the tubes for 3 to 5 minutes.

   c. What is the purpose of the tube that does not contain plant material?

   ____

7. Record your observations in the table below.

RESULTS:

Material	Color at Start	Color After 3–5 min	Presence of Precipitate
Onion			
Potato			
No material			

What do you think happened?

Do your results support your hypothesis?

Your null hypothesis?

Benedict's reagent is a test for certain sugars (reducing sugars) such as glucose. In the presence of glucose, a red precipitate forms. In the presence of sucrose (not a reducing sugar), no precipitate forms. From your results, what do you **conclude?**

Do potatoes store starch? _______ sugar? _______

Do onions store starch? _______ sugar? _______

What types of sugars are stored in the plant material you have studied?

Plant Material	Types of Storage Sugar

Laboratory Review Questions and Problems

1. In order for photosynthesis to occur in green plants, the following must be present:

a. ______________________________ as the energy source.

b. ______________________________ as the carbon source.

c. ______________________________ for the absorption of light energy.

d. ______________________________ as the electron donor.

2. According to your spectrophotometric data in Exercise B, which colors of light are used in photosynthesis?

3. a. Explain the difference between an action spectrum and an absorption spectrum.

b. For chlorophylls, carotenes, and xanthophylls, what is the relationship of the action spectrum to the absorption spectrum?

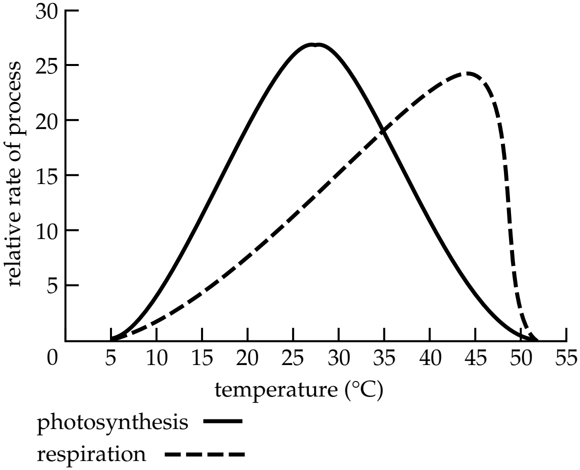
c. Why do plants contain so many pigments?

4. When using the I_2KI test in Exercise C, why did you extract the pigments from the leaf before adding I_2KI?

5. Many plants contain water-soluble red pigments called anthocyanins. Why were these not visible in the chromatogram of the chlorophyll extract?

6. The processes of photosynthesis and respiration were studied in separate labs, but, as you know, any cell that is carrying on photosynthesis is also carrying on respiration. On average, if a plant is to grow, the rate of photosynthesis must exceed the rate of respiration by a factor of at least three.

The following graph shows the effect of temperature on the rates of photosynthesis and respiration of one plant. The temperature at which the two rates are equal is referred to as the *compensation point* and is not the same for all plants.



a. At what temperature is the compensation point reached in this example? ______

b. At what temperature(s) would you expect growth to be most rapid? Explain.

c. As temperature rises, what happens to the rate of photosynthesis? Of respiration? Why?

d. Certain fruits, such as apples, are frequently stored under refrigeration in a carbon dioxide–rich atmosphere. Explain the reason for this.

7. Explain how each of the following could limit the rate of photosynthesis.

CO_2 concentration

Light quantity

Temperature

Water

Meiosis: Independent Assortment and Segregation

LABORATORY 13

OVERVIEW

Sexual reproduction allows the genes of two individuals to combine and provides the variability upon which evolution can work.

In animals that reproduce sexually, the production of sex cells, or gametes, requires that each parent's chromosomes be reduced to half the normal number. This halving of the

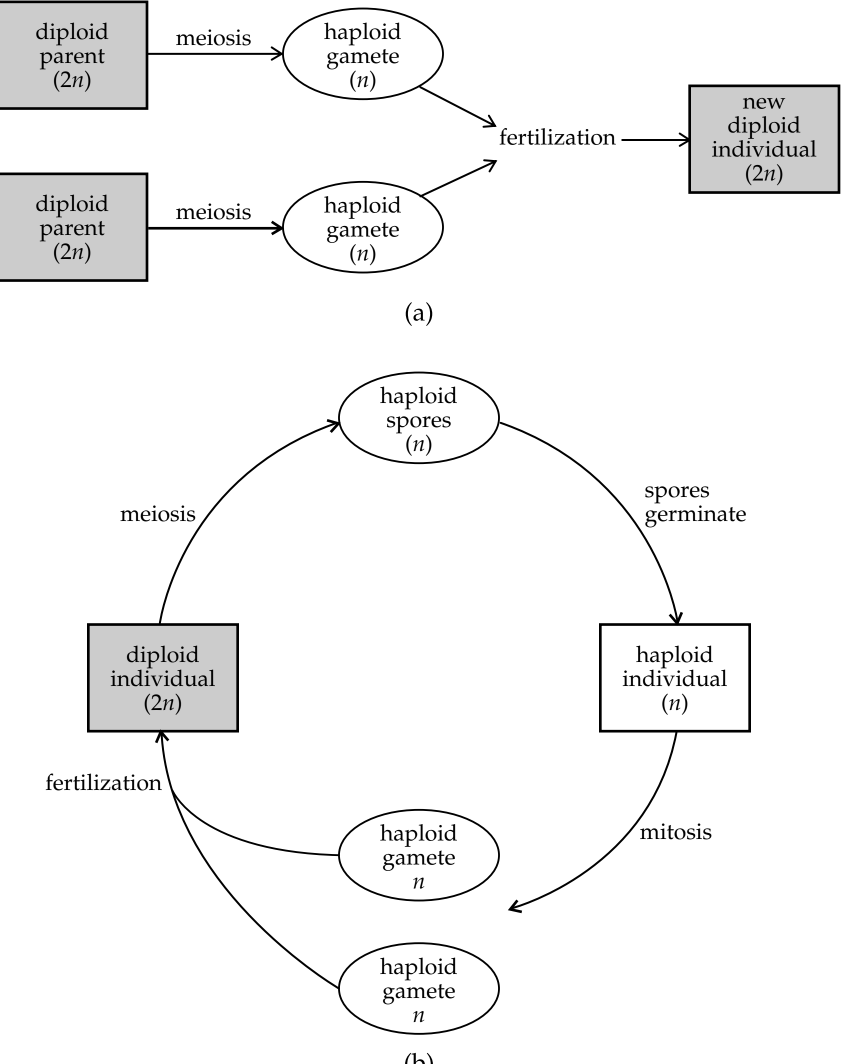


Figure 13-1 *Meiosis in (a) animals and (b) plants.*

parent's chromosome number from the **diploid,** or **2*n*,** number to the **haploid**, or ***n***, number is the result of **meiosis**. Combining two haploid (*n*) gametes during fertilization then restores the chromosome number to the number that is characteristic of the diploid (2*n*) organism (Figure 13-1).

In plants, meiosis results in the production of haploid (*n*) **spores,** which germinate to form haploid individuals. These individuals then give rise to haploid gametes by mitosis. Subsequent fusion of two haploid gametes during fertilization once again results in a diploid (2*n*) individual.

During this laboratory period, you will examine the process of meiosis and discover the genetic consequences of sexual reproduction.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

✔ EXERCISE A | Simulation of Chromosomal Events During Meiosis

Meiosis consists of two nuclear divisions (meiosis I and meiosis II) and results in the production of four daughter nuclei, each of which contains only half the number of chromosomes (and half the amount of DNA) characteristic of the parent (Figure 13A-1).

During meiotic reduction of the chromosome number to half, however, chromosomes are not just divided into two sets at random. In diploid organisms, chromosomes occur in matched pairs called **homologous chromosomes.** These are identical in size, shape, location of their centromeres, and types of genes present. One member of each homologous pair is contributed by the male parent and one is contributed by the female parent during sexual reproduction. Meiosis provides a precise mechanism for separating these homologous chromosomes so that daughter cells always carry one member, or homologue, of each chromosome pair.

Objectives

- ☐ List and explain the principal events of the stages of meiosis.
- ☐ Define and explain the following terms: diploid, haploid, homologous chromosomes, alleles, synapsis, and tetrad.
- ☐ Explain the difference between the first and second meiotic divisions.
- ☐ List and explain the similarities and differences between meiosis and mitosis.

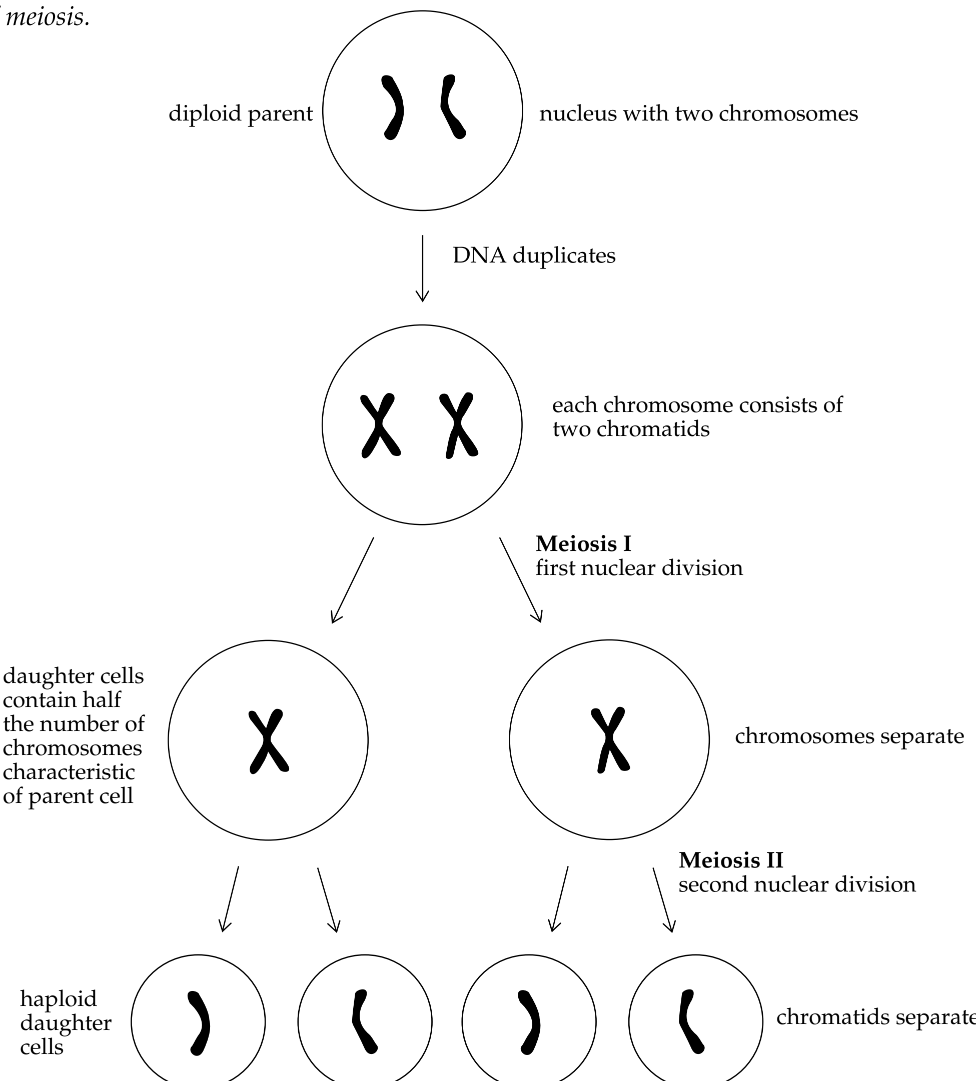
Procedure

You can study the process of meiosis using the chromosome simulation kit used earlier to demonstrate mitosis (Laboratory 9). The yellow and red strands of beads, both of the same length, represent two homologous chromosomes. A yellow strand represents the contribution of one parent and a red strand represents the contribution of the other parent. In other words, the yellow and red chromosomes are a homologous pair. The second yellow and red strands in your kit are to be used as chromatids for each of these chromosomes.

Interphase

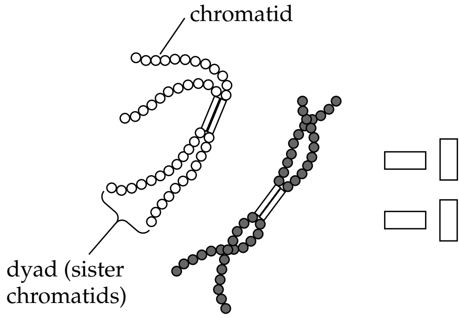
Place one strand of red beads and one strand of yellow beads near the center of your work area. (Recall that chromosomes at this stage would exist as diffuse chromatin and not as visible structures.) Position the two hollow, cylindrical beads at right angles to each other near the chromosomes. These hollow beads

Figure 13A-1 *An overview of meiosis.*



represent a pair of centrioles. DNA synthesis occurs during interphase prior to meiosis, and each chromosome, originally composed of one strand, is now made up of two strands, or **sister chromatids,** joined together at the **centromere** region. (Within the centromere region where two chromatids are most closely associated, a platelike protein structure or **kinetochore** is attached to each chromatid. Spindle fiber microtubules attach to the kinetochores during division.) A chromosome composed of two chromatids is called a **dyad** (or **bivalent**). Simulate DNA replication by bringing the magnetic centromere region of the second red strand into contact with the centromere region of the first red strand. Do the same with its homologue, the yellow strand (Figure 13A-2).

Figure 13A-2 *A homologous pair of chromosomes, each containing two chromatids, following DNA synthesis during interphase.*



Centriole replication also takes place prior to division. Use two additional cylindrical beads to simulate centriole replication. Place these next to the two original centriolar bodies.

Meiosis I

Prophase I

Homologous chromosomes come together and **synapse** (closely apply themselves to each other), pairing along their entire length. Here, you should recognize the first major difference between mitosis and meiosis.

a. Did homologous chromosomes synapse during prophase of mitosis? ________

A **tetrad,** consisting of four chromatids or two dyads, is formed. Entwine the two chromosomes as shown in Figure 13A-3. During this time, the pair of homologous chromosomes seems to shorten and thicken. Within the tetrad, a ladderlike protein structure, the **synaptonemal complex,** helps to align the tightly paired homologous chromosomes. At this site, segments of two non-sister chromatids (each belonging to a different homologue) may be exchanged by breaking and rejoining. This process, called **crossing-over,** further increases genetic variability (Figure 13A-3b). *Why?* ________________________________

You will not include crossing-over in this simulation, but you should be aware that it can happen during prophase I. You will examine this process in greater detail in Exercise D.

The centrioles that replicated prior to division begin to move to opposite sides (poles) of the nucleus as the nuclear membrane breaks down. Separate the two pairs of centrioles and move them to each side of the chromosomes (Figure 13A-3a). Spindle fibers also appear during prophase. You will not simulate spindle fibers. Imaginary spindle fibers are shown as dotted lines in all diagrams.

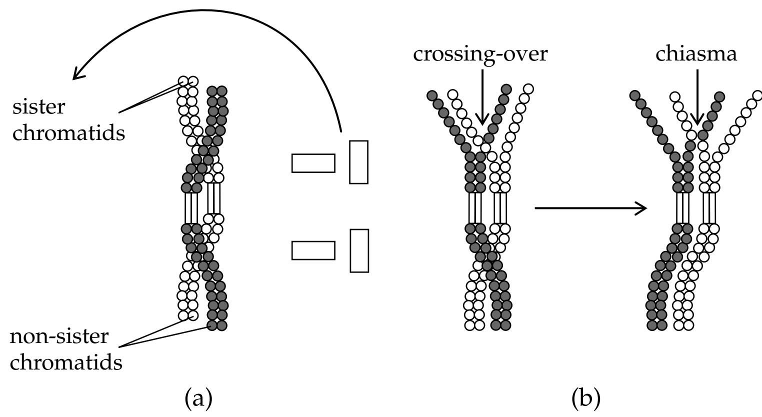


Figure 13A-3 *(a) Tetrad formation during prophase I. Centrioles begin to migrate to opposite sides of the nucleus as the nuclear membrane begins to break down. (b) Crossing-over occurs, and homologous chromosomes begin to untangle forming chiasmata. Note new gene combinations on the inner, non-sister chromatids.*

By the end of prophase I, each tetrad can be clearly seen to contain four separate chromatids. Sister chromatids are linked at their centromere, while non-sister chromatids that have crossed over appear to be held together at **X**-shaped locations called **chiasmata** (singular, **chiasma**) (Figure 13A-3b).

Metaphase I

Chromosomes have untwined by this time and can now be seen as dyad chromosomes. They now line up in the center of the cell in homologous pairs.

b. How does this arrangement of chromosomes differ from that in metaphase of mitosis?

____

Position the chromosomes near the midpoint between the centrioles and at right angles to the imaginary spindle fibers extending from the centrioles (Figure 13A-4).

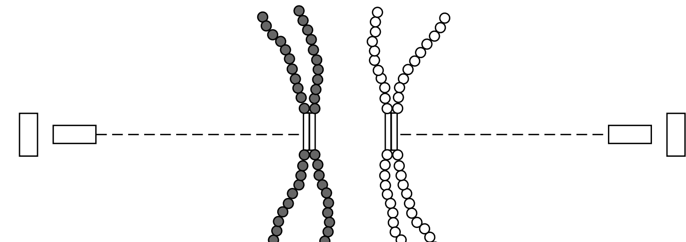
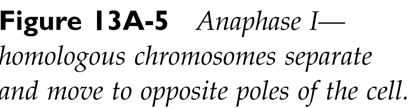


Figure 13A-4 *Metaphase I—homologous chromosomes line up in pairs.*

Anaphase I

During anaphase, the homologous chromosomes separate (Figure 13A-5) and are pulled to opposite sides of the cell by **kinetochore microtubules** (see Laboratory 9, Exercise B). This represents a second significant difference between the events of mitosis and meiosis.



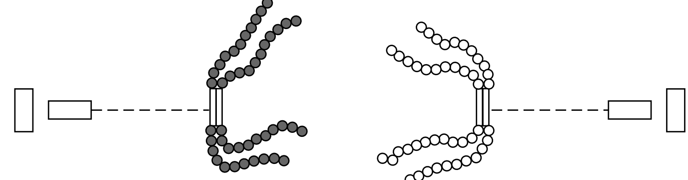


Figure 13A-5 *Anaphase I—homologous chromosomes separate and move to opposite poles of the cell.*

c. What is this difference? ____

____

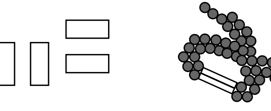
d. What happens to the chromatids of each chromosome during anaphase of meiosis?

____

Telophase I

Place each chromosome near its centriole pair (Figure 13A-6). Centriole duplication takes place at the end of telophase in preparation for the next division. Place a second pair of centrioles near the first and at right angles to it.

Formation of a nuclear membrane and division of the cytoplasm, **cytokinesis,** often occur at this time to produce two cells, but this is not always the case. Notice that each chromosome within the two daughter cells still consists of two chromatids.



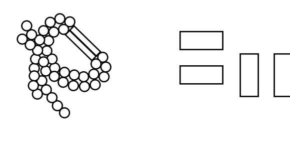


Figure 13A-6 *Telophase I—homologues are found at opposite ends of the cell. These may be separated into two cells by cytokinesis. In most organisms, centrioles duplicate at this stage.*

e. Compare the amount and arrangement of genetic material in each cell following telophase I of meiosis and telophase of mitosis. ____

f. How many of each type of chromosome do you see per cell? _______ *How many chromatids does each chromosome have?* _______

A second division is necessary to separate the chromatids of the chromosomes in the two daughter cells formed by this first division. This will reduce the amount of DNA (number of chromatids) to one double-helical strand per chromosome, typical for a nondividing cell. This second division is called **meiosis II.** It resembles mitosis except that (1) it is part of the continuing process of meiosis and thus is called meiosis II, and (2) only one homologue from each homologous pair of chromosomes is present in each daughter cell undergoing meiosis II.

Meiosis II

The following simulation procedures apply to *both* chromosome groups (daughter cells) produced by meiosis I.

Interphase II (Interkinesis)

The amount of time spent "at rest" following telophase I depends on the type of organism, the formation (or not) of new nuclear membranes, and the degree of chromosomal unwinding. Because interphase II does not necessarily resemble interphase I, it is often given a different name—**interkinesis.** DNA replication does not occur during interkinesis. This represents a third major difference between mitosis and meiosis.

Prophase II

Separate the pairs of duplicated centrioles and tape them down on opposite sides of each chromosome group (Figure 13A-7).

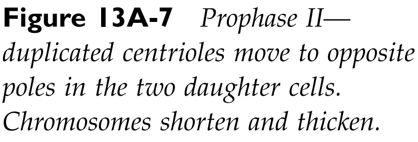
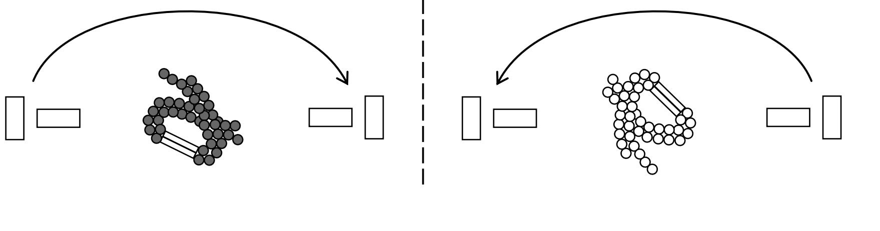


Figure 13A-7 *Prophase II—duplicated centrioles move to opposite poles in the two daughter cells. Chromosomes shorten and thicken.*



g. Does this action duplicate what you did during prophase I of meiosis? ________

h. What is different about prophase I and prophase II of meiosis? ____

____

Metaphase II

Orient the chromosome so that it is centered between the centrioles during metaphase II (Figure 13A-8).

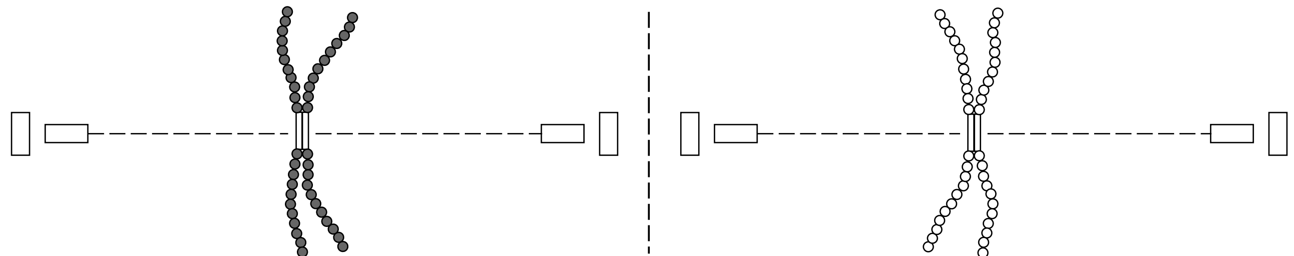


Figure 13A-8 *Metaphase II—chromosomes line up in single file. In this simulation, only one chromosome composed of two chromatids is present in each daughter cell, but in a cell containing many chromosomes, all of the chromosomes would now be lined up in the center of the cell, in single file.*

i. How does metaphase II differ from metaphase I? ____________________________

____

j. How does metaphase II of meiosis remind you of metaphase of mitosis?

____

Anaphase II

Sister chromatids now appear to be more loosely associated at the centromere region. When completely separated, each chromatid will have its own centromere region and can be referred to as a chromosome. Separate the sister chromatids of the chromosome and pull the new daughter chromosomes toward the centrioles on opposite sides of each daughter cell (Figure 13A-9).

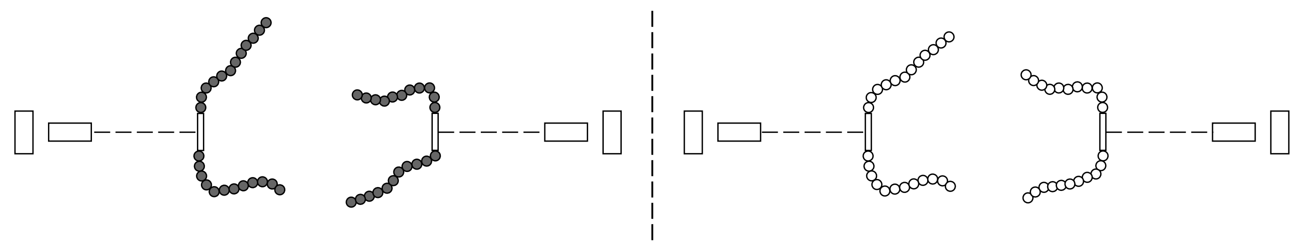


Figure 13A-9 *Anaphase II—the chromatids of each chromosome are separated and move to opposite poles of the cell.*

Telophase II

Pile each chromosome near its centriole. Formation of a nuclear membrane and division of the cytoplasm, cytokinesis, occur at this time (Figure 13A-10).

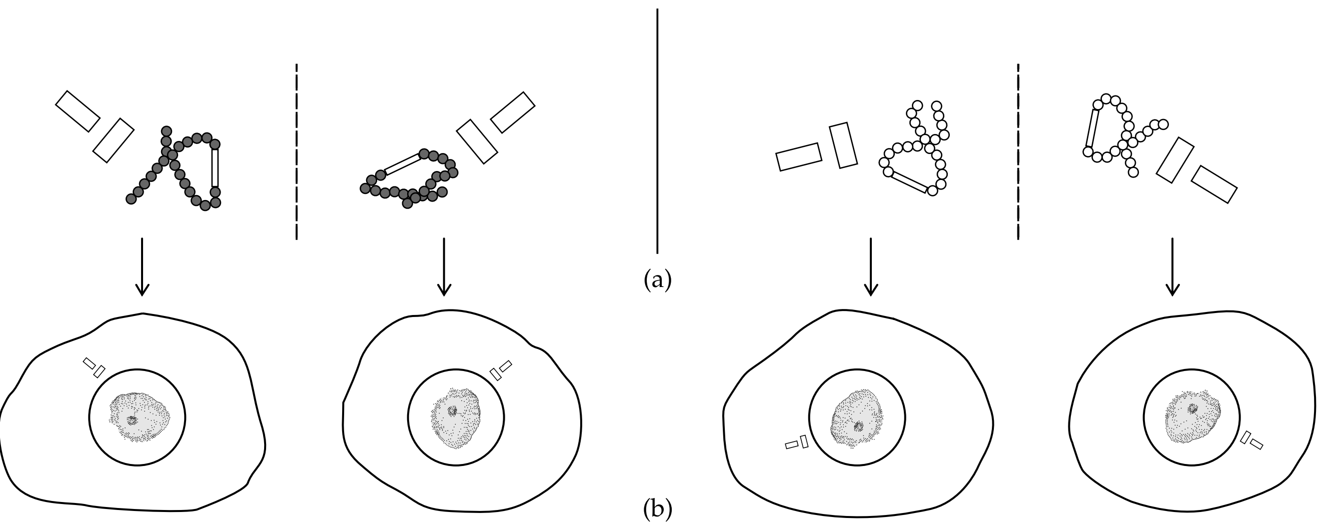


Figure 13A-10 *Telophase II—four haploid daughter cells are formed following cytokinesis. New nuclear membranes are formed within each daughter cell; one pair of centrioles is present outside the nuclear membrane. (a) Simulation. (b) Diagram of cells.*

k. How many cells have you formed during the process of meiosis? ______

l. How many cells were formed during the process of mitosis? ______

m. Are the cells formed in meiosis haploid (n) or diploid (2n)? ______________

n. If the same set of chromosomes with which you began this exercise were to undergo mitosis, would the resulting cells be haploid or diploid? ______________

o. List three major differences between meiosis and mitosis: ____

____

Place a single short red strand of beads and a single long yellow strand of beads on your laboratory table. *Could a cell like this exist?* ______ *Would it be haploid or diploid?* ______________ *Could this cell carry out mitosis?* ______ *Meiosis?* ______ Demonstrate these processes if they can occur. Use the strands of beads available in your kit.

Place a long red strand and a long yellow strand of beads on your laboratory table. *Could a cell like this exist?* ______ *Would it be haploid or diploid?* ______________ *Could this cell carry out mitosis?* ______ *Meiosis?* ______ Demonstrate these processes if they can occur. Use the strands of beads available in your kit.

EXERCISE B | Mendel's First Law: Alleles Segregate During Meiosis

Since there is a pair (the homologues) of each type of chromosome in a diploid organism, there will also be a pair of each type of gene: one gene on one chromosome and the second on its homologue. Genes for a particular trait are found at the same **locus** (physical place or location) on each of the homologous chromosomes. Each of these two genes is called an **allele.**

In some cases, the alleles on the two homologues are identical and the organism's **genotype** (gene content) is said to be **homozygous.** In other cases, the two alleles control alternative expressions of the same trait (for example, green and yellow are alternative expressions, or alternative forms, of the seed-color gene in corn). In this case the organism's genotype is said to be **heterozygous.**

During meiosis, homologous chromosomes are separated from each other, and only one may be carried in a particular gamete or spore. Thus the alleles carried on each of the homologous chromosomes are also separated or **segregated.** *Mendel's first law states that alleles segregate in meiosis* (Figure 13B-1). When two haploid gametes combine during fertilization, two alleles for each trait are again present in the offspring.

Objectives

- ☐ Define and explain Mendel's first law.
- ☐ Apply Mendel's first law to a simple monohybrid cross between two heterozygous individuals.

Procedure

Repeat the simulation of Exercise A, but first take a piece of label tape and mark one bead on each yellow strand (chromatid) as ***A*** (same location on each strand). Mark one bead on each red strand as ***a.*** Make sure that ***A*** and ***a*** appear at the same locus on the two homologues (Figure 13B-2).

*a. Why do the two chromatids of a chromosome have the same alleles (*A *and* a*) on one dyad?*

____

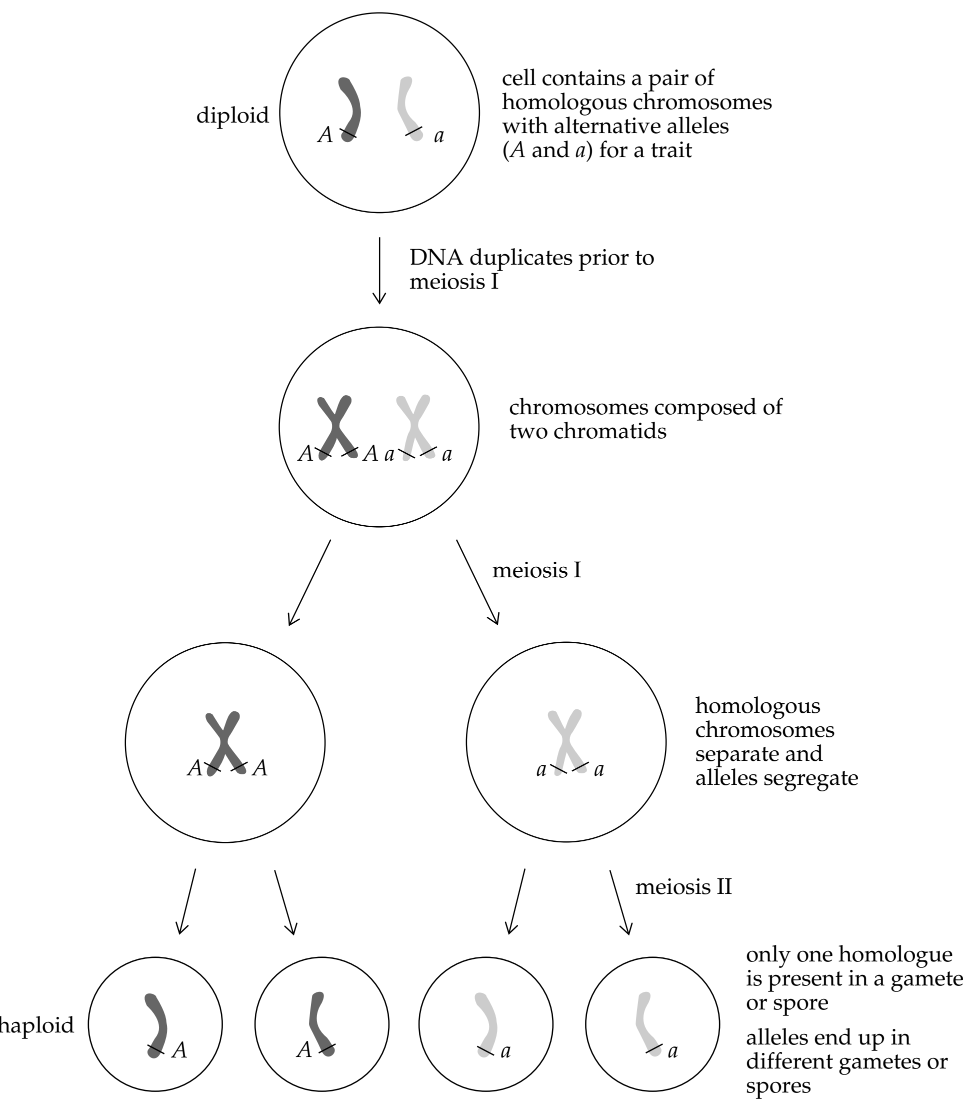


Figure 13B-1 *Alleles segregate during meiosis.*

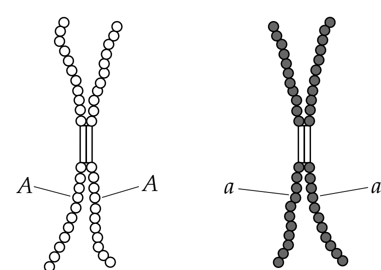
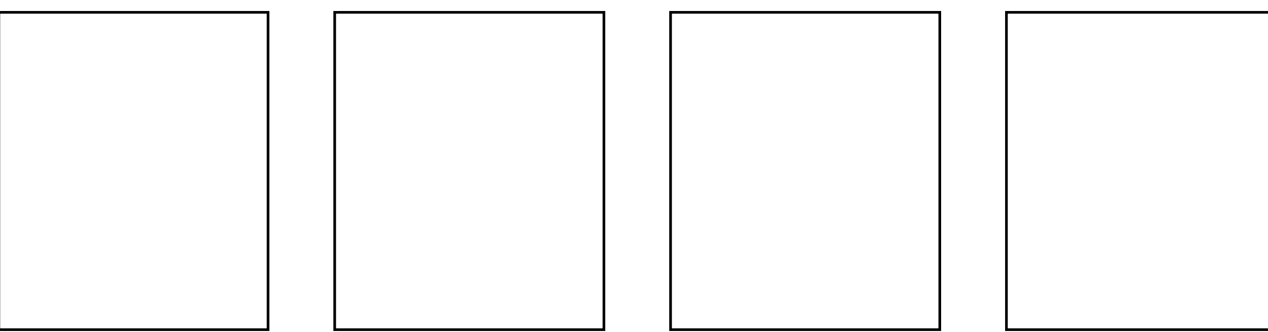


Figure 13B-2 *Alleles on homologous chromosomes.*

Diagram the products of this meiosis below. Use colored pencils to show individual chromosomes. Indicate ***A*** and ***a*** as they appear during your simulation.



b. What do you conclude about the final distribution of the A *and* a *alleles in the daughter cells?*

____

____

c. Do your observations support Mendel's first law? ________ *Explain.* ______________________________

____

You have just demonstrated that during meiosis the two factors (alleles) for any trait segregate so that each ends up in a different gamete or spore.

Mendel's first law can be verified by examining kernel color on an ear of **hybrid** corn (formed from the union of gametes from two parents that differ in one or more inheritable characteristics). Each kernel represents a seed developed from a fertilized egg of the corn plant. Thus kernel color is determined by the combination of alleles contributed by the gametes: the sperm and the egg (Figure 13B-3).

In corn an allele (*R*) produces purple-colored seeds. The allele *R* is **dominant** to the allele *r* for "no purple" or yellow seeds. If two *R* alleles or two *r* alleles are present in the seed, it is said to be **homozygous.** The seed will be purple (*RR*) or yellow (*rr*), respectively. If the two alternative alleles, *R* and *r*, are both

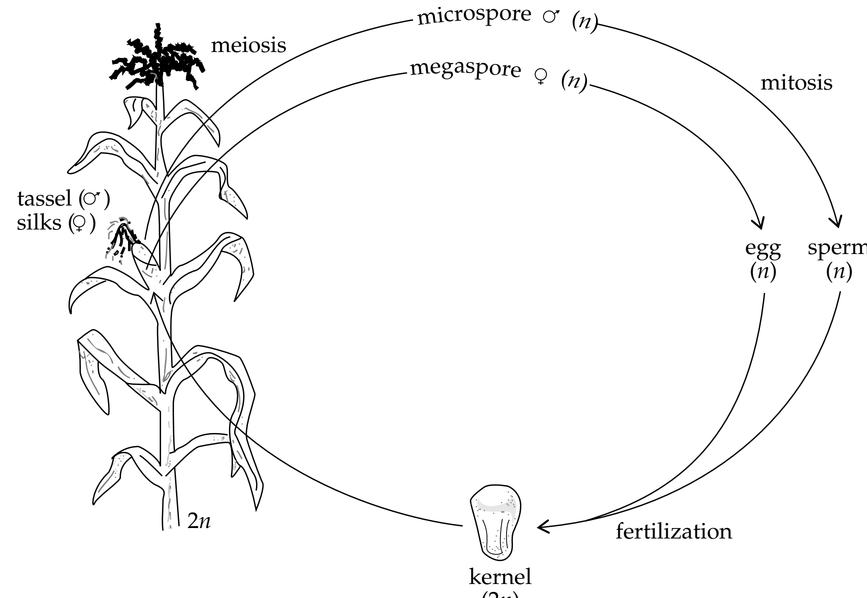


Figure 13B-3 *Corn life cycle.*

present in the seed, then the **heterozygous** kernel will be purple due to the dominance of the *R* gene. The **genotype** (genetic constitution of alleles) is *Rr*, but the **phenotype** (appearance) of the kernel is purple.

A cross is made between a homozygous dominant plant (*RR*) and a homozygous recessive plant (*rr*). The cross $RR \times rr$ represents the fertilization event in the parental (P_1) generation.

d. What color seeds would the RR *parent have?* ________________

e. What is the genotype of gametes produced by the RR *parent?* ______

f. What color seeds would the rr *parent have?* ________________

g. What is the genotype of gametes produced by the rr *parent?* ______

h. Consider the genotypes of gametes that can be produced by the homozygous parents, RR *and* rr. *All offspring resulting from the union of one gamete from each parent would have the genotype* ______

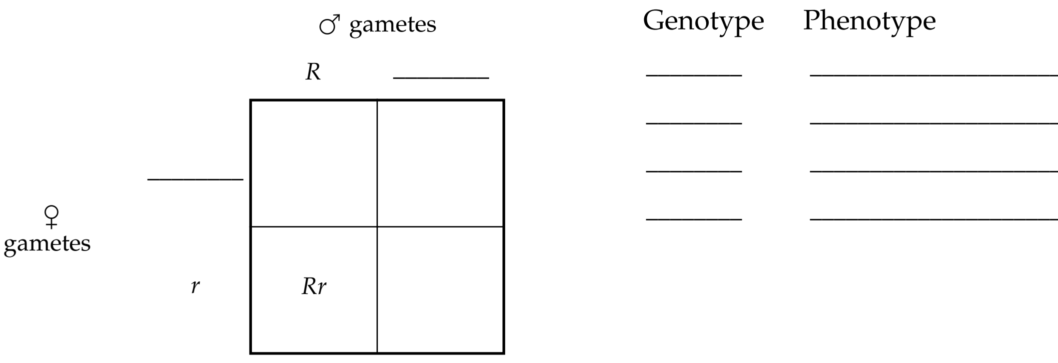
i. What is the phenotype of this F_1 *generation?* ________________

When F_1 individuals make gametes, their alleles for seed color will segregate.

j. What are the genotypes of gametes produced by F_1 *individuals?* ________________

The consequences of this segregation of alleles will become apparent in the next (F_2) generation (offspring produced by F_1 individuals) when one examines all the possibilities for the genotypes that would be present in the F_2 individuals.

The possible combinations of alleles that may be produced in each parent's gametes, and the results of these combinations in the genotypes of the offspring, can be determined by using a **Punnett square.** All of the possible genotypes of gametes that can be produced by one parent are listed across the top of the square; all genotypes of gametes that can be produced by the other parent are listed along the side. In the Punnett square below, one type of gamete from each F_1 parent has already been listed, and one possible combination is shown. Fill in the blanks for the other gamete genotype for each parent, and then complete the other three combinations in the square to determine the possible genotypes of the offspring. (*Note:* By convention, the dominant allele for each trait is written first: for example, *Rr,* not *rR.*) Next to the Punnett square, list the genotypes and phenotypes of the four types of individuals produced in the F_2 generation.



k. How many different kinds of genotypes are present in the F_2 *generation?* ______

l. Indicate below the proportion (ratio) of individuals with these genotypes:

genotype (homozygous dominant)	number		genotype (heterozygous)	number		genotype (homozygous) recessive)	number
______	______	:	______	______	:	______	______

m. How many different kinds of phenotypes are present in the F_2 *generation?* ______

n. What is the proportion (ratio) of individuals showing these phenotypes?

number	phenotype	number	phenotype
_______	purple :	_______	yellow

Your instructor will provide a plastic box containing a series of corn ears that demonstrate crosses. Obtain one of the corn ears labeled "Monohybrid Cross F_2." Count and record the total number of purple kernels from at least four rows. Repeat with the yellow kernels.

Number of purple kernels _______ Number of yellow kernels _______

Proportion of purple kernels to yellow kernels _______ : _______.

The phenotypic ratio of purple to yellow kernels in the F_2 generation should be 3:1.

o. How do your results compare with this expected phenotypic ratio? _______________________________________

p. Can you determine the genotypic ratio by examining the ear of corn? _______ *Why or why not?*

____

EXERCISE C Mendel's Second Law: Alleles of Unlinked Genes Assort Independently

Now let us consider meiosis involving two sets of homologous chromosomes. Alleles for trait A (*A* or *a*) are found on one pair of homologues. Alleles for an entirely different trait B (*B* or *b*) are found on the other pair of chromosomes. Assume that two parents are each heterozygous for both genes. Each parent would have the genotype *AaBb.* It is possible for these parents to produce gametes *AB* and *ab* or *aB* and *Ab,* depending on how the pairs of homologous chromosomes are arranged at metaphase I of meiosis. The alleles for the two genes sort themselves out independently. The behavior of *A* is not linked to that of *B* (**unlinked genes**) because the genes are on separate chromosomes. So, for example, the combination *AB* is as likely as the combination *ab. Mendel's second law states that alleles of unlinked genes assort independently* (Figure 13C-1).

Since many gametes are produced at one time, a parent can produce gametes of all four genotypes: *Ab, ab, aB,* and *Ab.* When considering the possible genotypes for offspring, all gamete genotype possibilities for each parent must be considered. *a. Why?* _______________________________________

Objectives

- ☐ Demonstrate the alternative arrangements of homologous chromosomes during metaphase I of meiosis.
- ☐ Relate the arrangement of homologous chromosomes in metaphase I to the number of types of genetically different gametes that can be produced.
- ☐ Define and explain Mendel's law of independent assortment.
- ☐ Verify that the law of independent assortment holds true for alleles in a dihybrid cross between two heterozygous individuals.

Procedure

Combine your simulation kit with that of the person sitting next to you. Remove five beads from one side of the centromere on all four strands of one kit to form chromosomes of a different length from those in the other kit.

Using two pairs of homologous chromosomes, repeat all the steps of meiosis. Remember that your homologues are alike in length, not color. You have one homologous pair (one red dyad and one yellow dyad) in which the centromere is in the middle and the four chromosome arms are of equal length, and a second homologous pair (one red dyad and one yellow dyad) with short arms on one side of the centromere region and long arms on the other. Mark the chromosomes with tape to indicate the alleles they are carrying, as shown in Figure 13C-2.

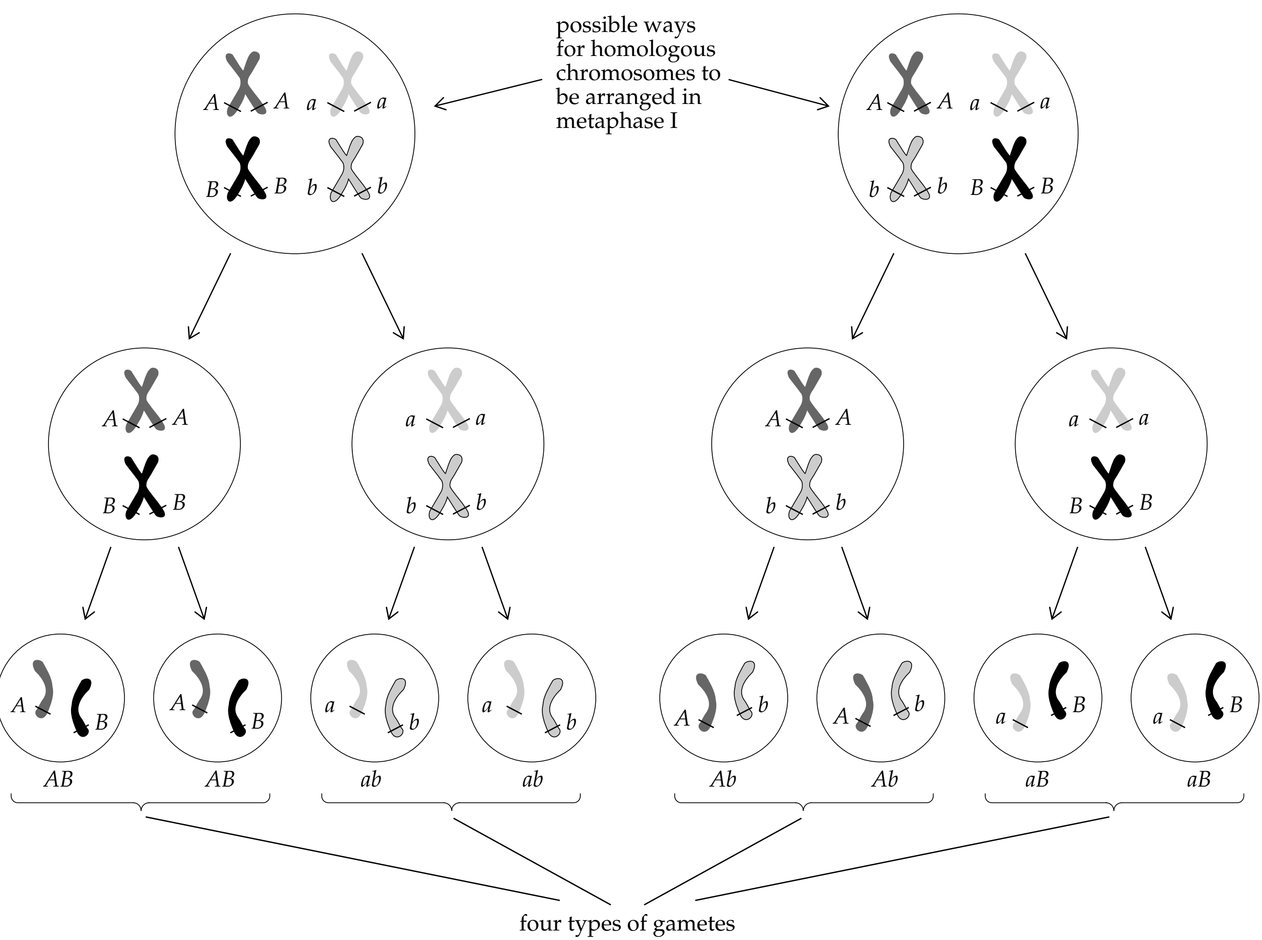


Figure 13C-1 *Possibilities for allele combinations in haploid gametes or spores are dependent upon independent assortment of alleles present on different chromosomes.*

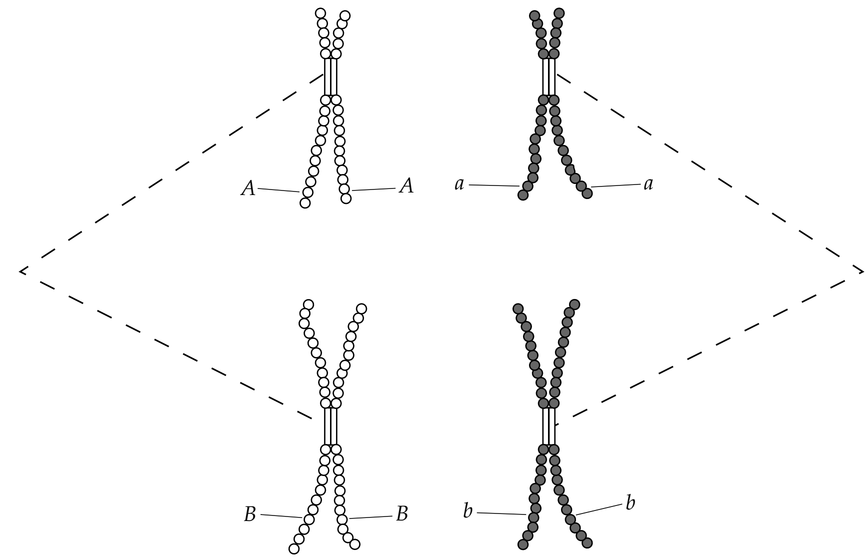
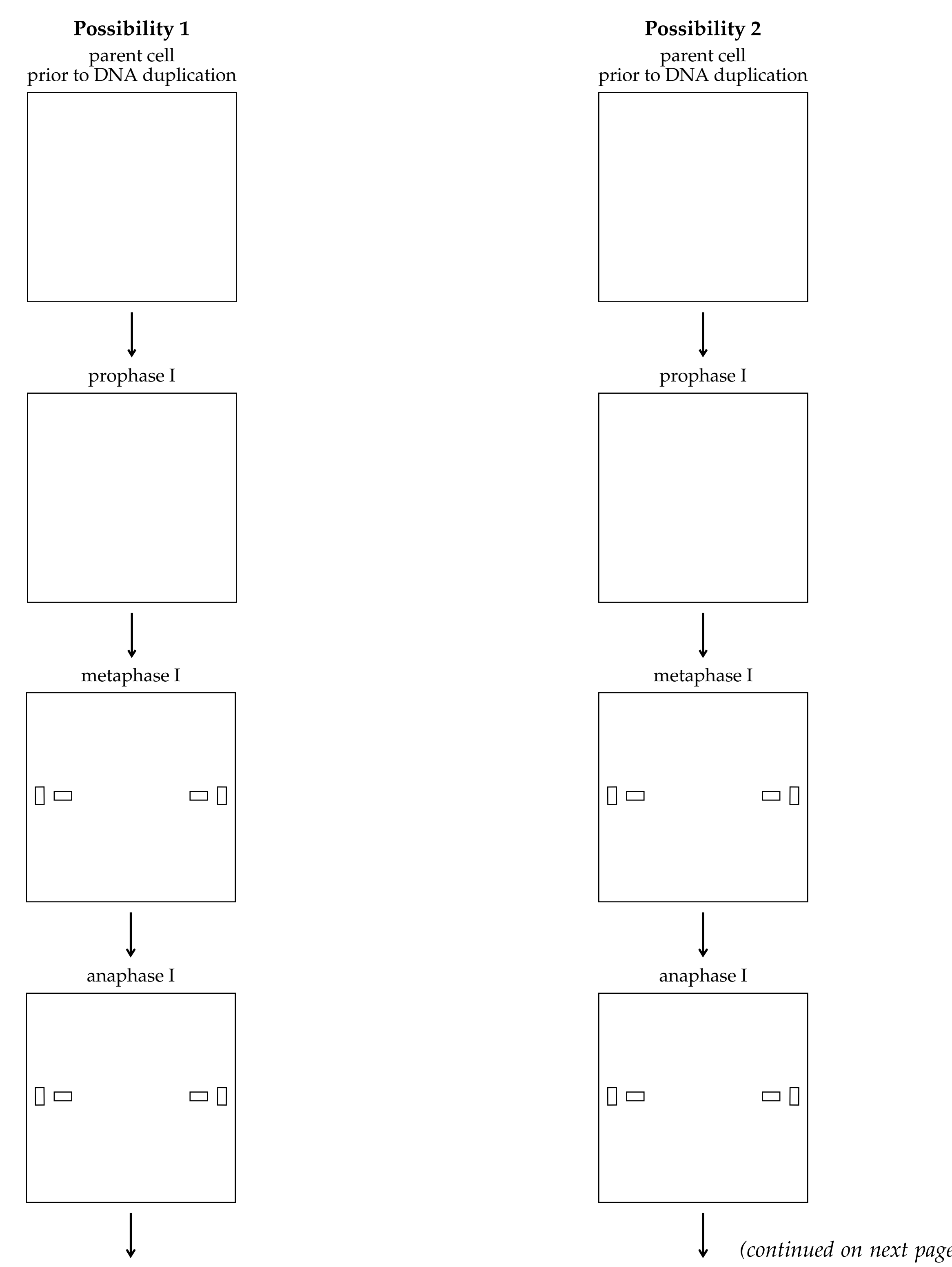


Figure 13C-2 *Mark alleles at the same locus on each chromatid and on homologous chromosomes.*

If you begin with two pairs of homologous chromosomes, there are two possibilities for their alignment during metaphase I of meiosis, depending on which side you place the yellow and red homologues. Draw both of these possibilities in Figure 13C-3. Indicate the alleles present on each chromosome.

Follow possibility 1 through meiosis, using the red and yellow beads. As you proceed through your simulation for possibility 1, figure out how the chromosomes and alleles would be distributed in



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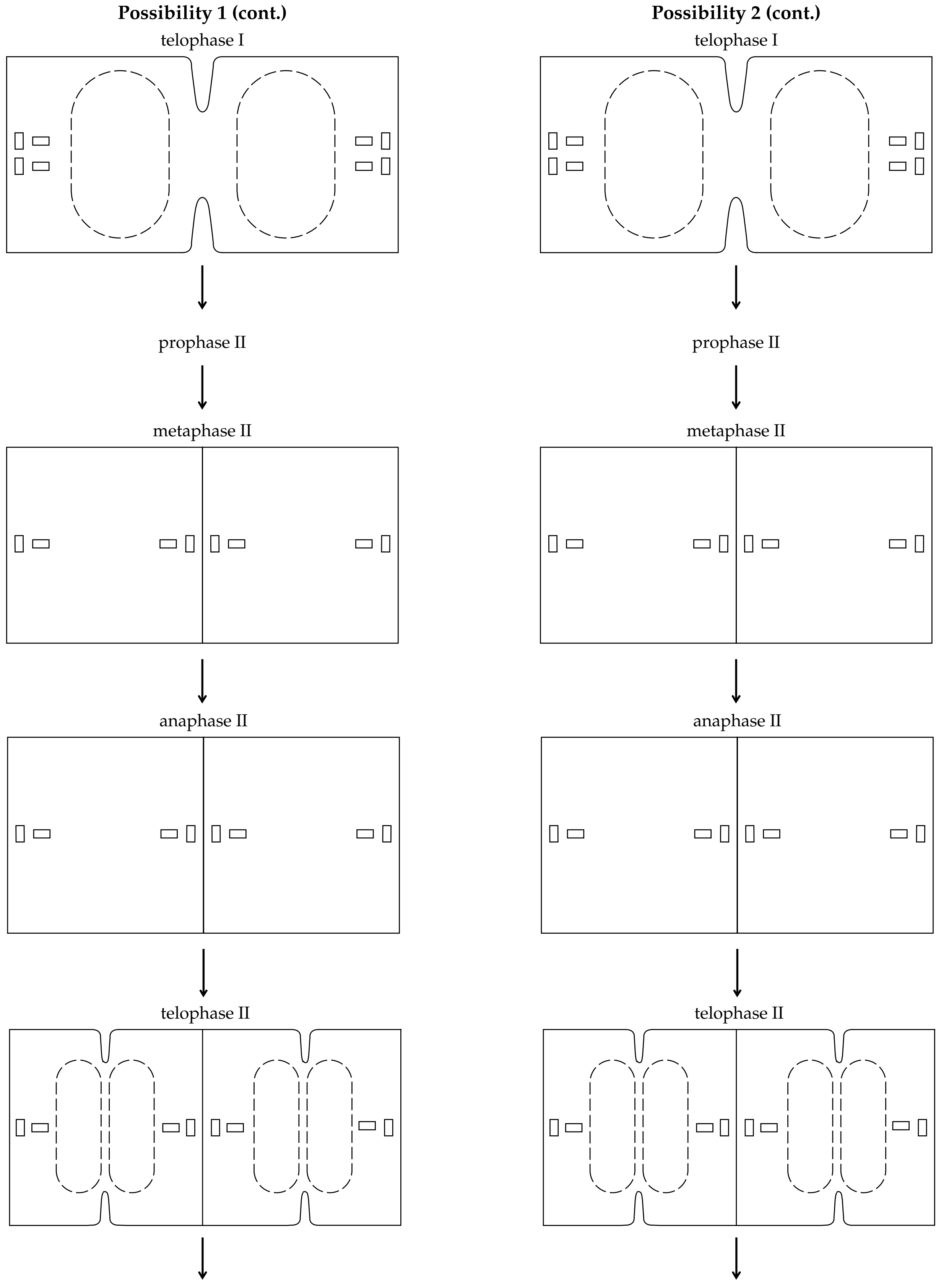


Figure 13C-3 *Meiosis with two pairs of homologous chromosomes.*

possibility 2. In the spaces provided in the diagrams in Figure 13C-3, draw the chromosomes as they appear for both possibilities at all stages indicated. Use colored pencils to show the color of each chromosome at each step. Always indicate the alleles present on each chromosome. Your laboratory instructor should check your bead setup for metaphase I and metaphase II.

You have now demonstrated Mendel's second law: alleles of unlinked genes assort independently.

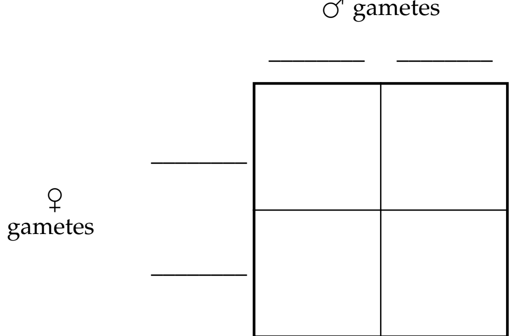
b. *How many possible combinations of alleles exist if you consider the results from both possibilities?* ________

c. *How many* different *types of gametes can be made by an individual of genotype* AaBb? ________

Mendel's second law can be verified by tracing the fate of two unlinked genes in corn through a series of crosses. In addition to the gene for seed color (alleles *R* and *r*), there is a gene that controls seed shape (alleles *S* and *s*). The *S* allele is responsible for one of the enzymes involved in the conversion of sugar to starch in developing corn kernels. The presence of starch in *SS* and *Ss* seeds gives the mature seeds a full, smooth appearance, and their phenotype is considered to be "smooth." However, the *ss* seeds lack this enzyme and contain a much higher proportion of sugar. As a consequence, they taste sweeter than "smooth" kernels and appear "wrinkled." The ears of corn with which you will work were produced by first crossing a plant from a homozygous purple, smooth-seeded plant (*RRSS*) with a homozygous recessive yellow, wrinkled-seeded plant (*rrss*).

d. *Which alleles are present in the gametes of these parent types:* RRSS *(purple, smooth)* ________ rrss *(yellow, wrinkled)?* ________

Find the possibilities for the genotypes that would be present in individuals of the F_1 generation by filling in the Punnett square below.



e. *What is the genotype of all the individuals in the* F_1 *generation?* ________

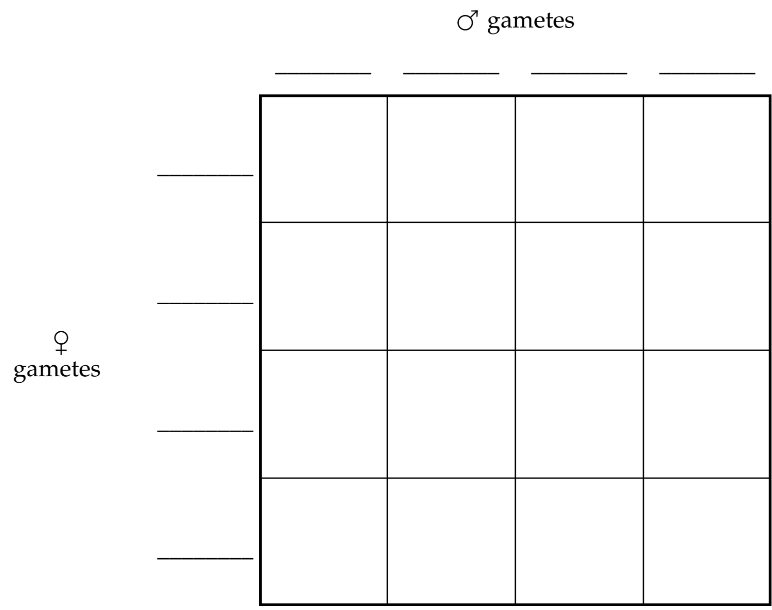
f. *What is the phenotype of all the individuals in the* F_1 *generation?* ____________________

g. *Which alleles are present in gametes produced by the* F_1 *plants?* ____________________

Use the Punnett square to find the proportions of different genotypes in the F_2 progeny resulting from all the possible unions of the various gametes produced by the F_1 generation.

h. *How many different genotypes are represented?* ________

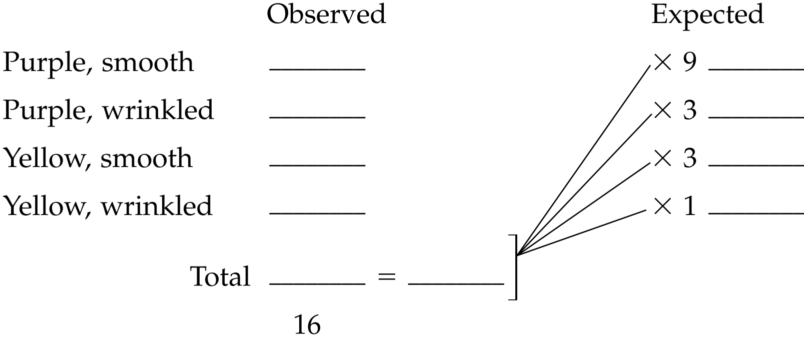
Use colored pencils to identify each of the different phenotypes produced by this cross. Circle all genotypes that result in a particular phenotype with the same color.



i. Indicate the proportions (ratios) of individuals showing the following phenotypes: purple, smooth _______:
purple, wrinkled _______: yellow, smooth _______: yellow, wrinkled _______

On demonstration you will find ears of the P, F_1, and F_2 generations of this cross. In the F_2 of a dihybrid cross involving dominant unlinked genes, one expects to find a phenotypic ratio of 9:3:3:1. Select an ear of corn labeled "F_2 of dihybrid cross." There are four types of kernels present on each ear.

Count at least five rows of kernels and record the number of each type found under "Observed" below. To compare the observed data with the numbers expected of each phenotype, divide the total number of all seeds counted by 16 (round to the nearest whole number) and multiply this value by 9, 3, 3, and 1, respectively. Record this information under "Expected."



j. Do your results support what you predicted from your Punnett square? _______

k. If the genes were linked, rather than unlinked, would the alleles assort independently? _______

l. Would you expect to see the same genotypic-to-phenotypic ratios in genetic crosses if the genes were linked? _______
Why or why not? ____

____

EXTENDING YOUR INVESTIGATION: MEIOSIS AND LINKED GENES

Suppose that two genes, *A* and *B*, are located on the same chromosome. These are **linked** genes and tend to stay together during meiosis. Would they obey Mendel's second law? If two individuals, *AABB* and *aabb*, are crossed, all F_1 individuals are *AaBb*. Formulate a hypothesis to predict how the genotypes of offspring in the F_2 generation would be determined.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will happen when you cross two *AaBb* individuals?

Identify the **independent variable** in this experiment.

Identify the **dependent variable** in this experiment.

Design a procedure to test your hypothesis. (Use the bead kits from Exercise A.)

PROCEDURE:

RESULTS: Diagram your results.

From your results, describe how the genotypes in the F_2 generation are determined.

Do your results support your hypothesis?

Your null hypothesis?

Was your prediction correct?

What do you **conclude** about the effects of linked genes on the genotypes of offspring in the F_2 generation?

EXERCISE D | Meiosis and Crossing-Over in *Sordaria (Optional)*

The fungus *Sordaria fimicola* is often used to study the processes of gene segregation and crossing-over during meiosis. This common fungus spends most of its life cycle in the haploid condition (Figure 13D-1). Its body is composed of haploid (*n*) cells attached end to end to form hairlike filamentous hyphae that intertwine to form a mass (*mycelium*). When hyphal cells of two different mycelia come together, they fuse and the haploid nuclei from one hypha migrate into the other. The haploid nuclei combine within the dikaryotic cells to form a diploid (2*n*) zygote nucleus. The diploid nucleus in the hyphal tip immediately undergoes meiosis to form four nuclei, returning the organism to its haploid state. These haploid nuclei then divide mitotically to yield a total of eight haploid cells. The cells develop thick, resistant cell walls and are called **ascospores.** The ascospores are arranged in a linear array within a sac called an **ascus** (plural, *asci;* see Figure 13D-2). Many such asci grouped together line the inside of a fruiting body (**ascocarp**), formed from tightly fused hyphae. In *Sordaria* the ascocarp, called a **perithecium,** is flask-shaped with a small hole through which mature spores escape when the asci rupture.

Ascospores of *Sordaria* are normally black. However, several different genes can be involved in determining spore color, and each of these genes can have several allelic forms. Black spores can only be produced if both of two genes controlling color are normal or wild-type (g^+ and t^+). A mutation in one such gene can result in gray spores (t^+ and g^-), whereas a mutation in a different gene can result in tan spores (t^- and g^+). If both tan and gray mutant alleles are present (t^- and g^-), the cumulative effect of both mutations is that the ascospores are colorless (Figure 13D-3). Ascospores are haploid, so only one allele is present for each gene. (There is no homologous chromosome carrying an alternative or duplicate allele at the same locus.) As a result, the spore's phenotype (physical characteristic) is equivalent to its genotype—the expression of the allele cannot be "masked" by a different (and perhaps dominant) allele at the same locus on the homologous chromosome. This equivalence is one of the major reasons that fungi such as *Sordaria* (an ascomycete) are used extensively in genetic research. It is also of critical importance in this exercise.

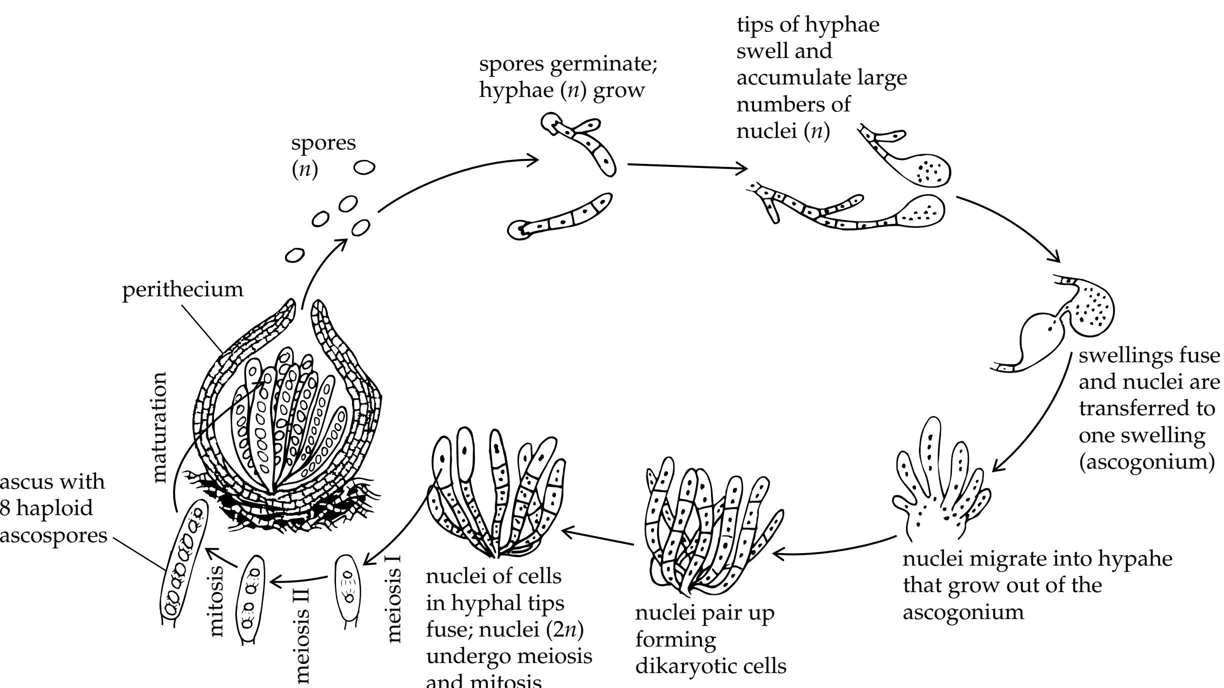


Figure 13D-1 Sordaria fimicola *life cycle.*

Figure 13D-2 *Photomicrograph of* Sordaria fimicola *asci.*

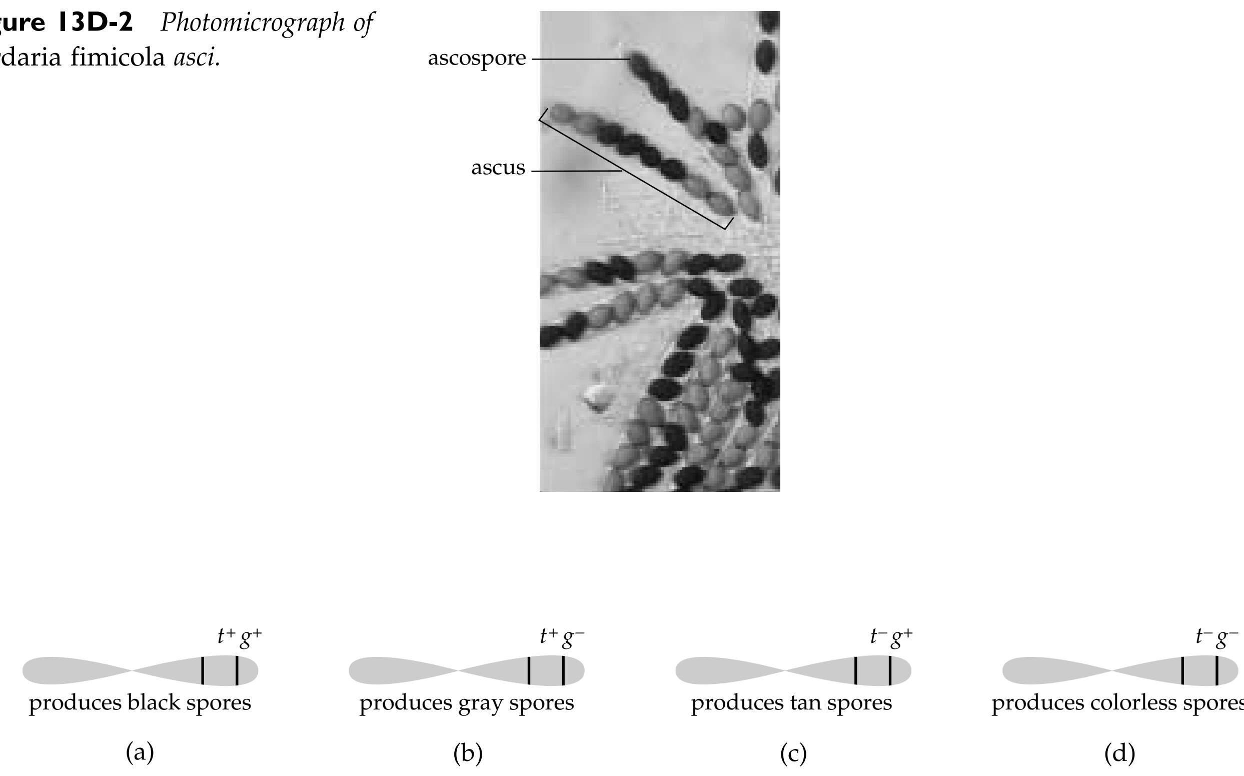


Figure 13D-3 *Two different genes control ascospore color. When both genes are represented by the wild-type allele, the spores are black (a). A mutation in either one of the genes can cause gray color (b) or tan color (c) if the allele at the second locus remains wild type (+). If both genes are mutant, the ascospores are colorless (d).*

The genetics of both the process and the results of crossing-over in *Sordaria* can be studied by observing the phenotype (color) and arrangement of ascospores in asci of hybrids that are produced by mating a wild-type black strain (t^+g^+) and a mutant strain (either t^+g^- or t^-g^+). In this exercise, you will begin your study with the behavior of the gene at the tan locus (remember, this is a mutation, t^-, of the wild-type gene, t^+). Thus, you will cross a black strain (t^+g^+) with a tan strain (t^-g^+). This study will require 7 to 10 days for completion. You will set up crosses between wild-type and tan-type mutant *Sordaria* during this laboratory period and then analyze your results during next week's laboratory period (Laboratory 14).

Objectives

- ☐ Describe the life cycle of *Sordaria fimicola.*
- ☐ Describe the role of mitosis and meiosis in the formation of asci in an ascomycete fungus such as *Sordaria fimicola.*
- ☐ Demonstrate how to make a hybrid cross between two different strains of *Sordaria fimicola.*
- ☐ Explain how meiosis and crossing-over result in different arrangement of ascospores in asci.
- ☐ Identify nonhybrid and hybrid MI and MII asci.

✔ PART 1 Crossing *Sordaria* Strains (*Week 1*)

If mycelia from two different strains of *Sordaria* are placed on the same agar plate, the mycelia will fuse and hybrid asci will form (however, it is important to understand that a genetic strain may also mate with itself).

Note: Your instructor may have already made crosses for you. If so, the crosses have been allowed to incubate for 7 days and the plate you receive will contain mature perithecia. In this case, proceed to the directions for Part 2. Your instructor may also ask you to proceed with the analysis of crossovers and mapping of the *Sordaria* chromosome. If this is the case, continue with Exercise A in Laboratory 14.

If you are asked to set up your own crosses, proceed as follows.

Procedure

In this exercise you will cross a wild-type black strain with a mutant tan strain. You may wish to refer to these as "+" and "*t*," respectively, to simplify notation. (This means that the black strain is normal or t^+ for the tan allele, the tan strain is mutant or t^- for the tan allele, and both strains are normal for the *g* allele, or g^+.

1. Obtain wild-type black (+) and tan (*t*) cultures of *Sordaria fimicola.* The agar plate will be covered with a mycelial mat of hyphae and will already have been cut into small squares. *Do not open the lid yet!*
2. Obtain a sterile Petri dish of crossing agar and label it with your initials.
3. **Use aseptic technique.** Place the end of a small metal spatula into 95% ethanol and, holding the end downward (so that alcohol does not run up the handle and onto your fingers), heat the spatula in the flame of a Bunsen burner or alcohol lamp. Remove the spatula and allow the flame to extinguish. Let the spatula cool for 10 to 15 seconds.
4. Lift up the lid of the wild-type (+) culture plate (hold the lid above the agar surface to prevent contaminants from falling onto the agar surface) and touch the spatula to the agar at the side of the dish to make sure the spatula is cool. Remove a block of agar containing a portion of the mycelium.
5. Transfer the agar block to the Petri dish of crossing agar, turning it upside down so the mycelium contacts the surface of the crossing agar. Place the block at one of the positions marked + in Figure 13D-4. Reflame the spatula and repeat this procedure with a second block of agar from the wild-type (+) black strain and place the block in the second position marked + in Figure 13D-4. Blocks should be approximately 2 cm apart.

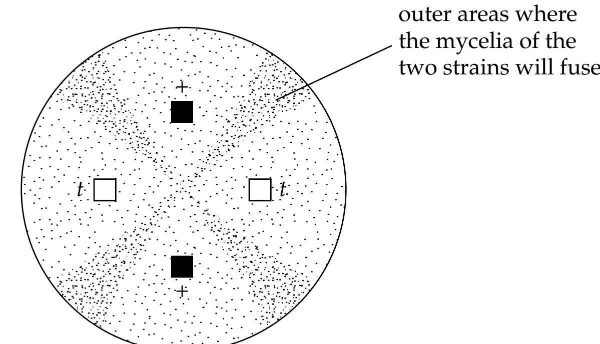


Figure 13D-4 *Blocks of agar covered with mycelia from different genetic strains of* Sordaria *are placed on crossing agar. In week 2 of this experiment (Laboratory 14), you will collect perithecia from the areas where the two strains will have fused.*

6. Reflame the spatula and transfer two blocks from the mutant (*t*) tan strain to the marks indicated in Figure 13D-4.
7. Allow the plates to incubate in the dark at 22 to 24°C for approximately 7 days.

PART 2 Analyzing Hybrids and Crossovers (*Week 2*)

Your instructor will provide you with a plate of *Sordaria fimicola* that contains a cross of black and tan strains. The plate has been incubated for a week and a mycelial mat covers the surface. You will notice areas covered by black and tan hyphae and a dark **X**-shaped area where hyphae growing out from the original agar blocks have now fused (Figure 13D-4). The small black structures (that look much like poppy seeds) are the perithecia or fruiting bodies of the fungus (see Figure 13D-1).

Recall that when mycelia of the black strain (referred to here as + to simplify notation) fuse with mycelia of the mutant tan strain (referred to simply as *t*), their haploid nuclei fuse to form diploid nuclei at the tips of specialized elongated hyphae that will develop into asci. A diploid nucleus divides by meiosis (I and II) to form four nuclei (tetrad stage). Each of these nuclei then replicates by mitosis to form two new haploid nuclei, for a total of eight haploid nuclei. Each of these serves as the nucleus for a spore called an **ascospore.** There are eight ascospores in each ascus of *Sordaria fimicola* (Figure 13D-5).

A key factor that makes *Sordaria fimicola* so useful for genetic studies is that the eight ascospores within the ascus are ordered; they are always lined up in a pattern that is related to the way in which they

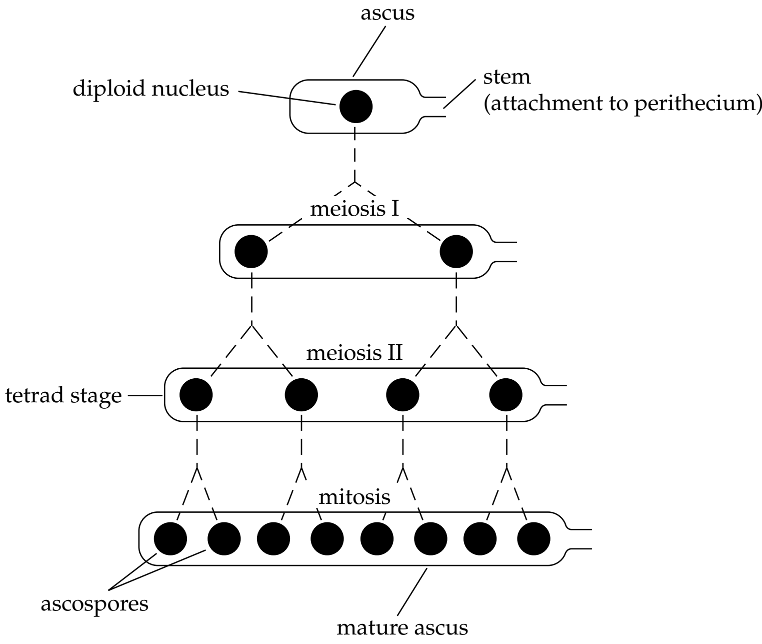


Figure 13D-5 *A diploid* Sordaria *nucleus resulting from the fusion of two haploid nuclei undergoes meiosis I and II to form four haploid nuclei (tetrad stage). Each of these nuclei will, by mitosis, form two haploid nuclei, for a total of eight ascospore nuclei in a single ascus.*

were produced by meiosis. Because of the elongated nature of the ascus and the fact that the spindles of the meiotic and mitotic divisions do not overlap, you can identify the nucleus from which each ascospore was made. (Trace the lineage of the third ascospore from the left in Figure 13D-5.)

If a black strain has hybridized with a tan strain and no crossing-over has occurred during meiosis I, the asci produced will contain four black and four tan ascospores. Segregation of the wild-type black allele and the tan mutant allele to two different nuclei has occurred at meiosis I (see Figures 13D-5 and 13D-6a). Each of these nuclei will give rise to four haploid ascospores as a result of meiosis II and mitosis. To designate the point at which segregation of alleles occurred, these "4+4" asci are called **MI asci.**

(a) **Formation of Non-Crossover Asci**

Meiosis I — Meiosis II — Mitosis

Two homologous chromosomes lining up at metaphase I of meiosis. The two chromatids of one chromosome each contain the allele for tan spore color (t), and the two chromatids of the other chromosone each contain the allele for wild-type spore color (+).

The first meiotic division (MI) results in two cells each containing just one type of spore-color allele (either tan or wild-type.) Therefore, segregation of these alleles has occurred at the first meiotic division.

The second meiotic division (MII) results in four cells, each with the haploid number of chromosomes (1*n*).

A mitotic division simply duplicates these cells resulting in 8 spores. They are arranged in the 4+4 pattern.

(b) **Formation of Crossover Asci**

Meiosis I — Meiosis II — Mitosis

In this case, crossing-over occurs in the region between the gene for spore color and centromere as the homologous chromosomes line up at metaphase I.

This time, MI results in two cells each containing both alleles (1 tan, 1 wild-type), therefore, the alleles for spore color have not yet segregated.

MII results in segregation of the two alleles for spore color.

The mitotic division results in 8 spores arranged in the 2+2+2+2 or 2+4+2 pattern.

Figure 13D-6 *(a) Formation of MI non-crossover asci in* Sordaria. *(b) Formation of MII crossover asci. The symbol + has been substituted for* t^+ *to indicate the wild-type allele.*

a. *Which alleles are responsible for producing black spore color in* Sordaria? ____________________

b. *Which alleles are responsible for producing tan spore color in* Sordaria? ____________________

c. *In the space below, show how the chromosomes must be aligned to produce an ascospore pattern of four black spores at the top of the ascus and four tan spores at the base of the ascus. (See example in Figure 13D-6a.)*

If crossing-over occurs at meiosis I, the result is two nuclei in which both alleles t^+ and t^- (or + and *t* in our notation) are present in each daughter nucleus. These alleles do not segregate until meiosis II, when the chromatids of each chromosome separate. Depending on the arrangement of the chromatids when they separate in meiosis II, four different color patterns of ascospores within the asci are possible (Figure 13D-6b). Asci showing these 2+2+2+2 or 2+4+2 color patterns are designated **MII asci.**

Since in *Sordaria* a genetic strain can mate with itself, asci containing spores of only one color (all black or all tan) are also possible, but these are not referred to as hybrids.

Figure 13D-7 shows meiosis I crossovers between different pairs of non-sister chromatids (these belong to homologous chromosomes present in a diploid fusion nucleus). Complete the information in the figure to show the results of meiosis II and mitosis, then match each ascospore to one of the patterns shown in Figure 13D-6b. (The first possibility has been completed for you.)

Procedure

1. Obtain a plate of *Sordaria* that contains a cross of black and tan strains (a cross plate). Identify the locations where mycelia of the two strains have overlapped and fused. Dark lines of tiny perithecia should be visible in these areas. Use a toothpick to gently scrape the surface of the plate to collect perithecia. (It is usually best to collect perithecia toward the outer rim of the dish.) Do *not* scrape up any of the agar onto your toothpick!
2. Place the perithecia in a drop of water on a slide. Cover with a coverslip and gently press on the coverslip (use a small cork or pencil eraser) to rupture the perithecia. Be gentle so that the ascospores remain in the asci (see Figure 13D-2). If you press too hard, you will observe (in step 3) single ascospores dispersed throughout the slide. In this case, discard your slide and try again.
3. View the slide using the 10× objective and locate a group of asci containing at least some hybrid asci (asci that contain both black and tan ascospores). In the space below, draw a diagram of each type of ascus that you observe. Indicate whether the ascus is hybrid and if so, whether it is MI or MII. Try to find an example of each type of ascus shown in Figure 13D-6a, b. You may have to inspect several perithecia and perhaps make several slides.

d. *What type of ascus is most common?* ____________________ *Why?* ____________________

____

e. *Occasionally you will see asci with a 2+3+1+1+1 pattern or a 2+2+1+2+1 pattern.*
What might be the explanation for these patterns? ____________________

____

f. *In your own words, explain the difference between an MI and MII ascus.*

____

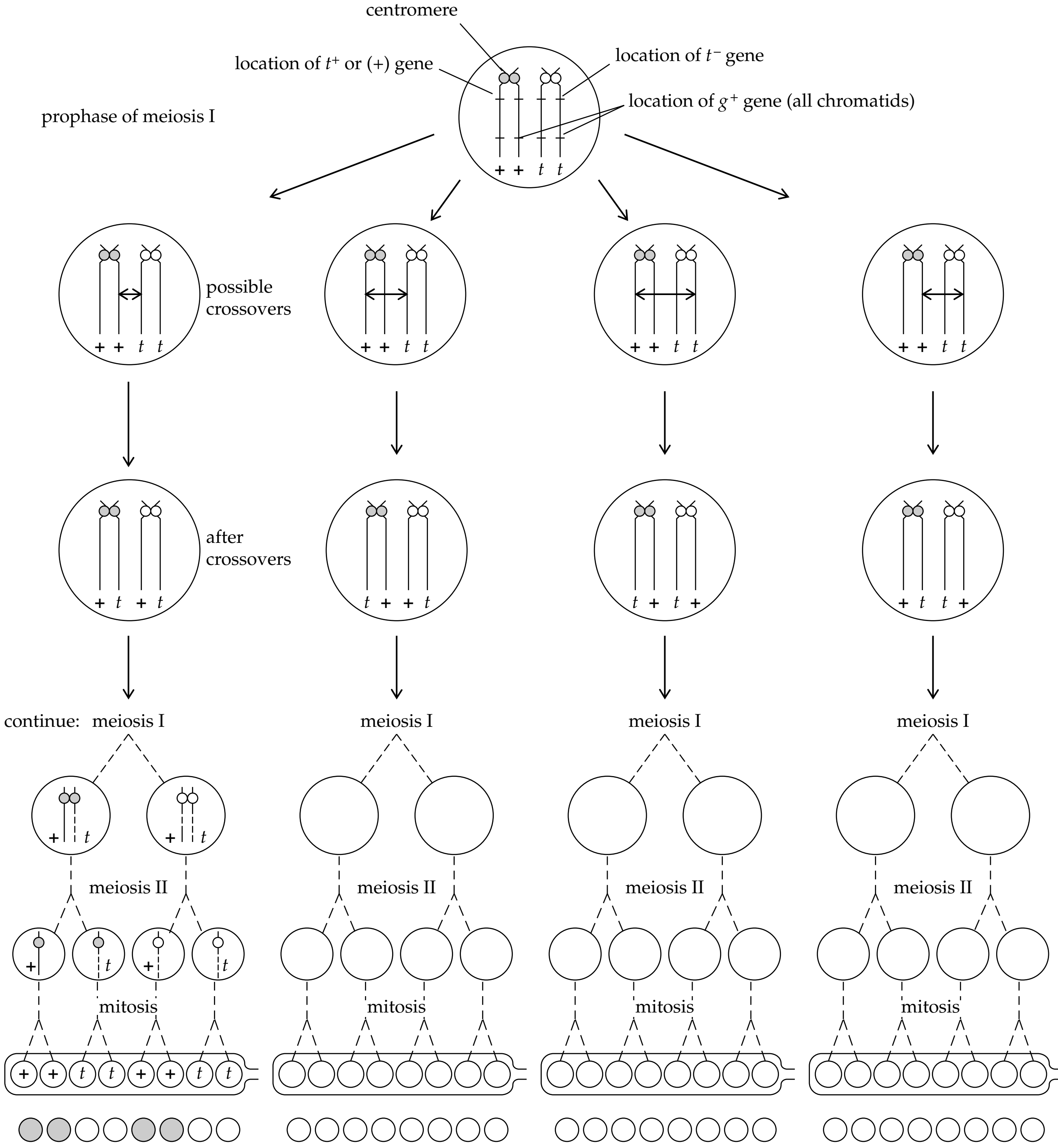


Figure 13D-7 *A diploid fusion nucleus contains a homologous pair of chromosomes carrying alleles for ascospore color. Each chromatid has a gene for tan (normal +, or mutant* t*) and a normal gray gene (*g^+*). (Centromere color indicates future spore color for the combination of alleles present on that homologue.) Crossing-over occurs during prophase I. Non-sister chromatids exchange alleles, with several different patterns of recombination being possible (as shown by arrows ⟷). After crossing-over is complete, meiosis I continues, followed by meiosis II. The results of different patterns of recombination can be seen in the patterns of spore color in resultant asci. (Modified from Richard P. Nickerson,* Genetics*, p. 161, Scott Foresman/Little Brown, 1990.)*

Be sure that you can identify all of the types of MI and MII asci. During the next laboratory period you will calculate the frequency of crossover events by determining the percentage of MI and MII asci on your cross plate. Using the crossover frequencies, it is possible to map the *Sordaria* chromosome (see Laboratory 14, Exercise A).

Laboratory Review Questions and Problems

1. Indicate the stage of meiosis during which each of the following events or situations occurs.

_______ Chromosomes synapse

_______ Chromosomes line up in single file

_______ DNA duplicates

_______ First cell stage to contain only one homologue from each chromosome pair

_______ Homologous chromosomes separate

_______ Chromatids separate

_______ Crossing-over

2. List five ways in which meiosis differs from mitosis.

3. Complete the table below.

	Number of Chromatids per Chromosome	Number of Chrosomes per Cell
G_1 } Interphase preceding meiosis		
S } Interphase preceding meiosis		
G_2 } Interphase preceding meiosis		
Prophase I		
Metaphase I		
Anaphase I		
Telophase I		
Interphase		
Prophase II		
Metaphase II		
Anaphase II		
Telophase II		

4. During gamete formation, if alleles did not segregate at meiosis I, what might the consequences be for a zygote resulting from the union of such gametes?

5. Because of independent assortment, it is possible for a single human to produce 2^{23} different types of gametes. Explain how this is possible.

6. During gamete formation, genetic variation, in addition to that due to independent assortment, can be increased by crossing-over events during meiosis I. Explain what consequences this could have for possible offspring.

7. How might recombination of genes due to crossing-over be important to the process of evolution?

8. Two pea plants, *Rr* and *RR,* are crossed. What are the genotypes and phenotypes of the F_1 offspring? Did segregation of alleles occur? Did independent assortment of alleles occur?

9. Two pea plants, *RrSs* and *rrSs,* are crossed. What are the genotypes and phenotypes of the offspring? Did segregation of alleles occur? Did independent assortment of alleles occur?

10. What does it mean to say that two genes are "linked"? Can linked genes assort independently? Why or why not?

11. In a certain species of flower, genes for petal color, petal shape, and plant height are linked by their presence on the same chromosome.

Dominant	Recessive
Blue (*B*)	White (*b*)
Frilly petal (*F*)	Smooth petal (*f*)
Tall (*T*)	Short (*t*)

A tall, blue, frilly plant, heterozygous for each trait is crossed with a white, smooth, short plant. Among the offspring are some unexpected blue, smooth, short plants as well as some white, frilly, tall plants. Explain how this could have happened.

12. Two strains of *Sordaria* (black and gray) are crossed, and asci have the following color patterns. (+ represents wild-type, t^+):

Group	Ascopore Pattern
I	++++*gggg*
II	*gggg*++++
III	*gg*++*gg*++
IV	++*gg*++*gg*
V	++*gggg*++
VI	*gg*++++*gg*

a. Which asci result from the absence of crossing-over?

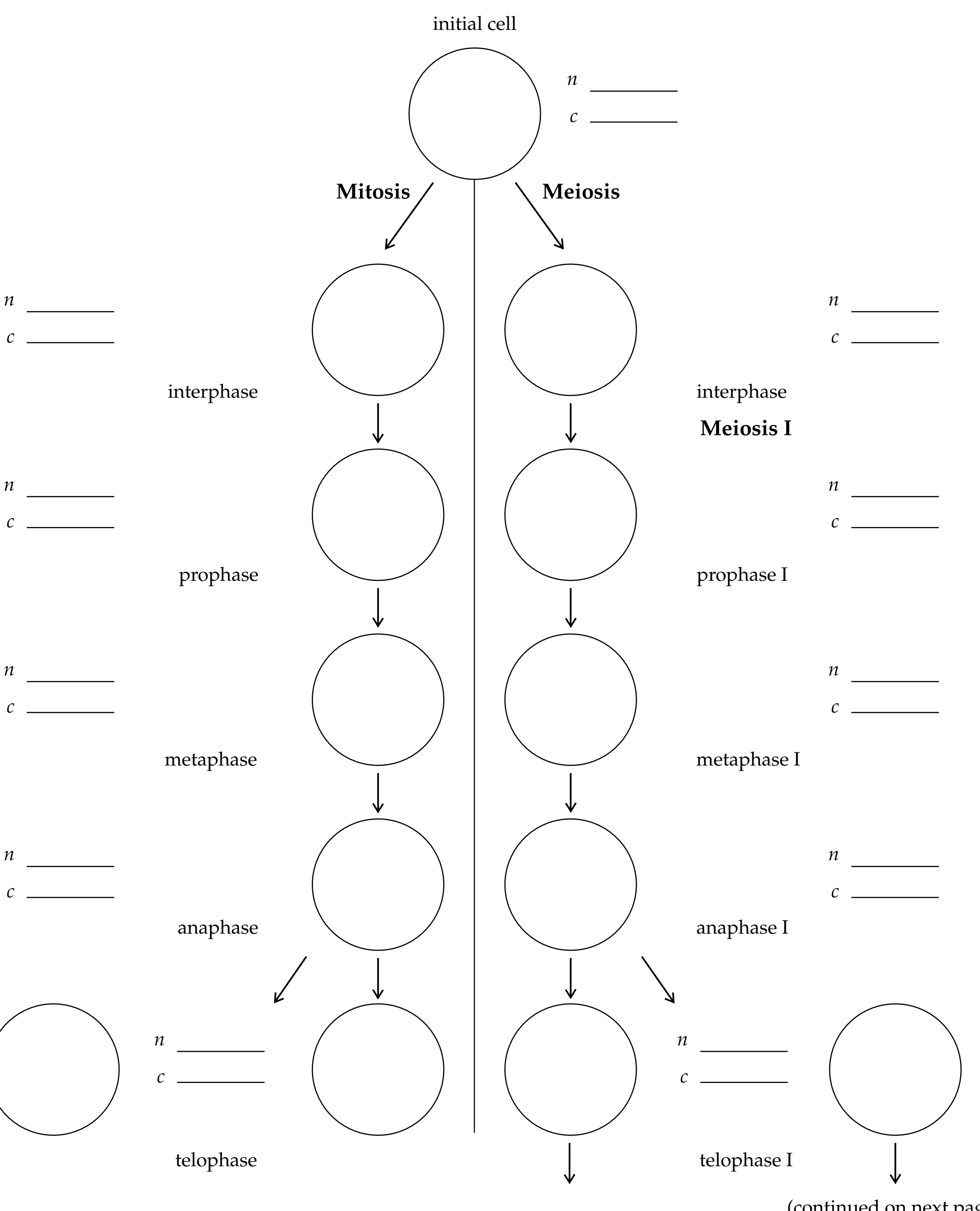
b. Which asci are MI asci?

c. Which asci are MII asci?

d. Why can a single cross produce all of the types of asci shown?

e. Explain why only half of the ascospores in groups III, IV, V, and VI are the result of recombination (*think*, and review Figure 13D-7).

13. From your study in Laboratory 9 and Laboratory 13, you should understand the basic differences between the processes of mitosis and meiosis and the genetic consequences of each. To summarize your understanding, start with a cell having a diploid chromosome number equal to six ($2n = 6$) and draw the processes of mitosis and meiosis. (You may wish to use colored pencils.) Note the n number ($1n$, $2n$, $4n$, etc.), where n represents a unique set of chromosomes (e.g., if diploid, there are two sets of homologous chromosomes, or $2 \times n = 2n$). Also note the c number, which refers to the number of chromatids (or copies of DNA) per chromosome.



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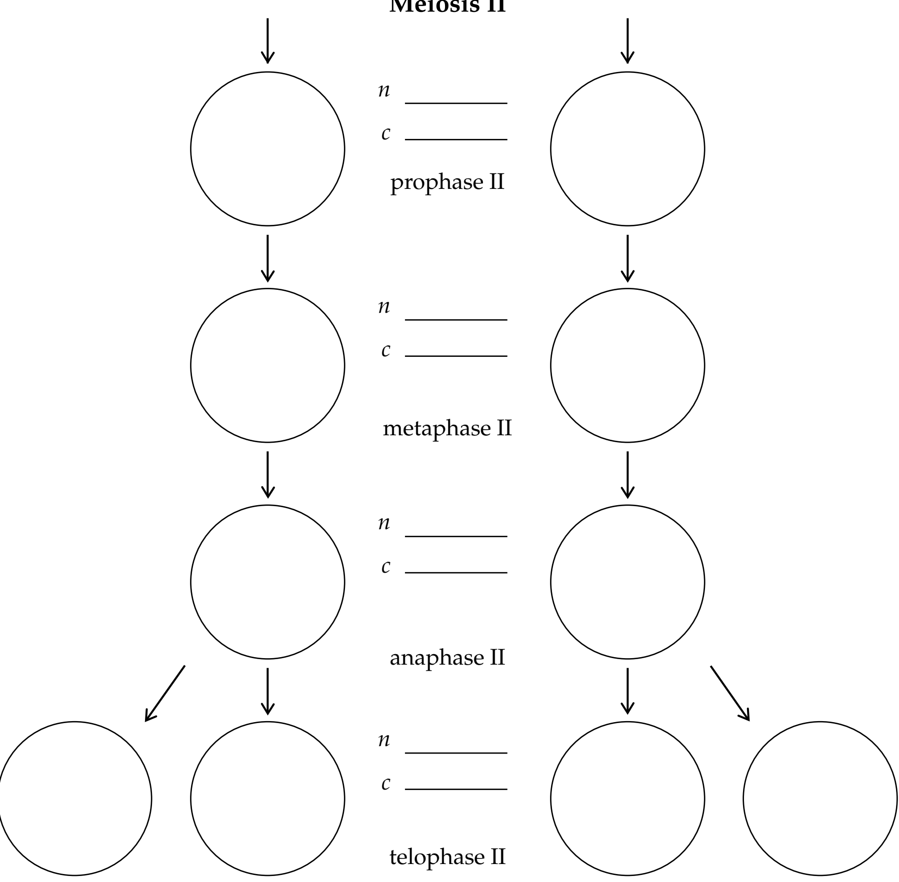


Genes and Chromosomes: Chromosome Mapping

LABORATORY 14

OVERVIEW

Genes are located on chromosomes in particular positions called **loci.** Genes can be assigned to the sex chromosomes (*X* and *Y*) by studying inheritance patterns among family groups, but assigning genes to specific autosomes (non-sex chromosomes) is more difficult. Since segregation of alleles for linked genes is not independent, linkage studies make it possible to assign two genes to the same chromosome, but this information does not usually allow us to identify a particular gene as belonging to a specific chromosome. Several methods, including the use of restriction enzymes and recombinant DNA techniques (Laboratory 18), have made it possible not only to assign a gene to a particular chromosome, but to determine, or **map,** its location with respect to other genes on that chromosome.

In this laboratory, you will map the genes for spore color in the fungus *Sordaria.* Using linkage analysis, you will be able to determine the relative distance between the centromere and known mutant genes. You will also study the giant chromosomes of the fruit fly *Drosophila,* which will allow you to visualize genes on a eukaryotic chromosome. By using bacterial conjugation as a means of genetic recombination, you will be able to map several genes on the circular chromosome of the bacterium *Escherichia coli.* Finally, you will have the opportunity to use restriction enzymes and electrophoresis to compile a restriction map of the phage lambda (λ).

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

EXERCISE A Mapping the Genes of *Sordaria fimicola*

In *Sordaria fimicola,* ascus formation requires both meiotic and mitotic divisions to produce eight haploid ascospores. During the first nuclear division of meiosis (meiosis I, or MI), crossing-over often occurs between non-sister chromatids of homologous chromosomes, resulting in recombination of the genes on the two homologues (Figure 14A-1). The greater the distance between the two genes on the chromosome, the more likely it is that crossing-over will occur; the genes are "loosely" linked. If the genes are close together, then crossing-over is less likely; the genes are said to be "tightly" linked. Thus the frequency with which two genes recombine is related to how far apart the two genes are on the chromosome. By using recombination frequencies, it is possible to produce a *linkage map* for genes on a chromosome.

Figure 14A-1 *(a) Two homologous chromosomes carrying different alleles of a gene for a specific trait. The alleles are located at the same loci on the homologues. (b) Crossing-over occurs. (c) Alleles are exchanged, and each chromosome carries alleles that are different from those on the original chromosome.*

B B b b → B b B b → B b B b

(a) (b) (c)

PART 1 Mapping *Sordaria* Chromosomes *(Week 2; see Laboratory 13, Exercise D)*

During ascus development, if no crossing-over occurs in meiosis I, a 4+4 pattern of ascospores is produced. These asci have an **MI pattern.** If crossing-over does occur, then the pattern of ascospores is referred to as **MII** and is either 2+2+2+2 or 2+4+2.

From the numbers of asci with MI and MII patterns following a cross, you can "map" the location of the gene for tan color (*t*) on the *Sordaria* chromosome. You will determine the distance of the tan gene locus from the centromere (centromere-to-locus distance). Note that this exercise considers just the gene for tan color, since the presence of a normal allele (t^+) or a mutant allele (t^-) will alter ascospore color. For this reason you will map the distance from the centromere, not the distance from another gene. (In more typical linkage mapping, recombination between two genes is studied to determine map distance between the genes.)

Review basic information about *Sordaria* genetics in Laboratory 13, Exercise D, before continuing. Be sure that you can recognize MI and MII ascus patterns.

Objectives

- ☐ Explain how meiosis and crossing-over result in different arrangements of ascospores within asci.
- ☐ Calculate the map distance between a gene for ascospore color and the centromere of the chromosome on which the gene is found.

Procedure

1. Your instructor will provide you with several brown paper bags used to simulate *Sordaria* perithecia from a black × tan cross. Each bag contains 10 asci. Remove the asci and determine whether they are nonhybrid or hybrid, MI or MII asci. Your instructor will ask you to report the number of MI and MII asci in your bag; your response should be verified (or corrected) by your laboratory partner. (You do *not* count the nonhybrid asci containing all like-color ascospores. *Why?*) After totaling the number of MI and MII asci for the entire class, you will be asked to determine the frequency of crossing-over and the map distance from the centromere to the tan gene, using steps 7 and 8 of this procedure. Record class data in Table 14A-1.

Table 14A-1 Class Data

Total asci counted	
Total MI asci	
Total MII asci	

a. What is the map distance of the tan gene from the centromere on the Sordaria *chromosome as simulated in this cross?* ______________________

2. Now, obtain your *Sordaria* cross (from Laboratory 13). Alternatively, your instructor has set up the *Sordaria* crosses and will provide you with a culture.
3. Where the mycelia of the two strains overlap and fuse, dark lines of tiny perithecia will be visible. Use a toothpick or spatula to gently scrape the surface of the agar to collect perithecia (see Figure 13D-4). It is usually best to collect perithecia toward the outer rim of the dish.
4. Place the perithecia in a drop of water on a slide. Cover with a coverslip and gently press on the coverslip (use a small cork) to rupture the perithecia. Be gentle so that the ascospores remain in the asci (see Figure 13D-2).
5. View the slide using the 10× objective and locate a group of hybrid asci (recall that asci produced by fusion of two identical strains, both black or both tan, will result in ascospores that are all of the same color within an ascus—disregard these asci). Hybrid asci contain both black and tan ascospores within each ascus.
6. Count at least 50 hybrid asci and score them as either MI asci (4+4 arrangement) in which alleles segregated in meiosis I, or MII asci (2+2+2+2 or 2+4+2) in which alleles segregated in meiosis II. (Remember, do *not* count the nonhybrid asci.) Record your results in Table 14A-2. Determine the number of M1 and M2 asci counted by all students and record class results in Table 14A-3.

Table 14A-2 Your Data

Number of MI Asci Showing No Crossover (4:4)		Number of MII Asci Showing Crossover (2:2:2:2) or (2:4:2)		Total MI + MII Asci	Percentage of Asci Showing Crossover	$\frac{\text{Frequency}}{2}$ (Map Units)
○○○○●●●●		○○●●○○●●				
●●●●○○○○		●●○○●●○○				
		○○●●●●○○				
		●●○○○○●●				

Recall that in this exercise, you are studying only one gene, and you will map its distance from the centromere by determining the frequency of crossover events involving that gene—crossovers that occur somewhere between the centromere and the gene and result in its recombination with the chromatid of a different chromosome.

The frequency of crossing-over between two genes is largely controlled by the distance between genes (or between gene and centromere, as in this case); the probability of a crossover occurring between two particular genes on the same chromosome increases as the distance between those genes increases. The frequency of crossing-over is, therefore, proportional to the distance between genes. An arbitrary unit of measure, the **map unit,** is generally used to describe distances between linked genes. A map unit is equal to a 1% frequency of crossovers. For instance, when there is a 30% frequency of crossing-over between two genes, these genes are said to be 30 map units apart.

7. Determine the frequency of crossing-over (percentage of crossovers) by dividing the number of MII crossover asci by the total number of asci counted, and multiplying by 100:

$$\text{Frequency of crossing-over} = \frac{\text{MII}}{\text{MI + MII}} \times 100$$

8. In *Sordaria,* since only 4 of the 8 ascospores carry recombinations, the frequency of recombination is one-half of the frequency of crossing-over.

b. Why do only half of the ascospores carry recombinant chromosomal strands?

____

____

The relationship between frequency of crossing-over and map distance is expressed as:

$$\text{Number of map units} = \frac{4 \times \text{number of recombinant (MII) asci}}{8 \times \text{total number of asci}} \times 100$$

or

$$\text{Number of map units} = \frac{\text{frequency of crossing-over}}{2}$$

9. Record the map distance of gene t or t^{+} from the centromere. ________ Record this map distance in Table 14A-2. Published results indicate that the map distance of the tan spore-color gene from the centromere in *Sordaria fimicola* is 26 map units.

c. How closely do your data fit this measurement? ______________________________

PART 2 The Chi-Square Test (*Optional*)

To test how closely your class crossover data agree with or "fit" the value for the map distance of the mutant tan gene obtained by others (26 map units), you can perform a chi-square test (see Appendix I). The chi-square test is a statistical test that allows you to determine if differences between your data (what was *observed*) and known or hypothetical values (what was *expected*) are significant.

Objectives

☐ Perform a chi-square test to determine the probability that your data "fit" the expected map distance.

Procedure

1. As you determine whether or not your observed data "fit" within the limits of what is expected, the chi-square analysis is actually testing the validity of your **null hypothesis** (Laboratory I). Your null hypothesis states: There will be no difference between the predicted (expected) map distance of 26 units and the observed map distance calculated from class data.

If there *is* a difference (your map distance is not 26 units), you want to know how significant this difference is. Is the difference due just to chance (perhaps sampling errors)? Or is it due to something real (a chromosomal rearrangement or deletion, etc.)? If the results of the chi-square test indicate that the observed data do *not* vary significantly from the expected, then you accept the null hypothesis: there is no difference. If, however, the results indicate that the

Table 14A-3 Class Data

Number of MI Asci		Number of MII Asci		Total MI + MII Asci	Percentage of Asci Showing Crossover	$\frac{\text{Frequency}}{2}$ (Map Units)
○○○○●●●●		○○●●○○●●				
●●●●○○○○		●●○○●●○○				
		○○●●●●○○				
		●●○○○○●●				

observed data vary significantly from the expected, then you *reject* the null hypothesis and accept your **alternative hypothesis** (see Laboratory I).

Your instructor will use the data from the *Sordaria* simulation to demonstrate how to perform the chi-square test following the steps outlined below.

a. How well do the data from the class simulation fit the expected result of 26 map units?

____

2. Use the class data on *Sordaria* cross plates from Table 14A-3.

Chi-square is calculated as shown in the following example.

Example If 26 map units is the expected locus-to-centromere distance for the tan gene on the *Sordaria* chromosome, you should expect 52% of all asci you observe to be crossover asci. (Recall that you divided the percentage of crossovers by 2 to calculate map distance; 26% × 2 = 52%.) Suppose 1,000 asci are counted by the class; 52% of 1,000 = 520. Thus, 520 of the asci should be crossovers and 480 should be non-crossovers. This is what is *expected.* What was actually observed by the class was

Crossovers = 510

Non-crossovers = 490

Using the formula

$$\chi^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

for both crossovers and non-crossovers, the expected (or hypothetical values) are filled in as follows:

$$\chi^2 = \frac{(\text{observed crossovers} - 520)^2}{520} + \frac{(\text{observed non-crossovers} - 480)^2}{480}$$

Since 510 crossovers and 490 non-crossovers were observed in class,

$$\chi^2 = \frac{(510 - 520)^2}{520} + \frac{(490 - 480)^2}{480}$$

$$\chi^2 = \frac{(-10)^2}{520} + \frac{(10)^2}{480} = \frac{100}{520} + \frac{100}{480} = 0.192 + 0.208 = 0.400$$

Do observed and expected results differ in this case, where $\chi^2 = 0.400$? To decide, you must refer to a Critical Values of χ^2 Table (Appendix I). Table 14A-4 is an abbreviated table for use in this laboratory.

Table 14A-4 Critical Values of Chi-Square

Degrees of Freedom (*df*)	Probability (*p*)				
	0.9 (9 in 10)	0.5 (1 in 2)	0.2 (1 in 5)	0.05 (1 in 20)	0.001 (1 in 100)
1	0.016	0.46	1.64	**3.84**	6.64
2	0.21	1.39	3.22	5.99	9.21
3	0.58	2.37	4.64	7.82	11.35

First, you must determine the **degrees of freedom** (*df*) for your experiment. In this example, the degrees of freedom are 1 less ($n - 1$) than the number of attributes being observed ($n = 2$, since crossover and non-crossover are the only attributes being studied). Next, you determine a **probability value** (*p* value). For most scientific studies, the minimum probability for rejecting a null hypothesis is usually $p = 0.05$. In selecting a probability of $p = 0.05$, you set a "level of rejection" for your null hypothesis. If you

reject your null hypothesis, you have a 1 in 20 (or 5%) chance of being wrong in doing so (a fairly low probability of making the wrong decision!). Having determined the *df* and *p* values, you next find the **critical value** from the chi-square table, in this case 3.84.

- *If the calculated chi-square value is* ***greater than or equal to*** *the critical value, then you* ***reject*** *the null hypothesis (and accept the alternative hypothesis).* You conclude that deviations from the expected are sufficiently large to be meaningful (significant), so there must actually be a difference (you reject the null or no-difference statement).
- *If the calculated chi-square value is* ***less than*** *the critical value, then you* ***accept*** *the null hypothesis.* You conclude that deviations from the expected are sufficiently small that there is no difference (you accept the null or no-difference statement).

The chi-square value for this example ($\chi^2 = 0.40$) is much smaller than 3.84. This means that you *accept* the null hypothesis: that class observations agree with (there is no difference from) the published or known value of 26 map units for the distance of the tan gene from the centromere on the *Sordaria* chromosome. [*Note:* If you rejected the null hypothesis based on $\chi^2 = 0.40$, you would have a *greater* chance of being wrong in making this decision—almost a 1 in 2 chance (see $p = 0.5$ for a critical value of 0.46), and this is not an acceptable degree of error.]

3. Now, apply the chi-square test to your class results recorded in Table 14A-3. Perform your calculations in the space below.

b. What is your null hypothesis for this investigation? ______________________

What is your alternative hypothesis? ______________________

c. How well do your class data fit the expected value of 26 map units? ______________________

d. Do you accept or reject your null hypothesis? ______________________

e. What do you conclude about the distance of the tan gene from the centromere on the Sordaria *chromosome?* ______________________

____

✔ EXERCISE B | Examining the Giant Chromosomes of *Drosophila*

During development, cells of the *Drosophila* salivary glands pass through the cell cycle many times, their DNA replicating in preparation for division. However, the cells do not divide and as a result, the number of DNA strands comprising each chromosome continues to increase; the chromosomes become multistranded and are called *polytene* chromosomes. The DNA content of polytene chromosomes is approximately 1,000 times greater than the normal DNA content of chromosomes containing a single helical strand of DNA.

The many DNA strands of a polytene chromosome condense and fold in the same manner as the single helical strand of DNA in other chromosomes. Highly condensed or folded areas stain darkly and give chromosomes a banded appearance. Since all of the DNA strands in the giant polytene chromosome are duplicates of one another, the folded portions are in register with one another and the bands appear to stretch across the entire chromosome, giving the chromosomes a very dramatic "striped" appearance (Figure 14B-1).

During transcription of messenger RNA, some of the banded regions of the polytene chromosomes uncoil and expand to form "puffs." Thus, it is possible to visualize genes on these giant chromosomes. If the protein product synthesized by the gene can be identified, so can the gene. Proof of its location can also be obtained by isolating the mRNA produced by the "puff" region, labeling it in some way, and

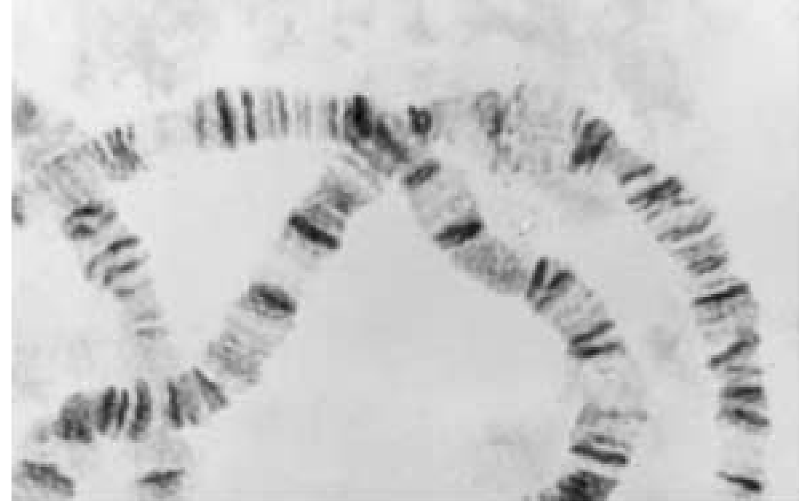


Figure 14B-1 *The polytene chromosomes of* Drosophila melanogaster.

hybridizing this mRNA with the chromosome. Where the bases are complementary to the DNA, they bind, allowing the genes to be identified by autoradiographic or immunofluorescent techniques.

In this exercise you will prepare chromosome "squashes" of *Drosophila* polytene chromosomes and observe their banded nature.

Objectives

- ☐ Describe how the banded pattern of the giant chromosomes of the *Drosophila* salivary gland is related to the arrangement of genes on the chromosome.

Procedure

1. Use the dissecting microscope. If your microscope does not have a built-in illuminator, use a black background or a mirror and a transparent glass plate. Obtain a clean slide and place a drop of 0.7% saline toward one end.
2. Place a *Drosophila* larva in the saline, and with two dissecting needles decapitate the larva. Place one needle at the middle of the larva and the other just behind the head. Often you can see the two salivary glands separating from one another in a **V** at this point, and it is best to place the point of the needle in the middle of the **V.** Pull the needle at the head with a quick

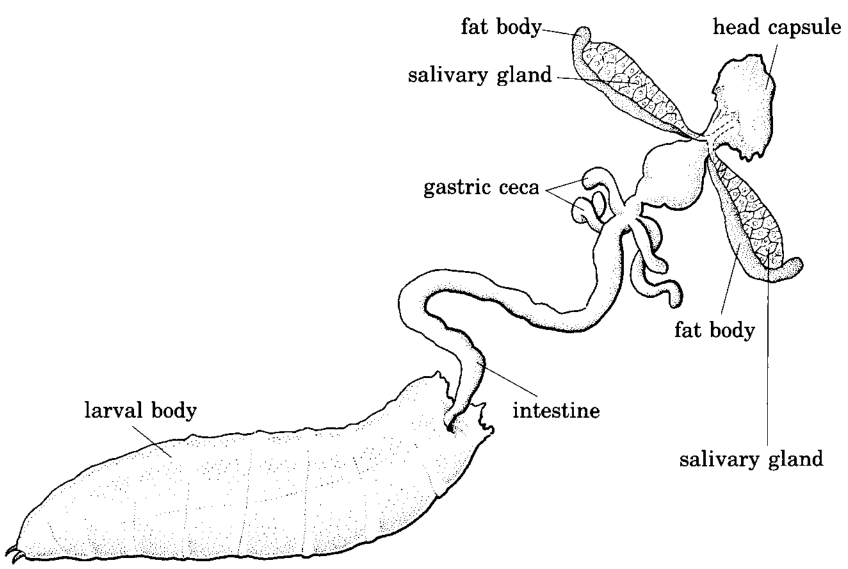


Figure 14B-2 *Dissection of the salivary glands of a* Drosophila melanogaster *larva.*

jerk and then relax, and the salivary glands will slowly be pushed out of the body. The glands are elongated and semitransparent—the individual cells are large (grapelike in appearance) and are lined up along the lumen of each gland. The lumen connects to the digestive tract through a duct (Figure 14B-2).

3. Remove the opaque fat body material adhering to the glands. If you are using transmitted light, the fat body material will appear grayish. With reflected light, it will appear white.
4. Place a drop of acetocarmine or aceto-orcein stain next to the drop of saline and, with a dissecting needle, transfer the glands from the saline to the stain. Examine the drop of stain to make sure the glands were transferred.
5. Stain for 10 minutes. Make sure that the drop of stain does not dry up.
6. Place a coverslip on the preparation.
7. Place the slide between the folds of a paper towel and press down on the coverslip firmly. The eraser end of a pencil can also be used for pressing.
8. Examine the slide using low power (10×) to locate the chromosomes. Examine the chromosomes to observe the banded pattern.

   a. *Do you see any bulges along the length of the chromosome?* _______ *What do they represent?*

   ____

   b. *How many chromosomes do you see?* (Note: *Homologous chromosomes are synapsed along their entire length, so what appears as one chromosome is actually two.)* _______

Note: The chromosome studies in Exercises A and B have introduced you to some of the basic, and more classical, techniques for locating genes on eukaryotic chromosomes. Rapid advances in biotechnology have made it possible to map eukaryotic genes more quickly and more accurately. To do this, special enzymes, **restriction endonucleases** (see Exercise D), are used to chop DNA into small fragments. These enzymes recognize specific nucleotide sequences in the DNA and always cut the DNA at the same sites. (Mutations in the DNA may alter the nucleotide sequence of a site so that the restriction endonuclease no longer cuts the DNA at the original position: a fragment with a "new length" is produced.)

Fragments produced by restriction endonucleases are called RFLPs (pronounced "rif-lips"). (See Laboratory 15, Exercise F.) By studying how frequently characteristic RFLPs appear in several generations of families that exhibit a particular genetic trait or disorder, geneticists can determine the approximate location of the gene for that disorder on a particular chromosome. Just as in linkage studies, where two genes are said to be linked if they constantly appear together (they are so close together on the chromosome that no crossing-over can occur), the RFLP and a particular trait are assumed to be "linked" and, thus, in close proximity to one another on the chromosome. If the RFLP is hybridized to the DNA of the chromosome, the physical location of the gene can be determined. In addition, the RFLP can serve as a "marker" for a specific trait. If the DNA of a patient contains the RFLP associated with a particular disorder, it is likely that the person will manifest that disorder. This is especially important as a diagnostic tool for disorders that are characterized by late onset.

EXERCISE C Mapping the Chromosome of *Escherichia coli*

Reproduction in bacteria is primarily an asexual process involving fission, but a type of sexual reproduction known as **conjugation** can also occur. During this process, genetic material is transferred from one bacterium (the + or donor strain) to another bacterium (the − or recipient strain). Donor cells contain a fertility factor, or *F* factor, carried by a plasmid (a small extrachromosomal, circular piece of DNA), which can be transferred during conjugation. These donors (males) are designated as F^+. If the *F* factor becomes integrated into the bacterial chromosome of the donor, the chromosome (although usually not the entire chromosome) can be transferred to the recipient bacterial cell. If recombination takes place, the recipient

may express characteristics originally unique to the donor. Bacteria containing the *F* factor integrated into the chromosome (as an **episome**) are called **Hfr** (high frequency of recombination) **cells.**

The amount of chromosomal DNA that can be transferred from an Hfr cell to a recipient cell is determined by how long the cells remain in contact (during conjugation, the cells are attached by **pili;** DNA transfer occurs through cytoplasmic "bridges"). The farther away a given gene is from the leading point of the chromosome being transferred, the less chance the gene has of being transferred before the bridge is broken. By interrupting mating at specific times, it is possible to construct a circular map of the bacterial chromosome. The map distances are in "minutes," referring to the time it took to transfer certain genes (Figure 14C-1).

In this exercise, you will work with two strains of *E. coli.* The donor, the Hfr strain, is streptomycin-sensitive: it does not carry the gene for streptomycin resistance and thus will be killed by this antibiotic. This "wild type" also carries alleles for synthesizing the amino acids proline, leucine, and threonine, and the vitamin thiamine. Thus this strain is designated $Str^s\ pro^+\ leu^+\ thi^+\ thr^+$. The wild-type strain can live on minimal medium that contains only glucose, ammonia, and inorganic salts, because it can make all of the amino acids needed for growth. (Remember: if streptomycin is present in the medium, these cells will die.)

The mutant strain, which is the recipient, carries the gene for streptomycin resistance, but does not carry the alleles for synthesizing proline, leucine, threonine, and thiamine. This mutant strain is designated $Str^r\ pro^-\ leu^-\ thi^-\ thr^-$. For the recipient to survive (prior to recombination), the minimal medium must be supplemented with the amino acids proline, leucine, and threonine and the vitamin thiamine; streptomycin in the medium will not harm the recipient strain.

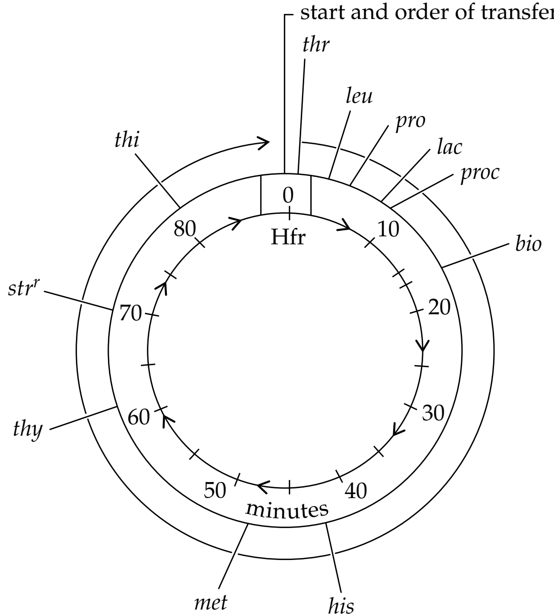


Figure 14C-1 *Genetic map of* Escherichia coli *showing several genes.*

You can determine whether particular genes have been transferred from the donor to the recipient by testing to see if the recipient has acquired the ability to synthesize any of the nutrients that had previously been synthesized only by the donor strain. For example, the transfer of the thr^+ gene can be detected in the recipient by the fact that the recipient, previously thr^- (unable to synthesie threonine), is now thr^+, due to recombination. [Since this gene is close to the origin of replication and transfer (Figure 14C-1), it is the most likely gene to be transferred to the recipient.] The recipient can now synthesize its own threonine and no longer requires a medium containing threonine for its survival.

a. *If you had unlabeled cultures of* Str^s pro$^+$ leu$^+$ thi$^+$ thr$^+$ *and* Str^r pro$^-$ leu$^-$ thi$^-$ thr$^-$, *what could you do to tell them apart?* ______________________________

____

____

b. *What kind of test could you devise to tell whether recombination had occurred between* Str^s pro$^+$ leu$^+$ thi$^+$ thr$^+$ *and* Str^r pro$^-$ leu$^-$ thi$^-$ thr$^-$? ______________________________

____

____

Procedure

1. Work in pairs. Review Appendix III, Preparing Serial Dilutions. Obtain a broth culture of *E. coli* strain *Str^s pro$^+$ leu$^+$ thi$^+$ thr$^+$* labeled "D" for "donor strain."
2. Obtain a broth culture of *E. coli* strain *Str^r pro$^-$ leu$^-$ thi$^-$ thr$^-$* labeled "R" for "recipient strain."
3. Also obtain from your instructor four agar plates containing minimal medium supplemented with streptomycin and thiamine (STR/THI) and two agar plates containing minimal medium (M) supplemented with the amino acids proline, leucine, threonine, and the vitamin thiamine, but without streptomycin (M/PLTT).

   Notice that both types of plates you are using are supplemented with thiamine. Examine Figure 14C-1.

   c. *Where is the thiamine (*thi*) gene located?* ______________ *What is the chance that it will be transferred from donor to recipient during conjugation?* ______________ *Why would you supplement plates with this vitamin?* ______________________________
4. Before proceeding, gather the following equipment and set up your work area on a clean surface:

   1 sterile, *empty* capped test tube (the "conjugation" tube)

   5 tubes containing 9.0 ml of sterile distilled water

   1 beaker containing 95% ethyl alcohol

   1 glass spreading rod

   10 sterile 1-ml pipettes
5. Using aseptic technique, first flaming the mouth of the tube and using a sterile pipette, transfer 1.0 ml of "D" suspension into the conjugation tube. Aseptically transfer 1.0 ml of "R" suspension into the conjugation tube. *Note the time* of addition of culture "R" to culture "D" in the conjugation tube: __________ Gently agitate the mixture by rotating the tube between the palms of your hands. Allow the culture to incubate at 37°C for 30 minutes. Be sure that the mating mixture is *not* disturbed during this time.
6. Aseptically pipette 1.0 ml of "D" culture into a 9.0-ml sterile water blank and mix thoroughly. Note that the donor culture is now diluted to 1:10. Label the tube "D 1:10."
7. Using the diluted donor culture, prepare a spread-plate (see Laboratory 6, Exercise C). Use a sterile 1-ml pipette to remove 0.1 ml of dilute culture and transfer it to the center of the STR/THI plate. Dip a glass spreading rod in 95% ethyl alcohol and flame it. Let it cool for a

few seconds and touch it to the outer edge of the agar plate—if it sizzles, it is still too hot to use. When the rod has cooled, spread the inoculum over the surface of the plate. To avoid contamination, hold the lid above the plate as you are working with the bacteria.

8. Use a sterile 1-ml pipette to remove 0.1 ml of dilute "D" culture and transfer it to the center of a M/PLTT plate. Spread the bacteria as described in step 7.

d. Do you expect the donor strain to grow on the STR/THI plate? _______ *Why or why not?*

____

e. Do you expect the donor strain to grow on the M/PLTT plate? _______ *Why or why not?*

____

f. Why did you prepare both the STR/THI and the M/PLTT plates using only donor cells?

____

____

9. Now repeat the procedure outlined in steps 6 through 8, but this time use the recipient strain and fresh STR/THI and M/PLTT plates. (Label the diluted recipient strain culture "R 1:10.")

g. Do you expect the recipient strain to grow on the STR/THI plate? _______ *Why or why not?*

____

h. Do you expect the recipient strain to grow on the M/PLTT plate? _______ *Why or why not?*

____

i. Why did you prepare both the STR/THI and M/PLTT plates using only recipient cells?

____

____

10. After conjugation has proceeded for 30 minutes (step 5), remove the culture from the 37°C water bath or incubator and vigorously agitate the mixture by rotating the tube between the palms of your hands (create a vortex, if possible).

11. Aseptically transfer 1.0 ml of the conjugation mixture to a sterile water blank (9.0 ml). Mix thoroughly (roll between your palsm) and label the tube "D × R 1:10."

12. Aseptically pipette 1.0 ml of the D × R 1:10 suspension into a second sterile water blank (9 ml). Mix thoroughly (roll between your palms) and label the tube "D × R 1:100."

13. Aseptically transfer 0.1 ml of the D × R 1:10 dilution to the surface of one of the two unused STR/THI plates. Mark the plate "STR/THI 1:10" and label it with your name and the date.

14. Similarly, transfer 0.1 ml of the D × R 1:100 dilution to the surface of the other STR/THI plate. Mark the plate "STR/THI 1:100" and label it with your name and the date.

15. Sterilize a glass spreading rod by dipping it in alcohol and passing it quickly through a flame. Spread the bacteria on the STR/THI 1:10 plate as in step 7. Resterilize the spreading rod and spread the bacteria on the STR/THI 1:100 plate.

16. Be sure that all six of your plates are clearly labeled. Tape them together. Invert them and place them in an area designated by your instructor. They will be incubated for 2 days at 37°C and then refrigerated until the next laboratory period.

17. During the next laboratory period, complete Table 14C-1 using ✔ to indicate growth and 0 to indicate no growth. Interpret your results.

j. Did conjugation occur? _______ *How do you know?* _______________________

____

Table 14C-1 Record of Growth (✓) and No Growth (0) of *E. coli*

	Donor (1:10)	Recipient (1:10)	D × R 1:10	D × R 1:100
STR/THI				
M/PLTT			X	X

k. With reference to the circular map of E. coli, *which genes$^{(+)}$ are found in the recombinant cells that were mutant$^{(-)}$ in the recipient cells?* ______________________________

____

l. It is unusual for the entire E. coli *chromosome to be transferred during the process of conjugation. What is the consequence of this fact for genes that are farther away from the origin of replication and transfer?* ______________________________

____

m. Why was streptomycin included in the agar used for plating recombinant cells?

____

If the recipient bacterial strain had also been *met*$^-$ (lacking the ability to synthesize the amino acid methionine) and recombination with the same wild-type donor cell occurred, what would have happened under the following conditions? (Refer to Figure 14C-1.)

- Conjugation is interrupted after 10 minutes and recombinants are plated onto minimal medium containing streptomycin. *n. Would recombinants grow?* ________

  o. Would these recombinants grow on medium containing both streptomycin and methionine? ________

- Conjugation is allowed to continue for 60 minutes and recombinants are plated onto minimal medium containing streptomycin. *p. Would these recombinants grow?* ________

  q. What does this indicate about the relative positions of the genes for proline, leucine, threonine, methionine, and thiamine? ______________________________

  ____

✓ EXERCISE D Restriction Endonucleases: Mapping Bacteriophage Lambda

Restriction endonucleases are essential tools in recombinant DNA methodology. Several hundred have been isolated from a variety of prokaryotic organisms. In the nomenclature of restriction endonucleases, the letters refer to the organism from which the endonuclease was isolated. The first letter of the name stands for the genus name of the organism. The next two letters represent the initial letters of the second word of the species name. The fourth letter (if there is one) represents the strain of the organism. Roman numerals indicate whether the particular endonuclease was the first isolated, the second, and so on. For example:

*Eco*RI
- *E* = genus *Escherichia*
- *co* = *coli*
- R = strain RY 13
- I = first endonuclease isolated

*Hin*dIII *H* = genus *Haemophilus*
in = *influenza*
d = strain Rd
III = third endonuclease isolated

Each restriction endonuclease "recognizes" a specific DNA sequence (usually a 4- to 6-base-pair sequence of nucleotides) in double-stranded DNA and digests phosphodiester bonds at specific sites in the sequence. For example, the restriction endonuclease *Eco*RI cuts double-stranded DNA as follows:

```
  ↓
–G–A–A–T–T–C–
–C–T–T–A–A–G–
           ↑
```

Since this endonuclease cuts at specific sites on each strand, "**sticky ends**" are produced. (Sticky ends make it possible to insert DNA pieces, usually containing a gene of interest, into plasmids or viral vectors that have been cut with the same endonuclease, providing complementary sites for attachment—see Laboratory 18.) Other restriction endonucleases cut DNA in the same position on both strands to produce fragments with "**blunt ends.**"

Fragments of DNA produced by restriction enzyme cleavage can be separated by gel electrophoresis. When any molecule enters an electrical field, its mobility, or the speed at which it will move, is influenced by the charge on the molecule, the strength of the electrical field, the size and shape of the molecule, and the density of the medium (gel) through which it is migrating. Consequently, it is possible to separate heterogeneous populations of molecules (such as fragments of DNA). When all molecules are positioned at a uniform starting site on a gel, the gel is placed in a chamber containing a buffer solution and electrodes, and an electric current is applied, the molecules will migrate and form bands (concentrations of homogeneous molecules). Since the phosphates in the DNA backbone are negatively charged at neutral pH, DNA fragments will migrate through the gel toward the positive electrode.

After electrophoretic separation and staining of the gels with colored or fluorescent dyes, the sizes of DNA fragments can be determined by comparing them with markers of known molecular weight or other fragments of known sizes. Once this information is available, it is possible to construct a **restriction map** of the DNA in question.

✓ PART I Estimating DNA Fragment Size Using Gel Electrophoresis

In this exercise, you will use an agarose gel to separate a mixture of bacteriophage lambda (λ) DNA fragments produced using two restriction enzymes, *Eco*RI and *Hin*dIII. (The density of an agarose gel can be varied to improve the resolution of similar-sized molecules.) In agarose, the migration rate of linear fragments of DNA is inversely proportional to their size; the smaller the DNA fragment, the faster it migrates through the gel. The size of the fragments is measured in number of base pairs (bp).

The size of fragments produced by a specific endonuclease (*Eco*RI in this exercise) can be determined by using *standard* fragments of known size (fragments produced by *Hin*dIII in this case). These fragments are electrophoresed together and, after visualizing the bands by staining with dyes such as methylene blue or ethidium bromide (a fluorescent dye), a standard curve can be plotted for *Hin*dIII bands of known size. The sizes of the standard fragments are plotted on the *Y*-axis and the distances they migrated are plotted on the *X*-axis. By plotting the migration distances of the fragments of unknown size on the *X*-axis, you will be able to determine the size of the *Eco*RI fragments from the standard curve.

Objectives

- ☐ Explain the principles of electrophoresis as they pertain to separating and identifying DNA fragments.
- ☐ Determine DNA restriction fragment sizes using a standard curve.

Procedure

Work in groups of four.

A. Casting Agarose Gels

1. Tightly tape the ends of the gel bed with masking tape to make a good seal (Figure 14D-1).
2. Place the gel comb across the gel bed, putting the ends of the comb into the notches on the side of the bed nearest the end of the bed. (The comb creates "wells" in the gel into which you will load various samples for testing.)
3. Obtain a bottle of 1% agarose (50 ml) from your instructor. ***Caution:*** The bottle will be hot since it has just been heated to melt the agarose. (Add 2 drops of Carolina Blu stain if required by your instructor.)

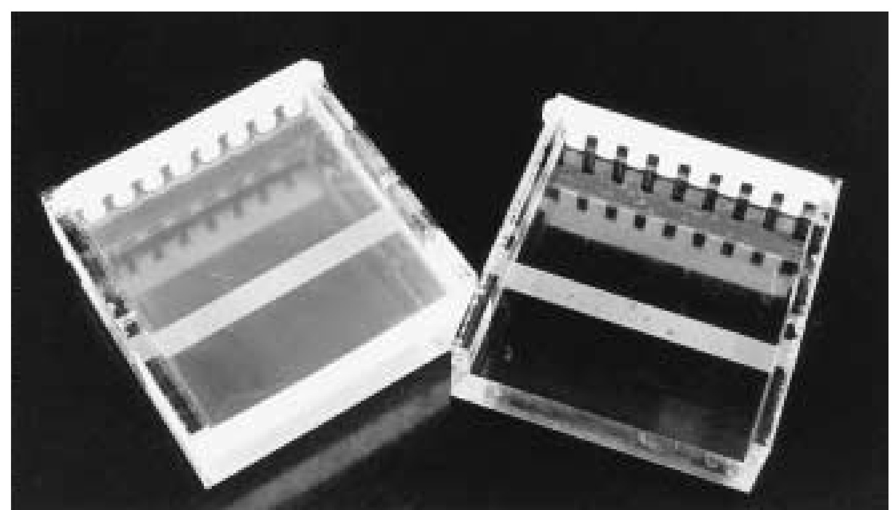


Figure 14D-1 *Preparation of the gel bed. The bed on the left has been taped and the agarose gel has been poured into it.*

4. Use a Pasteur pipette to transfer some agarose solution into the gel bed at the edges of the tape to make sure there are no leaks, but do this quickly and do not let it solidify. Now, carefully pour the remaining agarose into the gel bed. The agarose should cover only about one-third the height of the teeth (about 5 to 7 mm thick; you do not want your gel to be too thick, or too thin). If bubbles appear around the teeth of the comb, remove the comb and reinsert it. If bubbles appear in the gel, use a Pasteur pipette to draw them off to the sides.
5. Keep the gel bed completely immobile while the gel is setting (approximately 15 minutes). The final appearance of the gel will be cloudy.
6. When ready, remove the tape from the ends of the gel bed.
7. Carefully lower the gel bed into the chamber with the comb nearest to the *negative* (black) electrode (Figure 14D-2). Make sure that the gel bed is properly seated and centered.
8. Fill the electrophoresis chamber with running TBE buffer (tris-borate-EDTA) until the buffer covers the surface of the gel. (Make sure the 10× stock buffer has been diluted 1:10.) (Add 12 drops of Carolina Blu stain to 1 liter of buffer, if required by your instructor.)
9. Carefully remove the comb (pull it straight up). Make sure that the sample wells are filled with buffer. Remove any bubbles by using a Pasteur pipette to blow gently into the buffer above the wells. *Do not stick the end of the pipette into the wells*—you might puncture the gel!

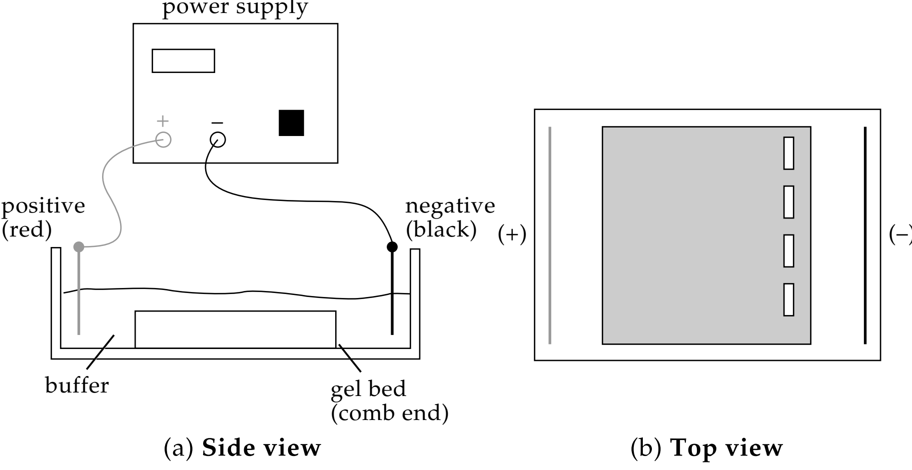


Figure 14D-2 *Setup for the electrophoresis system.*

B. Loading Samples

1. If a practice gel is available, practice loading the samples using the gel-loading solution only.
   a. To load practice samples on the gel, use a small micropipette or plastic transfer pipette. Pull a small amount of the practice gel-loading solution into the end of the pipette. (Do not allow the solution to move up into the body of the pipette or bubbles will be introduced into the well of the agarose gel during loading.)
   b. Dip the pipette into the buffer and hold the tip of the pipette slightly *above* the well in the gel. Gently dispense the solution. The loading dye is denser than the buffer and will move into the well. (Do *not* place the tip of the pipette into the well or you might puncture the gel.)
2. After practicing, you are ready to load the gels. Obtain a microtest tube containing phage lambda DNA digested with *Eco*RI endonuclease.* FIll one well of the electrophoresis apparatus with approximately 20 μl of this solution. The DNA is mixed with a solution containing tracking dye that will make it possible to trace the process of the DNA migration in the agarose gel.
3. Obtain a microtest tube containing phage lambda DNA digested with *Hin*dIII endonuclease. Follow your instructor's directions and fill a second well with 20 μl of this *Hin*dIII digest. The DNA fragments from this digest are of known size and will serve as a "standard" for measuring the size of the *Eco*RI fragments from step 2.
4. Load 20 μl of undiluted phage lambda DNA (control) into a third well.

C. Electrophoresis

1. Place the top on the electrophoresis chamber and connect the electrical leads (black to black and red to red). If using an Edvotek chamber, set the voltage to 50 volts. If using a Cabisco apparatus, set to 80 volts and check for a current reading of 50 to 100 milliamperes. When the current is flowing, you should see bubbles on the electrodes.
2. Allow electrophoresis to continue for a minimum of 1½ hours, or until the loading dye has moved at least 5 to 7 cm from the wells. The tracking dye will eventually form two bands of color. A purplish band (bromophenol blue) will be seen farthest from the wells. A slower-moving aqua-colored band (xylene cyanol) will migrate through the gel at a rate

---

**DNA for this procedure has been predigested to save time. However, your instructor may wish to have you perform the digests rather than use predigested material. If this is the case, your instructor will give you separate directions.*

equivalent to that of a DNA fragment approximately 2,000 bp long. This aqua band will be migrating just in front of your smallest DNA fragments. Turn off the electrophoresis apparatus when the bromophenol blue band (purple band) reaches the opposite end of the gel.

3. After electrophoresis is completed and the power supply is turned off, disconnect the leads and remove the cover of the electrophoresis chamber.

D. Staining

Wear gloves!

1. Fill a staining tray (or large Petri dish) with methylene blue staining solution (or Carolina Blu Final Stain, if required).
2. Carefully remove the gel bed from the chamber and gently transfer the gel to a staining tray. Use the scooper provided with your kit or keep your hands under the gel during the transfer. You may wish to remove a small piece of gel from the upper right-hand corner to keep track of the gel's orientation. *Do not stain in the electrophoresis apparatus.*
3. Stain for 30 minutes.
4. Carefully transfer the gel to a tray containing approximately 500 ml of distilled water to destain. Rinse several times and then let the gel destain for 1 to 24 hours. *Do not* change the water during this time or the bands will fade.
5. Transfer the gel to a visible-light box or overhead projector for examination.

E. Determining Fragment Size

1. After observing the gel on the light box, carefully wrap the gel in plastic wrap and smooth out all the wrinkles, or overlay with a transparency sheet.
2. Use a permanent marking pen to trace the outlines of the sample wells and the location of the bands.
3. Remove the plastic wrap and flatten it out on a white piece of paper on the laboratory bench. Save the gel in a plastic bag. Add several drops of the water used for destaining. Close the bag tightly and store at 4°C.
4. If the exercise was done as a demonstration, your instructor will transfer the marks onto an overhead transparency and will make copies of the transparency for each student. If you have run your own gel, you can make measurements directly from the plastic wrap.
5. For the *Hin*dIII fragments, measure the migration distance in centimeters (to the nearest millimeter). Measure from the front edge of the sample well to the front edge of each band on your gel.

Table 14D-1

*Hin*dIII Fragment (bp)	Distance Traveled (cm)
23,130	
9,416	
6,557	
4,361	
2,322	
2,027	

The distance a fragment migrates is related to its molecular weight. The greater its molecular weight, the shorter the distance the fragment will travel through the gel. For simplicity, we will use base-pair length instead of molecular weight. The known *Hin*dIII fragment lengths are given in Table 14D-1. Indicate the distance in centimeters that each has traveled. You will identify each fragment by the distance traveled: the shortest fragment will have traveled farthest, the next shortest will be just behind, and so on.

Note: You will observe six bands (Figure 14D-3). The band closest to the origin may appear to be diffuse—it is actually composed of pieces of DNA of two different sizes, 27,491 and 23,130 bp. For graphing purposes, you will use a base-pair size of 23,130. Two additional bands, 564 and 125 bp, are usually not observed. The larger (564 bp) usually does not contain enough DNA to be visible using methylene blue stain; the smaller (125 bp) usually runs off the end of the gel.

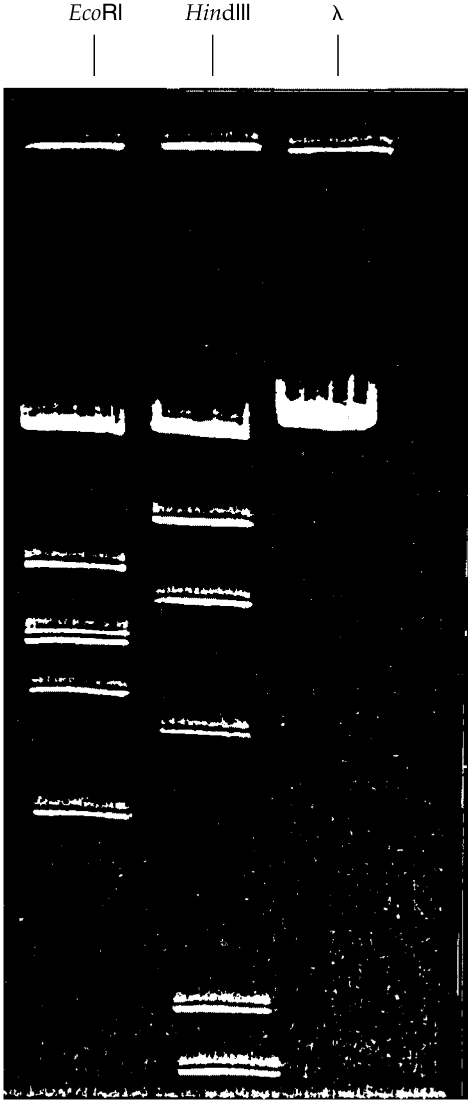


Figure 14D-3 *Agarose gel (photo not to scale) from electrophoresis of fragments produced by restriction endonuclease digestion of lambda DNA. Lane 1, digestion with* EcoRI*; lane 2, digestion with* HindIII*; lane 3, undigested lambda DNA.*

6. Use Figure 14D-4b at the end of the lab to graph your results. [The horizontal (*X*) axis of semilog paper is divided into a linear scale; the vertical (*Y*) axis is divided into a logarithmic scale.] Mark the *X*-axis at 1 cm, 2 cm, 3 cm, 4 cm, and 5 cm. Label this axis "migration distance."

7. Size in base pairs is plotted along the *Y*-axis. Assume that the first section or cycle of semilog paper represents 0 to 1,000 bp, the second represents 1,000 to 10,000 bp (see Figure 14D-4a). (On semilog paper, each section along the vertical axis is used to represent an increase by the power of 10.) On the *Y*-axis, mark the approximate position of each of the phage lambda *Hin*dIII standard fragment sizes in base pairs.

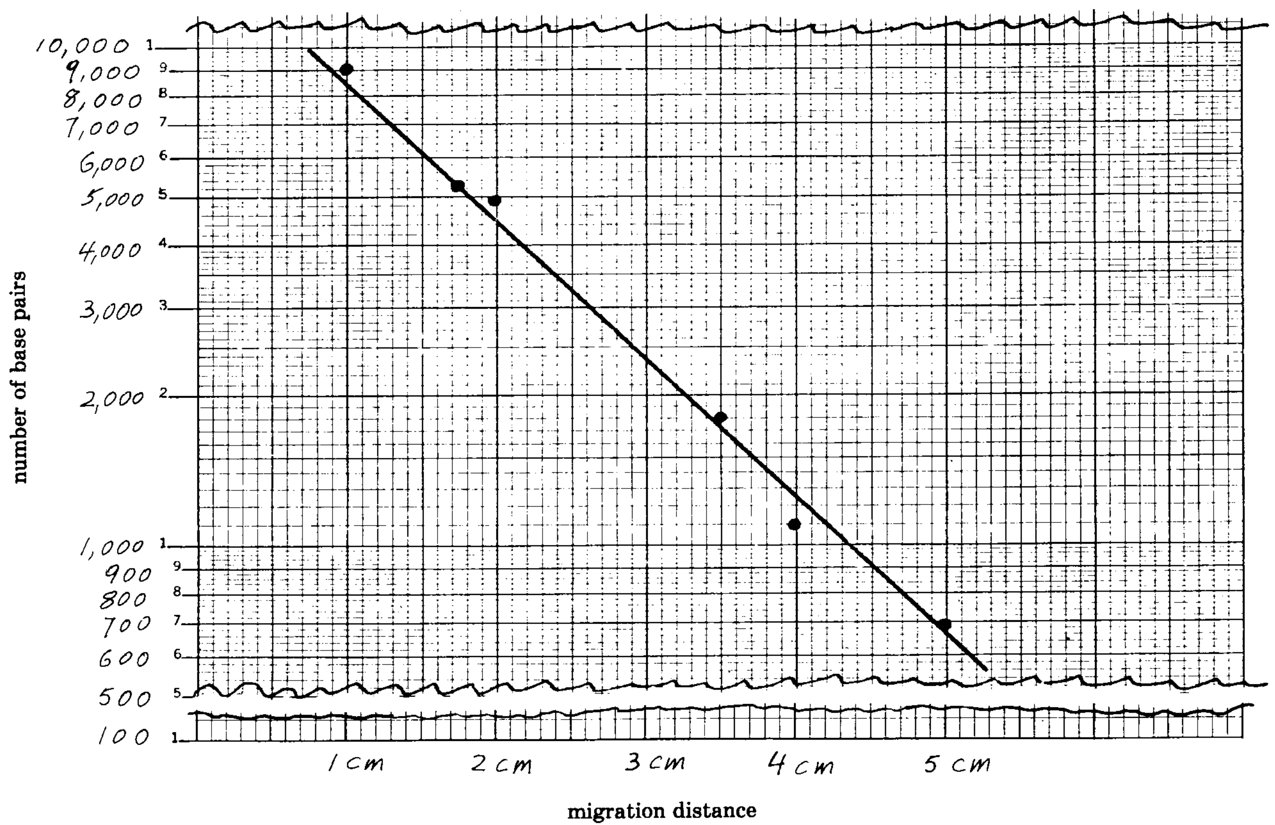


Figure 14D-4a *Example of a standard curve used to determine DNA fragment size. Note: The electrophoresis running time specified in your experiment is different from the running time used to generate the example standard curve shown here, so your standard curve will differ from this one.*

8. Each band on your gel of the *Hin*dIII digest should correlate with one of the fragment sizes in Table 14D-1. To plot your curve, locate the base-pair length you marked for each fragment on the *Y*-axis, then move horizontally along the *X*-axis according to the distance the fragment has traveled. When you have plotted all your points, draw a straight line that fits as close as possible to all the points (although it will not intersect all of them). This line describes the trend of the data and will serve as what is called a *standard curve,* similar, but not identical, to the one shown in Figure 14D-3.

9. Use this standard curve to determine the sizes of the fragments of phage lambda DNA digested with *Eco*RI. You should observe five bands (Figure 14D-3). Measure the migration distance for each *Eco*RI fragment. Locate that distance on the *X*-axis of your graph and use a ruler to extend a line upward until it crosses your standard curve. Mark the point where the lines cross, and use a ruler or the edge of a piece of paper to find where this point lies on the *Y*-axis, which gives you the number of base pairs.

a. *What is the relationship between DNA fragment size and rate of travel through the agarose gel?*

____

____

Expected *Eco*RI fragment sizes in base pairs are listed in Table 14D-2. Compare your observed results with the expected sizes by entering the base-pair sizes you observed beside the corresponding expected fragment size. *Note:* This technique is not exact—you should expect as much as a 10% to 15% error.

Table 14D-2 *Eco*RI Fragment Sizes for Phage Lambda DNA

Expected	Observed
21,226	
7,421	
5,643	
4,878	
3,530	

PART 2 Constructing Restriction Maps

A **restriction map** shows the location of each restriction site (place where the restriction endonuclease "cuts" the DNA) in relation to other sites. A restriction map of a viral or bacterial chromosome can be constructed by comparing the sizes of DNA fragments produced when the chromosomal DNA is digested by a combination of restriction enzymes. First, individual enzymes are used to cut the DNA into fragments of a certain size. A mixture of the same enzymes is then used to cut the DNA into fragments of different sizes (Figure 14D-5a). By determining fragment size and sequencing (establishing the order of) the overlapping fragments, the restriction sites on the DNA can be mapped in relation to the linear sequence of the DNA fragments (Figure 14D-5b). Note that "kb" indicates kilobase pairs; 1,000 base pairs.

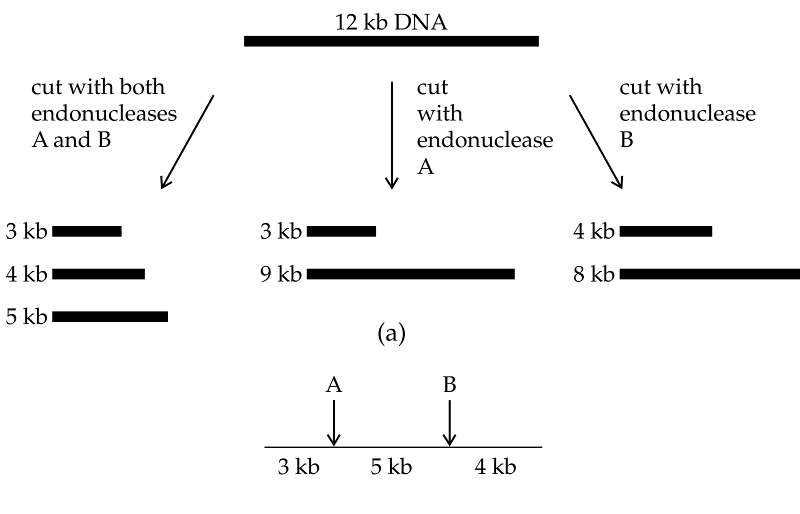


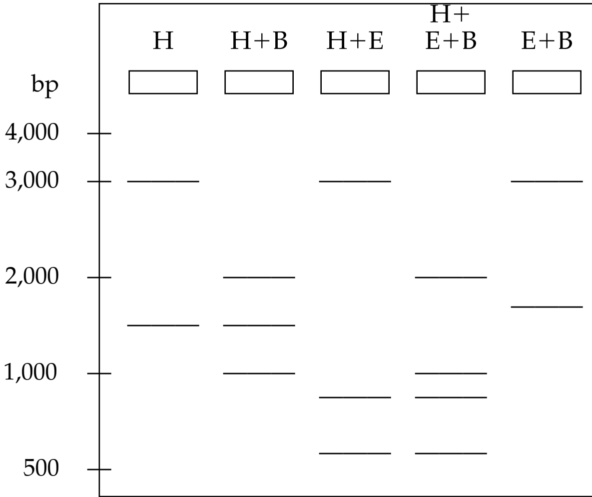
Figure 14D-5 *(a) Enzyme A cuts at one end of the DNA molecule while enzyme B cuts at the other end. The fragments could NOT be arranged in sequence as 3/4/5 kb because enzyme B produced two pieces of 4 kb and 8 kb and there is no way to get an 8-kb piece unless the 3-kb piece is next to the 5-kb piece. (b) For this reason the fragments must be arranged as 3/5/4 kb.*

Objectives

- ☐ Construct restriction maps of circular plasmid DNAs.
- ☐ Construct restriction maps of linear DNA molecules.

Procedure

1. A circular plasmid has been cut with restriction enzymes H, B, and E alone and in combination. Electrophoresis of restriction fragments produces the gel shown below. Base pair (bp) sizes are shown on the scale to the left of the gel. Estimate the size of each fragment using this scale. Start with enzyme H and assume that it produces two fragments.

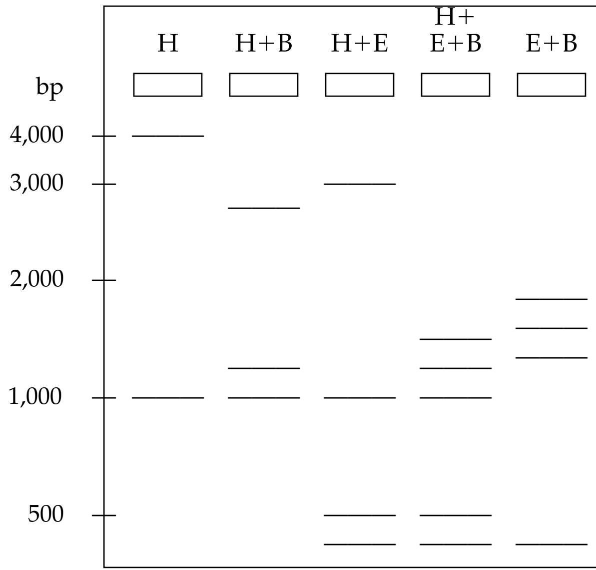


a. How many restriction sites were present for enzyme H? ______

2. Draw a circle in the space below and locate the enzyme H restriction sites in relation to approximate sizes of the restriction fragments.

3. Continue to add restriction sites at the appropriate locations by determining the approximate sizes of the restriction fragments in the H+B lane on the gel. Follow with each additional set of fragments until you have established a complete restriction map; a restriction map for which the correct fragment sizes would be produced by digestion with restriction endonucleases H, E, and B.

4. Next, a linear piece of DNA is cut using enzymes H, B, and E alone and in combination. Electrophoresis of restriction fragments produces the gel shown on page 14-21. Base-pair sizes are shown on the scale to the left of the gel. Estimate the sizes of each fragment using this scale. Start with enzyme H and assume that it produces two fragments.



b. How many restriction sites were present for enzyme H? ______

5. Draw a line in the space below and indicate the position of the restriction site(s) for enzyme H.

6. Now continue to add restriction sites at the appropriate places along the line by estimating the fragment sizes produced by the other enzymatic digestions represented on the gel. By sequencing the overlapping fragments, you will create a complete restriction map for the linear DNA molecule.

PART 3 Mapping the Bacteriophage Lambda (λ) Chromosome

Bacteriophage lambda (λ) is a temperate phage; it can replicate autonomously or can convert *E. coli* to the **lysogenic** cycle by inserting into the *E. coli* chromosome as a prophage. Phage λ exists as a double-stranded DNA molecule of 48,502 base pairs. It can be either a linear or circular molecule because each end of the chromosome has a single-stranded tail (called the COS site). The tails are complementary (like sticky ends), allowing the linear molecule to easily convert to a circle. Because of its relatively small size, restriction enzyme digestion of phage λ DNA can be used to construct a restriction map.

Objectives

- ☐ Construct a restriction map of phage λ DNA from restriction digest fragment sizes.
- ☐ Use electrophoretic data to construct a map of phage λ DNA.

Procedure

1. Lambda DNA (48,502 bp) is cut using restriction enzymes *Afl*II (from *Anabaena flos-aquae*) and *Apa*I (from *Acetobacter pasteurianus*), as shown on the following page.

*Afl*II –C–T–T–A–A–G (cut ↓ between C and T on top strand)
–G–A–A–T–T–C– (cut ↑ between G and A on bottom strand)

*Apa*I –G–G–G–C–C–C– (cut ↓ between C and C on top strand)
–C–C–C–G–G–G– (cut ↑ between C and C on bottom strand)

The enzymes are used both alone and in combination, and fragments of the following approximate sizes (in bp) are produced:

*Afl*II	*Apa*I	*Afl*II + *Apa*I
5,872	10,086	2,532
6,078	38,416	3,546
6,540		5,872
30,012		6,540
		30,012

Use these data to establish the sequence of fragments and the restriction sites of *Afl*II and *Apa*I on λ DNA. Record this sequence as a linear restriction map (see Figure 14D-5) of bacteriophage λ on the line below.

|————————————————————————————————| 42,502 bp

2. In Part 1 of the exercise you determined the restriction fragment sizes for lambda DNA digested by *Hin*dIII and *Eco*RI. Using the *Hin*dIII map (Figure 14D-6) for practice, determine the sizes of the known fragments (the length of DNA in bp between two restriction sites) by subtracting the restriction site bp designation at the left of the fragment from the bp designation on the right. For example, the fragment marked (*) is 25,157 bp − 23,130 bp = 2,027 bp in length. Record this known size below the *Hin*dIII map on the lines provided (____ bp).

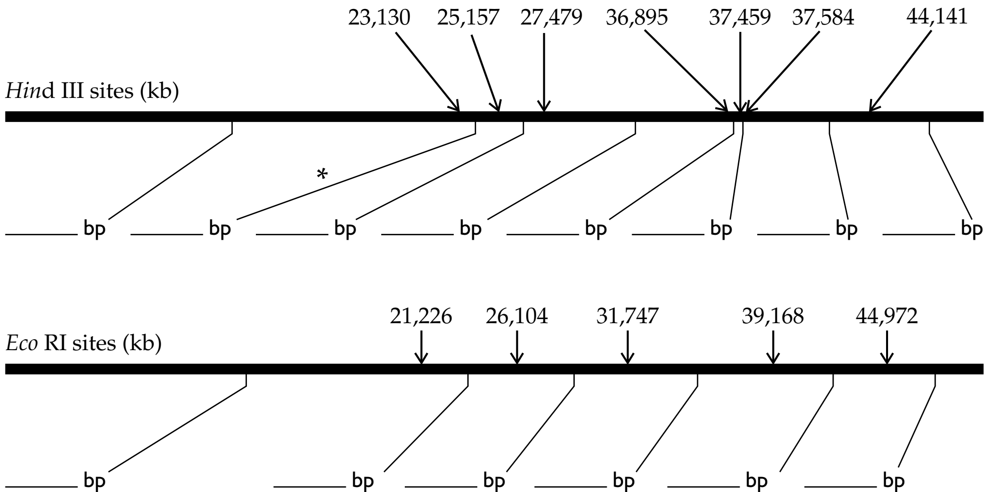


Figure 14D-6 *Lambda DNA restriction enzyme sites. The locations of "cuts" (arrows) are indicated in base pairs (bp) from the origin.*

Using your data from Part 1, compare your restriction fragment sizes determined by electrophoresis (see Tables 14D-1 and 14D-2) with those in the restriction-site maps of λ DNA in Figure 14D-6.

a. *Are there any fragments shown on the map that are missing from your gel?* ________

How could this be the case? ____

3. Now, using your own electrophoresis data for *Eco*RI fragments (Part 1), compare the restriction fragment sizes you determined by electrophoresis to the actual *Eco*RI fragment sizes recorded on the bacteriophage λ restriction map in Figure 14D-6.

b. *How do the fragment sizes for* Eco*RI that you determined from your electrophoresis standard curve compare to those of known length as determined from the* Eco*RI map in Figure 14D-6?*

____

4. Use your *Eco*RI data from Part 1 to construct your own λ DNA map. By matching your fragment sizes (as determined after electrophoresis from your standard curve) to the known data for *Eco*RI (Figure 14D-6), you should be able to establish the correct order of the fragments. Some bands of similar size may migrate together.

(λDNA)

Recall that the base sequences of the fragments of DNA produced by restriction enzyme digests can be determined by several means. This makes it possible to develop a complete genetic sequence of the chromosome. For larger pieces of DNA, a procedure called **"chromosome walking"** can be used. Two different restriction digests are used to cut identical pieces of DNA into fragments. Each of these DNA fragments is introduced into a bacterium via a plasmid vector. Each bacterium then clones its fragment as it replicates and forms a colony. Thus, each bacterial colony contains many copies (clones) of the same fragment, and

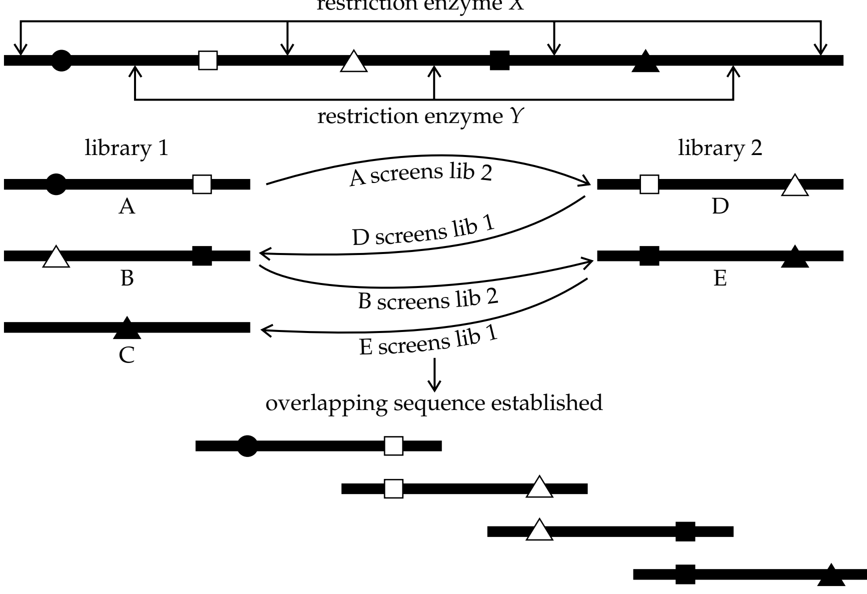


Figure 14D-7 *Chromosome walking. Restriction enzyme fragments are introduced into plasmids for cloning. All fragments formed by digesting DNA with Enzyme* X *form a library 1. All fragments formed by digesting DNA with Enzyme* Y *form library 2. By searching for complementary ends, hybridizing probes from library 1 clones with library 2 clones, it is possible to determine the linear sequence of DNA on a chromosome.*

there are many colonies, each containing a different "clone" fragment. The group of cloned colonies that develops from the fragments created by one restriction enzyme represents a **library.** The two resulting libraries can be used as probes to screen each other for overlapping complementary sequences; the right-hand end of one piece complements the left-hand end of the next piece and the right-hand end of the second piece complements the left-hand end of the next piece as if "walking" down the chromosome (Figure 14D-7).

Laboratory Review Questions and Problems

1. Ascospores of the fungus *Sordaria* are haploid. Why is this an advantage in studying the genetics of the organism?

2. Two genes for shell color, genes *A* and *B*, are on the same arm of a chromosome in a rare species of clam. Mating yellow-shelled individuals produced 800 yellow-shelled clams and 225 orange-shelled clams. Orange shells result from recombination of alleles in crossing-over events that occurred during meiosis and gamete production. What is the map distance between genes *A* and *B?*

3. There are four genes, *A, B, C,* and *D,* on a chromosome that you wish to map. These are the recombination frequencies among these genes: $B \times D = 4\%$, $B \times C = 10\%$, $D \times A = 2\%$, $C \times A = 16\%$, $C \times D = 14\%$. Map the chromosome.

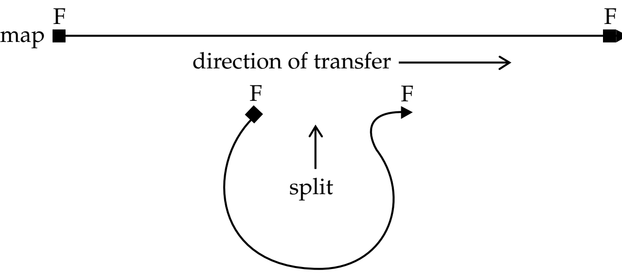
4. In a series of breeding experiments among frogs, a linkage group composed of genes *A, B, C,* and *D* was found to show the following crossover frequencies. Map the chromosome. (Use the matrix like a Punnett square to show recombinations.)

	A	*B*	*C*	*D*	
A	—	10%	4%	9%	
B	10%	—	6%	19%	
C	4%	6%	—	13%	
D	9%	19%	13%	—	

5. You are trying to map a newly isolated bacterial chromosome. After allowing different mutant strains to conjugate for different lengths of time, you test whether a certain gene (for example, leu^-) that was previously nonfunctional in the mutant is now functional (leu^+) due to transfer

of the gene from the donor strain and subsequent recombination. Only recombinants will grow on minimal medium. Use these data to map the *leu*, *pro*, *bio*, and *thi* genes on the chromosome. (*Note:* When the bacterial chromosome breaks open for replication and transfer, the *F* factor is split. The leading edge of the chromosome being transferred is on the *right* in the diagram below.)

Donor	Recipient	Growth on Minimal Medium			
		5 min	10 min	30 min	45 min
leu^+ bio^-	× leu^- bio^+	Yes	Yes	Yes	Yes
bio^+ pro^-	× bio^- pro^+	No	Yes	Yes	Yes
pro^+ leu^-	× pro^- leu^+	No	No	Yes	Yes
pro^- leu^+	× pro^+ leu^-	Yes	Yes	Yes	Yes
bio^- thi^+	× bio^+ thi^-	No	No	No	Yes



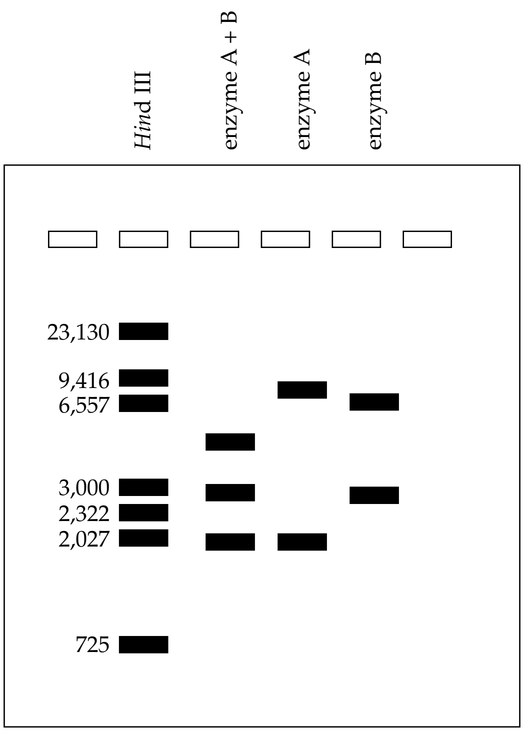
6. When samples of DNA are subjected to electrophoresis, why should the samples be loaded onto the gel at the negative pole of the electrophoresis apparatus?

7. Restriction endonucleases are used by bacteria as a form of "protection." Explain how this occurs and why the endonucleases do not destroy the bacterial cells themselves.

8. A circular bacterial DNA plasmid is cut using two restriction endonucleases. Restriction enzyme A yields a single linear molecule of 45,000 base pairs (4.5 kilobase pairs, or 4.5 kb). Enzyme B produces two restriction fragments of 1.2 kb and 3.3 kb. A combination of enzymes A and B produces three restriction fragments of 1.2 kb, 1.3 kb, and 2.0 kb. Map the plasmid, showing restriction sites for A and B and relative fragment lengths.

How many recognition sites are present for each restriction enzyme?

9. A length of human DNA from chromosome 2 has been cut by two restriction enzymes, A and B. Electrophoresis is carried out using *Hin*dIII fragment markers for a standard. The resulting gel is shown below. Note that enzyme A cuts the DNA into two pieces, and enzyme B cuts the DNA into two pieces, but of different sizes. When A and B are used together as a double digest, three pieces are produced.



Use the *Hin*dIII fragments to make a standard curve, using Figure 14D-4b. Then determine the lengths of the fragments produced by enzymes A and B alone and A and B in combination.

To determine the base sequence of the fragments, you first need to know their order in the single piece of human DNA (this is like knowing the letters in three words but not knowing the order of the words in a sentence). In trying to find the order, you are mapping the gene. This is called a restriction map. How are the pieces of DNA ordered in the original piece of human DNA?

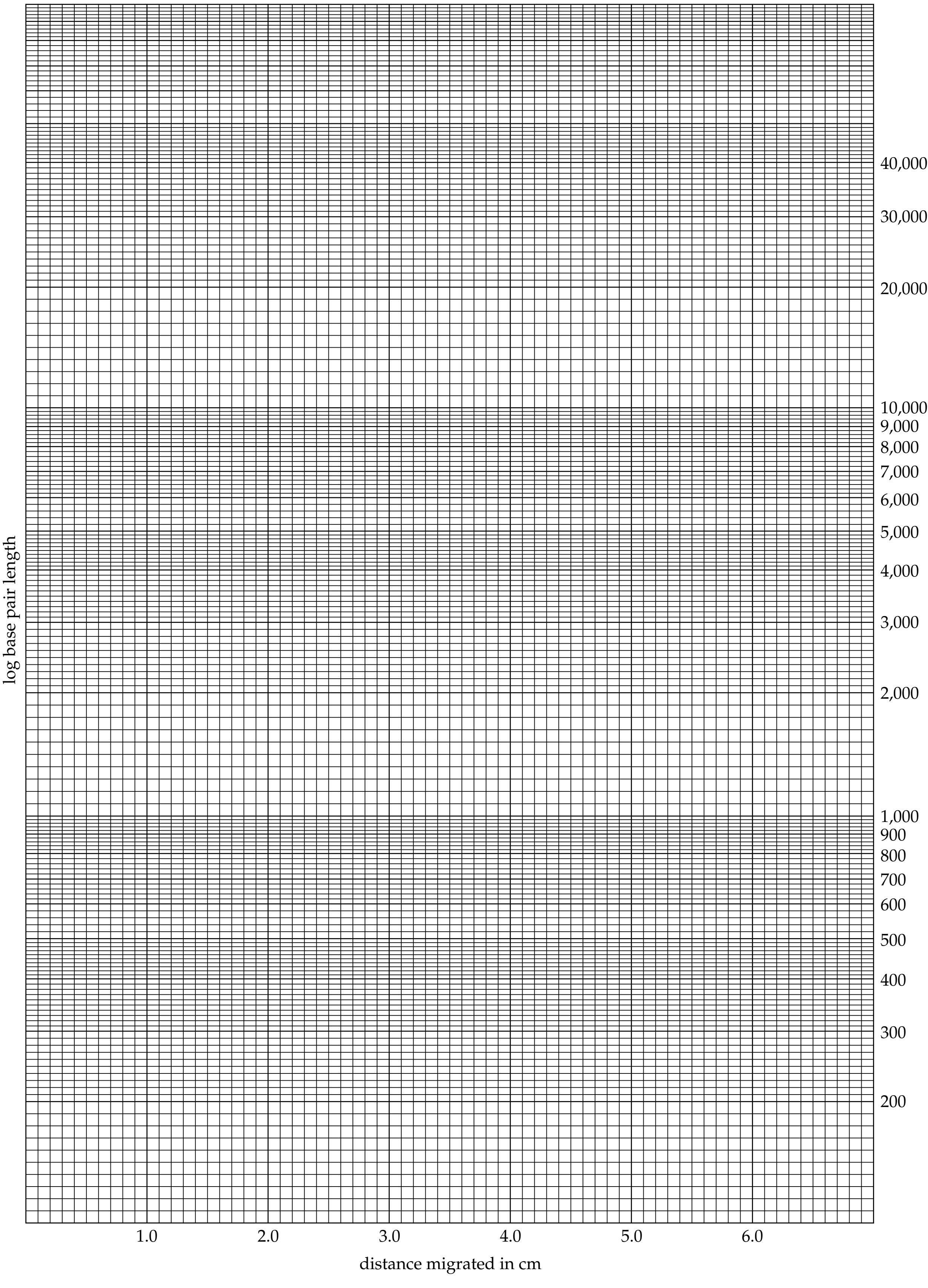


Figure 14D-4b *Semilog graph paper.*

LABORATORY

15

Human Genetic Traits

OVERVIEW

All people are recognizably human, but no one is exactly like anyone else, not even an identical twin. The basis for the similarity and the reasons for the diversity that coexist in all species have puzzled and intrigued people for thousands of years. Recently, experiments with a wide variety of plants, animals, and microorganisms have yielded detailed knowledge of how traits are passed from one generation to the next, how genetic information is decoded and expressed during development, and how genetic variability can account for gradual evolution.

STUDENT PREPARATION

To prepare for this laboratory, read the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency. Work through Exercise A before coming to the laboratory.

✓ EXERCISE A Human Cytogenetics

Many human hereditary defects caused by chromosomal abnormalities may be identified by examining human chromosomes from cells that have been arrested in metaphase of mitosis—a stage when chromosomes are very short and compact. Leukocytes (white blood cells) or fetal cells obtained by amniocentesis or chorionic villus sampling are often used for diagnosis.

The cells are cultured (to increase their number), treated with a chemical that disrupts the mitotic spindle apparatus, and placed in a hypotonic salt solution to swell their nuclei. The mixture is then centrifuged (to increase the concentration of cells) and transferred to a glass slide. As a drop of the cell

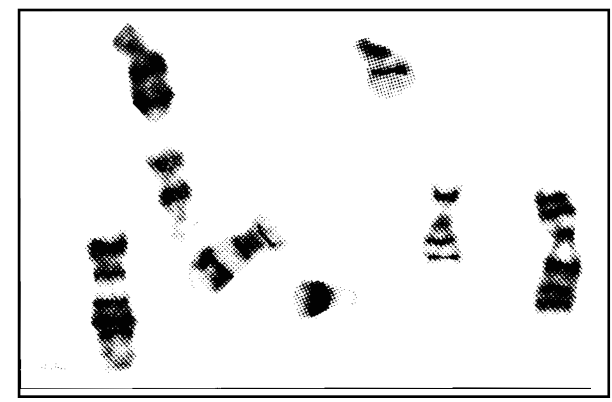


Figure 15A-1 *G-banded chromosomes. This pattern is produced by using Giemsa stain. Bands do not represent genes, but they do serve as markers for locating genes and gene families.*

suspension hits the slide, the nuclei break open and the chromosomes spread apart; usually chromosomes from a single cell remain in an identifiable group. The cells are then stained, sometimes using special procedures that result in banded chromosomes (Figure 15A-1).

The "metaphase spread" produced by a single cell is then photographed. The photograph can be cut apart and homologous chromosomes can be arranged in pairs according to size, location of the centromeres, and length of the chromosome arms. Chromosome pairs are arranged in a specific order and labeled. The result is called a **karyotype.**

Objectives

- ☐ Match and order pairs of human chromosomes to make a karyotype.

Procedure

1. Figure 15A-2 is a diagrammatic representation of G-banded human chromosomes (only one chromosome from each homologous pair is represented). Figure 15A-3b (page 15-20) is a metaphase preparation of banded chromosomes. Cut out these chromosomes and match them to the chromosomes shown in Figure 15A-2. Match homologous chromosomes by size, length of arms, and location of the centromere. Place the homologous pairs together above corresponding numbers in Figure 15A-3a (page 15-3). A sample karyotype, Figure 15A-4, will also assist you in matching chromosome pairs by size and banding.
2. Note that the *X* chromosome has a single thick band on its upper end and four bands on its lower end. The *Y* chromosome is very small and has a single thick band on the tips of its arms at one end.
3. Once you have matched the chromosomes, tape them onto the blank karyotype sheet (Figure 15A-3a).

   a. Are these chromosomes from a male or a female? ______________

Bring the completed karyotype with you to the laboratory.

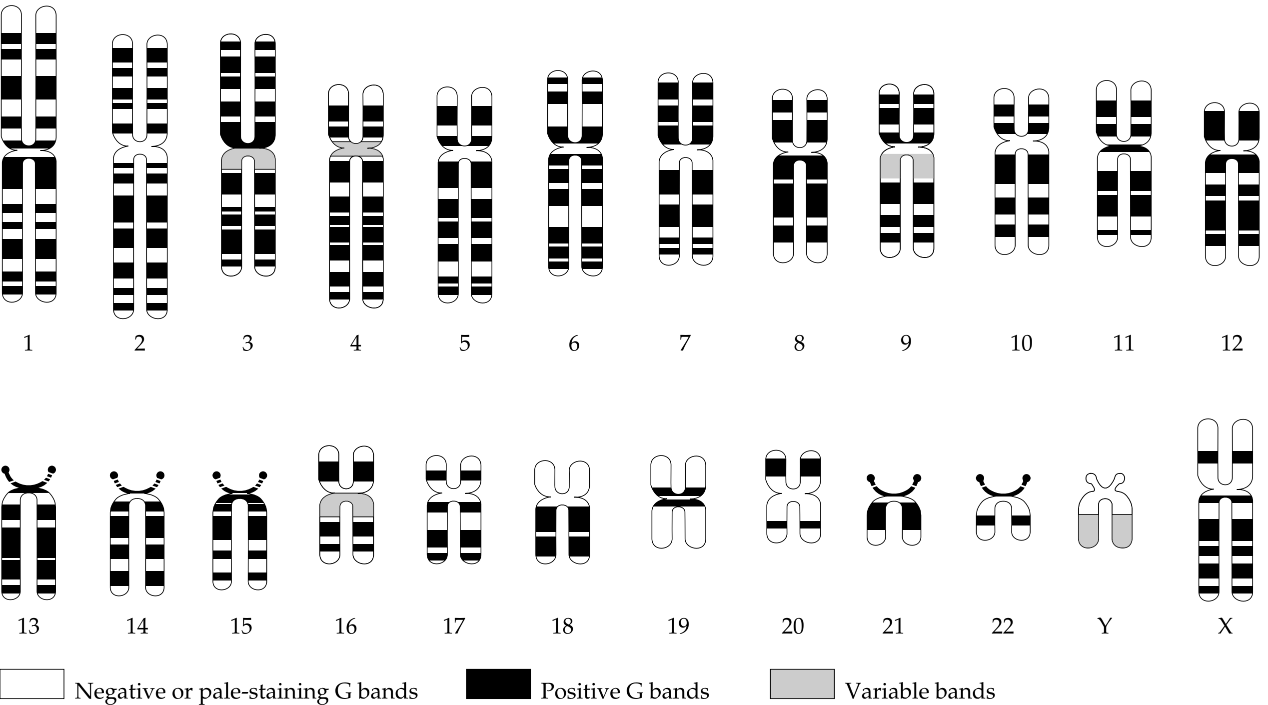


Figure 15A-2 *Diagram of G-banded human chromosomes.*

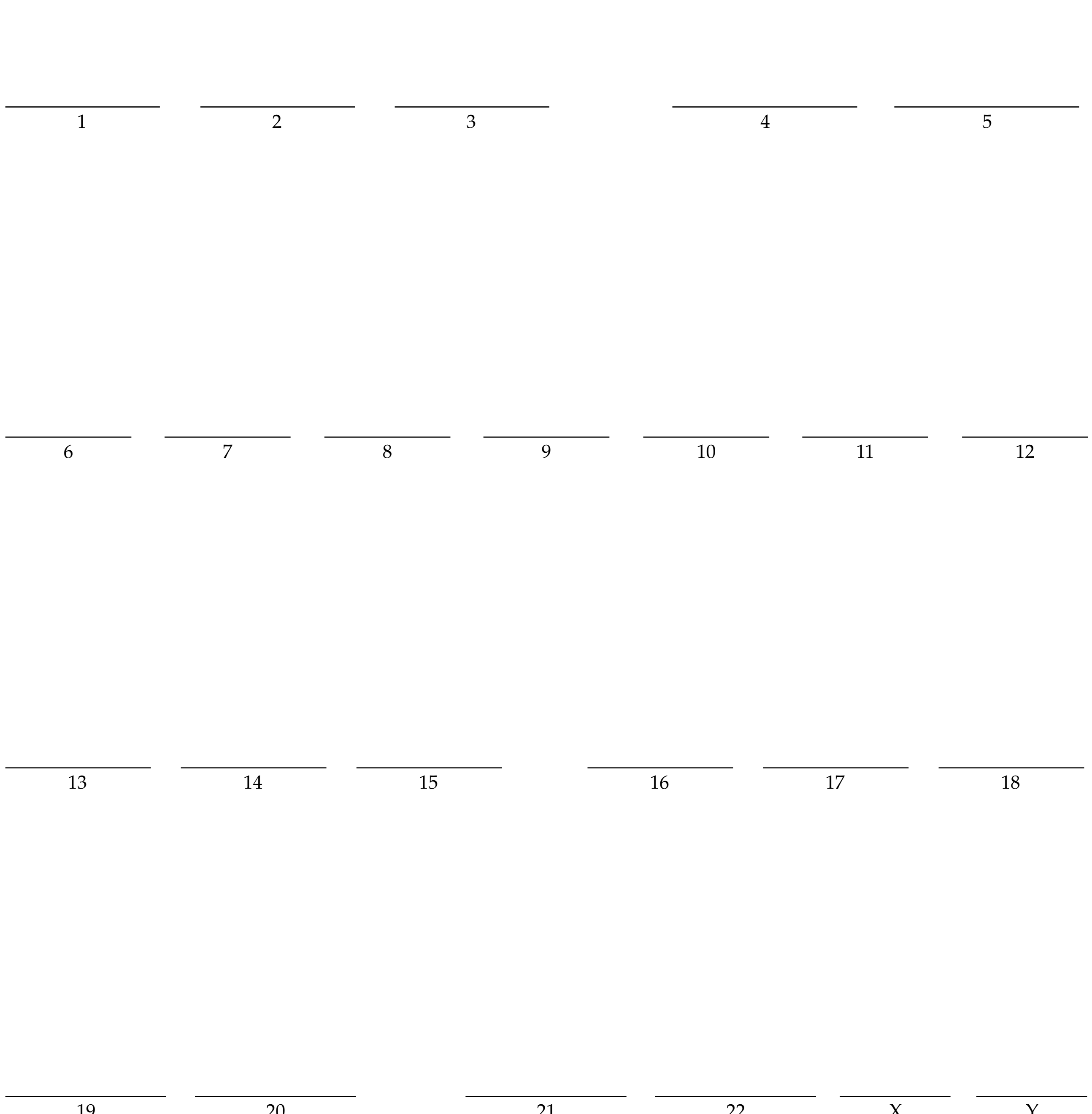


Figure 15A-3a *Match homologous chromosome pairs from Figure 15A-3b (page 15-20) and attach them in appropriate spaces above. Use the karyotype (Figure 15A-4) to help you match the pairs by size and banding patterns.*

Figure 15A-4 *Karyotype of G-banded human chromosomes.*

✔ EXERCISE B | *X* and *Y* Chromosomes

An individual's sex chromosomes can be identified more simply than by karyotyping. Cells scraped from the inside of the mouth can be stained with a dye specific for DNA. If the individual is female, a darkly staining **Barr body,** or sex-chromatin body, appears near the nuclear membrane (Figure 15B-1). This structure is one of the *X* chromosomes that has condensed; only one *X* chromosome remains active in females. No such body is found in male cells.

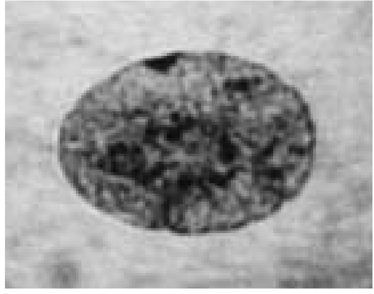


Figure 15B-1 *Part of a cell from the squamous epithelium of a female, showing nucleus with Barr body (top).*

a. How many X chromosomes are found in male cells? ______

Both males and females ultimately have just one active *X* chromosome, which provides the correct amount of *X* chromosome products required for the normal functioning of cells in both males and females. In cases where more than two *X* chromosomes are present (see Exercise D, Part 1), the number of Barr bodies in a cell is $N - 1$, where N is the number of chromosomes in the cell.

While Barr-body stains reveal the number of *X* chromosomes present, they do not reveal anything about the presence or number of *Y* chromosomes. Instead, a special stain, acridine orange, is used to stain the *Y* chromosome, which then stands out as a very bright fluorescent structure in the cell.

Objectives

☐ Describe how, without karyotyping, an individual's sex chromosomes composition can be determined.

Procedure

Examine the two slides on demonstration. Both slides show cheek scrapings, one from a female and the other from a male. In the space provided below, draw what you see on these slides. Determine which slide shows tissue from a female and which shows tissue from a male.

EXERCISE C Mendelian Inheritance in Humans

PART 1 Monohybrid Crosses in Humans

Several human traits may be used to demonstrate Mendel's law of segregation. The traits you will study are controlled by a single gene that can occur in two forms (alleles). One allele is dominant, the other is recessive. A person's phenotype is determined by the combination of alleles present. An individual can be homozygous dominant, heterozygous, or homozygous recessive.

Objectives

☐ Describe the genetic basis for several human traits evident in the classroom population.

☐ Determine the extent to which individuals are different from one another based on the presence or absence of selected genetic traits.

Procedure

For each of the following 11 traits, determine your phenotype and enter it in Table 15C-1. Based on your phenotype, record your possible genotypes for each of the traits. Remember that if you have a recessive characteristic you must have both recessive alleles, but if you have a dominant characteristic you may be either homozygous dominant or heterozygous. If you have a dominant phenotype, you may have no way of knowing whether you carry a recessive allele. In this case, use a dash (—) to represent the unknown second gene.

With the help of your laboratory instructor, compile the data for your section. Calculate the percentages for each trait.

1. **Dimpled chin.** A cleft in the chin is a dominant trait. (*D*, allele for dimpling; *d*, allele for absence of a dimple.)
2. **Free ear lobe.** In most people the ear lobes hang free (dominant allele, *E*), but in a person with two recessive alleles (*e*), the ear lobes are attached directly to the head. Use a mirror or the opinion of your classmates to determine your phenotype.

3. **Widow's peak.** The action of the dominant allele (*W*) results in a hairline that forms a distinct point, known as a widow's peak, in the center of the forehead. The recessive allele (*w*) produces a continuous hairline. Omit this tabulation if a gene for baldness has had some effect on your hairline.
4. **Ability to taste PTC.** Some persons detect a distinct bitter taste in small concentrations of the chemical phenylthiocarbamide (PTC), while others do not taste it. A dominant allele *T* confers the ability to taste this chemical; those who are homozygous for the recessive allele *t* are nontasters. Place a PTC paper strip on your tongue and allow it to remain there for about 10 seconds. If you are a taster you will know it. If you have any doubt about your ability to taste the substance, you are a nontaster.
5. **Interlocking fingers.** When the fingers are interlocked, some people will almost invariably place the left thumb on top of the right (dominant allele *F*), whereas others will place the right over the left (recessive allele *f*).
6. **Bent little finger.** A dominant allele *B* causes the last joint of the little finger to bend inward toward the fourth finger (*b* is the recessive allele for a straight finger). Lay both hands flat on the table, relax your muscles, and note whether you have a bent or a straight little finger.
7. **Hitchhiker's thumb.** This characteristic, more precisely called distal hyperextensibility of the thumb, can be determined by bending the distal joint of the thumb back as far as possible. While there tends to be some degree of variation, certain individuals can bend it back until there is almost a 90-degree angle between the two joints. This characteristic is an effect of a recessive allele *h* (dominant allele, *H*).
8. **Long palmar muscle.** A person homozygous for a recessive allele *l* has a long palmar muscle that can be detected by examination of the tendons running over the inside of the wrists. Clench your first tightly and flex your hand. Now feel the tendons. If there are three, you have the long palmar muscle. If there are only two tendons (the large middle one will be missing) you do not have this muscle. Examine both wrists—if you find this trait in one or both wrists you have two recessive alleles. If not, you have the dominant allele *L.*
9. **Pigmented irises.** When a person is homozygous for the recessive allele *p,* there is no pigment in the front part of the eyes and a blue layer at the back of the iris shows through, resulting in blue eyes. A dominant allele of this gene, *P,* causes pigment to be deposited in the front layer of the iris, thus masking it blue to varying degrees. Other genes determine the exact nature and density of this pigment, thus there are brown, hazel, violet, green, and other eye colors. Here, you are concerned only with the presence or absence of such pigment.
10. **Mid-digital hair.** Some people have hair on the second (middle) joint of one or more of the fingers, while others do not. The complete absence of hair on this joint for all fingers is due to a recessive allele *m* and the presence of hair is due to a dominant allele *M.* There seem to be a number of alleles determining whether hair will grow on one, two, three, or four fingers. This hair may be very fine, so you should use a hand lens to look carefully on all fingers before deciding whether this hair is present on any one of your fingers, indicating the presence of dominant allele *M.*
11. **Second (index) finger shorter than the fourth.** This is a characteristic that appears to be sex-influenced. Use the symbol S^S for a shorter second finger and the symbol S^L for a longer second finger. Tabulate your results by sex, since the frequency should vary by sex.

   a. *For the traits observed, did you find that the dominant alleles were expressed most often in your laboratory section?* ________

   b. *For which traits was this not true?* ______________________________

   ____

   c. *How do you explain your results?* ______________________________

   ____

Table 15C-1 Genetic Traits

Characteristic	Your Phenotype	Your Possible Genotypes	Data for Your Laboratory Section: Number of Each Phenotype	Data for Your Laboratory Section: Percentage
1. Dimpled chin (*D*)				
Nondimpled chin (*d*)				
2. Free ear lobes (*E*)				
Attached ear lobes (*e*)				
3. Widow's peak (*W*)				
No widow's peak (*w*)				
4. Taster of PTC (*T*)				
Nontaster (*t*)				
5. Left thumb on top (*F*)				
Right thumb on top (*f*)				
6. Bent little finger (*B*)				
Finger not bent (*b*)				
7. Hitchhiker's thumb (*h*)				
Normal thumb (*H*)				
8. Long palmar muscle (*l*)				
Two tendons only (*L*)				
9. Pigmented iris (*P*)				
Unpigmented iris (*p*)				
10. Mid-digital hair (*M*)				
No mid-digital hair (*m*)				
11. Shorter second finger (S^S)			♂	♂
Longer second finger (S^L)			♀	♀

PART 2 How Individual Is Each Individual?

Procedure

1. Pick a member of your class to serve as an "individual."
2. Everyone in the class should stand up.
3. Have the "individual" call out his or her phenotype for each of the traits studied. As each phenotype is called out, all those who do not have that phenotype should sit down.

   a. How many characteristics must be considered before the "individual" stands out as a unique individual? ______

   b. In the United States, a great mixing of genotypes has taken place through immigration and intermarriage. In a country where there has been little immigration, would you expect an individual to stand out sooner or later than occurred in your class? ______

4. Instead of considering the inheritance of only one of the traits just studied, consider the inheritance of two of these traits. All of these traits are unlinked, thus Mendel's principle of independent assortment applies.

c. What is the phenotype of a person with the genotype EETT? ______________________

d. What alleles would be present in gametes produced by this individual?

____

e. What is the phenotype of a person with the genotype eett? ______________________

f. What alleles would be present in gametes produced by this individual?

____

*g. If the two homozygous individuals above (*EETT *and* eett*) produce offspring, what would be the expected genotypes and phenotypes of their offspring?*

Genotypes ______________ *Phenotypes* ______________

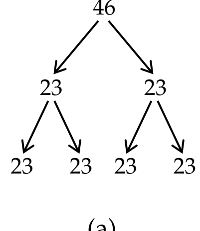
*h. Assume that two individuals heterozygous for both of these traits (*EeTt*) marry and produce offspring. What would be the expected genotypes and phenotypes of their offspring?*

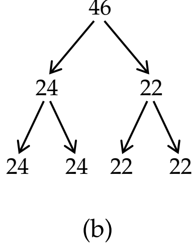
Genotypes ______________ *Phenotypes* ______________

✔ EXERCISE D | Chromosomal Abnormalities—Nondisjunction and Translocation

Mitosis and meiosis are usually very exact processes that result in the correct distribution of chromosomes to the daughter cells. However, mistakes occasionally result in an abnormal number of chromosomes or pieces of chromosomes in the daughter cells. Usually, zygotes with abnormal chromosomal compositions are spontaneously aborted. However, if the combinations are not lethal at an early stage in development, the phenotype and viability of the resulting individual may be seriously affected.

Recall that during meiosis, homologous chromosomes synapse during prophase I and then separate from each other during anaphase I (Figure 15D-1a). Sometimes a pair of chromosomes may adhere so tightly that they do not pull apart during anaphase I. This will result in one of the daughter cells receiving duplicate chromosomes and the other receiving none of that type of chromosome (see Figure 15D-1b). This failure of chromosomes to separate is called **nondisjunction.** Nondisjunction may also occur during the second meiotic division if the chromatids of a dyad chromosome do not separate from each other (Figure 15D-1c).





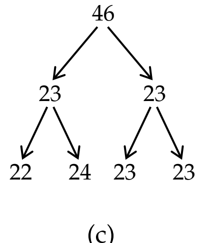


Figure 15D-1 *Meiosis in humans (n = 23). (a) Normal meiosis. (b) Nondisjunction at meiosis I. (c) Nondisjunction at meiosis II. Numbers indicate the number of chromosomes present in the cell after each division.*

Other chromosomal abnormalities are caused by chromosome breakage and redistribution of chromosome parts. Actual breakage of the chromosome can be caused by a variety of factors, including ionizing radiation and certain drugs and chemicals. The transfer of a portion of one chromosome to another, usually nonhomologous, chromosome is called a **translocation.** The abnormal chromosome may then be passed on from parent to offspring. Some cases of Down syndrome are caused by a translocation in which a large portion of chromosome 21 becomes attached to chromosome 15.

Objectives

- ☐ Define and distinguish between nondisjunction and translocation.
- ☐ Explain the chromosomal basis of Klinefelter's, Turner's, triple-*X*, Jacob's, and Down syndromes.

✔ PART I Nondisjunction: Sex Chromosomes

Normal meiosis in a human female results in the production of egg cells with 22 autosomes and one *X* chromosome. Normal meiosis in a human male results in the production of two types of sperm: one half with 22 autosomes and one *X* chromosome and one half with 22 autosomes and one *Y* chromosome.

a. *If an* X*-containing egg is fertilized by an* X*-containing sperm, an* XX *zygote will be formed. Will this individual be male or female?* ______________

b. *How many Barr bodies (sex-chromatin bodies) will the zygote have (see Exercise B)?* ________

c. *If an* X*-containing egg is fertilized by a* Y*-containing sperm, an* XY *zygote will be formed. Will it be male or female?* ______________

d. *How many Barr bodies will it have?* ________

If nondisjunction of the *X* chromosomes occurs during meiosis in a human female, some of the eggs will contain two *X* chromosomes and others will contain no *X* chromosome. In a male, nondisjunction can result in four kinds of sperm: nondisjunction during meiosis I will result in sperm with both the *X* and *Y* chromosomes and sperm with no sex chromosome, designated *0*. Nondisjunction during meiosis II may result in sperm with *XX* and sperm with no sex chromosome (*0*) or in sperm with *YY* and sperm with *0*. Gametes produced by nondisjunction usually fertilize, or are fertilized by, normal gametes.

Procedure

Several syndromes resulting from nondisjunction in humans are listed below.

Triple-*X* syndrome The individual will develop into a normal-appearing female, but may be sterile. She may also be mentally retarded.

Klinefelter's syndrome The individual will develop as a male. During early development he appears normal, but abnormalities become apparent at puberty. Testes do not fully develop. The person is usually taller than average, his muscular development may be somewhat feminine, breast development may occur, and his voice may be higher-pitched than normal. Although the individual may develop a "female" appearance, fluorescent cell staining always reveals the presence of a *Y* chromosome.

Turner's syndrome The individual will be female. She appears normal during early development, but at puberty does not menstruate, breasts do not develop, and no eggs are produced by the ovaries.

Table 15D-1 Nondisjunction in a Human Female

Abnormal Gamete (egg)	Normal Gamete (sperm)	Zygote (genotype)	Expected Sex	Number of Barr Bodies	Name of Syndrome
XX	*X*				Triple-*X* syndrome
XX	*Y*				Klinefelter's syndrome
0	*X*				Turner's syndrome
0	*Y*				Nonviable, not seen

Table 15D-2 Nondisjunction in a Human Male

Abnormal Gamete (egg)	Normal Gamete (sperm)	Zygote (genotype)	Expected Sex	Number of Barr Bodies	Name of Syndrome
X	*XX*				Triple-*X* syndrome
X	*XY*				Klinefelter's syndrome
X	*0*				Turner's syndrome
X	*YY*				Jacob's syndrome

Jacob's syndrome The individual appears to be sexually normal.

Note that all of these syndromes, except Jacob's syndrome, may result from nondisjunction in either the male or the female parent.

Based on the information given in this and previous exercises, complete Tables 15D-1 and 15D-2.

✔ PART 2 Nondisjunction: Autosomes

Autosomal nondisjunction involves chromosomes other than the sex chromosomes. The incidence of Down syndrome caused by autosomal nondisjunction of chromosome 21 increases with the age of the mother (although evidence suggests that the father may sometimes be responsible for providing the extra chromosome).

a. What explanations might be proposed for the relationship between increasing maternal age and the increasing frequency of Down syndrome? ____________________

____

Down syndrome—trisomy 21 Three number 21 chromosomes are present. Individuals are characterized by a fold of the upper eyelid, short stature, broad hands, stubby feet, a wide, rounded face, a large tongue, and mental retardation.

Trisomy 18 This syndrome involves an extra chromosome 18. Individuals are characterized by a misshapen skull, eye problems, overlapping fingers, heart defects, feeding problems, and severe mental and developmental retardation.

Procedure

Work in pairs. Obtain a blank karyotype form and a copy of a photograph of human chromosomes. The chromosomes may be banded or simply stained with Feulgen stain. Carefully cut out the individual chromosomes. Using as a guide the sample karyotype that you prepared in Exercise A, arrange each homologous pair of chromosomes on the blank karyotype form. (Remember that the comparison of bands is helpful.) Do not fasten the chromosomes until they have been checked by your instructor. Keep all scraps until you have identified each chromosome. Now tape the chromosomes to the karyotype form. Identify the karyotype you have made. The possible choices are normal female; trisomy 21 (Down syndrome) male; trisomy 21 (Down syndrome) female; trisomy 18 male or female.

✔ EXERCISE E Constructing a Human Pedigree

Genetic analysis of the inheritance pattern for a specific human trait often requires collecting information about a family's genetic history and using it to construct a pedigree chart, which traces the occurrence of particular characteristics through several generations, or "lines." Such pedigrees enable genetic counselors

to derive facts about a couple's genetic makeup and thereby accurately calculate the chances that their offspring will inherit detrimental genes.

The pedigree chart provides a systematic and convenient method for recording data. One set of symbols and arrangements commonly used in pedigree construction is given in Table 15E-1. Individuals are designated by specific symbols, ○ for female and □ for male. Phenotypes are represented by shading the symbols. Familial relationships must also be indicated on pedigree charts: marriages, offspring, siblings, and identical or fraternal twins. For easy reference, symbols are often numbered, and birth or death dates are recorded beside the symbols.

Objectives

☐ Construct a human pedigree from case history information.

Procedure

1. Read the true case history that follows. Analyze and complete the pedigree chart in Figure 15E-1 using the symbols given in Table 15E-1.

Case History 147*

Jane M. requested genetic counseling because she and her husband were planning to begin a family and were concerned because Jane's brother, Brian, was moderately retarded.

The family history indicated that Brian was the third of four children. The pregnancy was uncomplicated, but delivery required the use of forceps. Shortly after his birth, the family noticed that Brian was fussy and was slow to crawl and sit. After a neurological evaluation, Brian's parents were told that he had brain damage, probably due to the forceps delivery. Brian had attended special schools and now, at the age of 22, lived at home and had no major physical problems.

Jane's and Brian's mother, Mrs. S., indicated that her daughter, Carol, had a 6-year-old daughter who required a special education program. Another daughter, Nancy, had a son, 4 years old, considered to be developmentally delayed. Brian's mother denied that any of her three daughters, Jane, Carol, or Nancy, were mentally retarded or had any learning disability, but it was noted during counseling that Brian's mother, as well as Jane and Carol, appeared to have below-average intelligence.

After genetic screening tests, Brian was diagnosed as having a condition known as "fragile *X*" syndrome. This *X*-linked recessive condition can be identified by karyotype studies of cells cultured in media deficient in specific nutrients. Under these conditions, the *X* chromosome breaks very close to the end of its long arm and releases a fragment. Males who carry the fragile *X* chromosome are usually severely retarded. If the carrier of the fragile *X* chromosome is female, it is likely that, in the heterozygous condition, the presence of a normal *X* chromosome will prevent severe mental retardation. Protection by the normal *X* chromosome is not complete, however, for heterozygous female carriers usually exhibit mild forms of mental retardation.

After further screening of family members, it was explained to the family that Brian's mother carried the fragile *X* chromosome, as did Jane and Carol, but Nancy's karyotype did not reveal the fragile *X* chromosome. Women who carry the fragile *X* are at 50 percent risk of having affected sons and 50 percent risk of having girls who are "carriers." Nancy would not be at risk for having affected children and fragile *X* was not the explanation for her child's developmental delay.

Further investigation revealed additional information about the family background as noted in the partial pedigree (Figure 15E-1).

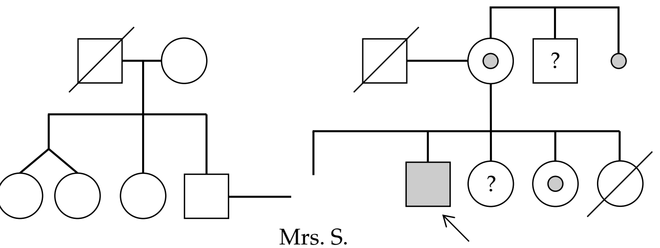
2. Examine Figure 15E-1. On a separate sheet of paper, write a paragraph describing the family history prior to that for Mrs. S.

**Case history information was provided by Dr. Nina Caris, Department of Biology, Texas A & M University.*

Table 15E-1 Symbols Used in Pedigree Analysis

Symbol	Meaning	Symbol	Meaning
	Male		Offspring of unknown sex
	Female		Aborted or stillborn offspring
	Mating between individuals		Deceased offspring
	Mating between close relatives	or	Affected individual
I II 1 2 3	Parents (top row) and their offspring (bottom row) listed in birth order. Roman numerals indicate generations; arabic numbers indicate birth order within a generation	or	Propositus (male) or proposita (female). First case in family that was identified.
		or	Heterozygotes
	Identical (monozygotic) twins		*X*-linked carrier
	Nonidentical (fraternal) twins	d. 1968 d. 1982	Date of death
		?	Questionable whether individual had trait

Figure 15E-1 *Partial pedigree for the fragile X syndrome in Mrs. S.'s generation and in the generation preceding hers. Complete the chart by drawing the symbol for Mrs. S. in the position indicated, then add symbols (see Table 15E-1) for the family members described in the case study.*



3. Complete the pedigree (Figure 15E-1) for Mrs. S., her children, and grandchildren according to the information given in the family history.

a. How would you counsel Jane? And, if Carol sought genetic counseling about having further children, how would you counsel her? (Write your answer on a separate sheet.)

b. If Carol's daughter marries and decides to have children, what would you tell her if she sought your counsel? (Write your answer on a separate sheet of paper.)

c. What would you infer about the genotype of Mrs. S.'s grandparents? ______________

d. What might have been the genotype of Mrs. S.'s uncle? ______________

EXERCISE F Forensic Science: DNA Fingerprinting

In human DNA, there are many long sequences of base pairs that are similar in all individuals. Some of these sequences code for proteins, but others do not. (In fact, because most of the human genome does *not* code for protein, a large amount of variation can occur without consequence.) Mutations in coding regions may randomly eliminate restriction sites (places where restriction enzymes cut DNA) or form new ones. Thus, if the same restriction enzyme is used to cut the DNA from two individuals, the resulting fragments from corresponding allelic regions may be of different lengths. These different-length pieces are called **RFLPs** (pronounced "riflips"), **restriction fragment length polymorphisms** (Figure 15F-1a).

Restriction fragments of different lengths (RFLPs) can be separated on a gel by electrophoresis to produce a **DNA fingerprint;** the pattern of bands on the gel will be different for each individual (except for identical twins). The steps involved in analyzing DNA fingerprints, used to identify or match individuals (Figure 15F-2), can be summarized as follows:

1. Once RFLPs are separated on a gel, the gel is treated with an alkaline solution to denature the DNA. The two strands of the DNA separate, forming single strands.

2. A sheet of nitrocellulose paper or a nylon membrane is blotted onto the gel to pick up the single-stranded DNA fragments.

3. Artificially synthesized, radioactive, single-stranded, DNA fragments called **probes,** complementary in sequence to portions of specific RFLPs, are applied to the blotting sheet. They base-pair only with complementary sequences. Excess probe is rinsed off.

4. The blotting sheet is used to expose X-ray film. Since only radioactive materials will expose the film, only those sequences that have base-paired with the radioactive probes will show up on the film. The resulting pattern is called a DNA fingerprint.

Since mutations in DNA are cumulative over time, more closely related individuals and groups can be recognized by their genetic fingerprints: similar banding patterns of RFLPs. Thus, DNA fingerprinting can be used not only to identify genetic material from the same individual, but also to establish paternity and to discern evolutionary relationships.

For added precision, especially when forensic evidence is needed, RFLP analysis is accompanied by another type of DNA fingerprinting that analyzes the **variable number of tandem repeats (VNTRs)** present in genomic DNA. Within the human genome, short nucleotide sequences of 5 to 10 base pairs may be repeated over and over again (in tandem, and often head to tail) to form longer sequences of 20 to 200 base pairs. Often these tandem nucleotide sequences occur in front of or behind single-copy genes that code for proteins.

The sequence of base pairs in tandem repeats shows little variation from individual to individual, but the *number* of repeats can vary greatly, leading to different fingerprint patterns among individuals. Differences in length among arrays of tandem repeats result from unequal crossing-over during meiosis. VNTRs protect genes from damage by allowing for some "slippage" in front of and behind a gene being "cut out" of one chromosome and "pasted into" or recombined with another during crossing-over. As a consequence, the lengths of tandem repeat arrays located close to a particular allele on maternal and paternal homologous chromosomes belonging to the same individual also may differ (Figure 15F-3).

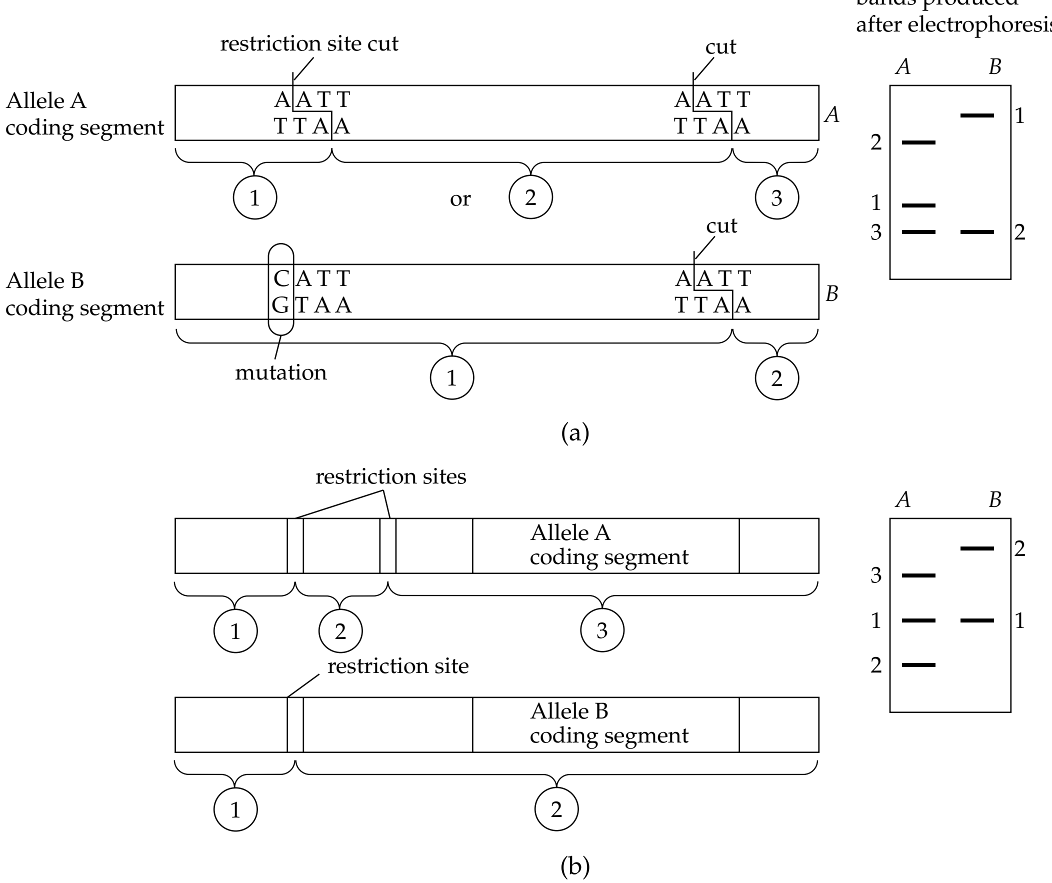


Figure 15F-1 *DNA fingerprinting using RFLP sequences. Mutations in DNA can result in eliminating or forming new restriction sites where restriction endonucleases can cut the DNA to form fragments of varying lengths (RFLPs).*

(a) Elimination of a restriction site within the coding sequence of an allele due to a base-pair change (converting allele A *to allele* B*) results in only two fragments being formed during enzymatic digestion, rather than three. Since the fragments vary in length, a different fingerprint is obtained for alleles* A *and* B.

(b) If restriction sites in a DNA sequence preceding an allele are affected, rather than the allele itself, differences can also be detected from DNA fingerprints. Often, a unique fingerprint pattern results from a mutation in a noncoding sequence found in front of a DNA sequence (allele) suspected of causing a specific genetic disorder. If this pattern is consistently found among those with the genetic disorder (pattern B *in this case), this fingerprint pattern can be used for diagnostic purposes even though identification of the gene is not conclusive and its specific location is not known.*

To obtain a DNA fingerprint using VNTR regions, the DNA is cut with restriction enzymes that do *not* cut within the tandem repeat area. Instead, they cut in front of and behind the repeat sequence. The polymerase chain reaction (PCR) is used to augment the number of copies of the isolated repeat areas. (For accuracy, several different repeat areas are analyzed by cutting with different restriction enzymes and are then amplified using PCR.) The PCR reaction uses short DNA primers to synthesize many copies of the VNTR regions to be used for electrophoresis and fingerprinting (Figure 15F-3). PCR makes it possible to obtain the required amount of DNA from even the smallest traces of blood, semen, and other tissues and cells of an individual.

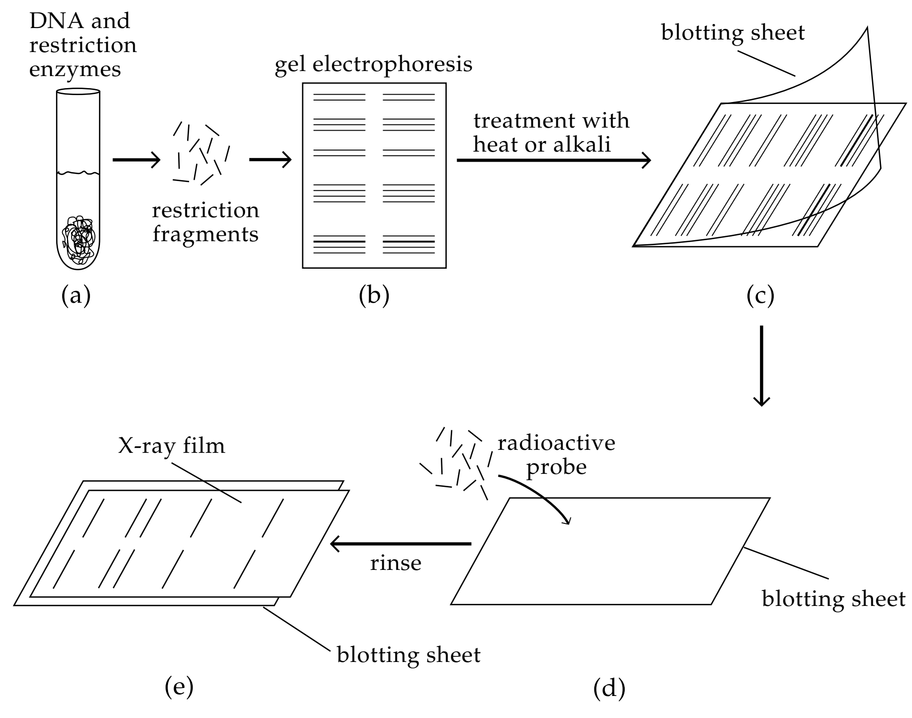


Figure 15F-2 *DNA fingerprinting. (a) DNA is broken into fragments by restriction endonucleases. (b) The fragments are separated by gel electrophoresis, then the gel is treated by heat or an alkaline solution to separate the strands. (c) A sheet of nylon or nitrocellulose film is used to "blot" the gel and pick up DNA. (d) When radioactive DNA probes are added to the sheet, they base-pair only with complementary strands in various regions, producing a DNA fingerprint. (The sheet is then rinsed to wash away any probes that do not hybridize so that these pieces will not interfere with the pattern.) (e) The blotting sheet is allowed to expose X-ray film. The radioactive probes will identify the RFLPs of interest—only these will be visible on the film.*

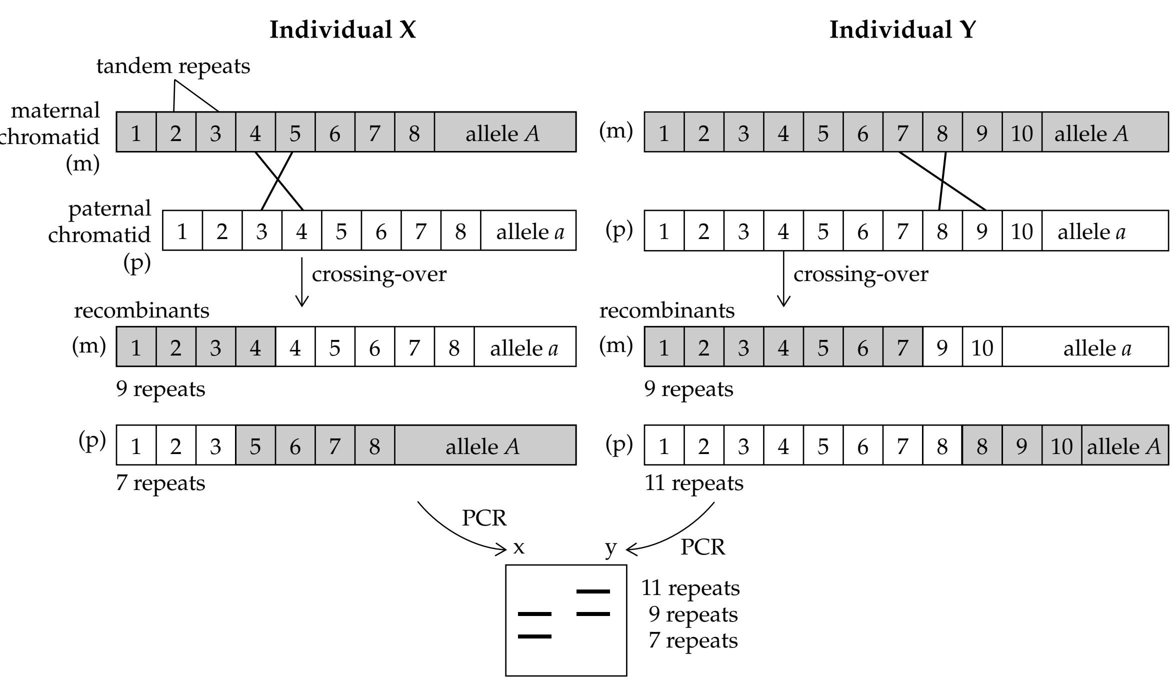


Figure 15F-3 *VNTR differences in homologous chromosomes. The number of tandemly repeated sequences of DNA associated with particular genes on a chromosome differs widely among individuals. These differences are generated by unequal crossing-over, so that even maternal and paternal homologues of the same individual, as well as different individuals, have different numbers of sequences associated with the same allele.*

Objectives

- ☐ Describe how restriction enzymes are used in DNA fingerprinting.
- ☐ Interpret results from a DNA fingerprint.

Procedure*

A crime has been committed. A brief scenario of the crime is given below.

> *A woman jogging through a mid-city park at dusk was accosted by a man who dragged her into a stand of trees and bludgeoned her with a baseball bat. The man fled when interrupted by a fellow jogger who heard the woman's screams. The jogger who witnessed the crime saw the man flee from the scene but could not identify the suspect for certain because it was fairly dark at that time of evening. In the police lineup, the witness tentatively identified a suspect, but the man standing next to him also looked a lot like the alleged attacker. Fortunately, the victim had pulled some hair from the attacker's head. DNA was extracted from the papillae of the hairs and a DNA fingerprint was developed to compare the DNA from the evidence (hairs), and the two suspects, X and Y. (Electrophoresis of PCR-amplified VNTRs was used to create the needed forensic evidence.)*

1. Insert a gel comb into an electrophoresis gel tray and cast an agarose gel as described in Laboratory 14, Exercise D. (Add 2 drops of Carolina Blu stain to 50 ml of agarose, if instructed to do so.)
2. When the gel has solidified (about 10 minutes), place it in the gel box so that the comb is at the negative (black) end.
3. Fill the gel box with TBE (tris-borate EDTA) buffer until the buffer covers the gel. (Add Carolina Blu stain to the buffer if instructed to do so.)
4. Gently remove the gel comb and check for bubbles.
5. Add DNA samples to the wells using a microcapillary pipette. Load the contents of each tube—Suspect X-1, Suspect X-2, Evidence 1, Evidence 2, Suspect Y-1, and Suspect Y-2—into separate wells. Use a clean pipette for each. Position the pipette above the well to load. *Do not put the pipette tip into the well* or you may punch a hole in the gel! (See Laboratory 14, Exercise D, for details.)

   Note: During PCR, two different primers were used to produce multiple copies of the suspects' DNA and the evidence DNA. Primer 1 was used to produce the DNA of Suspect X-1, Evidence 1, and Suspect Y-1. Primer 2 was used to produce the DNA of Suspect X-2, Evidence 2, and Suspect Y-2. To match a suspect to crime scene evidence, the PCR products from Primer 1 must match Evidence 1 and the products from Primer 2 must match Evidence 2.
6. Close the electrophoresis apparatus, connect the leads, and turn on the power supply to 80 volts. Allow the electrophoresis to run for approximately 2 hours or until the bromophenol blue band nears the end of the gel (see Laboratory 14, Exercise D, for details). If Carolina Blu stain has been added to the agarose and buffer, you will also see faint blue bands in the "fingerprint" pattern.
7. Turn off the power supply, disconnect the leads, and open the gel box.
8. Remove the gel and stain it using the procedure described by your instructor (staining procedures vary with the preparation of agarose), using either methylene blue or Carolina Blu Final Stain.
9. Analyze the gel by placing it on a light box or transilluminator. You may wish to use plastic wrap or transparency film to make "copies" of the gel and its bands for analysis. (See Laboratory 14, Exercise D, for details.)

**This exercise is adapted from the Carolina Biological Supply Kit 21-121-, PCR Forensic Simulation Kit.*

a. *Which "suspect" DNA matches the "evidence" DNA? Who committed the crime?*

____

b. *If identical twins had been included among the suspects, what would you have observed in the DNA fingerprint pattern?* _______________

Laboratory Review Questions and Problems

Use a separate sheet of paper to answer the following questions. Show all of your work. Indicate all genotypes and phenotypes, where appropriate. If more than one type of offspring is produced, indicate the proportion of each type.

1. A man with attached ear lobes marries a woman who is heterozygous for free ear lobes. What types of offspring and what proportion of each type would be expected from this mating?

2. Polydactyly is an inherited human trait in which the affected individual has extra fingers or toes. This defect results from the dominance of the defective gene (*P*) over the normal allele (*p*).

a. If a man with polydactyly (assume that he is heterozygous) marries a normal woman, what are the possible genotypes of their children?

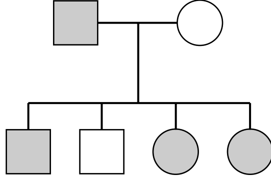
b. What are the phenotypes of these genotypes?

c. If two individuals heterozygous for this trait marry, what are the possible genotypes of their children?

d. What are the phenotypes of these genotypes?

3. Formation of Barr bodies takes place when a zygote has already divided to form a multicellular embryo. Because of this, the body of a female can be a "mosaic of sex-linked phenotypes." Explain.

4. Color blindness is an *X*-linked recessive trait. The phenotypes within a family pedigree are shown below. Determine the genotypes of each individual.



5. A color-blind man marries a woman whose father was color-blind but whose mother had normal vision. Would it be possible for the couple to have a color-blind son? A color-blind daughter? What is the probability of each of these events happening?

6. The inheritance of human blood types is dependent on multiple alleles. The ABO blood system, characterized by three alleles, is commonly used to determine the suitability of donors and recipients for blood transfusions.

Normally, the body does not store antibodies for proteins it has never encountered. The ABO blood system, however, is an exception to this rule. In this system, antibodies to other blood types occur naturally whenever the antigen (A or B) is not present. An individual with

type A blood possesses type A antigen and type B antibody; an individual with type B blood possesses type B antigen and type A antibody; type O blood contains neither the A nor the B antigen, but both antibodies; type AB blood contains both antigens and neither antibody. As a result, if, for instance, a type A person receives type B blood, the B antigen will be agglutinated by the B antibodies present in the type A blood. Antibodies in the donor's blood are generally of little consequence because they are so diluted in the recipient's blood.

The general rule in transfusions is never to allow an individual to receive an antigen that does not occur in his or her blood. Since type O blood contains neither antigen A nor antigen B, this blood type is considered as the "universal donor" and may be transfused to any blood type. To study the inheritance of the ABO alleles, the symbols I and i with superscripts are used, since more than two alleles must be considered (capital and lowercase letters will not suffice). I^A, I^B, and i represent alleles of antigens A and B and the allele for blood type O (which produces no antigens), respectively.

An individual homozygous for type A blood (I^AI^A) will produce I^A gametes; a heterozygote (I^Ai) will produce I^A and i gametes. (Remember that in this case the genotype cannot be determined from the phenotype.) A similar situation holds for type B individuals. Individuals with type AB blood will produce both I^A and I^B gametes. Only i gametes will be produced by an individual with type O blood. Remember that each parent contributes only one allele to its offspring, and if the parent produces more than one type of gamete, each allele has the same probability of being passed on to the offspring.

a. What types of gametes will a woman with type AB blood produce?

b. What types of gametes will a man with type O blood produce?

c. If these two people were to marry and have children, what would be the possible genotypes of their children's blood?

d. Which blood types could their children have?

e. Which blood types could not be inherited by the children of these parents?

7. The pattern of inheritance of blood groups has some practical application in medico-legal cases involving disputed parentage.

a. If a child has type A blood, could both parents be of type O? Why?

b. If the mother is type A and the father is type B, would blood tests help to determine whether a particular child belonged to them? Why?

c. If a woman has type O blood and her child has type A blood, could a man with type B blood possibly be the father of the child? Why?

d. If both husband and wife have type AB blood, what possible blood types could their offspring have?

8. A child of blood type O has a mother whose blood type is also O. Which of these men could be the father of this child: a man of blood type A, one of blood type B, or one of blood type O? Recall that blood tests reveal only the phenotype of the individual, not the genotype, so an individual with type A blood may carry the alleles for types A and O. (Refer to question 6.) Based on the results of blood typing alone, can you rule out any of the three men as possible fathers? Why or why not?

Conclusive evidence can be gained by DNA fingerprinting, using a sample of DNA from each of the three men and from the child and the mother. The child's DNA fingerprint should contain bands that are also present in the mother's fingerprint. If additional bands are present, they must be represented in the father's fingerprint. The following results are obtained after a restriction enzyme digest, followed by autoradiography.

Key to bands:

1 Mother

2 Child

3 Possible father 1, blood type A

4 Possible father 2, blood type B

5 Possible father 3, blood type O

1 2 3 4 5

Which of the three men is the father of the child? How do you know?

9. List the steps involved in DNA fingerprinting. Why would there be an advantage to using more than one probe when developing a fingerprint?

10. A cemetery next to a river has been flooded after a spring of continuous rain. Many of the coffins have ruptured and bones are scattered about. Members of three families are buried in the cemetery. The families would like to reclaim the bones of their ancestors and place them in a proper burial place. As a forensic scientist, you have been asked to assist. How might your proceed to determine to which families the bones belong?

Figure 15A-3b *Cut out chromosomes and use Figures 15A-2 and 15A-4 to help you match homologous chromosome pairs. Arrange these pairs above the appropriate positions in Figure 15A-3a.*

LABORATORY

16

DNA Isolation

OVERVIEW

To most students of biology, DNA is an abstraction. You can memorize the names and structures of the nitrogenous bases and know all about the history of DNA's discovery, but until you actually handle DNA, it remains a strange and mysterious substance.

The purpose of this laboratory is to give you firsthand experience with DNA by isolating it from plant tissue. (Optional exercises for isolating DNA from animal or bacterial cells are also provided.) You will start with whole onions and end with a relatively pure preparation of DNA, containing literally billions of genes. Once isolated, the DNA can be stored in alcohol or dried out. It will be possible for you to hold in your hands the key to an organism's development and structure.

STUDENT PREPARATION

Prepare for the laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency. Isolating DNA will require the entire period, so be prepared to begin immediately.

EXERCISE A | DNA Isolation Procedure

DNA can be isolated from onion cells using several techniques. The procedure in Part 1 requires the use of chloroform and yields DNA that is fairly clean (chromosomal proteins associated with the DNA have been removed). Part 2 introduces a quick method for isolating DNA without the use of chloroform, but yields are not as clean. The DNA may also be sheared into shorter lengths that are more difficult to recover.

If all steps of the procedures in Parts 1 and 2 are followed carefully and the molecular structure of DNA remains intact, the genetic material of onions will precipitate as a thick, stringy white mass that may be spooled out on a glass rod. Isolation of DNA involves three basic steps:

1. **Homogenization** Before DNA can be released from the nuclei of onion tissue, the cell walls, plasma membranes, and nuclear membranes must first be broken down. This is done by homogenizing the onion tissues in a blender. Detergents in the homogenizing medium help to solubilize membranes and denature proteins.
2. **Deproteinization** Chromosomal proteins must be stripped from the DNA. The proteins can then be denatured and precipitated from the homogenate containing the DNA.
3. **Precipitation of DNA** When ice-cold ethanol is added to the homogenate, all components of the homogenate stay in solution—except DNA, which precipitates at the interface between the alcohol and homogenate layers.

If the DNA is damaged during the isolation procedure, it will still precipitate, but as a white fuzzy mass that cannot be collected on a glass rod.

Objectives

- ☐ Become familiar with the physical properties of DNA by isolating it from living tissue.
- ☐ Learn the purpose of each step in the isolation procedure as it relates to the physical and biochemical characteristics of the genetic material.

PART I DNA Isolation (*Chloroform*)

Procedure

Work in pairs throughout the DNA isolation procedure. Do not proceed to a subsequent step until *both* of you agree that everything to be done in the present step has been completed correctly. The DNA molecule is easily degraded, or broken down, so it is important to follow all instructions closely. If available, wear gloves to prevent nucleases (present on your skin) from contaminating the glassware.

HOMOGENIZATION

1. Dice a medium-sized onion into cubes no larger than 3 mm. (This step may already have been done for you to save time.)
2. Obtain a mass of 50 g of diced onion. Transfer *all* of this material to a 250-ml beaker.
3. Add 100 ml of homogenizing medium to the diced onion and incubate the beaker in a 60°C water bath for 15 minutes (No longer!). This heat treatment softens the onion tissue and allows penetration of the homogenization solution. It also denatures many enzymes that could interfere with the isolation procedure. The detergent-like action of the sodium lauryl sulfate present in the homogenizing medium helps to dissolve cell membranes and denature proteins.
4. Quickly cool your preparation to 15 to 20°C in an ice bath (a slush of ice and water). This step prevents the denaturation of DNA.
5. Pour your cooled preparation into a blender and fasten the lid. Homogenize for 45 seconds at low speed, followed by 30 seconds at high speed. Homogenization breaks open the cells and releases their contents (carbohydrates, proteins, fats, and nucleic acids). The NaCl/sodium citrate buffer stabilizes the DNA by forming a Na^+ shell around the negatively charged phosphates of the DNA. The citrate inactivates the DNase that would otherwise break down the DNA.
6. Pour the homogenate from the blender into a 1-liter beaker. Allow it to stand in an ice bath for 15 to 20 minutes.
7. Filter the homogenate through four thicknesses of cheesecloth into a 500-ml beaker, taking care to leave the foam behind.
   a. *Where is the DNA at this point?* ____________________

DEPROTEINIZATION

8. Pour 80 ml of 95% ethanol into a clean 250-ml flask and pack ice completely around it. The alcohol must get very cold before it is used in the procedure (step 16). (Your instructor may provide you with alcohol that has been kept in a freezer; pack this alcohol in ice to keep it cold.)
9. Pour *only* 50 ml of your filtered homogenate into a clean 250-ml flask.

10. Use a 5-ml pipette to gently add 2 ml of chloroform to the homogenate.

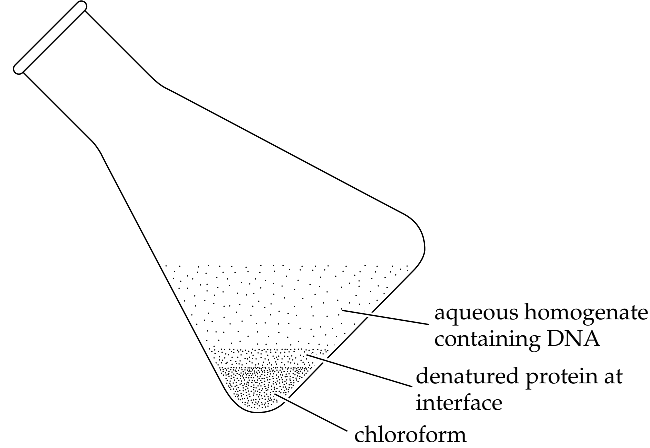
CAUTION: ***Your laboratory instructor will demonstrate how to do this safely!***

11. *Gently* swirl the contents of the flask. The purpose of this step is to increase the contact between the chloroform layer and the homogenate layer, but not to mix them (Figure 16A-1). Swirling too vigorously may result in the formation of an emulsion in which no layers are discernible. (If this occurs, see the procedure given below for treatment of an emulsion.)

b. What is the white material in the interface layer between the homogenate and the chloroform?

____

Figure 16A-1 *Denatured protein collects at the interface between the two nonmiscible layers of homogenizing medium and chloroform.*



12. Carefully pour the homogenate into another clean 250-ml flask, leaving the chloroform and protein layers behind. Rinse out the flask from which you poured the homogenate so that you may use it as the clean flask when this step is repeated.

13. Repeat steps 10 through 12 four more times. By the fifth time, much less denatured protein will be separating from the homogenate. If, at any time, an emulsion is formed, follow the procedure given below for treatment of the emulsion.

TREATMENT OF EMULSIONS

If you have swirled your flask too vigorously and created an emulsion, you will have to centrifuge the preparation.

A. Pour the emulsion into a centrifuge tube. Leave about 2 cm unfilled at the top of the tube. Use water to prepare a balance tube of equal volume. The balance tube may be another group's preparation if it is exactly the same volume as yours. Your preparation and its balance tube should be placed opposite one another in the centrifuge head.

B. Advance the centrifuge slowly from one step to the next (a few seconds at each step) until top speed is attained. If the centrifuge begins to shake violently, turn it off immediately and check the balance of your tubes.

C. Centrifuge for 5 minutes at top speed. When you turn the centrifuge off, let it coast to a stop. *Do not open the lid until it has stopped. Under no circumstances should you try to stop the moving head with your hand.*

D. Carefully pour the homogenate from the tube into a clean 250-ml flask, leaving the chloroform and protein layers behind.

Repeat steps 10 through 12 for the remaining number of times, as required by step 13.

14. After the fifth deproteinization treatment, gently pour the homogenate into a 250-ml beaker. It is very important that the homogenate not be contaminated by chloroform, so it may be necessary to leave a small amount of homogenate behind when you pour it off from the chloroform and protein layers this final time.

PRECIPITATION OF DNA

15. Place your beaker with its deproteinized homogenate in an ice bath. Let it cool until it reaches 10 to 15°C.

16. Slowly add ice-cold ethanol (prepared in step 8) down the side of the beaker until the white, stringy DNA precipitate appears. It may not take all 80 ml of alcohol to precipitate the DNA.

17. Spool out, or wind up, the stringy DNA onto a glass rod by rotating the rod in *one direction only* in the beaker of DNA. Continue to rotate the rod as you move it in large circles through the beaker.

c. Why is it important always to rotate the rod in the same direction?

____

18. If you want to keep the DNA, gently ease it off the end of the glass rod into a vial filled with 50% ethanol. Be sure the cap is tight enough to prevent leakage.

PART 2 DNA Isolation (*No Chloroform*)

Procedure

Work in pairs throughout the DNA isolation procedure. Do not proceed to the next step until *both* of you agree that everything to be done in the present step has been completed correctly. The DNA molecule is easily degraded, or broken down, so it is important to follow all instructions closely. If available, wear gloves to prevent nucleases (present on your skin) from contaminating the glassware.

Your instructor will perform steps 1–3. After these preparation steps have been completed, proceed with steps 4 through 8.

1. Cut a yellow onion into wedges and place them in a blender.

2. Add 100 ml of chilled buffer/detergent solution. Homogenize the mixture at low speed for 45 seconds, then at high speed for 30 seconds. Homogenization breaks open the onion cells and releases their contents (carbohydrates, proteins, fats, and nucleic acids).

3. Filter the homogenized mixture through cheesecloth (place the filter in a funnel) into a beaker on ice. Make sure everything is kept cold!

4. Each student should obtain approximately 4 ml of the onion preparation in a clean test tube.

5. Add 2 ml of meat tenderizer to the solution in the test tube and mix the contents slightly with a Pasteur pipette.

a. What color is the solution? ____________

b. What does the meat tenderizer do? ______________________________

____

6. Use a Pasteur pipette to slowly add an equal volume (approximately 6 ml) of ice-cold 95% ethanol down the side of the test tube. Tilt the tube slightly. You should see a distinct layer of ethanol over the colored filtrate.

7. Gently swirl the Pasteur pipette at the interface of the two layers. This process is called "spooling" the DNA. Always rotate the pipette in the same direction. The stringy, slightly

gelatinous material that attaches to the pipette is DNA. (If the DNA has been damaged, it will still precipitate, but as white flakes that cannot be collected on the glass rod.)

c. Why does isolated DNA appear stringy? ____________________

____

8. If you want to keep the DNA, gently ease it off the end of the pipette into a vial of 50% ethanol. Cap the vial tightly.

EXERCISE B Isolation of DNA from Animal Cells *(Optional)**

The procedure for isolating DNA from animal cells is similar to that for isolating DNA from plant cells (Exercise A). In this exercise, you will isolate DNA from mammalian testicular tissue. Tissue from the testes is a good choice for DNA isolation because of its high nucleus-to-cytoplasm ratio, which prevents lipid or protein from interfering with the procedure. Another advantage is that fresh tissue can be obtained without sacrificing the organism: testes are readily available from your local veterinarian or from spay/neuter clinics.

Objectives

- ☐ Isolate DNA from animal tissue.
- ☐ Use a colorimetric test to identify DNA.

PART I Isolation of DNA

Procedure

1. Chill, on ice, a tube of homogenization buffer.

2. Slice or mince a small piece (2 to 3 mm^3) of testis tissue and place it in a cold tissue homogenizer. Add 1 ml of homogenization buffer and two to three drops of sodium lauryl sulfate solution.

3. Homogenize the tissue over ice, using a tissue homogenizer attached to a variable-speed electric drill (a chilled mortar and pestle may be used instead, but a drill is preferable).

4. Transfer the slurry into a centrifuge tube. Obtain a 2 M solution of NaCl. To the volume of slurry in the tube add twice that volume of 2 M NaCl (an equal volume of chloroform can be used instead of NaCl, but, in this case, be sure to use a fume hood). Stopper the tube and shake vigorously for at least 2 minutes.

a. Has the color or viscosity of the contents in the tube changed? ________ *If so, how?*

____

5. Centrifuge the tube at least 5 to 7 minutes at top speed in a clinical centrifuge. If 2 M NaCl was used, the tube will contain a precipitate; the liquid portion will contain the DNA. If chloroform was used, the tube will contain three layers following centrifugation; the top layer contains the DNA.

6. Decant the portion of the liquid containing the DNA into a chilled beaker.

7. Slowly add two volumes of cold ethanol to the liquid containing the DNA. Use a glass rod to spool out the whitish DNA fibers (scoring the end of the glass rod with a diamond pencil or file will make it easier to spool out the DNA).

The DNA can be redissolved in 0.1 M EDTA, or, more slowly, in a salt solution (4% NaCl).

**This exercise was developed by Peggy O'Neill Skinner, Bush School, Seattle, Washington.*

PART 2 Colorimetric Detection of DNA

You can demonstrate that the whitish material you have isolated is, in fact, DNA by using a colorimetric test; **diphenylamine** will turn blue when it reacts with DNA.

Procedure

1. Pour 3 ml of 4% NaCl into a clean test tube.
2. Use an applicator stick to remove some of your isolated DNA from the glass stirring rod and redissolve this material in the NaCl.
3. Mark the tube with an "I" to indicate that it contains your *isolated* material.
4. Place 3 ml of a DNA standard solution into a second test tube and 3 ml of water into a third test tube. Mark these with an appropriate indication of their contents. These tubes will provide you with a standard and a control with which to compare your results.
5. Add 3 ml of diphenylamine reagent to each tube.
6. Using a beaker of water, boil the three tubes for 15 minutes. Cover the tops of the test tubes with marbles and be sure to use boiling chips in the beaker of water.
7. Compare colors in the tubes and record your results. Tube I ____________________;

   DNA standard ____________________; (H_2O) ____________________

   a. Why did you use a standard in this experiment? ____

   ____

   b. Why does the standard not serve as a control in this experiment? ______________________________

   ____

EXERCISE C Isolation of DNA from Bacteria (*Optional*)

Isolation of DNA from the bacterium *Escherichia coli* is similar to isolation of DNA from plant cells (Exercise A). Since *E. coli* are prokaryotic cells and do not contain nuclei, it is fairly simple to isolate DNA from ruptured cells.

Objectives

☐ Isolate DNA from bacterial cells.

☐ Determine the absorbance characteristics of DNA.

PART 1 Isolation of DNA

Procedure

1. Suspend 2 g of bacterial paste in 25 ml of 0.15 M NaCl and 0.1 M EDTA. (Use a screw-top glass tube if you do not have access to a clinical centrifuge for later steps.)
2. Add 1 ml of lysozyme solution.
3. Incubate in a water bath at 37°C for 30 minutes.
4. Add 2 ml of 25% sodium lauryl sulfate.
5. Incubate at 50°C for 10 minutes or until the mixture is clear.
6. Add 7.5 ml of 5 M $NaClO_4$ and stir.
7. Add an equal volume (35 ml) of a chloroform/isoamyl alcohol mixture (50:1).

8. Shake well.
9. If you have access to a refrigerated centrifuge, centrifuge the mixture at 10,000 rpm for 10 minutes. Alternatively, you may use a clinical centrifuge. Spin at top speed. A protein pellet will form at the interface between the aqueous buffer and chloroform/isoamyl alcohol solutions. Remove the clear aqueous upper phase, which contains the DNA, and save it in a beaker on ice. If you do not have access to a centrifuge, allow the solution to settle out (this will take approximately 45 minutes), then pipette out the clear aqueous phase (upper layer) and place it in a large glass test tube or beaker.
10. Add two volumes of cold 85% ethanol slowly down the side of the beaker or tube containing the DNA solution.
11. Stir with a glass rod (acid-washed or heated and cooled to remove any nucleases). Stir gently and spool out the DNA. Scoring the end of the glass rod with a diamond pencil will make it easier to spool out the DNA.

PART 2 Measuring Absorbance of DNA

If an ultraviolet spectrophotometer is available, you can test the purity of the DNA you have isolated. The DNA can be dissolved and absorbance readings can then be taken at 280, 260, and 230 nm. For nucleic acids, absorbance is maximum at 260 nm and minimum at 230 nm. Most proteins have a strong absorption at 280 nm. Thus, the higher the ratio of the absorbance at 280 to the absorbance at 260 ($A_{280/260}$), the higher the protein content.

Procedure

1. Use a wooden applicator stick to remove some of the isolated DNA from the glass rod.
2. Redissolve the isolated DNA in 5 ml of saline citrate buffer.
3. See Laboratory 4 for directions on using the spectrophotometer. To prepare an absorption spectrum for DNA, first determine the absorption at 160 nm. If it is “off scale,” dilute your sample. Then determine the absorbance at 220 nm, and thereafter at 10-nm increments up to 300 nm. Graph the absorption spectrum on a separate piece of graph paper. (For instructions on graphing absorption spectra, see Laboratory 4.)
4. If a pure DNA standard is available, repeat the procedure in step 3 using this standard. Plot this curve on the same graph.

   a. How do the two absorption spectra compare? ____________________

   ____

5. From your graph, determine the $A_{280/260}$ ratio for both the DNA you have isolated and the pure DNA. Remember that proteins absorb strongly at 280 nm.

   b. Which sample is purer (contains a smaller amount of protein contamination)?

   ____

PART 3 Colorimetric Detection of DNA

You can use a colorimetric test to demonstrate that you have isolated DNA. Follow the directions in Exercise B, Part 2.

PART 4 Preparing a Standard Curve

If time permits, use dilutions of the DNA standard (known concentrations in μg/ml) to prepare a standard curve (see Laboratory 4) by reacting samples of specific concentrations of DNA with diphenylamine. Use the spectrophotometer to read absorbance at 660 nm. To determine concentration without a

spectrophotometer, simply compare the color of the unknown to the colors of the DNA standard dilutions. Plot the standard curve on a separate piece of graph paper. From this standard curve you should be able to determine the DNA concentration in your sample. DNA concentration = _______ μg/ml

Laboratory Review Questions and Problems

Some of the following questions can be answered from your laboratory experience. Other questions may require that you use the knowledge gained from lectures or from reading the text.

1. What did you learn about the properties of DNA during this laboratory period?

2. As your text explains, scientists once believed that proteins constituted the genetic material because of the great number of variations that are possible in their composition and structure. Why would these qualities be important in the genetic material? How does DNA meet this requirement?

3. What structural characteristic of DNA allows it to be spooled out on a glass rod? Why is it not possible to spool out precipitated proteins? (*Hint:* Compare the relative lengths of DNA and protein molecules.)

4. Construct a flow chart that illustrates the steps you took in isolating DNA from onion tissue. At each step, briefly indicate the purpose of the procedure or the solution used.

5. If you wanted to determine the DNA concentration in the tissue you used for the extraction procedure, how might you proceed? (*Hint:* If you did not perform Exercise C, read through Parts 2 and 4 of Exercise C and apply what you learn to the procedure you used in Exercise A.)

DNA—The Genetic Material: Replication, Transcription, and Translation

LABORATORY

17

OVERVIEW

Exact, yet variable and mutable—these are the characteristics of our genetic material, **DNA (deoxyribonucleic acid).** DNA is composed of subunits called **nucleotides** which bond together to form long polynucleotide strands. When nucleotides in one strand pair specifically (by hydrogen bonding) with nucleotides in a second strand, a double-stranded molecule—the DNA helix—is formed.

Each DNA nucleotide consists of a sugar (deoxyribose), a phosphate, and a nitrogenous base. An enormous amount of information is encoded in DNA using only four nitrogenous bases (adenine, guanine, cytosine, and thymine) in DNA nucleotides. Variability in DNA results from the arrangement (or sequence) of nucleotide bases along the polynucleotide strands. This sequence is transmitted faithfully, as exact copies, through DNA synthesis (**replication**) at each cell division, and from generation to generation, in all organisms. Occasionally, however, changes (**mutations**) occur in the nucleotide sequence; these are the basis of evolution.

According to the "central dogma of molecular biology," DNA does not act directly, but rather codes for the synthesis of **RNA (ribonucleic acid)** molecules in a process called **transcription.** These RNA "messages" are decoded in the process of protein synthesis (**translation**). Thus DNA regulates cell activity and determines the phenotype of organisms by determining the type of proteins produced by the cell (Figure 17-1).

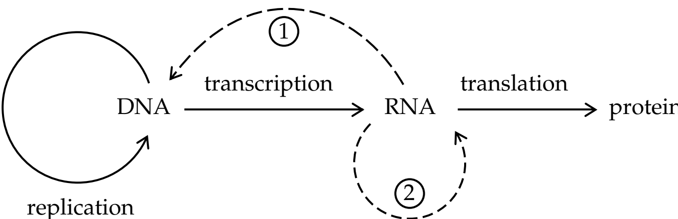


Figure 17-1 *The "central dogma of molecular biology" states that DNA is transcribed to form messenger RNA which can the be translated into protein. In this way, information stored in DNA is encoded in RNA and then is decoded to form a protein product. However, it is now known that exceptions to the central dogma exist: certain RNA viruses ① can produce DNA by reverse transcription (RNA → DNA) and ② can also replicate their RNA molecules (RNA → RNA).*

STUDENT PREPARATION

Cut out the "nucleotides" on both the green and blue sheets of paper distributed by your instructor. Keep these in two separate envelopes. Also, cut out the yellow "amino acids." Bring these materials to the laboratory with you.

Familiarize yourself with the processes of replication, transcription, and translation by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

✔ EXERCISE A Replication

The DNA molecule is composed of two strands of nucleotides (polynucleotides) hydrogen-bonded together and twisted to form double-stranded DNA. The double-stranded DNA helix is regular, linear, and stable because small nucleotide bases called **pyrimidines** always pair specifically with larger nucleotide bases called **purines.** Thymine (T) and cytosine (C) are the pyrimidines and adenine (A) and guanine (G) are the corresponding purines. Adenine always pairs with thymine, forming two hydrogen bonds (A═T), and cytosine always bonds with guanine, forming three hydrogen bonds (G≡C) (Figure 17A-1).

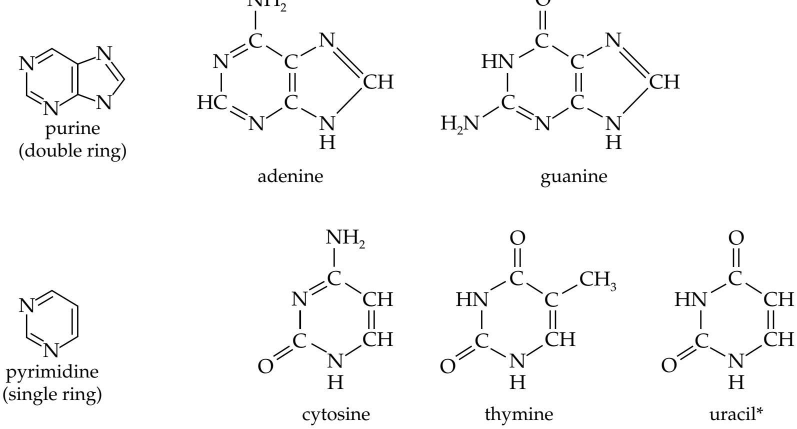


Figure 17A-1 *The purine and pyrimidine bases present in the nucleotides of DNA and RNA.*

*Replaces thymine in RNA molecules; the CH_3 group present in thymine is absent in uracil.

DNA polynucleotide strands have beginnings and ends, just like sentences. At one end of each strand is a nucleotide bearing a phosphate group that is linked to carbon number 5 (5′ carbon) of the sugar deoxyribose. This is called the 5′ end. At the other end of the chain, a hydroxyl (—OH) group extends from carbon number 3 (3′ carbon) of the deoxyribose in the last nucleotide. This end is called the 3′ end. Similarly, within the DNA molecule, each bond between two adjacent nucleotides in the polynucleotide strand is formed between the 3′ hydroxyl of one nucleotide and the 5′ phosphate of the next. These bonds are called 3′ → 5′ phosphodiester bonds (Figure 17A-2).

One polynucleotide chain in the double-stranded molecule always runs in a 5′ ⟶ 3′ direction while the other runs in a 3′ → 5′ direction: the chains are said to be arranged **antiparallel** to each other (Figure 17A-3). You may not think this is important, but before you read a book you must know which direction to read. Before DNA can work, it too must know its directions.

Replication of DNA is **semiconservative.** (*Semi-*, like *hemi-*, means half.) It is possible to break the hydrogen bonds of double-stranded helical DNA molecules, separating the two polynucleotide strands. Because the base pairing is specific (A═T and G≡C), each single strand can then serve as a **template** or

Figure 17A-2 *Nucleotides are added to a DNA chain, one at a time, by attaching the 5′ phosphate of an incoming nucleotide to the 3′ hydroxyl of the last nucleotide in the lengthening DNA chain. A phosphodiester bond is formed and inorganic pyrophosphate (PP_i) is released. The overall direction of synthesis is 5′ → 3′ for the new polynucleotide strand.*

Figure 17A-3 *Polynucleotide strands in the double-stranded helical DNA molecule run antiparallel to one another. Dotted lines represent hydrogen bonds.*

pattern for the formation of a **complementary strand.** Two new double-stranded DNA molecules are produced—each composed of an old polynucleotide strand and a newly synthesized polynucleotide strand. In other words, each of the "daughter" DNA molecules is half new and half old (Figure 17A-4).

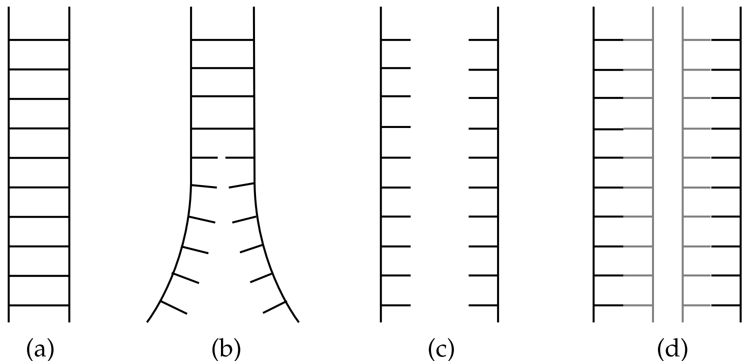


Figure 17A-4 *DNA replication is semiconservative. (a) Nucleotides in DNA are specifically paired and held together by hydrogen bonds. (b) During replication, the hydrogen bonds break and (c) the two original halves of the DNA molecule can serve as templates for the synthesis of complementary strands made from new nucleotides. (d) The two "daughter" molecules of DNA are duplicates of the original DNA. One polynucleotide strand of each molecule consists of nucleotides from the original DNA molecule; the other strand is composed of newly synthesized DNA.*

Objectives

- ☐ Describe the process of semiconservative replication.
- ☐ Explain how the structure of the DNA molecule (including hydrogen bonding and base-pairing specificity) makes semiconservative replication possible.
- ☐ Describe how semiconservative replication duplicates the parent DNA molecule.
- ☐ Describe the structure of a double-stranded DNA molecule and explain the nature of its antiparallel-stranded structure.

Procedure

A model kit has been prepared to help you understand the basic mechanisms of replication, transcription, and translation. Check to see that your kit includes the following materials:

1 DNA molecule (white)

1 sheet of deoxyribonucleotides (blue): dA, dG, dT, dC (*d* indicates *deoxy*ribonucleotide)

1 sheet of ribonucleotides (green): A, G, U, C

1 sheet of amino acids (yellow)

1 ribosome (black)

4 aminoacyl-tRNA synthetase enzymes (green)

4 tRNA molecules (blue)

1 ATP molecule (orange)

1. Begin with the white DNA molecule. Cut out the two strips and paste them together as indicated on the strips. Label the 5′ and 3′ ends of each of the two strands.
2. Use scissors to cut the hydrogen bonds and separate the two nucleotide chains labeled I and II.

3. Using the blue nucleotides, semiconservatively replicate the DNA. Line up the blue nucleotides in the proper order along each of the original DNA strands (Figure 17A-5a).

a. Which four bases are present in the nucleotides used to synthesize DNA?

____

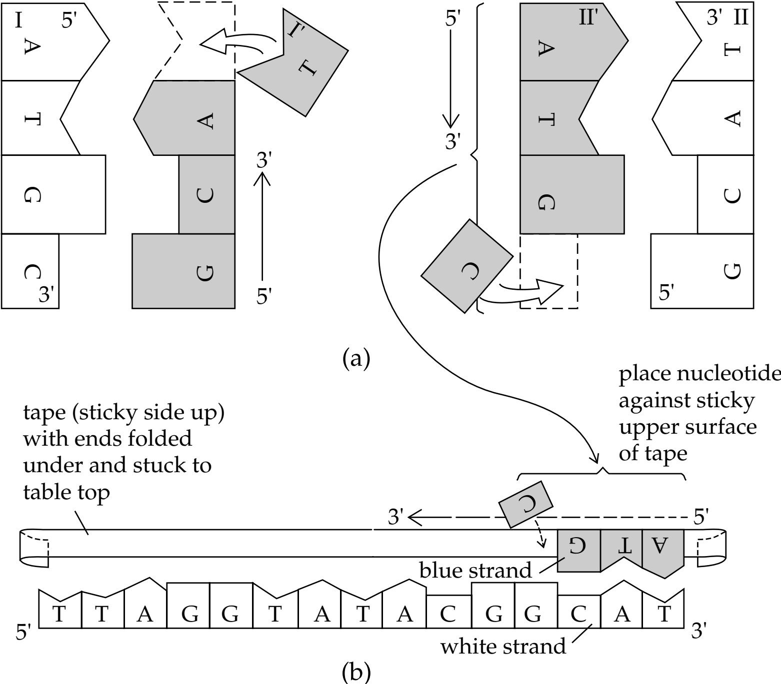


Figure 17A-5 *Replicating a DNA molecule. (a) Blue nucleotides (shown here in shading) complementary to those in the white DNA are lined up properly. (b) Nucleotides are taped to a piece of transparent tape to simulate polymerization of a polynucleotide strand in a 5′ → 3′ direction.*

4. Obtain a piece of transparent adhesive tape. Stick one end to the laboratory bench, then turn the tape over, keeping its sticky side up; turn the other end under and stick it to the bench (Figure 17A-5b). Attach each new nucleotide, letter side up, to the sticky side of the tape, aligning the straight bottom edge of each nucleotide along the straight edge of the tape so that most of the nucleotide covers the tape. Each blue nucleotide should correspond to its complementary nucleotide in the white strand (Figure 17A-5b). Be sure to synthesize the new DNA strand in the proper direction—starting at the 5′ end of each *new* strand (opposite the 3′ end of the original white DNA strand), you should add one nucleotide at a time until you reach the 3′ end of the new chain. This will be opposite the 5′ end of the original white DNA strand. Use the same method to tape together the nucleotides of the other new strand.

This process of bonding one nucleotide to the next within a lengthening or “growing” strand is called **polymerization** and is accomplished by an enzyme, **DNA polymerase.** Do *not* tape the blue and white strands to each other.

5. Indicate which new strand was made using strand I DNA as a template and which was made using strand II DNA as a template by writing I′ and II′ on them (Figure 17A-5a). Mark the 5′ and 3′ ends of each newly synthesized strand.

b. What types of bonds are made between nucleotides within the new strand?

____

For the purposes of making a model, you have kept the blue and white DNA strands separated. However, during replication, nucleotides in the original strand are bonded to complementary nucleotides in the new strand as it is being synthesized.

c. What types of bonds join the template and complementary polynucleotide strands together?

____

d. Compare the new strands (blue) with the old strands (white). To which white strand is the blue strand I′ identical? ______ *Complementary?* ______

6. Save these molecules to show your laboratory assistant, who will be coming around to check your work.

Note that the piece of DNA you have been working with is fairly short and represents only a small part of a replication "bubble" within a longer DNA molecule that is being replicated *bidirectionally* (Figure 17A-6a).

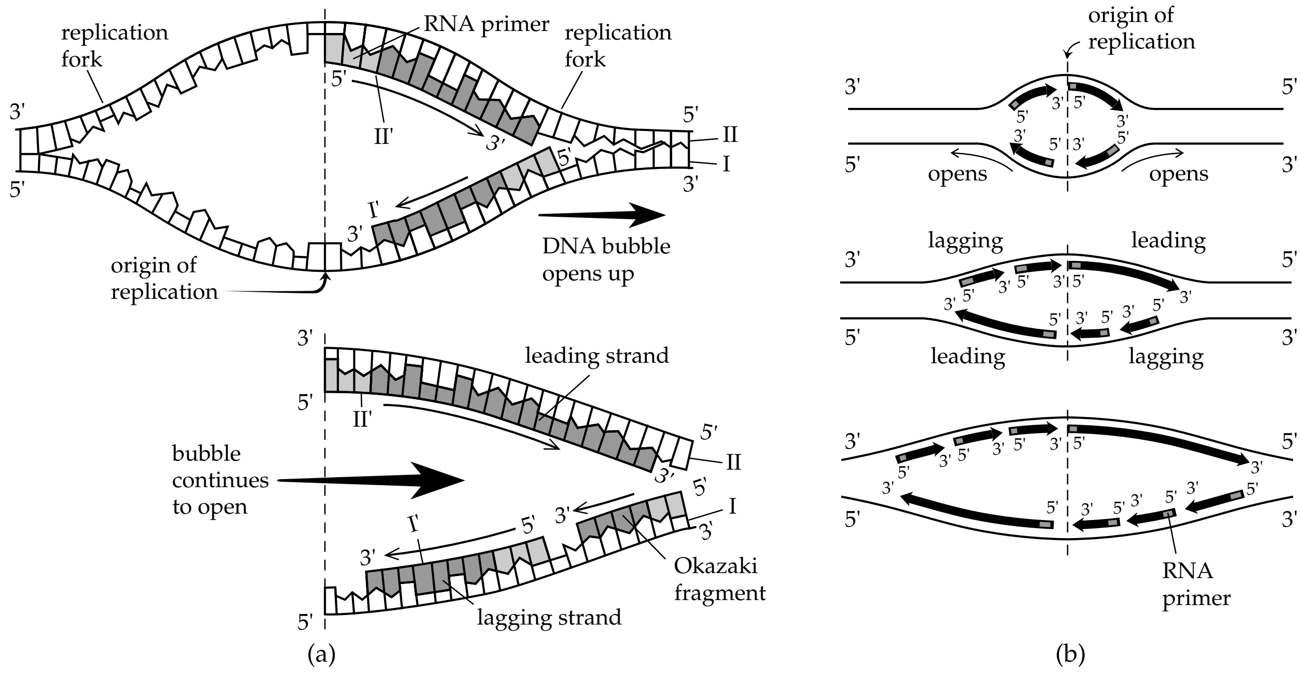


Figure 17A-6 *As a DNA replication bubble opens up bidirectionally, one strand of DNA at each replication fork can be used as a template for continuous synthesis of complementary DNA in a 5′ → 3′ direction, while the other DNA strand serves as a template for the discontinuous synthesis of a complement in short 5′ → 3′ segments that are eventually linked together.*

(a) In this replication fork, strand II serves as the complement for continuous synthesis of II′ in a 5′ → 3′ direction, corresponding to the direction in which the bubble is expanding. This strand is called the "leading strand." On the other side, however, the complement to strand I is being made discontinuously. Short I′ pieces are synthesized in a 5′ → 3′ direction that is opposite to that of bubble expansion. These pieces (called Okazaki fragments) will eventually be linked together by the enzyme DNA ligase. This new strand, synthesized more slowly and in small pieces, is called the "lagging strand." Small RNA primers are necessary to start the synthesis of both leading and lagging strands.

(b) Note that leading and lagging strands reverse in "top-bottom" orientation at opposite ends of the replication bubble because of the directions in which the opposite ends of the bubble are opening.

EXERCISE B Transcription and Translation

DNA directs the synthesis of proteins through the processes of **transcription** (DNA → RNA) and **translation** (RNA → protein). Replication, however, does *not* necessarily precede transcription and translation.

Although the genetic material, the DNA, ultimately codes for all the RNA and protein made by a cell, DNA is not *directly* involved in protein synthesis, or translation. DNA functions as a reference library from which books do not circulate. There is always an intermediate step between DNA and protein: the **messenger RNA (mRNA).**

For each particular protein to be synthesized, the DNA nucleotide sequence is first transcribed into mRNA. In eukaryotes, where a nuclear membrane separates the DNA from the cytoplasm, the mRNA is modified and then moves from the nucleus to the cytoplasm and attaches to ribosomes, where the machinery for protein synthesis is found. In prokaryotes, ribosomes can attach to mRNA even while it is being synthesized, and translation can begin immediately.

Objectives

- ☐ Differentiate between the chemical structures of RNA and DNA.
- ☐ Distinguish between the structures and roles of tRNA, rRNA, and mRNA.
- ☐ Describe the process of transcription.
- ☐ Describe the role of aminoacyl-tRNA synthetase enzymes.
- ☐ Distinguish between the functions of amino acid activation and charging of tRNAs.
- ☐ Define codon and explain why the genetic code is called a triplet code.
- ☐ Read the genetic code and identify the amino acid that corresponds to a given codon.
- ☐ List, in sequence, the steps involved in translation.
- ☐ Describe the events of elongation, translocation, and termination.
- ☐ Indicate the role of AUG, UGA, UAA, and UAG codons.

✔ PART 1 Transcription—RNA Synthesis

Using the I′ strand of blue DNA made from the I strand of white DNA, you will now synthesize a molecule of messenger RNA (mRNA) using the green ribonucleotides (Figure 17B-1). *Note:* You will be transcribing only one strand of the double-stranded DNA molecule. This strand serves as the template for mRNA synthesis. The messenger RNA made complementary to this strand of the DNA encodes the same information (sequence of nucleotides), with U substituted for T, as the nontranscribed strand of the original double-stranded DNA (often called the "sense" strand because it is the information in the nontranscribed DNA strand that makes "sense" and will ultimately determine the sequence of amino acids in the protein). The enzyme **RNA polymerase** is responsible for the polymerization reactions of transcription.

Figure 17B-1 *You will notice that the green nucleotides have the same shape as the blue nucleotides. The only difference in their structure is that* deoxyribonucleotides *are missing an oxygen on carbon number 2 (carbon 2′) of the sugar molecule (a). The sugar is deoxyribose in the nucleotides of DNA (a) and ribose in the nucleotides of RNA (b).*

Procedure

1. Line up the green ribonucleotides complementary to the I′ template DNA strand and tape them together, as in Exercise A, to form a polynucleotide strand. Like DNA, mRNA is always

synthesized in a 5 → 3′ direction. Make sure that you tape the nucleotides together in the proper direction to simulate the process of polymerization.

2. Label the 5′ and 3′ ends of the messenger RNA you have made.

a. What do 5′ and 3′ refer to? ___________________________

3. Record the nucleotide sequence of your mRNA.

5′ end __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ 3′ end

4. Each group of three nucleotides in a messenger RNA is called a **codon.** Using brackets, identify the codons of your mRNA sequence as written above. (Assume the first codon begins with the first nucleotide at the 5′end.)

b. What is the importance of these codons? ____

____

PART 2 Translation—Protein Synthesis

You are now ready to use your mRNA molecule to synthesize a protein. The first step in this process of translation is the **activation** of the amino acids, the addition of adenosine monophosphate (AMP) to amino acids so that they can be attached to tRNA molecules. (AMP is one part of the two-part orange ATP molecule in your kit.) Special enzymes called **aminoacyl-tRNA synthetases** (the large green molecules in your kit) then "charge" or bind specific "activated" amino acids onto the proper transfer RNAs (tRNAs)—one for each amino acid. Charged transfer RNAs carry amino acids to the ribosomes and serve as "adapters" between the code built into the nucleotide sequence of the mRNA and the sequence of amino acids in the protein. (Keep in mind that amino acids and nucleotides are two very different kinds of molecules that must be paired during the process of protein synthesis. Just as you use an adapter to put a three-pronged plug into a two-pronged outlet, a tRNA molecule pairs amino acids and nucleotides.)

Each tRNA (there are four blue tRNA molecules in your kit) has a nucleotide triplet, an **anticodon,** at a specific site in the molecule's three-dimensional structure. Eventually, this anticodon sequence will pair with a specific codon (also a nucleotide triplet) on an mRNA molecule that is being translated. Amino acids are attached to another specific site at one end of the tRNA molecule. As the anticodons of tRNA molecules pair, one at a time, with the sequence of codons in mRNA, the proper amino acids are aligned in the order dictated by the sequence of mRNA nucleotide triplets. Peptide bonds are formed between the amino acids to synthesize the polypeptide chain coded for by the mRNA.

Procedure

1. Find the four green aminoacyl-tRNA synthetase enzymes in your kit (see Figure 17B-2). Place one of the enzymes on your desk.

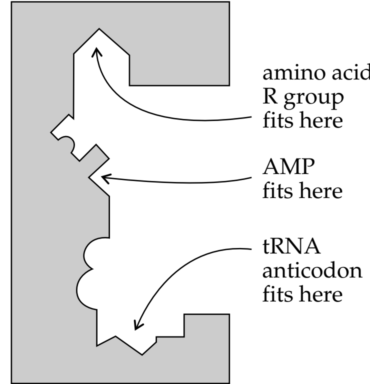


Figure 17B-2 *Each aminoacyl-tRNA synthetase enzyme contains a binding site for the unique R group of a specific amino acid and a binding site for the anticodon of a specific tRNA. In this way, a specific amino acid will be attached to a prescribed tRNA. A third binding site for the adenosine portion of AMP is also present.*

2. Find the proper amino acid that fits into the enzyme. The enzyme is shaped so that the anticodon bases of a tRNA fit into one end and the R group of a specific amino acid fits into the other. This is how a particular tRNA molecule ends up carrying its specific amino acid: each aminoacyl-tRNA synthetase enzyme contains binding sites for a specific amino acid and a specific tRNA (Figure 17B-2). Do *not,* however, insert the amino acid into the enzyme at this time.

ATP is used to "activate" the amino acid—that is, to convert it to a higher-energy form—so that it *can* be inserted into the enzyme and then attached to its tRNA. When this happens, ATP is split to form AMP and inorganic pyrophosphate (PP_i). The AMP, attached to the amino acid, fits into a groove on the enzyme (Figure 17B-3).

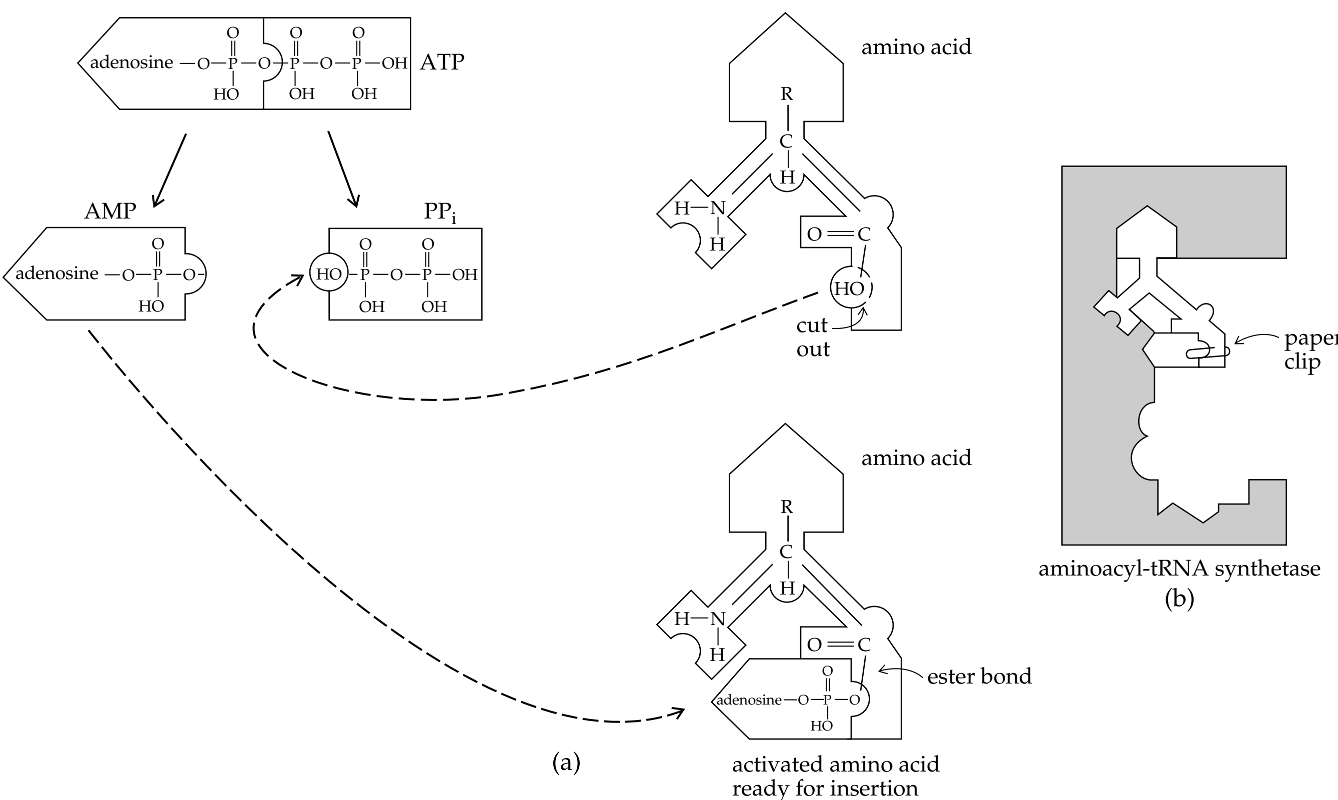


Figure 17B-3 *(a) An amino acid is activated by the splitting of ATP, resulting in the formation of AMP, inorganic pyrophosphate (PP_i), and the energy required to attach the activated amino acid to the aminoacyl-tRNA synthetase enzyme (b). During its activation, the amino acid loses an —OH group, which is incorporated into the pyrophosphate. (The nonionized forms of the amino acid, ATP, AMP, and PP_i are shown here.)*

3. Find the ATP molecule (orange). Cut the —OH group from the carboxylic acid group on the end of the amino acid you are working with. Use a paper clip to attach AMP at the same site (Figure 17B-3). Note that an ester bond is formed between the carbonyl group (C═O) of the amino acid and the phosphate group of AMP. The amino acid is now activated.

4. Insert the amino acid into the aminoacyl-tRNA synthetase enzyme.

Now tRNA can enter the synthetase enzyme (Figure 17B-4). Each tRNA has the same set of three nucleotides (CCA) on its 3′ end. This is where the amino acid will be attached. Since each aminoacyl-tRNA synthetase enzyme has a special binding site for a particular anticodon, only one tRNA can enter the enzyme to bind with the already-bound amino acid. The adenine nucleotide (A) of the 3′ CCA of tRNA fits into the same groove in the synthetase enzyme as does the adenosine of AMP. When AMP is released, the A residue of the CCA can be attached to the amino acid (Figure 17B-4).

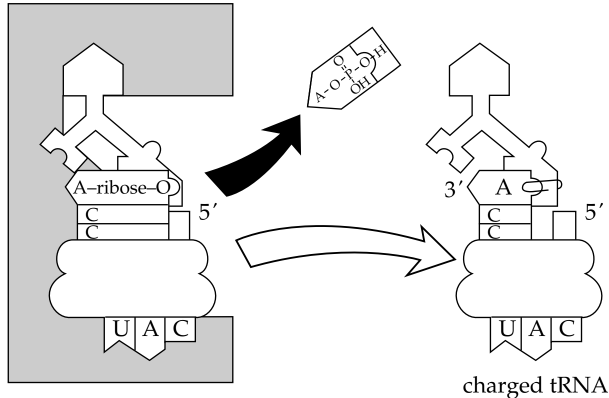


Figure 17B-4 *The amino acid attaches to the 3′ hydroxyl of the A nucleotide at the CCA end of tRNA.*

5. Find a tRNA bearing an anticodon that will fit into the proper binding site of the synthetase enzyme.
6. Insert the tRNA into the synthetase enzyme and remove the AMP molecule.
7. Attach the amino acid to the tRNA using a paper clip. The tRNA now has the correct amino acid hooked to it and is said to be a **charged** tRNA. Note that the amino acid is bound to the oxygen on the 3′ carbon of the ribose of the 3′-terminal A nucleotide of tRNA. An ester bond has been formed (Figure 17B-5).

Figure 17B-5 *Formation of an ester bond linking an amino acid to the 3′ hydroxyl of the A nucleotide at the CCA end of tRNA.*

activated amino acid

AMP

tRNA

ribose

charged tRNA

AMP

8. The tRNA carrying its appropriate amino acid now breaks loose from the aminoacyl-tRNA synthetase molecule to participate in the process of protein synthesis. The synthetase enzyme can be used repeatedly. Remove the charged tRNA from the enzyme.
9. Now charge your three other tRNAs with amino acids, as in steps 3–8. Once all four tRNA molecules are charged, you are ready to begin the process of protein synthesis.

During protein synthesis, messenger RNA attaches to a small ribosomal subunit. In most bacteria, a short sequence of nucleotides in the 16s RNA of the small ribosomal subunit binds to a special sequence of nucleotides (the Shine-Dalgarno sequence) in the mRNA near the "start" site for protein synthesis. In eukaryotes, the 5′ cap present on all mRNAs is involved in recognition of and binding to the small ribosomal subunit.

10. Attach your messenger RNA molecule to the ribosome by sliding it up through the right-hand slit. (For convenience, the large and small ribosomal subunits are already attached to one another, but this is not the case in the living cell.) Position the first two codons between the two slits, with the **AUG** or **initiation codon** on the left and the second mRNA codon on the right. (Although the AUG codon is the first one in the mRNA you are using, this is not usually the case in cells. The nucleotides to the 5′ side of the AUG—to its left, or upstream—represent the **leader sequence,** much like the leader on a movie film or VCR tape. Once the mRNA is bound, the AUG codon will be in register at the correct site for translation.) You will translate the message in the familiar 5′ → 3′ direction (Figure 17B-6).

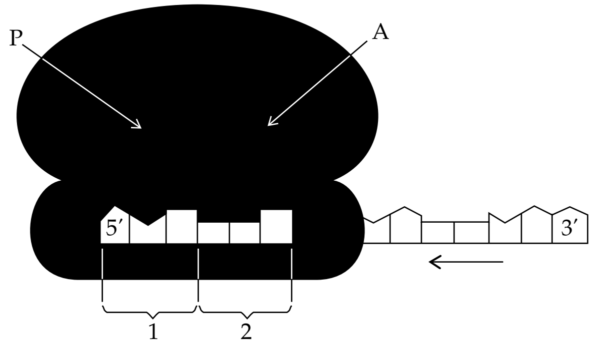


Figure 17B-6 *Attaching mRNA to the ribosome. When you first attach the mRNA, codon 1 is AUG, the initiation codon.*

Now, a charged tRNA, carrying an amino acid, will pair with the AUG initiation codon of mRNA. The 3′ end of the tRNA anticodon will pair with the 5′ nucleotide of mRNA—the tRNA and mRNA are antiparallel. (Two nucleotide strands, no matter how short, can interact only when they are antiparallel.) The combination of tRNA + mRNA + small ribosomal subunit is called the **initiation complex** (Figure 17B-7).

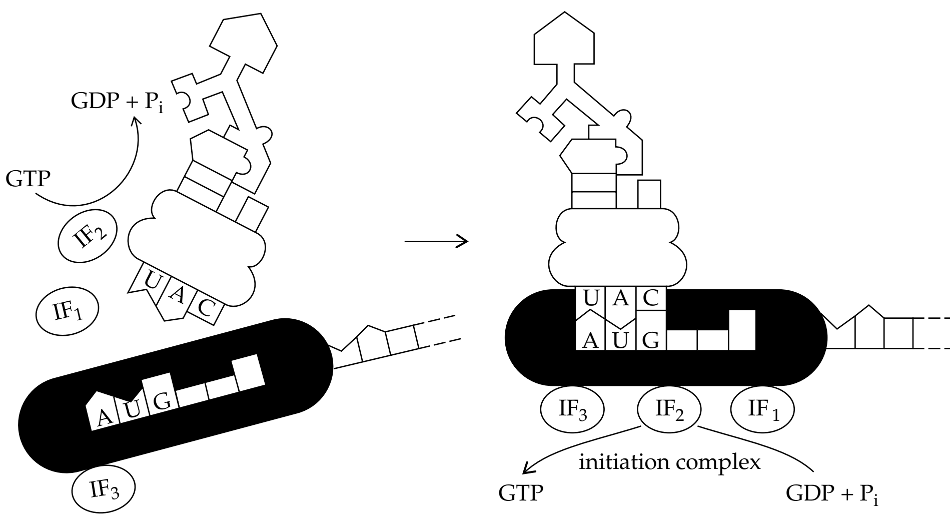


Figure 17B-7 *Formation of the initiation complex.*

A protein “factor” (small protein molecule) IF_3 is involved in binding AUG and in the attachment of mRNA to the small subunit during formation of this initiation complex. Factors IF_1 and IF_2-GTP are also involved in the attachment of the first tRNA molecule. (GTP, guanosine triphosphate, is used as a source of energy for most steps in protein synthesis.)

11. Find the blue tRNA that has an anticodon complementary to the AUG initiation codon. Pair it to the mRNA on the ribosome.

The large ribosomal subunit now attaches to the small ribosomal subunit of the initiation complex. Hydrolysis of the GTP of IF_2-GTP is required for this step. The large subunit is configured to form two major sites of activity: the **P site** (for peptidyl-tRNA), where new peptide bonds are formed, on the left, and the **A site** (for amino-acyl-tRNA), where new charged tRNAs arrive, on the right (Figure 17B-6). A third site, the **E site** (not shown), is occupied for a short time by the CCA end of the tRNA about to be ejected from the P site following removal of its amino acid during peptide bond synthesis.

12. Using the genetic code table (Table 17B-1), look up the initiation codon, AUG. Note that both methionine (Met) and formylmethionine (fMet) are specified for this codon. In prokaryotes, the initiation codon, AUG, always specifies fMet; Met is used in response to internal AUG codons in the mRNA. This means that, in prokaryotes, all proteins originally begin with fMet (which can be removed at a later time). In eukaryotes, methionine is also used for initiation, but it is not formulated. Write fMet on the yellow amino acid attached to the blue tRNA.

Important: **The genetic code specifies amino acids for codons in messenger RNA. Never look up the anticodon in the genetic code table.**

Table 17B-1 The Genetic Code: Codons as They Appear in mRNA

First Nucleotide	Second Nucleotide: U	Second Nucleotide: C	Second Nucleotide: A	Second Nucleotide: G	Third Nucleotide
U	UUU, UUC — phenylalanine	UCU, UCC, UCA, UCG — serine	UAU, UAC — tyrosine	UGU, UGC — cysteine	U, C
U	UUA, UUG — leucine		UAA — stop	UGA — stop	A
U			UAG — stop	UGG — tryptophan	G
C	CUU, CUC, CUA, CUG — leucine	CCU, CCC, CCA, CCG — proline	CAU, CAC — histidine	CGU, CGC, CGA, CGG — arginine	U, C
C			CAA, CAG — glutamine		A, G
A	AUU, AUC — isoleucine	ACU, ACC, ACA, ACG — threonine	AAU, AAC — asparagine	AGU, AGC — serine	U, C
A	AUA — methionine		AAA, AAG — lysine	AGA, AGG — arginine	A
A	AUG — methionine (start); formylmethionine (start)				G
G	GUU, GUC, GUA, GUG — valine	GCU, GCC, GCA, GCG — alanine	GAU, GAC — aspartic acid	GGU, GGC, GGA, GGG — glycine	U, C
G			GAA, GAG — glutamic acid		A, G

13. A second tRNA now attaches to the ribosome-tRNA-mRNA complex. This tRNA fits into the A site of the large ribosomal subunit and its anticodon is complementary to the second mRNA codon (Figure 17B-8). Insert the second tRNA, carrying amino acid 2, into the A site on the mRNA-ribosome complex. Which amino acid is carried by this second tRNA? Write the name on the amino acid.

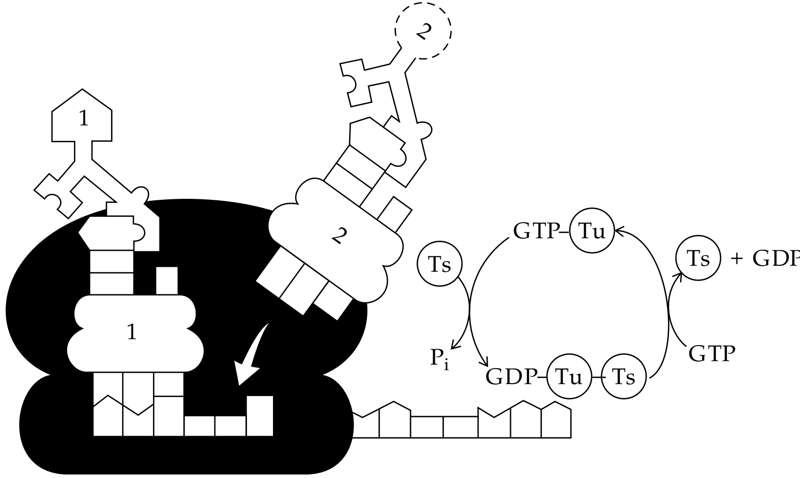


Figure 17B-8 *The large ribosomal subunit attaches to the initiation complex. Note the P and A sites in the large subunit (see Figure 17B-6). A second charged tRNA pairs with the codon located at the A site. The elongation factors shown here (Tu, Ts) are those present in bacteria (prokaryotes).*

A **peptide bond** is now formed between the —C(=O)—O— of amino acid 1 and the —NH_2 of amino acid 2. When this happens, the bond between the first tRNA (in the P site) and its amino acid breaks. Amino acid 1 is now held by the peptide bond to amino acid 2 on the second tRNA (Figure 17B-9). This step is called **peptidyl transfer.**

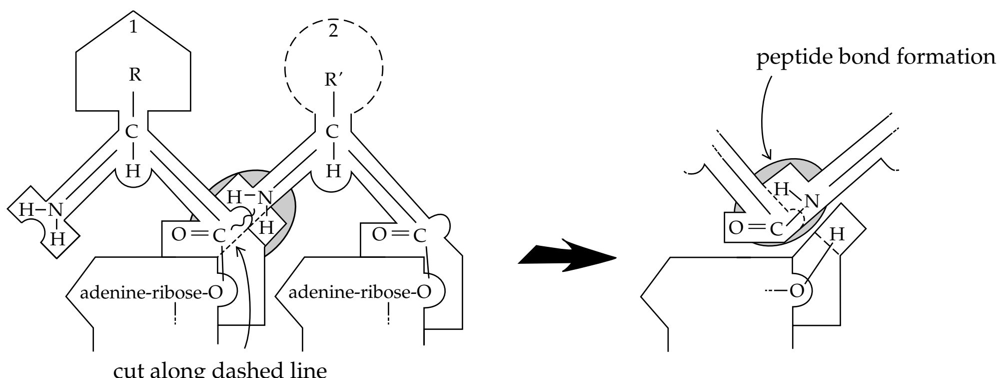


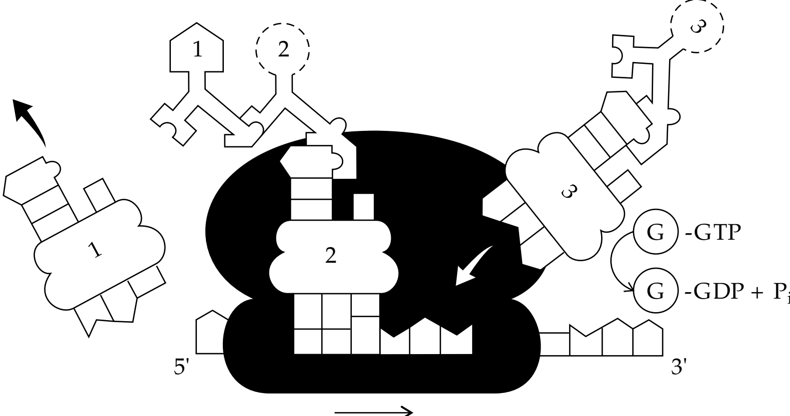
Figure 17B-9 *Formation of a peptide bond between two amino acids. The enzyme peptidyl transferase catalyzes the reaction. The H from —NH_2 of amino acid 2 is transferred to the oxygen in the 3′ position on the A residue of the first tRNA, restoring it to a 3′ —OH group.*

14. Attach the two amino acids together with a piece of tape. Using your scissors, remove the extra H on —NH_2. A peptide bond has been formed and the protein chain is now two amino acids long.

Each time a tRNA carrying an amino acid is added and a peptide bond is formed, the chain gets longer. Thus, this process is known as **elongation.** A protein elongation factor complexed with GTP aids in insertion of charged tRNAs into the A site on the ribosome. In prokaryotes, the elongation factor is a protein complex of Tu-GTP and Ts (which reactivates Tu after hydrolysis of GTP). In eukaryotes, elongation factors EF_1 and $EF_{1\beta}$ are used. Hydrolysis of GTP to GDP and P_i provides the energy for elongation (Figure 17B-8). The peptidyl transfer reaction is accomplished by an enzyme complex (**peptidyl transferase**) which is part of the large ribosomal subunit. However, recent evidence suggests that rather than ribosomal proteins possessing the enzymatic activity necessary for this reaction, it is RNA that is responsible: the RNA acts as a **ribozyme** (an RNA molecule with enzymatic activity).

15. The ribosome now moves along the message in a 5′ → 3′ direction. Place your fingers on the mRNA and move the ribosome to the right. The tRNA associated with the first codon is released since it is no longer bound to its amino acid. The process of ribosome movement from one codon to the next is known as **translocation** (Figure 17B-10). Another elongation factor (G in prokaryotes, EF_2 in eukaryotes) complexed with GTP is involved in the movement of the ribosome. Once again, GTP is hydrolyzed to provide energy for this movement.

Figure 17B-10 *Translocation and insertion of a new charged tRNA. The elongation factor (G) is that present in prokaryotes.*



16. Now, tRNA 2 is on your left with the first two amino acids attached to it. Match the anticodon of tRNA 3, carrying amino acid 3, to the next codon and repeat steps 14 and 15. You should now have three amino acids attached to tRNA 3. Which amino acid has been added in this third position? Write the name on the amino acid.

17. Repeat steps 14–16 until the last mRNA codon is in the A site. There is no tRNA having an anticodon to match this mRNA codon. The codons UAA, UAG, and UGA are **termination** or "stop" **codons.** The bond between the tRNA in the P site and the protein chain attached to it is hydrolyzed with the addition of H_2O (Figure 17B-11). This releases the newly synthesized

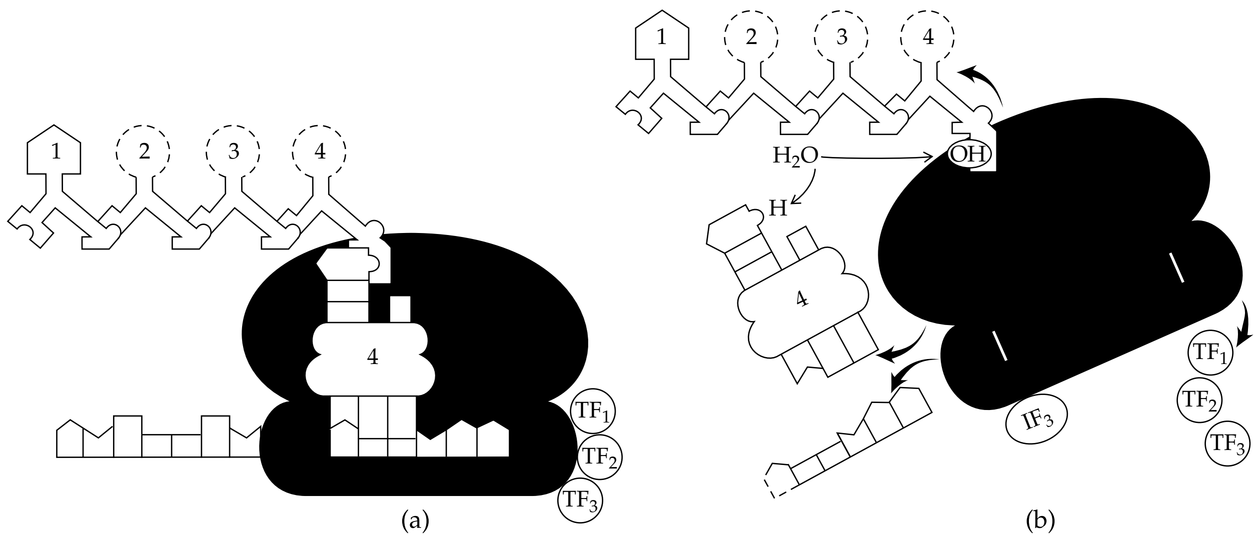


Figure 17B-11 *Chain termination. A termination or "stop" codon on mRNA is located in the A site and peptidyl transferase hydrolyzes the bond between the last amino acid of the protein and tRNA.*

protein. Several protein termination factors (TF) aid in the recognition of "stop" codons and termination of the peptide chain.

18. Release your protein chain, which should now be four amino acids long. The tRNA in the P site is also released, and the ribosomal subunits and mRNA separate.

Note that as a ribosome moves across a message, additional ribosomes can attach to the freed codons (codon 1, 2, 3, etc.). Each of these ribosomes can then serve as a site to start the synthesis of a protein. In this way, several molecules of a protein can be made simultaneously from one message. A complex of several ribosomes attached to a messenger RNA is called a **polysome.**

19. Make sure that you have identified all amino acids. Have all peptide bonds been formed correctly? Your laboratory instructor will check your work to see that you have completed the peptide chain correctly.

EXERCISE C Point Mutations in DNA

Point mutations are small changes in the DNA, such as base substitutions, base additions, and base deletions, but they may have profound effects on the protein formed by the gene, depending on where the mutations occur.

Objectives

☐ Determine the effect on the amino acid sequence of a point mutation (base substitution, base addition, or base deletion) in DNA.

PART 1 Base Substitutions—Possible Effects

Procedure

Refer back to the blue DNA strand that you used to make the messenger RNA for your model (Exercise B, Part 1).

a. Assume that a base substitution has occurred such that the ninth nucleotide has been changed from an A to a G. What is the sequence of the nucleotides in the third codon of the mRNA now?

____

b. What amino acid, if any, does this codon specify? ______________

c. What effect will this have on the protein formed from the DNA? ______________

____

d. If the ninth nucleotide had been changed from an A to a C, what effect would this have had on the protein formed? ______________

e. If the ninth nucleotide had been changed from an A to a T, what effect would this have had on the protein formed? ______________

f. Which of the base substitutions specified in questions a, d, *and* e *would be most likely to cause the production of a defective protein?* ______________

PART 2 Base Substitution Resulting in Sickle-Cell Anemia

Sickle-cell anemia is a genetic disease caused by a base substitution in the DNA of the gene coding for one of the polypeptides that makes up hemoglobin, the oxygen-carrying molecule of the red blood cells.

A normal individual has two alleles for the production of normal hemoglobin. The red blood cells of this individual will have the typical "doughnut shape." An individual with **sickle-cell anemia** has two

alleles (i.e., is homozygous) for the production of sickle-cell hemoglobin. The presence of sickle-cell hemoglobin causes red blood cells to take on sickle shapes, causing difficulty in passing through small blood vessels. These cells may clump and clog the blood vessels to the internal organs, thus depriving them of needed oxygen. Individuals with sickle-cell anemia may often be pale, tired, and short of breath. They may have pain in their arms, legs, back, and abdomen, and their joints may swell. Their low resistance to infections can trigger severe worsening of their condition and may eventually be fatal. (The anemia results from the fragility of the red blood cells.)

Procedure

1. Examine the prepared slide of normal human blood that is on demonstration. Draw a representative area of the slide in the space below.

2. Examine the prepared slide of blood from a person with sickle-cell anemia. Draw a representative area of the slide in the space below.

An individual who has one allele for sickle-cell hemoglobin and one normal allele produces both normal and sickle-cell hemoglobin; the amount of normal hemoglobin is enough to prevent the individual from becoming anemic. This individual is classified as a **carrier** since he or she can pass the recessive allele to offspring. In most ways, a carrier is normal and healthy. However, when the individual is in an environment that is low in oxygen, some red blood cells will undergo sickling. Such individuals are said to have **sickle-cell trait** (*not* sickle-cell anemia).

Sickle-cell hemoglobin differs from normal hemoglobin by only one amino acid in each of two β chains. Normal hemoglobin contains glutamic acid but sickle-cell hemoglobin contains valine.

a. What are the codons for glutamic acid? ______________________________

b. What are the codons for valine? ______________________________

c. Based on this information, what base substitution had to occur in the DNA in order to produce the allele for sickle-cell hemoglobin? ______________________________

Valine is a nonpolar amino acid, whereas glutamic acid is an acidic amino acid. Valine produces hydrophobic areas on the β chains that interact with hydrophobic areas on the β chains of other hemoglobin molecules, causing the hemoglobin molecules to clump. This produces the deformed red blood cells characteristic of sickle-cell anemia.

PART 3 Frame-Shift Mutations: Base Additions and Deletions

Refer back to the blue DNA strand that you used to make your mRNA (Exercise B, Part I). Assume that an A has been added between the fourth and fifth nucleotides. Indicate the sequence of the bases in the mRNA which will be transcribed from the DNA.

5′__ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ 3′

In the space below, indicate the sequence of amino acids in the protein coded for by this mRNA.

a. How does the above sequence of amino acids compare with the protein formed from the original DNA?

____

b. What effect would the removal *of a nucleotide from the original DNA have on the protein formed?*

____

____

Laboratory Review Questions and Problems

1. If DNA contained only the bases adenine and thymine, how long a code word would be necessary to enable coding for each of 20 different amino acids?

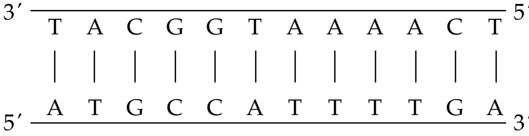
2. A particular DNA base sequence transcribed into messenger RNA is TTATCTTCGGGAGAGAAAACA. (a) If reading begins at the left, what amino acids are coded by this sequence? (*Note:* The initiation sequence is disregarded in this example.)

(b) If proflavine treatment caused the deletion of the first adenine nucleotide on the left, what changes would occur in the first six amino acids coded by this sequence?

3. Streisinger and co-workers studied amino acid sequences in the lysozyme protein produced by the T4 phage. One sequence is Lys-Ser-Pro-Ser-Leu-Asn-Ala, but as a result of a deletion of a single nucleotide and subsequent insertion of another nucleotide, this amino acid sequence was found to change to Lys-Val-His-His-Leu-Met-Ala. Using the codons in Table 17B-1, determine the nucleotide sequences that produced (a) the original amino acid sequence and (b) the subsequent changes.

4. A single (+) strand of DNA (base composition: A, 21 percent; G, 29 percent; C, 29 percent, T, 21 percent) is replicated by DNA polymerase to yield a complementary (−) strand. The resulting duplex DNA is then used as a template by RNA polymerase, which transcribes the (−) strand. Indicate the base composition of the RNA formed.

5. a. Given the following DNA molecule, write the sequence of the messenger RNA synthesized from the upper strand.



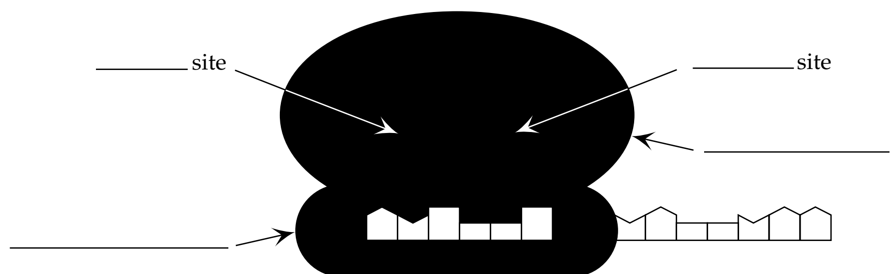
b. Indicate the 5′ and 3′ ends of the message.

c. Groups of three letters on the mRNA molecule are called ________________.

d. What is the special significance of the *first* group of three letters in the message?

e. What is the special significance of the *last* group of three letters in the message?

6. a. In the diagram, label the indicated parts.



b. Label the 5′ and 3′ ends of the messenger RNA in the diagram.

c. The triplet of tRNA nucleotides responsible for insertion of the correct amino acid into a protein chain by complementing with a triplet of mRNA is known as the ________________.

d. What are the last three nucleotides always found at the 3′ end of tRNA?

e. Use the space below to draw two tRNA molecules with attached amino acids. Two amino acids will be joined by a ________________ bond to form a dipeptide. The enzyme responsible for this reaction is ________________. Explain how the bond between the two amino acids is formed and show the final result by drawing an additional diagram.

7. You have the following DNA strand. Synthesize a protein from this strand. (Recall that a leader sequence may precede the AUG initiation codon.)

3′ AGATTACTCGAGCCGGGTAATCGGC 5′

mRNA

Protein

8. Make a strand of DNA complementary to the DNA strand in question 7. Mark the new strand's 5′ and 3′ ends. Now synthesize mRNA and a protein from this strand. Is the message the same as in question 7? Is the protein the same? (Recall that mRNA is read in a 5′ → 3′ direction!)

Complementary DNA

mRNA

Protein

9. You have synthesized the following protein: fMet-Pro-Asp-Gly-Thr. You accomplished this in a cell-free system containing tRNA molecules with the anticodons listed below:

3′ CCG 5′

5′ UGU 3′

5′ CGG 3′

5′ CAU 3′

3′ CUG 5′

mRNA

Construct the double-stranded DNA molecule from which this protein was synthesized. Show all of your reasoning.

DNA (2 strands)

LABORATORY

Molecular Genetics: Recombinant DNA

OVERVIEW

During the last decade there has been a technological revolution in the field of molecular genetics. Scientists can now explore and "engineer" changes in the genomes of a variety of organisms by obtaining pieces of DNA molecules and **recombining** them in different ways.

One of the key developments in **recombinant DNA technology** was the discovery of special enzymes called **restriction endonucleases.** These "restriction enzymes" have been isolated from a variety of prokaryotic organisms, especially bacteria. They protect bacteria by restricting foreign DNA, particularly viral DNA, from entering and functioning within cells. Restriction enzymes cut the foreign DNA at specific base sequences (restriction sites). The small pieces are then easily destroyed by other bacterial enzymes. Scientists have learned to use the same restriction enzymes as "molecular scissors" to cut all types of DNA molecules into smaller segments at specific locations.

The small pieces of DNA snipped from bacterial cells, fruit flies, frogs, or even humans can be recombined with other DNA. Often, the pieces are inserted into viruses that have been disabled or into bacterial **plasmids** (small, double-stranded DNA molecules located outside the bacterial chromosome). The plasmids or viruses act as **vectors** or carriers to transfer the DNA into the cell of a host—perhaps another bacterial cell or a eukaryotic cell. Bacterial host cells and some eukaryotic cells can multiply to form **clones** (a collection of copies of themselves) that can express the new genetic information and make new gene products. The cells have been **transformed** and may even express a new phenotype as a result of the added gene products. Commercially we can produce large quantities of rare proteins or other specific gene products, such as insulin or growth hormone, using recombinant DNA techniques. **Gene therapy,** the transfer of beneficial genes into the human body is also possible.

In this laboratory, you will investigate some of the basic principles of genetic engineering. Plasmids containing specific fragments of foreign DNA will be used to transform *Escherichia coli* cells, conferring both antibiotic (ampicillin) resistance and *lac*$^{+}$ phenotype (ability to metabolize lactose) to recipient cells.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

EXERCISE A | Bacterial Transformation: Constructing Recombinant Plasmids

The bacterium *Escherichia coli (E. coli)* is an ideal organism for genetic manipulation and has been used extensively in recombinant DNA research. It is a common inhabitant of the human colon and can easily be grown in standard nutrient mediums.

The single circular chromosome of *E. coli* contains 5 million DNA base pairs (1/600th the total amount of DNA in a human cell). In addition, the cell contains small, circular, *extrachromosomal* (outside the chromosome) DNA molecules called **plasmids.** These fragments of DNA, 1,000 to 200,000 base pairs in length, also carry genetic information. Some plasmids replicate only when the bacterial chromosome replicates and usually exist only as single copies within the bacterial cell. Others replicate autonomously and often occur in as many as 10 to 200 copies within a single bacterial cell. Certain plasmids, called R plasmids, carry genes for resistance to antibiotics such as ampicillin, kanamycin, or tetracycline.

In nature, genes can be transferred between bacteria in three ways: conjugation, transduction, or transformation. **Conjugation** is a mating process during which genetic material is transferred from one bacterium to another "sexually" different type. (See Laboratory 14, Exercise C.) **Transduction** requires the presence of a virus to act as a **vector** (carrier) to transfer small pieces of DNA from one bacterium to another. **Bacterial transformation** involves transfer of genetic information into a cell by direct absorption of the DNA from a donor cell.

Through the process of bacterial transformation, a bacterium can acquire a new trait by incorporating and expressing foreign DNA. In the laboratory, the DNA used most commonly for transformation experiments is bacterial plasmid DNA. These plasmids often carry a gene for antibiotic resistance. The presence of the antibiotic-resistance gene makes it possible to **select** bacteria containing the plasmid of interest; the bacteria that contain the plasmid will grow on a medium that contains the antibiotic, whereas bacteria lacking the plasmid will not be resistant to the antibiotic and will die.

Transformation can occur naturally, but the incidence is extremely low and is limited to a relatively few bacterial strains. During the growth cycle of these strains, there exists a short period of time when the bacteria are most receptive to uptake of foreign DNA. At this stage the cells are said to be **competent.** (Competence to absorb DNA usually develops toward the end of the logarithmic growth phase, just before cells enter the stationary phase in culture.) The mechanism by which competence is acquired is not completely understood, but in the laboratory, the competent state can be induced by treating bacterial cells with divalent cations such as Ca^{2+} and Mg^{2+}.

In this exercise, you will simulate the construction of a recombinant plasmid. Plasmids can transfer genes such as those for antibiotic resistance which are already a part of the plasmid, or plasmids can act as carriers for introducing foreign DNA from other bacteria, plasmids, or even eukaryotes into bacterial cells. Restriction endonucleases are used to cut and insert pieces of foreign DNA into the plasmid vectors (Figure 18A-1).

Each restriction endonuclease "recognizes" a specific DNA sequence (usually a 4- to 6-base-pair sequence of nucleotides) in double-stranded DNA and digests phosphodiester bonds at specific sites in the sequence (recall that phosphodiester bonds link one nucleotide to the next in a DNA polynucleotide chain). If circular DNA is cut at only one site, an open circle results. If the restriction endonuclease recognizes two or more sites on the DNA molecule, two or more fragments will result. The length of each DNA fragment corresponds to the distance between restriction sites (restriction sites flank the fragment at its ends). Some restriction endonucleases cut cleanly through the DNA helix at the same position on both strands to produce fragments with blunt ends. Other endonucleases cut specific nucleotides on each strand to produce fragments with overhangs or "sticky ends" (Figure 18A-2). Using the same restriction endonuclease to cut DNA from two different organisms produces complementary sticky ends, which can be realigned in a "template–complement" manner, thus recombining the DNA from the two sources (Figure 18A-2).

In bacteria, restriction enzymes provide protection by breaking and destroying the DNA of invaders, such as that of bacteriophage viruses. However, since the recognition sites for restriction endonucleases also occur within the bacterial DNA itself, bacteria have a mechanism for preventing their own restriction enzymes from digesting their own DNA. For each restriction endonuclease produced by a bacterium, there is a corresponding enzyme that methylates the bacterial DNA at that enzyme's specific recognition

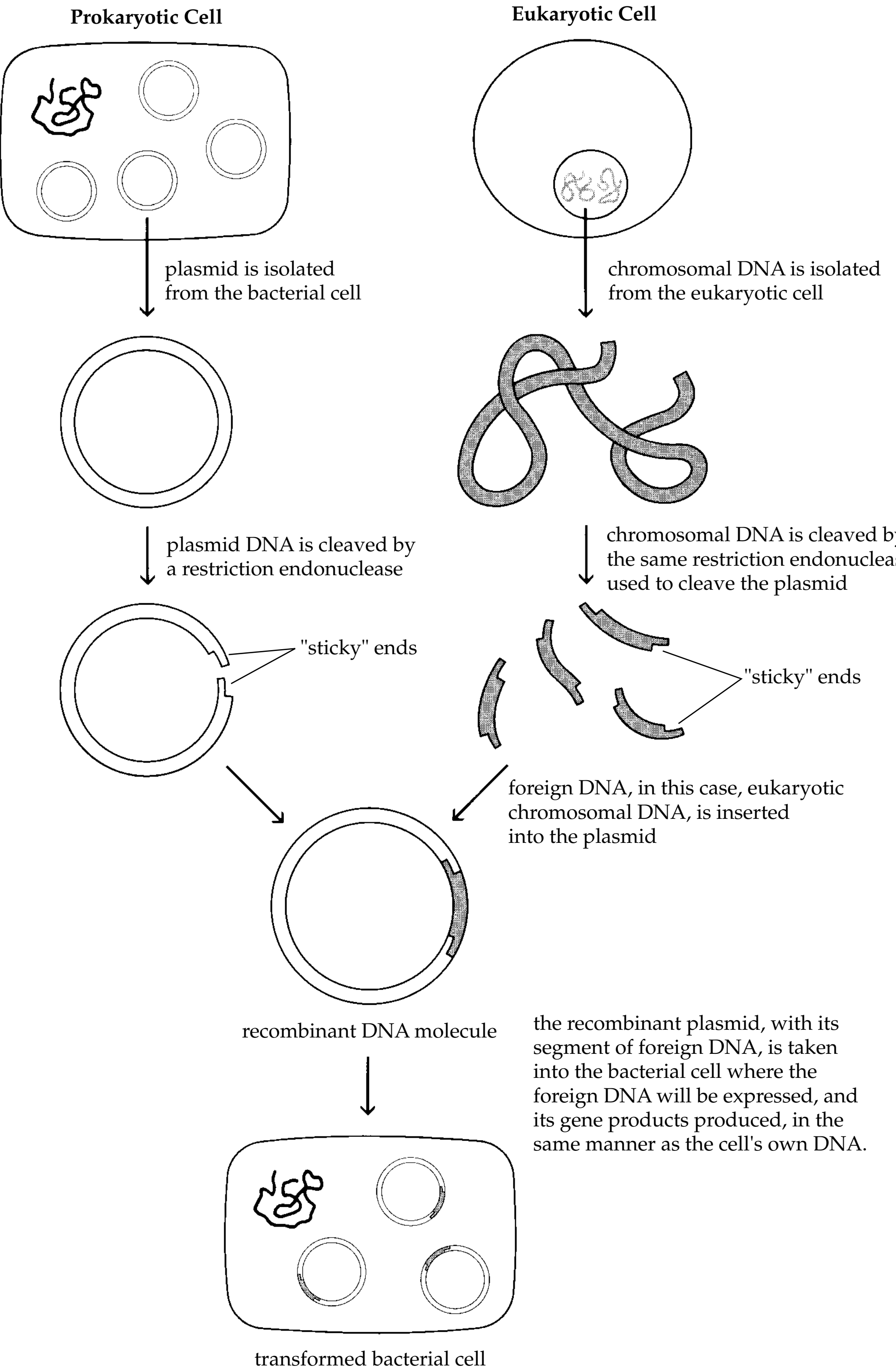


Figure 18A-1 *A typical recombinant DNA experiment.*

site: addition of a methyl group to the nucleic acid base prevents a close association from forming between the restriction endonuclease and the recognition site. In this way, bacteria can break down the DNA of invaders while protecting their own genetic material from destruction.

Objectives

- ☐ Describe how to construct a recombinant DNA plasmid.

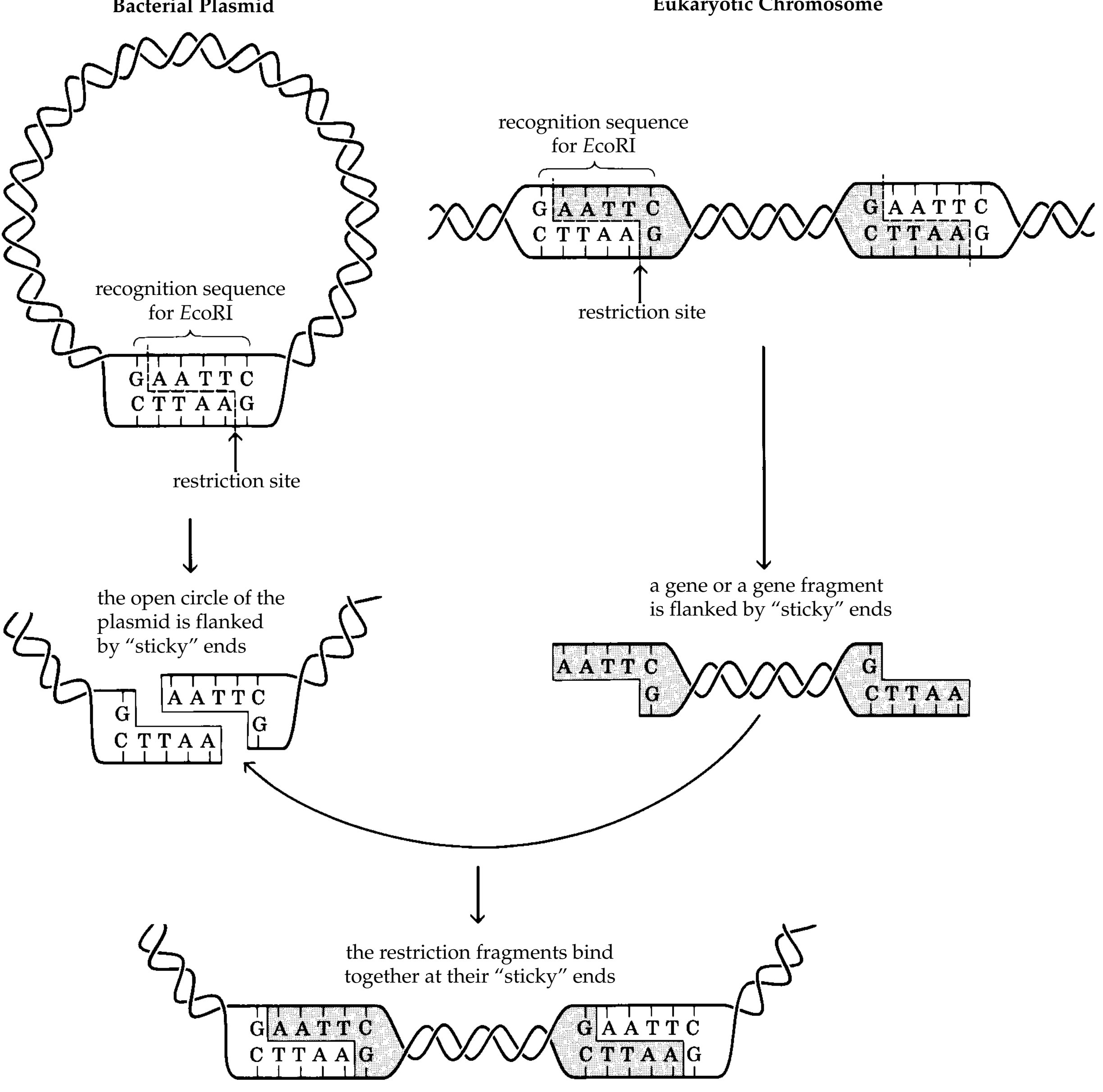


Figure 18A-2 *An example of how a bacterial plasmid and a fragment of eukaryotic chromosomal DNA are cleaved by the* EcoRI *endonuclease and then recombined.*

Procedure

The restriction endonuclease *Hin*dIII, isolated from *Haemophilus influenza,* recognizes the following restriction site and produces fragments with "sticky ends."

```
      cut
       ↓
5′  –A–A–G–C–T–T–  3′
3′  –T–T–C–G–A–A–  5′
                ↑
               cut
```

1. The shaded pieces of DNA in Figure 18A-3 (page 18-15) represent a segment of DNA from a human chromosome. The white piece of DNA represents a circular *E. coli* plasmid. Tape the

nucleotide strands together as indicated in order to form a circular plasmid of bacterial DNA and a long linear strand of eukaryotic DNA.

2. Use a pair of scissors to cut the DNA of both the *E. coli* plasmid and the human DNA sequence as they would be cleaved by *Hin*dIII.
3. Insert the human DNA into the plasmid and tape the fragments together.
   a. *DNA ligase is used to join the fragments. What do you notice about the 3′ and 5′ ends of the restriction fragments as they are recombined?* ____________________________
4. Suppose you wish to insert a second gene into the plasmid. You have *Eco*RI and *Bam*HI restriction endonucleases available. They cleave the DNA as follows:

```
            ↓
EcoRI   5′  G–A–A–T–T–C  3′
        3′  C–T–T–A–A–G  5′
                      ↑

            ↓
BamHI   5′  G–G–A–T–C–C  3′
        3′  C–C–T–A–G–G  5′
                      ↑
```

Design a method for recombining the second gene with your plasmid.

b. *Which restriction endonuclease would you use?* _________ *Why?* ____________________________

____

c. *What would be the required characteristics of the human DNA sequence?*

____

____

5. Use the "empty" DNA fragment in Figure 18A-3 to construct an appropriate human DNA fragment and insert it into the plasmid.

EXERCISE B | Rapid Colony Transformation with pAMP: Ampicillin Resistance*

Normally, *E. coli* cells are destroyed by the antibiotic ampicillin. In this exercise, you will induce competent *E. coli* cells to take up the plasmid pAMP, which contains a gene for ampicillin resistance. Only *E. coli* cells that have been transformed will be able to grow on agar plates containing ampicillin. Thus we can **select** for transformants: those cells that are not transformed will be killed by ampicillin; those that have been transformed will survive.

Objectives

- ☐ Discuss the principles of bacterial transformation.
- ☐ Describe how to prepare competent *E. coli* cells.
- ☐ Outline the general procedure for gene transfer using plasmid vectors.
- ☐ Carry out the transfer of the antibiotic gene *Amp*^{r} and describe how to select for transformed cells that contain the *Amp*^{r} gene.

**Exercise B was developed by Dr. David Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, and Dr. Greg Freyer, Columbia University College of Physicians and Surgeons.*

Procedure

Formulate a hypothesis on which to base an investigation of how *E. coli* cells can be transformed by the pAMP plasmid.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will happen when ampicillin-sensitive E. coli cells are transformed by pAMP?

What is the **independent variable** in this investigation?

What is the **dependent variable** in this investigation?

Use the following procedure to test your hypothesis.

1. Use a sterile micropipette to add 250 μl of ice-cold 0.05 M $CaCl_2$ to two Eppendorf microcentrifuge tubes.
2. Sterilize an inoculating loop by flaming it and then cool it by sticking it into the agar plate in an area where no bacteria are growing. Use the sterile inoculating loop to transfer a large (3-mm) colony of *E. coli* to one of the tubes. Be careful not to transfer any agar.
3. Vigorously tap the loop against the wall of the tube to dislodge the cell mass.
4. Suspend the cells immediately by vigorous pipetting using a 100-μl micropipette with a sterile tip or a sterile plastic transfer pipette.
5. Mark this first tube "(+)" and return it to the ice.
6. Repeat steps 2 to 5 for the second tube. Mark the tube "(−)."
7. Use a sterile inoculating loop to transfer 1 loopful (10 μl) of pAMP plasmid directly into the cell suspension in tube (+). At the correct angle, you will be able to see the plasmid solution form a film across the loop (much like what happens on a toy bubble-maker loop). Immerse the loop in the (+) cell suspension and mix well. Be sure to introduce the plasmid solution directly into the cell suspension—do not touch the wall of the tube as you insert the inoculating loop. Mix by tapping the tube with your finger.
8. Return the tube to ice for 15 minutes.
9. While the tubes are incubating, obtain two LB agar and two LB/Amp agar (LB agar containing ampicillin) plates. Label one LB agar plate "LB+" and the other "LB−." Label one LB/Amp plate "LB/Amp+" and the other "LB/Amp−." Mark your name on the lids.
10. A brief pulse of heat facilitates entry of foreign DNA into the *E. coli* cells. Heat-shock cells in both the (+) and (−) tubes by placing the tubes in a 42°C water bath for 90 seconds. (Tubes can be floated on the water by making an appropriate-sized hole in the center of a thin piece of Styrofoam to suspend the sample tube.) It is essential that cells be given a sharp and distinct shock, so work quickly.
11. Immediately return cells to ice for 2 minutes.
12. Use a sterile micropipette to add 250 μl of Luria broth to each tube. Mix by tapping with your finger and set at room temperature for recovery. Let sit for 10 minutes. During this period, the *Amp*^{r} gene, newly introduced into the transformed cells, codes for the synthesis of β-lactamase (an enzyme that destroys the antibiotic properties of ampicillin by cleaving its

β-lactam ring). The transformed cells are now resistant to ampicillin: they possess the gene whose product renders the antibiotic ineffective.

13. Place 100 μl of (+) cells onto the "LB+" plate and 100 μl of (+) cells onto the "LB/Amp+" plate. Place 100 μl of (−) cells onto the "LB−" plate and 100 μL of (−) cells onto the remaining "LB/Amp−" plate.

14. Immediately spread the cells using a sterile spreading rod. (Remove the spreading rod from ethanol and briefly pass it through a flame. Cool by touching it to the agar on a part of the dish away from the bacteria. Spread the cells and once again immerse the rod in alcohol and flame it.) Repeat the procedure for each plate.

15. Allow plates to set for 5 minutes. Tape your plates together and incubate *inverted* overnight at 37°C.

16. After 12 to 24 hours, indicate on which plates you observe growth.

LB− ______ LB/Amp− ______

LB+ ______ LB/Amp+ ______

a. What is the purpose of the (−) plates? ____________________

b. Why was no growth observed on the LB/Amp− plates? ____________________

Do your results support your hypothesis? ______ *Your null hypothesis?* ______

What do you ***conclude*** *about the ability of ampicillin resistance to be transferred from one bacterium to another?*

____

17. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per microgram of pAMP.

a. Determine the total amount of pAMP used: ______ μg. [You used 10 μl of pAMP (0.005 μg/μl); see step 7.]

b. Determine the concentration of pAMP (in μg/μl) in the total suspension of cells plus Luria broth used for recovery (250 μl $CaCl_2$ + 10 μl pAMP + 250 μl Luria broth; see steps 1, 7, 12): ______ μg/μl

c. Determine the total amount of pAMP in the 100-μl spread on the plate (see step 13): ______ μg pAMP/100 μl.

d. Count the number of colonies on the plate: ______ colonies. (If there are too many, divide the plate into quarters, count one quarter, and multiply by 4.)

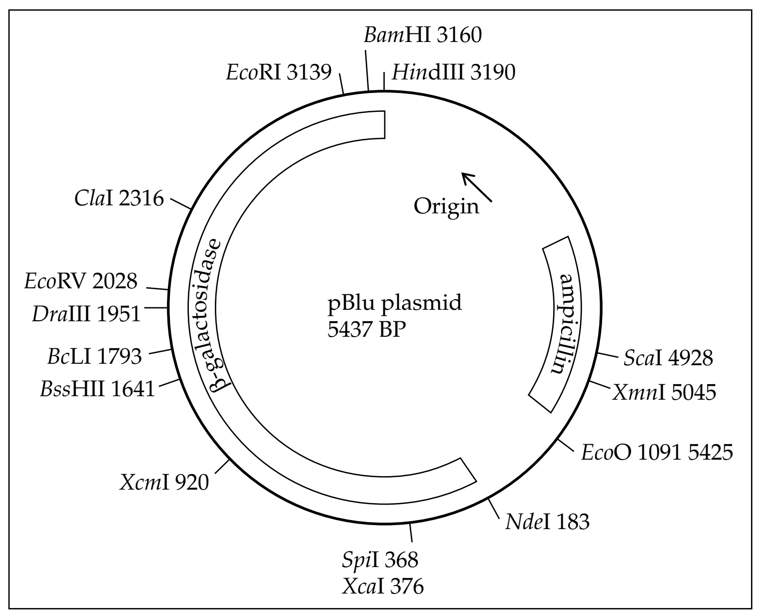
e. Divide the number of colonies by the amount of pAMP in the 100 μl of cell suspension spread on the plate (step c) to give colonies/μg pAMP (use scientific notation): ______ colonies/μg pAMP. This is the transformation efficiency.

EXERCISE C Transformation of *E. coli* with pBLU: The *lac*$^+$ Phenotype

In this exercise, you will work with the plasmid pBLU.™* In addition to the gene for ampicillin resistance (Amp^r), this plasmid carries a gene for production of the enzyme β-galactosidase (Figure 18C-1). This is one of the enzymes necessary for the complete breakdown of lactose, a carbohydrate that can be used in place of glucose as a source of nutrition for bacteria if glucose is unavailable. Restriction enzymes were used to insert the β-galactosidase gene into the pBLU plasmid.

**The pBLU™ plasmid was developed by Dr. Greg Freyer, Columbia University, College of Physicians and Surgeons, expressly for Carolina Biological Supply Company. Exercise C is adapted from the work of Dr. David Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, and Dr. Greg Freyer.*

Figure 18C-1 *The pBLU plasmid.™ A restriction map for the pBLU plasmid shows sites for cleavage by restriction enzymes used to insert the β-galactosidase gene into a plasmid carrying the* Amp^r *gene.*



The host cells used for the study are bacterial cells, *E. coli* strain JM101. These bacteria are *not* resistant to ampicillin. They are also *unable* to metabolize lactose (they are *lac*$^-$), due to a mutation in the *lac z* gene—the gene in the *lac* operon (Figure 18C-2) that produces β-galactosidase. JM101 cells, however, can acquire resistance to ampicillin (be transformed) by insertion of the pBLU plasmid carrying the *Amp^r* gene. And, at the same time, JM101 cells can acquire an undamaged copy of the β-galactosidase gene from the pBLU plasmid, transforming the cells to *lac*$^+$. Transformed (*lac*$^+$) cells can use lactose if it, instead of glucose, is supplied in the agar medium. *Lac*$^+$ cells can also use X-gal, a substitute for lactose that, when broken down by β-galactosidase, turns blue. As the transformed cells multiply to form **colonies,** the colonies will appear blue. The expression of the blue phenotype verifies that the gene for β-galactosidase (the *lac z* gene), contained in the pBLU plasmid, is being expressed.

Note that *selection* of transformed cells is actually based on their acquired ampicillin resistance and not on their ability to digest lactose. The agar used to select transformed cells contains ampicillin (in addition to X-gal), and only transformed cells (cells that now contain the *Amp^r* gene also carried on the plasmid) are able to grow. Selection for the *lac*$^+$ phenotype would require that the bacteria be grown on a medium containing *only* lactose as a food source, so that only those cells that are transformed to *lac*$^+$ could survive. This is not the case in this experiment, since some glucose is present in the agar medium.

Objectives

- ☐ Describe how to "engineer" a plasmid to include a piece of foreign DNA that confers the *lac*$^+$ phenotype to transformed cells.
- ☐ Carry out the transformation of *E. coli* using a plasmid that confers both ampicillin resistance and the *lac*$^+$ phenotype.

Procedure

Work in pairs. Formulate a hypothesis on which to base an investigation of how *E. coli* cells can be transformed by the pBLU plasmid.

HYPOTHESIS:

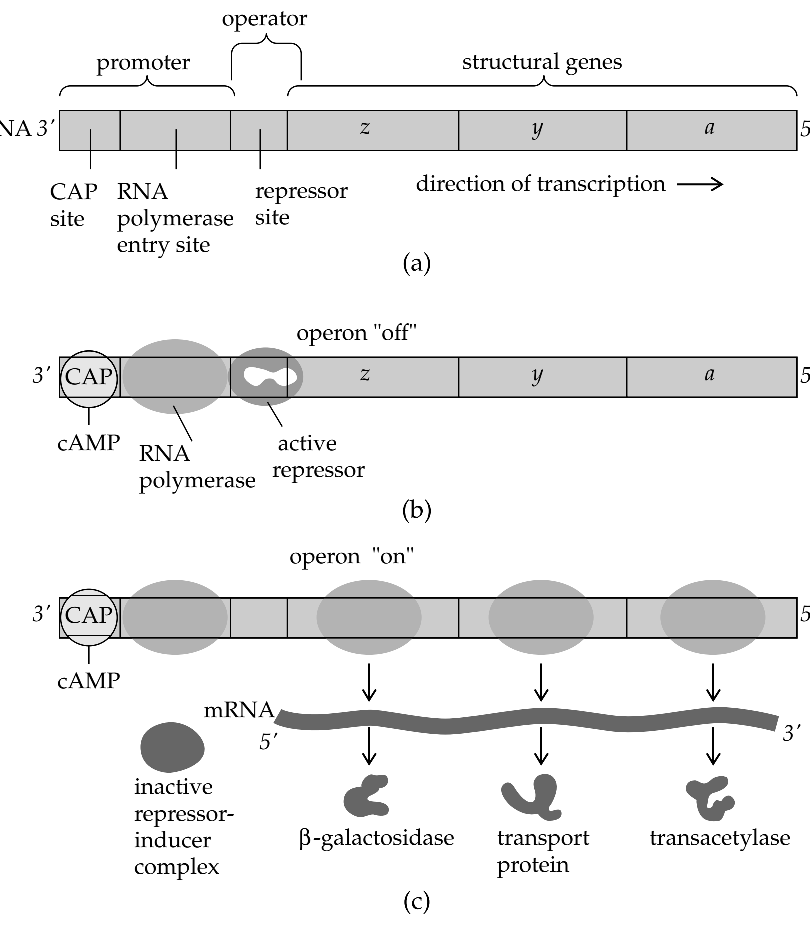


Figure 18C-2 *The* lac *operon. (a) The gene for production of β-galactosidase is the* lac z *gene. This is one of three structural genes in the* lac *operon. In combination with the* lac y *and* lac a *genes, the* lac *operon produces the enzymes necessary for the breakdown of lactose. The operon consists of a* promoter *region for the binding of RNA polymerse (as well as a binding site for catabolite activator protein/cyclic AMP, or CAP-cAMP, which enhances transcriptional activity) and an* operator *site where repressors can bind. The* lac *operon is under negative control by an active repressor that binds to the operator region where it blocks the movement of RNA polymerase. (b) The operon will remain "off" when glucose is readily available and lactose concentrations are low. (c) The* lac *operon turns "on" when allolactose (a derivative of lactose) acts as an inducer to inactivate the repressor (causing the repressor to fall off the operator region). This occurs when lactose concentration in the medium is high and glucose concentration is low. RNA polymerase can then transcribe a polycistronic messenger RNA that codes for the three gene products of the* lac *operon.*

NULL HYPOTHESIS:

What do you **predict** will happen when JM101 E. coli cells that are not able to use lactose as a food source are transformed by pBLU?

What is the **independent variable** in this investigation?

What is the **dependent variable** in this investigation?

Use the following procedure to test your hypothesis.

1. Obtain two sterile plastic test tubes (with caps) to serve as "transformation tubes." Mark one "(+)pLBU" and the other "(−)pBLU."
2. Pipette 250 μl of ice-cold 0.05 M $CaCl_2$ into each of the two plastic tubes (this will be used to make the cells competent) and place both tubes on ice. (Everything must be kept cold!)

3. Using a sterile transfer (inoculating) loop, transfer a large colony of *E. coli* JM101 cells from the agar plate supplied by your instructor into one of the tubes containing $CaCl_2$. (If colonies are small, use two colonies.) Be careful not to dig the loop into the agar! If you do, start over.
4. Immerse the loop in the $CaCl_2$ solution. Tap the loop vigorously against the sides or bottom of the tube—in the $CaCl_2$—until the cells have been dislodged.
5. Immediately, use a sterile transfer pipette to break up the lump of cells. Work the cells in and out of the *tip* of the pipette until, when held up to the light, no clumps of cells are visible.
6. Repeat steps 3, 4, and 5, adding cells to the second "transformation tube." Be sure to disaggregate cell clumps by vigorous pipetting.
7. Make sure to place both tubes back into the ice.
8. Use a sterile inoculating loop to transfer one loopful (approximately 10 μl) of pBLU plasmid solution (0.005 μg/μl as supplied by Carolina Biological Supply Company) into the (+)pBLU tube only. At the correct angle, you will be able to see the plasmid solution form a film across the loop (much like what happens on a toy bubble-maker loop). Immerse the loop in the (+)pBLU cell suspension and mix well. Be sure to introduce the plasmid solution directly into the cell suspension—do not touch the wall of the tube as you insert the inoculating loop.
9. Place on ice for 15 minutes.
10. While the cells are incubating, obtain the following set of agar plates:
    a. Two LB (Luria broth) agar plates. Mark the *bottom* of one plate "(+)" and the bottom of the second plate "(−)".
    b. Two LB/Amp agar plates. Mark one plate "(+)" and the other "(−)" as above.
    c. Two LB/Amp/X-gal plates. Mark one plate "(+)" and the other "(−)" as above.
11. After 15 minutes on ice, you must heat-shock the cells to assist with plasmid uptake. Remove both tubes and immediately place the tubes into 42°C water for 90 seconds. Immediately return the tubes to ice for *at least* 1 minute before proceeding.
    a. *Why do you think you must also heat-shock the cells in the (−)pBLU tube when no plasmid was added to the tube?* ______________________________

    ____
12. Use a sterile transfer pipette to add 250 μl (0.25 ml) of sterile Luria broth to each of the two tubes. Tap with your finger, gently, to mix and let the tubes stand at room temperature (place in a test tube rack) for 10 minutes. This is the recovery period—it will give cells, if transformed, a chance to start producing β-lactamase (see Exercise B) so that when exposed to ampicillin they will be able to degrade the antibiotic.
13. Use a sterile transfer pipette to add 100 μl of (+)pBLU cells to each of the three plates marked (+). Be careful *not* to touch the tip of the pipette to the agar—if you do, discard the pipette and obtain a clean one. Be sure to use an aseptic technique: only lift the lid above the plate—do not take it off or lay it down. You do not want air-borne bacteria and fungal spores to settle on your plates.
14. Use a second sterile transfer pipette to add 100 μl of the cell suspension from (−)pBLU to each of the three plates marked (−). Follow the same procedures and cautions given in step 13.
15. Use a sterile glass "spreader" to spread the cells across the surface of the agar plates. Dip the spreader in alcohol, and briefly pass it through the flame from a Bunsen burner or alcohol lamp. Always allow the alcohol to "burn off." Lift the lid on one of your Petri dishes and cool the spreader by placing it on the surface of the agar away from the cells—don't be surprised if it sizzles. When cool (but do *not* touch it with your fingers), use the spreader to

distribute the cells over the surface of the plate by gently rubbing back and forth at various angles. Lower the lid gently and return the spreader to the alcohol. (Do not flame the spreader before placing it back into the alcohol.) Repeat this procedure for the remaining dishes. If two spreaders are available, one partner should spread the cells on the (+) dishes, while the other partner spreads cells on the (−) dishes. Always put the spreader back into alcohol, and reflame it between using it on different Petri dishes.

16. Allow plates to stand for 5 minutes and then bundle the six plates into a stack. Tape the plates together and place *inverted* (top side downward) in a 37°C incubator. Incubate for 12 to 24 hours. If an incubator is not available, incubate at room temperature—it will simply take longer for the cells to grow and reproduce and for you to get your results!

b. Which plates will serve as experimental control plates? ______________ *Do you expect to see cells growing on these plates?* _______ *Why or why not?* ______________________________________

c. Why did you put both (+)pBLU and (−)pBLU cells on LB agar plates?

____

d. If growth occurs on both LB agar plates, what does this tell you? ______________________________

____

e. If growth does not occur on either LB agar plate, what might you conclude?

____

17. Indicate in Table 18C-1 what you expect to see, using G for growth and NG for no growth, on your plates after they have been incubated.

Table 18C-1 pBLU Transformation

Plate	Cells	Growth (G) or No Growth (NG)
LB	(+)pBLU	
LB	(−)pBLU	
LB/Amp	(+)pBLU	
LB/Amp	(−)pBLU	
LB/Amp/X-gal	(+)pBLU	
LB/Amp/X-gal	(−)pBLU	

Next Day

18. After incubation, record the number of colonies growing on the experimental LB/Amp/X-gal plate. If there are too many colonies to count, divide the plate into quarters using a marking pen. Count the number of colonies in one quarter and multiply by 4. Number of colonies:

_______. Color development indicates that the β-galactosidase gene is functioning and the cells have been transformed to *lac*$^{+}$.

f. What color are the colonies? ______________

If colonies are large, only their centers may be blue. X-gal is rapidly depleted from the medium as the colony grows.

g. Did you see any small white colonies at the edges of the blue colonies? _______

These white colonies are feeder colonies. Often, the destruction of ampicillin by the transformed bacteria forms an area around the colony where nontransformed cells can grow. The nontransformed cells, however, will appear white. *h. Why?* ________________________________

____

19. Note the results from the control plates.

i. Did cells grow on the LB agar plates? _______ *Why or why not?*

____

j. Did cells grow on the LB/Amp plates? _______ *Why or why not?*

____

k. Explain the reasons for growth of the transformed cells on the LB/Amp/X-gal plates. Why are the cells resistant to ampicillin? ________________________________

Do your results support your hypothesis? _______ *Your null hypothesis?* _______

What do you ***conclude*** *about the ability of transformed JM101.* E. coli *cells to use lactose and X-gal?*

____

20. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per microgram of pBLU.

a. Determine the total amount of pBLU used: _______ μg. (You used 10 μl of pBLU (0.005 μg/μl); see step 8.)

b. Determine the concentration of pBLU (in μg/μl) in the total suspension of cells plus Luria broth used for recovery (250 μl $CaCl_2$ + 10 μl pBLU + 250 μl Luria broth; see steps 2, 8, 12):

_______ μg/μl

c. Determine the total amount of pBLU in the 100-μl spread on the plate (see step 13):

_______ μg pBLU/100 μl

d. Count the number of colonies on the plate: _______ colonies. (If there are too many, divide the plate into quarters, count one quarter, and multiply by 4.)

e. Divide the number of colonies by the amount of pBLU in the 100 μl of cell suspension spread on the plate (step c) to give colonies/μg pBLU (use scientific notation): ________ colonies/μg pBLU. This is the transformation efficiency.

Because transformation is limited to those cells that are competent, increasing the amount of plasmid used does not necessarily increase the probability that a cell will be transformed. A sample of competent cells can usually be saturated with small amounts of plasmid, and excess DNA may actually interfere with the transformation process.

l. How does the transformation efficiency of pBLU compare with that of pAMP (Exercise B)?

____

Laboratory Review Questions and Problems

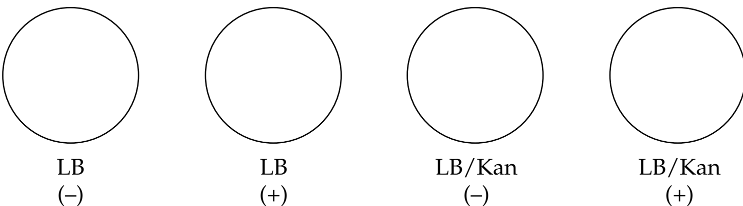
1. You are given the following assignment by a biotechnology firm. Transfer gene *A* from a mouse chromosome to plasmid pBR322 of *E. coli.* What would be your first consideration in choosing one or more restriction endonucleases to cut the plasmid and the chromosome? (*Hint:* Various genes on the eukaryotic chromosome are flanked by various nucleotide

sequences.) Gene *A*, by the way, is responsible for production of a protein that could be economically important if it could be produced in bulk.

2. You have successfully completed a transformation experiment. There are 800 colonies on your plate. You used 50 μl of a solution containing *E. coli* cells mixed with 1×10^{-3} μg/ml of plasmid DNA to inoculate the test plate. What was the transformation efficiency in this experiment?

3. Assume that a bacterial plasmid carries the gene for resistance to the antibiotic kanamycin. Using restriction enzyme A, you open the plasmid and insert a segment of a biologically important gene isolated from a mouse. The gene was excised from the chromosome as part of a fragment cut from whole DNA by using the same restriction enzyme A. After conducting the appropriate steps in a typical bacterial transformation, you plate the transformed cells (+) and control cells (−) on LB agar containing kanamycin and on LB agar alone.

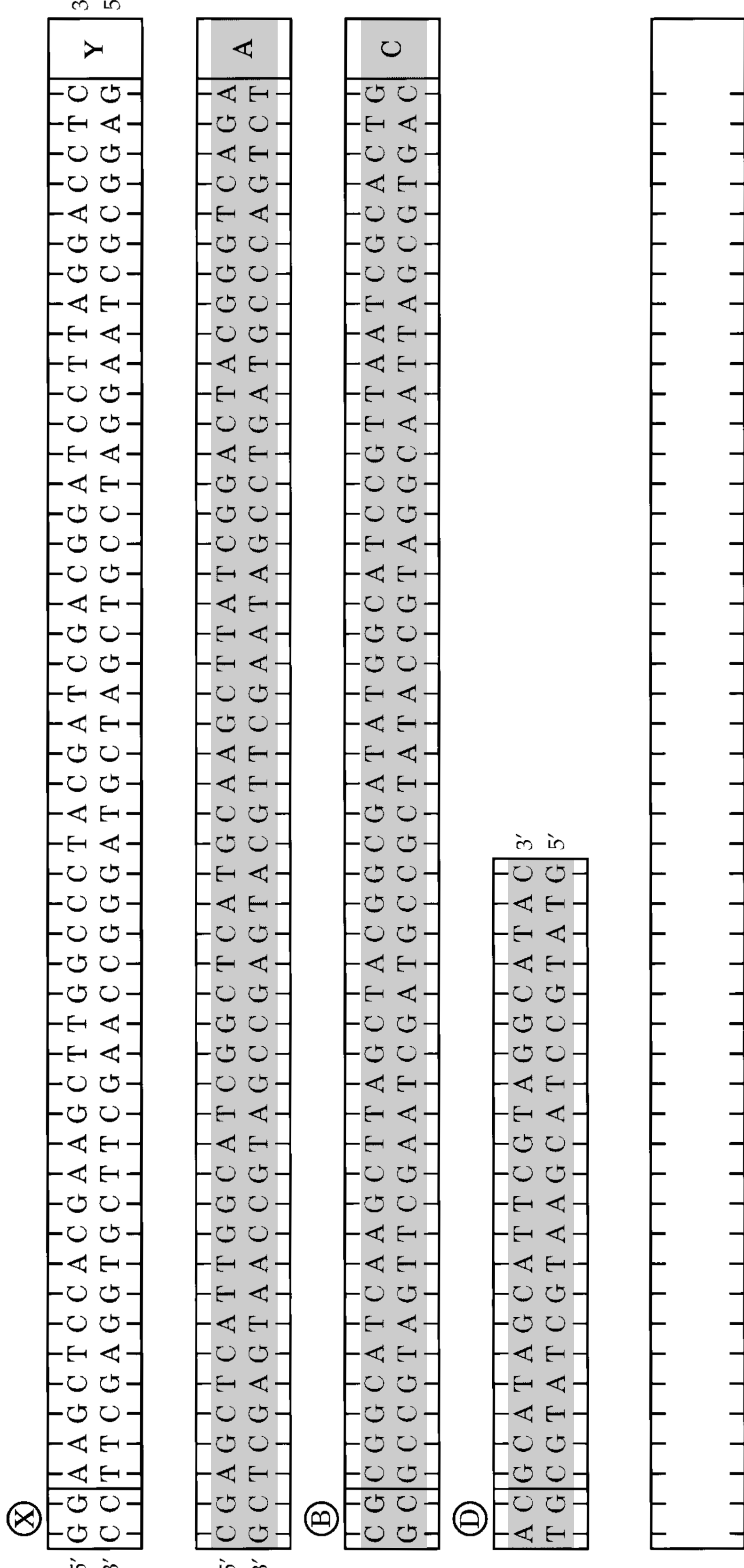
a. What do you *expect* to see? Indicate this on the plates below:



b. What you actually observe is no growth on either LB/Kan plate, but growth on both LB plates. You try the experiment again using a different restriction enzyme B. This time you get growth of transformed (+) cells on LB/Kan but no growth of control (−) cells on LB/Kan. You get growth of both transformed (+) and control (−) cells on LB plates. How might you explain these observations? Propose a map for the bacterial plasmid and the restriction sites for restriction endonucleases A and B.

4. You are working in a recombinant DNA laboratory and are asked to clone a gene from a very rare strain of bacteria. The gene produces an important protein used in the oil industry to clean up oil spills. You need large amounts of this product. Outline the steps you would take to get an ordinary bacterium such as *E. coli* (which does *not* normally make the protein) to produce large amounts of this protein.

Figure 18A-3 ***Directions:*** *Cut out the top white strand (the "plasmid") and position tab Y under X to form a circle; tape ends together. To form the eukaryotic "chromosome," cut out the two long shaded strands and tape tab B over tab A; cut out the short shaded strand and tape tab D over tab C.*



LABORATORY

Genetic Control of Development and Immune Defenses

OVERVIEW

The hereditary material within fertilized eggs, or zygotes, of animal organisms holds the key to both structure and function. DNA guides, through time, the structural and functional development of a single cell into an embryo and its morphogenesis into an adult. Even aging is a result of genetic programming.

Biologists are just beginning to understand the genetic control of development. Advanced technologies as well as molecular and recombinant DNA techniques have made it possible to discover more and more about the genomes of organisms and to map the entire genomes of some. Common patterns of gene function are beginning to emerge, suggesting that some day, perhaps, we will be able to explain the mysteries of animal development.

Protection for the organism is also built into the genetic code. A newborn mammal possesses a full set of genetic information for the synthesis of a vast number of different immunoglobulins (antibodies). During development of the B cells of our immune system, however, each cell is modified to produce only a single type of antibody. How could the limited amount of DNA in a single organism produce millions of different types of antibodies? Research in recent years has shown that functional immunoglobulins are assembled from DNA segments that are initially separated and then rearranged during B cell development. Once again DNA exhibits its programming functions—DNA holds the secrets not only for "what" it produces but for "how" and "when" it produces its products.

During this laboratory you will have the opportunity to explore the development of sea urchin, frog, and chick embryos. You will also have an opportunity to investigate antibody activity using the precipitin ring test.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

PART I DEVELOPMENT

All living things must be able to reproduce and develop. In animals, gametes produced by the process of meiosis unite during fertilization to form a single diploid cell, the zygote. The processes of cell division, cell movement, cellular differentiation, and morphogenesis result in the development of a multicellular embryo that will grow to form an adult.

✔ EXERCISE A | Fertilization and Early Development in Sea Urchins

Understanding how DNA regulates cell division, differentiation, and morphogenesis begins with observing the developing embryo. Sea urchins have long been the developmental biologist's favorite organism of study because they are relatively simple to obtain and culture in the laboratory. The cells of the developing sea urchin are also fairly transparent, providing us with a limited ability to "look inside" the embryo.

Early development of the sea urchin is under the genetic control not only of the zygote's DNA but of messenger RNA (mRNA) stored in the egg during its development. These messages include maternal mRNAs synthesized from maternal DNA prior to the meiotic events of oogenesis—mRNAs made from DNA that may not be included in the egg itself. Thus, the story of development begins before fertilization.

The unfertilized sea urchin egg is surrounded by a **vitelline membrane** that lies just above the surface of the cell's plasma membrane (plasmalemma). Within the cytoplasm, yolk granules (sea urchin eggs are microlecithal—they have very little yolk) can be observed. In addition to other cytoplasmic determinants and stored mRNAs, small **cortical granules,** composed of proteins and mucopolysaccharides, lie just beneath the plasmalemma (in the outer rim or cortex of the egg) (Figure 19A-1a). When a single sperm enters the egg plasmalemma, the membrane potential quickly changes as a wave of depolarization spreads from the site of sperm entry. This reaction is often referred to as a "fast block to polyspermy," since no additional sperm can gain entry following the change in membrane potential. Release of Ca^{2+} ions from the egg's endoplasmic reticulum, in response to G protein, causes the cortical granules in the egg's cortex to fuse with the plasmalemma (Figure 19A-1b).

The cortical granules discharge their contents into the space between the plasmalemma and the vitelline membranes. The excess mucopolysaccharide now present in the perivitelline space lowers the water potential of that area, and water flows in. This causes the perivitelline space to increase in diameter, making it appear as if the vitelline membrane is lifting off the surface of the zygote. Addition of proteins to the vitelline membrane hardens (or "tans") it as it is transformed into a **fertilization membrane** (Figure 19A-1c). Formation of the fertilization membrane (often called the "slow block to polyspermy") offers additional protection against multiple sperm entry. Hundreds of sperm can usually be observed still attached to the old vitelline membrane, now the fertilization membrane. These will be removed by the action of enzymes released from the cortical granules.

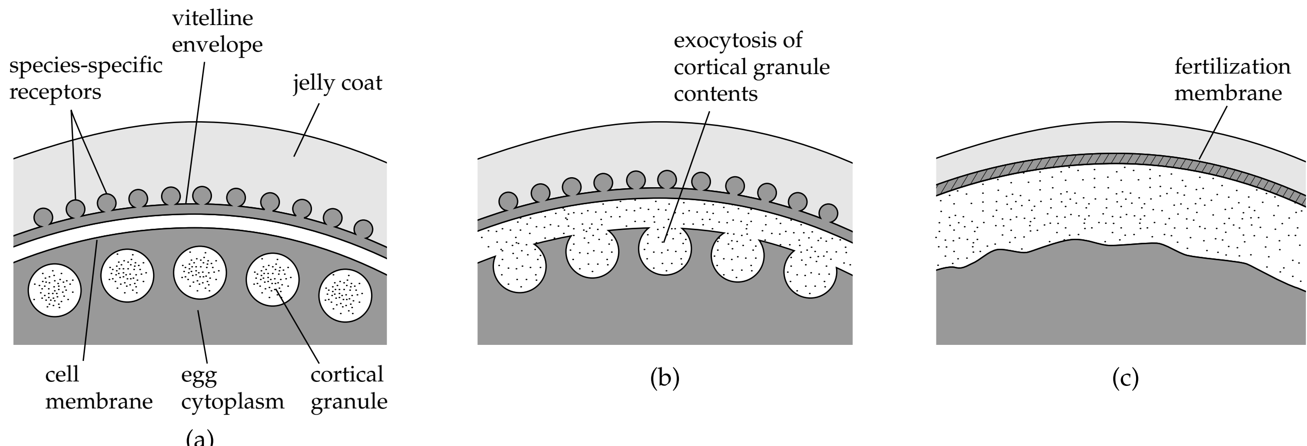


Figure 19A-1 *Formation of the fertilization membrane in the sea urchin. (a) The surface layers of an unfertilized egg include a jelly coat; the vitelline membrane or envelope, which bears species-specific receptors for sperm; and the cell membrane (plasmalemma). (b) Contact with a sperm triggers Ca^{2+} release and the fusion of cortical granules with the plasmalemma. (c) Cortical granules release their contents into the perivitelline space, causing the vitelline membrane to rise from the surface to form the fertilization membrane.*

Following fertilization, the stored mRNAs are responsible for directing protein synthesis during the early stages of development—cleavage, blastula formation, and gastrulation. In addition to the mRNA and protein already present in the fertilized egg, newly synthesized protein products are responsible for establishing regional differences in the egg cytoplasm. These proteins or **cytoplasmic determinants** are responsible for establishing the cytoarchitecture of the unfertilized egg, reorganizing the cytoplasmic elements in response to fertilization, controlling the direction of the first cleavage divisions, and establishing the axis of the embryo.

In this exercise, you will have the opportunity to observe the earliest stages of sea urchin development—beginning with fertilization. You will also have available later-stage embryos for observation of gastrulation and formation of pluteus larvae.

Objectives

- ☐ Describe the processes of fertilization and cleavage division in the sea urchin.
- ☐ Observe formation of the fertilization membrane as it expands to surround the sea urchin.

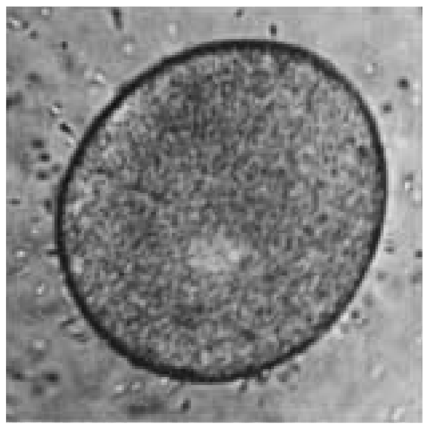
Procedure

1. Inject 0.1 to 1.0 ml of 0.5 M KCl intracoelomically through the soft tissue on the oral surface of the animal. Shake the animal gently after injection to distribute the KCl. (For some urchins, KCl injections will not work, but electrical stimulation is effective. This method requires an adjustable physiological stimulator set to a frequency of 60 Hz. Use 6 to 10 volts AC for smaller urchins and 20 to 30 volts AC for larger urchins. One electrode should be placed directly on the test (body surface) near the gonopore region and the other on a wet cotton ball or tissue on the oral surface.)
2. Gamete shedding should begin minutes after injection. (If you use electrical stimulation, gamete shedding will continue as long as the animal is stimulated, thus you can collect only the amount of material needed.) Once gamete shedding begins, the sex of the urchin can be determined. Semen is gray-white or cream colored. Eggs vary in color, depending on the species, and may be pale yellow, yellow-orange, or reddish.
3. To collect eggs, invert the female over a beaker of seawater and lower the urchin until it is covered by the seawater. Eggs will stream from the gonopores and fall to the bottom of the beaker. If electrical stimulation is used, place the female, after stimulation, right side up (gonopores up) in a beaker of seawater—the urchin should be completely covered with seawater.
4. Using a wide-mouth pipette (a turkey baster is useful), remove eggs that have settled to the bottom of the beaker and resuspend them in clean seawater.
5. Let the eggs settle, decant the seawater, and replace it with clean seawater. (This step removes any traces of coelomic fluid, which might interfere with the fertilization process.) The eggs are now ready to use.
6. It is best to collect sperm in the "dry" condition for maximum concentration. Place the male in a finger bowl of seawater, but make sure that the level of the water is below the gonopores. As the whitish semen is extruded from the gonopores, collect it with a Pasteur pipette. Store the semen in a test tube on ice (it will remain viable for several hours).
7. Return the urchins to a separate aquarium (if the animals continue to shed gametes, they may trigger other animals in the aquarium to shed gametes).
8. Obtain a depression slide and place a sample of egg suspension into the concave depression. Cover with a glass coverslip. Observe the eggs using the 10× and 40× objectives. If you wish to observe the jelly coats, add a drop of 0.1 Janus green before applying the coverslip.
9. Obtain a second depression slide and place another sample of the egg suspension into the concave depression. Cover with a coverslip, but leave a small opening on one side for the

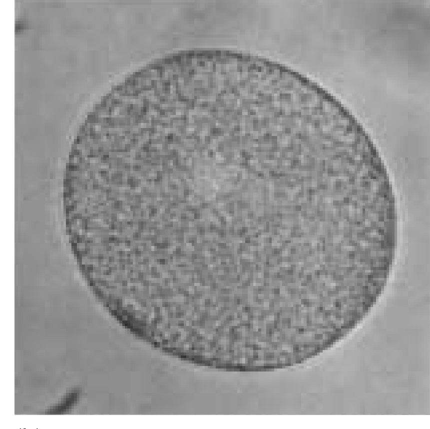
addition of sperm in step 10. Place the slide on the microscope stage and use the 10× objective to locate the eggs.

10. Use a Pasteur pipette to place a drop of concentrated sperm suspension on a clean glass slide. Add two drops of seawater. Mix with a toothpick and, using the blunt end of the toothpick, introduce some sperm suspension into the egg suspension on the slide. Observe immediately.
11. Watch for the fertilization membrane to form, then turn off the light on your microscope or remove the slide from the microscope and gently place it in a safe place (away from heat and hot light) for later observation. If the seawater begins to evaporate from your depression slide, add more using a Pasteur pipette.
12. Fusion of pronuclei usually occurs 30 to 45 minutes after gamete fusion. The first cleavage division occurs 60 to 90 minutes after gamete fusion, depending on the species. Check your slide in approximately 45 minutes and then at 10- or 15-minute intervals until you observe the first cleavage division.
13. Your instructor may have additional samples of embryos in other stages of development for you to examine. Prepare wet-mount depression slides of these. See if you can identify four- or eight-cell embryos, early blastulae, hatching blastulae, or gastrula embryos. In the sea urchin, the larval form is called a **pluteus larva.**
14. For a composite photographic description of sea urchin development, refer to Figure 19A-2.

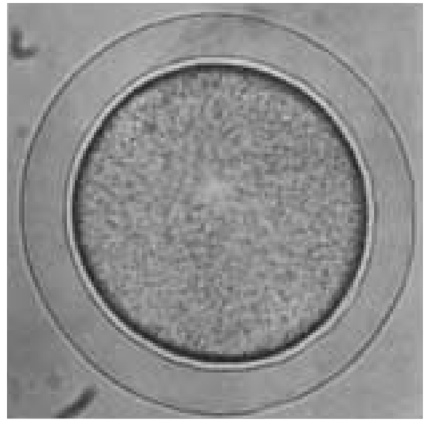
Figure 19A-2 *Sea urchin development. (a–h) As the egg divides, the cells become progressively smaller, so that by the blastula stage they are barely distinguishable. (i) Gastrulation begins with the formation of the blastopore, then (j, k) secondary mesenchyme cells break loose to migrate along the inner surface of the blastula. These form long pseudopodia that (l) help to "pull" the forming gut toward the opposite side of the embryo. (m) Spicules form from primary mesenchyme cells within the developing pluteus larva (n).*



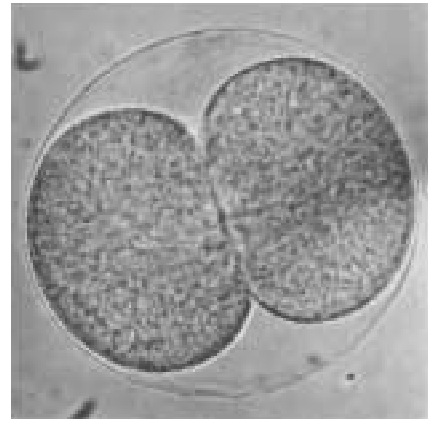
(a) Numerous spermatozoa can be seen surrounding an unfertilized egg.



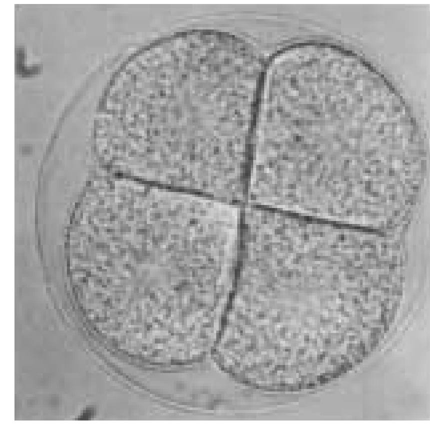
(b) Fertilized egg; the fertilization membrane has just begun to form. The light area slightly above the center is the diploid nucleus.



(c) The fertilization membrane is fully formed. The egg has begun to divide; if you look closely, you can see that there are two nuclei.

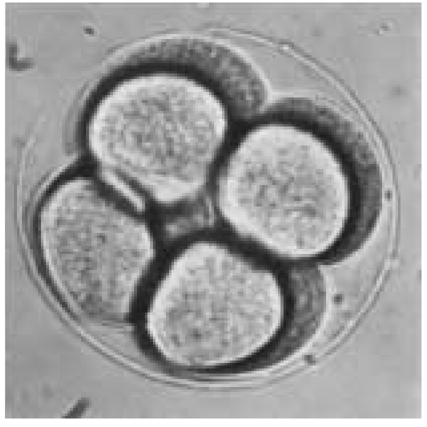


(d) The first division.

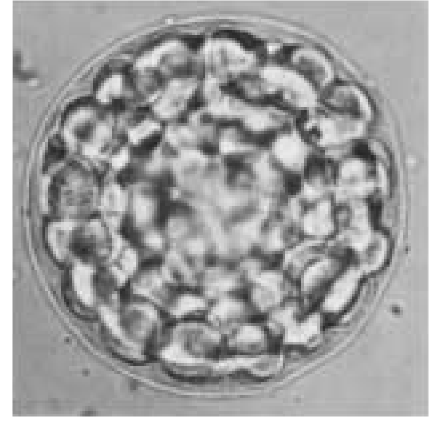


(e) Four-cell stage.

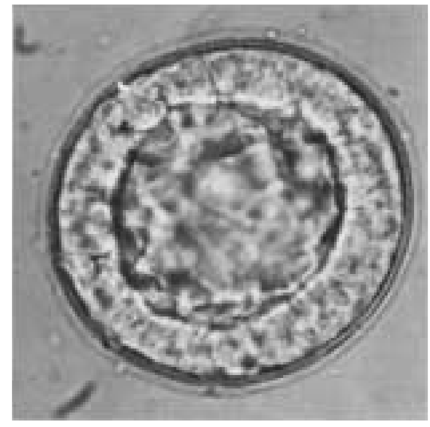
(continued)



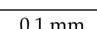
(f) Eight-cell stage.

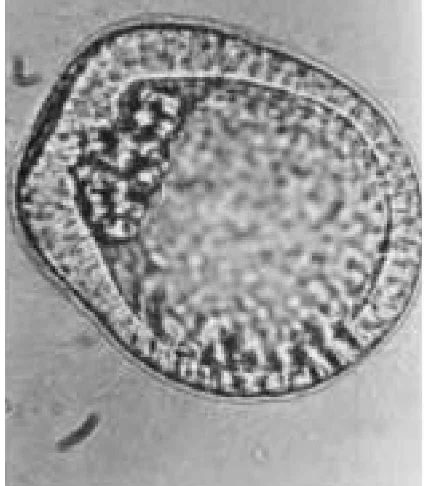


(g) The blastocoel forms.

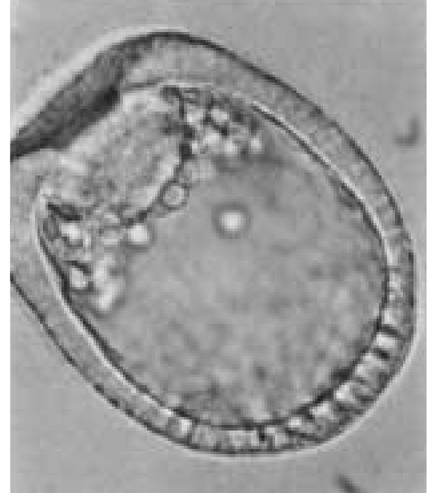


(h) The mature blastula.

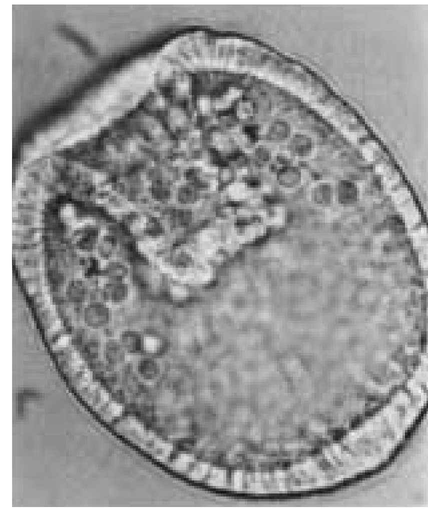




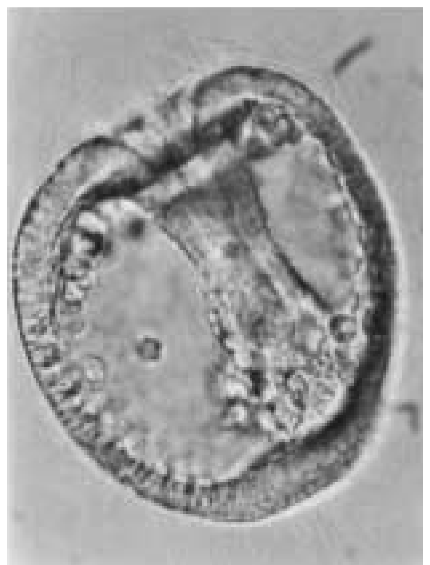
(i) The beginning of gastrulation; the blastopore has begun to form at the upper left, and cells near the blastopore have begun to migrate across the blastocoel.



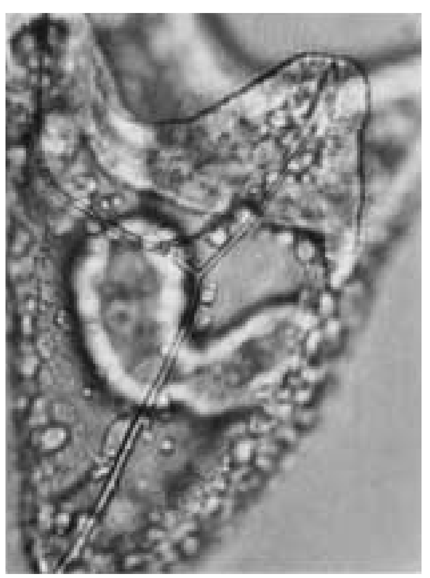
(j) The outer cell layer begins to fold inward at the blastopore, forming the archenteron.



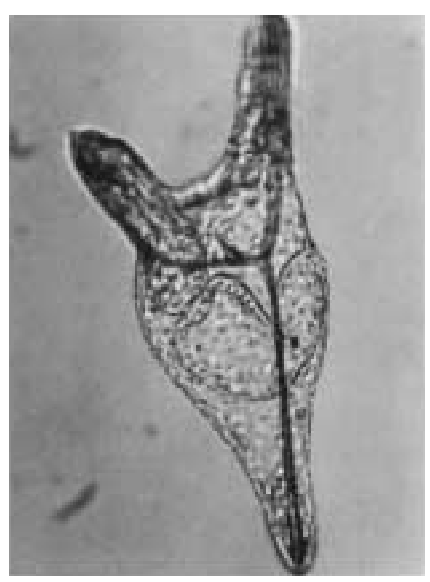
(k) The outer layer of cells continues to move across the blastocoel.



(l) The mature gastrula.



(m) Gastrula cells differentiate and organize to form the pluteus larva.



(n) Within 48 hours after fertilization, the egg has developed into a free-swimming multicellular organism, the pluteus.

✓ EXERCISE B | Cleavage

In order for a fertilized egg or zygote to become a multicellular organism, the zygote must divide by mitosis. During early development, this process of division is known as **cleavage.**

The fertilized egg is not a uniform sphere. Differential concentrations of cytoplasm and yolk (if present) can affect the cleavage process. The upper portion of the egg, usually richest in cytoplasm, is known as the **animal pole,** and the lower portion of the egg, containing more yolk, as the **vegetal pole** (Figure 19B-1a). The first plane of cleavage is vertical, bisecting both the animal and vegetal poles (Figure 19B-1b).

Depending upon the amount of yolk in the egg, the planes of cleavage may pass all the way through the zygote (holoblastic cleavage, typical of cells with small to medium amounts of yolk; sea urchin and frog) or through only a part of the zygote (meroblastic cleavage, typical of cells with large amounts of yolk; chicken).

A second cleavage division typically occurs at a right angle to the first, producing four cells. The third cleavage division cuts horizontally to form eight cells, four on the top and four on the bottom (Figure 19B-1c, d). The cells produced during these cleavage divisions are known as **blastomeres.** If the blastomeres in the top "tier" lie directly above those in the bottom tier, the pattern of cleavage is said to be **radial,** a pattern characteristic of echinoderms and chordates (deuterostomes).

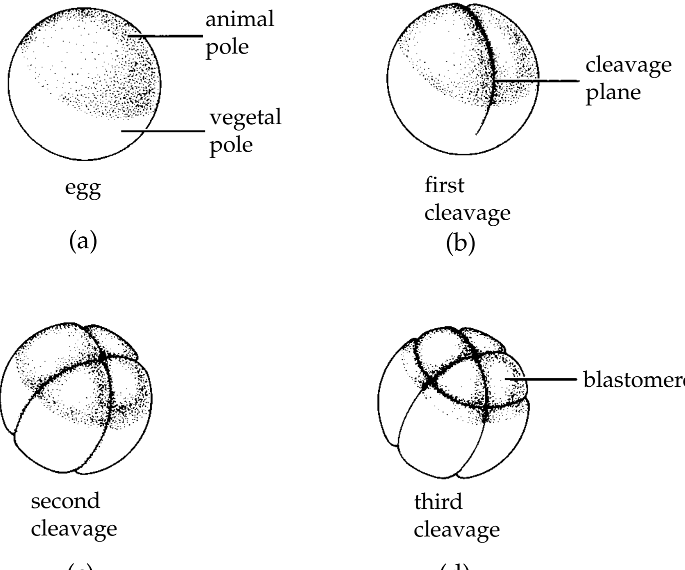


Figure 19B-1 *Eggs that contain a large amount of yolk in one hemisphere cleave unequally. The first two cleavages (b, c) split the egg through the poles. The third cleavage separates the yolkier (vegetal) part from the upper, less yolky (animal) part. The four cells in the animal hemisphere are much smaller than the four in the vegetal hemisphere.*

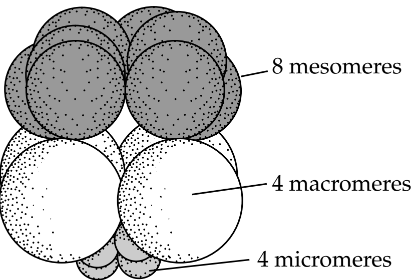
(d) The pattern of cleavage shown here is radial: the top four blastomeres are directly above the bottom four.

In the sea urchin, the fourth cleavage results in the formation of 16 blastomeres of three different sizes. Eight medium-size mesomeres are the product of the division of the four blastomeres in the animal hemisphere. The lower four blastomeres (vegetal hemisphere) produce four large macromeres and four small micromeres (Figure 19B-2). As a consequence of this cleavage pattern, cytoplasmic determinants are distributed in an unequal manner, laying the groundwork for future development (see Exercise C).

Objectives

- ☐ Describe how the amount and distribution of cytoplasm within a fertilized egg influence the patterns of cleavage.
- ☐ Describe how cleavage occurs in the starfish, frog, and chick.

Figure 19B-2 *Fourth cleavage division in sea urchin. An unequal fourth cleavage division results in 16 blastomeres of three different sizes. A top tier of eight medium-size blastomeres, called mesomeres, will eventually give rise to ectodermal structures. The four large macromeres will develop into endodermal structures associated with the gut. The four smallest blastomeres, micromeres, will produce mesodermal structures.*



Procedure

1. Obtain a composite slide of starfish (*Asterias*) development. This slide will have all stages of echinoderm development represented, including unfertilized eggs, fertilized eggs, and cleavage stages (Figure 19B-3). Some of the unfertilized eggs contain a **germinal vesicle**, a large swollen nucleus containing the nucleolus (appears as a black dot). The germinal vesicle breaks down at the end of prophase I and the egg appears as an opaque sphere during the remainder of its maturation.

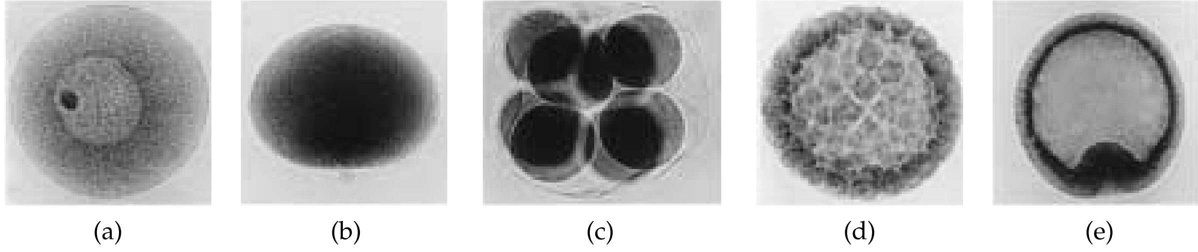


Figure 19B-3 *Embryonic development of the starfish: (a) germinal vesicle; (b) zygote with polar body; (c) 8-cell stage; (d) blastula; (e) early gastrula, blastopore apparent.*

Locate an unfertilized egg with a germinal vesicle and one without. Label a sheet of paper "Starfish Development" and draw these two examples of an unfertilized egg on the top half of the sheet. Label the nucleus and nucleolus.

a. Why is the nucleus of the developing zygote so big? ______

2. Find two-cell, four-cell, and eight-cell embryos on your slide of *Asterias* development. Notice that all of the cells in this embryo are the same size. On your sheet of paper, draw what you observe.

b. Is the mass of a four-cell or an eight-cell embryo any larger than the mass of the zygote? ______

c. What is the effect of cleavage on cell size? ______

On embryo size? ______

If live sea urchin material is available from Exercise A, observe the first cleavage division and other early cleavage stages. Compare your observations of starfish development with the stages of sea urchin development shown in Figure 19A-2.

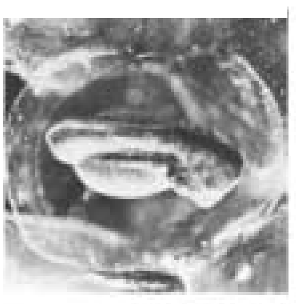
gray crescent

(a) (b) (c) (d)

(e) (f) (g) (h)

(i) (j) (k) (l)

(m) (n) (o) (p)



(q)

Figure 19B-4 *(a) The gray crescent of the frog zygote is bisected by the first cleavage plane so that each blastomere will contain animal-pole cytoplasm, gray-crescent cytoplasm, and vegetal-pole cytoplasm.*

Embryonic development of a frog: (b) egg; (c) 2-cell stage; (d) 4-cell stage; (e) 8-cell stage; (f) 16-cell stage; (g) 32-cell stage; (h) early blastula; (i) late blastula; (j) blastopore (the curved line where the dark region meets the light region); (k) early yolk plug; (l) late yolk plug; (m) neural plate; (n) early neural groove; (o) late neural groove; (p) neural tube; (q) tailbud stage.

d. What do you observe? Are the developmental stages similar? ________________________________

____

3. Examine a fertilized frog egg (preserved specimen) using a dissecting microscope. The frog egg shows how the arrangement of cytoplasm influences the placement of the first cleavage plane. In the frog, fertilization results in a shifting of the pigmented cytoplasm of the animal pole, establishing a grayish, crescent-shaped area (the **gray crescent**) on one side of the zygote (Figure 19B-4a). This area must be cut in half (bisected) by the first cleavage plane, thereby establishing the right and left halves of the future embryo. Use dissecting needles to move the egg around until you see the gray crescent. Do not poke the egg.
4. Now examine an embryo in the two-cell stage. *e. Does the cleavage plane cut the gray crescent in half?* ________

   Interestingly, if you separated these two halves of the embryo, each would develop into a normal tadpole (twins). If, however, you artificially divided the embryo into two halves (by slowing constricting it with a fine hair loop) so that all of the gray crescent material was contained in one blastomere, only the blastomere containing the gray crescent material would develop normally.
5. Examine a sagittal section through an early cleavage stage of the frog zygote. Notice the dark pigment at the surface of the animal pole of the egg. Are the top blastomeres the same size as the lower blastomeres? ________ Label a sheet of paper "Frog Development" and draw the representative section at the top of the sheet.

✔ EXERCISE C Formation of the Blastula

Repeated cleavages will result in formation of a hollow ball of cells called a **blastula.** The cavity inside the blastula is called the **blastocoel.** Even as early as this blastula stage, groups or layers of cells are already destined to become particular organs or organ systems; these layers of cells are known as **presumptive germ layers.** The major germ layers and their derivatives are listed in Table 19C-1.

Table 19C-1 Germ Layers and Their Derivatives

Germ Layer	Derivative
Epidermal ectoderm	Skin
Neural ectoderm	Brain, spinal cord, and neural crest cells
Chordamesoderm	Notochord and spinal disks in some organisms; ganglia
Mesoderm	Skeleton, circulatory system, excretory system, and parts of organs belonging to other systems
Endoderm	Gut and associated outpocketings

In the sea urchin, blastomeres that will give rise to cells of germ layers are already laid out at the 16-cell stage. A **fate map** can be assigned. Mesomeres will give rise to ectodermal structures including the cilia that develop on the blastula's surface. Macromeres will give rise to endodermal structures. Micromeres will be responsible for formation of the body cavity, many internal organs, and the skeletal elements (**spicules**) of the embryo (see Figures 19A-2 and 19B-2).

Objectives

- ☐ Describe the structure of a typical blastula.
- ☐ Relate the structure of the frog blastula to the establishment of the dorso-ventral and antero-posterior axes of the embryo.

Procedure

1. Reexamine the composite slide of starfish development. Locate an early blastula stage. The cells will be large enough to see and will appear as a dark ring of cells surrounding a lighter center. Remember that these are whole mounts and you are looking through a hollow sphere (see Figures 19A-2 and 19B-3). Draw your observations on the sheet of paper labeled "Starfish Development." Label blastomeres and blastocoel.

a. Why does the center appear to be lighter? ______________________

____

2. Locate a later blastula stage. The cells of the blastula are now so small that it will be difficult to distinguish individual cells (see Figures 19A-2 and 19B-3). Again, the outer rim appears to be dark. *b. Why?* ______________________

At this time, the cells of the blastula are covered by cilia which allow the blastula to spin and move. Draw your observations. Label blastomeres and blastocoel.

3. Examine a sagittal section through a frog blastula. Note the placement of the blastocoel.

c. Why is the blastocoel found closer to the animal pole of the embryo?

____

d. How does the size of the animal-pole blastomeres compare with that of the vegetal-pole blastomeres?

____

Draw the sagittal section on your sheet of paper labeled "Frog Development," and label blastomeres and blastocoel.

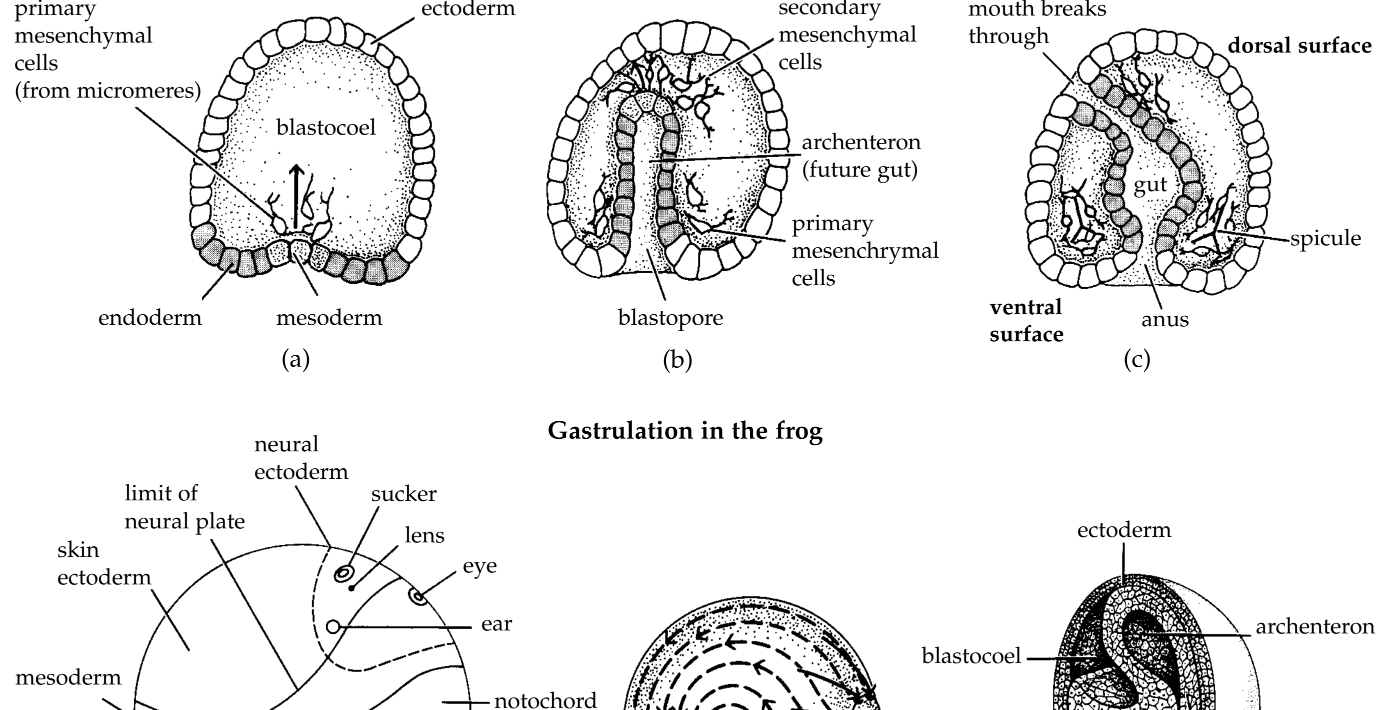
✔ EXERCISE D Gastrulation

As a blastula, most of the cells of the embryo (including those destined to become cells of internal organs and tissues) are on the outside of the hollow ball, and it is obvious that some of the sheets of cells must move, or migrate, to the inside of the blastula. This process of cell movement is called **gastrulation.**

In some organisms, such as the starfish and sea urchin, gastrulation is accomplished simply by buckling or pushing inward, forming a depression or **blastopore.** Endoderm and mesoderm reach the inside of the embryo in this manner. In other organisms, such as the frog or the chick, cells migrate to the interior by way of the blastopore, the portion of the embryo that will eventually contribute to the development of the anus (Figures 19A-2 and 19D-1). Mesoderm, endoderm, and chordamesoderm (notochord material) migrate to the inside. With the change in position of the germ layers, the blastocoel of echinoderms (starfish and sea urchin) and chordates (frog) is eventually obliterated and a new cavity, the **archenteron,** is formed within the gastrula. The archenteron is the primitive gut of the embryo (see Figures 19A-2, 19B-4, and 19D-1).

Note that during sea urchin development, secondary mesenchyme cells help to "pull" the endoderm of the newly forming archenteron (gut) toward the opposite side of the embryo, where it will fuse with the outer layer, or ectoderm, to form the mouth. (Hence the term "deuterostome" or second mouth, the anus forming at the blastopore or first opening.) See Figures 19A-2 and 19D-1.

Gastrulation in the starfish and the sea urchin

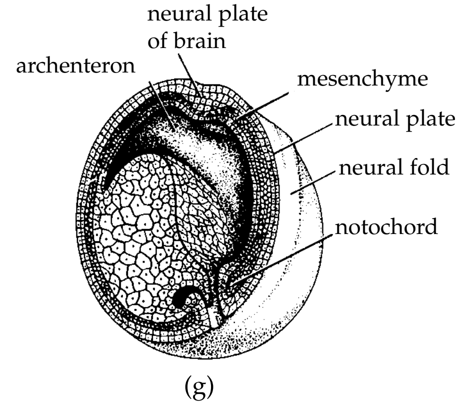


Gastrulation in the frog

neural
ectoderm
limit of
neural plate
sucker
lens
skin
ectoderm
eye
ear
mesoderm
notochord
visceral
pouches
somites
hindgut
(endoderm)
lip of
blastopore
(tail)
anterior
limit of
turned-in
material
anterior
limb bud
posterior
(d)

(e)

ectoderm
archenteron
blastocoel
endoderm
chordamesoderm
(f)



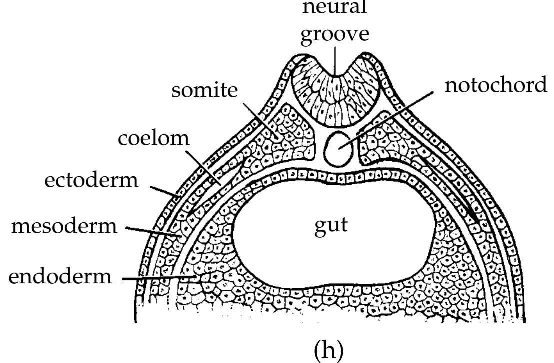


Figure 19D-1 *In some organisms, such as the sea urchin and starfish, gastrulation occurs as a simple buckling and pushing in of cells on one side of the embryo (a). This area of buckling becomes the blastopore, the entrance to the archenteron, or primitive gut (b). The archenteron will be lined by endoderm. The primary mesenchymal cells that arise from the mesoderm at the leading edge of the archenteron will accumulate in a ring near the vegetal pole, where they will form skeletal rods (spicules). Ectoderm will cover the entire embryo. The archenteron breaks through on its anterior end to form the mouth opening of the gut, and coelomic pouches bud from the gut (c).*

In other organisms, such as the frog, gastrulation occurs by an inward migration of cells by way of the blastopore (d, e). Sheets of ectoderm, mesoderm, and endoderm move around until they are in the correct position for organ formation. Immigration of cells and involution into the interior continue (f). The neural plate stretches in an anterior-posterior direction across the dorsal surface of the embryo (g). The edges of the neural plate will turn upward to form the neural folds along the neural groove, and eventually the entire structure will tubulate to form the brain and spinal column (h).

a. Why do the mesoderm, endoderm, and future notochord need to be on the inside?

---

b. To what organs or organ systems do these germ layers give rise?

Ectoderm and neural ectoderm remain on the outside of the frog or chick embryo. Eventually, the neural ectoderm will form a tube that is to become the brain and nerve cord, while the epidermal ectoderm spreads to cover this structure and the rest of the embryo.

Objectives

- ☐ Compare and contrast the structural components of the blastula and gastrula frog embryo.
- ☐ Describe the purpose of gastrulation.
- ☐ Compare the process of gastrulation in the starfish and the frog.

Procedure

1. Use high power (40×) to examine the composite slide of starfish (*Asterias*) development. Locate both early and late gastrula embryos. You will see the **archenteron** beginning to form as an inpocketing. The front end of the archenteron is expanded and will eventually fuse to the side of the embryo where the mouth will break through. The blastopore (region of inpocketing) becomes the anus. Refer to Figures 19A-2 and 19B-3. Draw the early and late gastrula embryos on the sheet of paper labeled "Starfish Development."
2. Locate a folded and twisted **bipinnaria** larva of *Asterias.* Note that the gut has become differentiated into an **esophagus, stomach,** and **intestine.** Two **coelomic pouches** may be seen attached to the gut wall. These are mesodermal and will eventually expand to form the mesodermally lined coelomic cavity. Draw a bipinnaria larva on your sheet of paper. Label the esophagus, stomach, intestine, and coelomic pouches.

   c. Recall from your study of diversity that echinoderms are enterocoelous coelomates. Explain how this description reflects the developmental events observed on the starfish composite slide.

   ---

3. Compare the structure of the starfish bipinnaria larva to that of the sea urchin pluteus larva (Figure 19A-2). If live specimens of sea urchin pluteus larvae are available from Exercise A, use a depression slide to observe that structure and movement. Observe the skeletal elements (spicules) and internal organs of the digestive system. Do you observe any coelomic pouches forming? In the space below, describe what you observe.

4. Study the preserved frog embryos on demonstration. Using the dissecting microscope, examine the blastopore region at the center of the lower edge of the gray crescent. Cells on the exterior stream toward the blastopore to turn inward (Figures 19D-1e and 19B-4j, k) and then spread out on the inside of the gastrula to take up their proper places. As cells converge on the blastopore, it appears first to form sides and then a ventral lip that completely encircles the yolky cells (the "yolk plug") of the interior (Figure 19B-4l).

   Note the changes in the blastopore region in eggs of increasing ages. Identify the yolk plug. Draw these embryo stages on the sheet of paper labeled "Frog Development."

5. Use scanning and low powers (4× and 10×) to observe a sagittal section through a late gastrula or **yolk plug** stage of the frog embryo. Identify the yolk plug and archenteron. Using Figure 19D-1, try to identify the cells of the notochord, ectoderm, and endoderm of the gastrula.

EXERCISE E Neurulation

During this stage of development, a strip of neural ectoderm on the outside of the dorsal surface of the embryo (Figure 19B-4m) turns upward to form a **neural tube** (Figure 19D-1h). The underlying mesoderm and notochord tissue induce formation of the neural tube. The folds of tissue forming the tube are the **neural folds** and the groove between them is the **neural groove.** Eventually the anterior end of the neural tube will expand to form the **brain;** the spinal cord will develop posterior to the brain.

Aggregations of mesoderm (mesodermal **somites**) behind the brain and alongside the spinal cord will form vertebrae (back bones) that protect and enclose the spinal cord. Mesodermal somites also give rise to dorsal skeletal muscles and to the dermis of the skin (Figure 19D-1d). The presence of somites is an indication of the segmented nature of vertebrate embryos. Biologists have recently shown that a series of genes control the development of segmentation in all segmented embryos, whether in fruit flies, mice, or in humans. These genes, called **homeotic genes,** often work in a cascade with other genes that control the basic head-to-tail and anterior-to-posterior architecture of the embryo. Within each homeotic gene, a special sequence or **homeobox** of 180 base pairs codes for a protein that is 60 amino acids long. This is a DNA regulatory protein that can “turn on” other genes involved in segmentation and segment identity (whether wings, antennae, legs, and so forth, are attached). The same homeobox is found in all homeotic genes of segmented organisms—its base sequence has been **conserved** throughout evolution.

The gut will tubulate during this later stage of development, and the ventral unsegmented mesoderm will split to form a mesodermally lined coelom (Figure 19D-1h).

Objectives

- ☐ Describe the process of neurulation.
- ☐ Describe the function of the chordamesoderm.
- ☐ Relate the development of the brain and the spinal column to the development of the neural tube and neural folds.

Procedure

1. Examine a cross section of a frog late neurula. Draw this on your “Frog Development” sheet. Identify and label the neural tube, notochord, somite mesoderm, gut cavity, epidermis, and mesoderm lining the coelom.
2. Examine a preserved late-neurula frog embryo. Iddentify the neural groove and neural folds.
3. Examine later stages of frog embryos (Figure 19B-4).

   a. *What happens to the shape of the embryo after the sides of the neural tube close?*

   ____

EXERCISE F Development of the Chick

Note that the same body-forming movements occur during gastrulation in the development of chickens as in frogs, except that the end product of the process is three flat layers of cells rather than three concentric rings in a sphere (Figure 19F-1). At the beginning of gastrulation, the disk of cells (blastodisc) on the top of the egg is composed of two layers—the upper layer containing ectoderm, neural ectoderm, notochord, and mesoderm tissue; the lower layer containing endoderm. During gastrulation, notochord and mesoderm cells are moved from the top layer to form a middle layer.

primitive streak
ectoderm
endoderm
migrating cells
mesoderm

(a)

Hensen's node
primitive pit
primitive streak forming

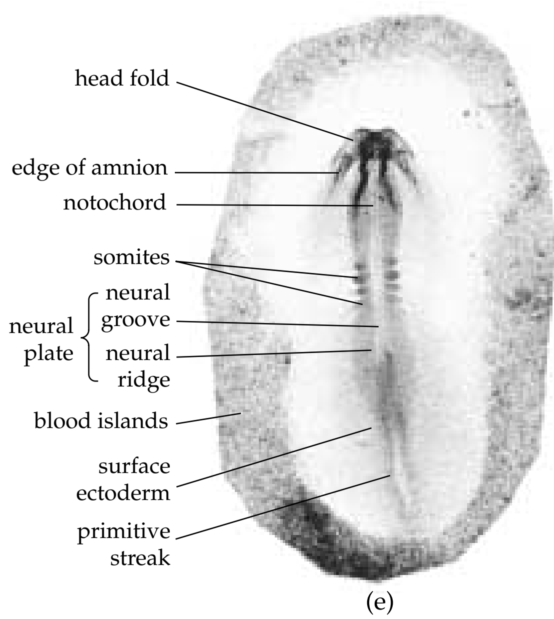
(b)

Hensen's node
primitive folds
primitive groove
cells migrate inward along sides of primitive streak (primitive folds)

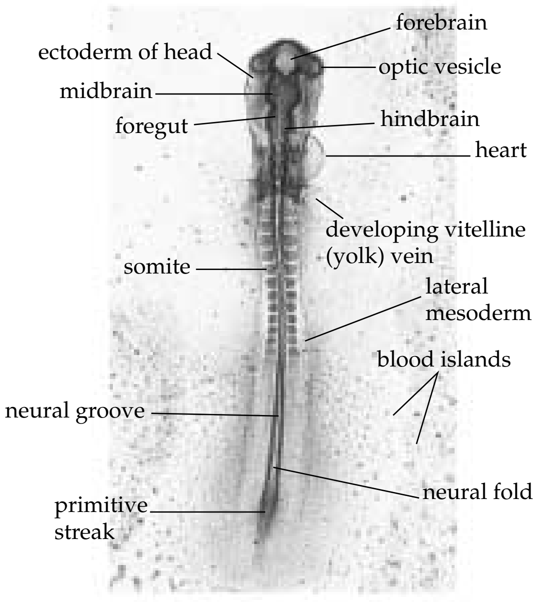
(c)

primitive streak
primitive fold
primitive groove

(d)



(e)



(f)

Figure 19F-1 *(Opposite.) Development of the chick blastodisc. (a) Cells migrate into and away from the primitive streak to form the mesoderm and some endoderm. (b, c) Formation of the primitive streak. Cells converge toward the midline of the blastodisc. (d) The chick embryo at 16 hours. The rounded mound of cells (Hensen's node) at the anterior end of the primitive streak represents an accumulation of chordamesoderm cells (notochord and cephalic mesoderm) that will invaginate. As cells move to the interior, the streak will recede toward the posterior end of the embryo, and the notochord cells will be pulled out into a strip in the middle layer (between the mesoderm that has already moved inward from the surface layer). Neural ectoderm cells will be pulled back as a sheet of tissue on the outside overlying the notochord. (e) The chick embryo at 24 hours. (f) The chick embryo at 33 hours. During the process of neurulation, the underlying mesoderm and notochord induce the formation of a neural tube in the strip of neural ectoderm lying above them.*

As cells migrate to form the underlying tissue layer, they converge toward the posterior portion of the midline of the blastodisc (Figure 19F-1c). There they sink downward (similar to the way cells sink inward at the blastopore of the frog gastrula). This area, at first triangular in appearance, is called the **primitive streak.** As more cells move into the area, the primitive streak appears to lengthen, forming the **primitive groove** bordered by **primitive folds,** formed by accumulations of cells converging on the area in preparation for invagination. If you made a tube out of the flat layers of the chick after completion of gastrulation, the structure would look surprisingly like a frog gastrula.

Objectives

☐ Compare gastrulation and neurulation in the chick and the frog.

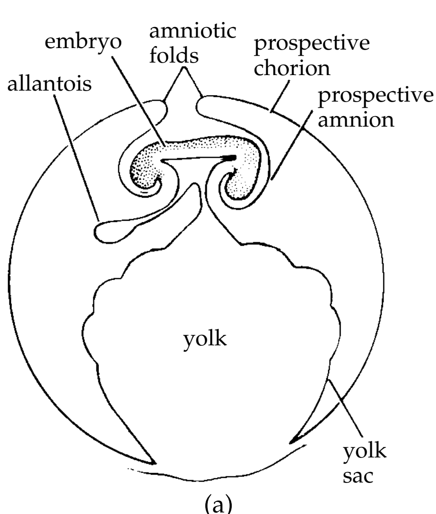
Procedure

1. Observe a prepared slide of a 16-hour-old chick and identify the primitive groove and primitive folds.
2. Observe later stages of chick development (Figure 19F-1d, e, f). Note that the neural folds and mesodermal somite blocks can easily be seen very early in the development of a chick (17 to 29 hours). Remember that the chick must tubulate in order for its "flat" layers of tissues to form a circular gut tube. The head is the first area to tubulate, and it appears to be lifted up off the surface of the blastodisc (Figure 19F-1f).

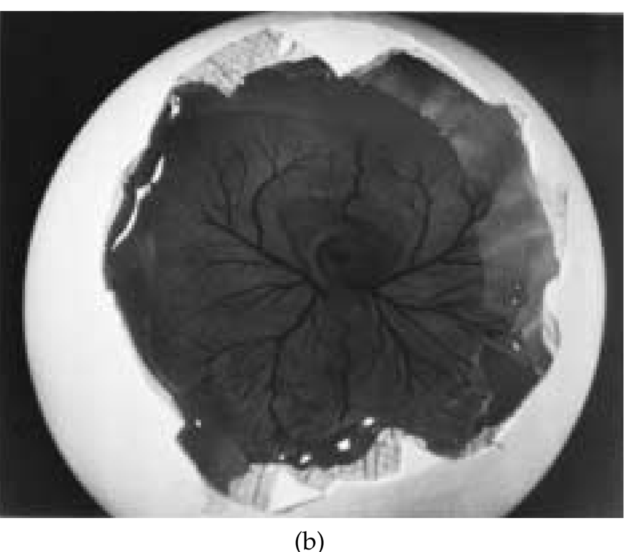
✔ EXERCISE G Formation of Extraembryonic Membranes in the Chick

As the chick continues to develop inside the egg, special vascularized membranes develop to serve the respiratory, protective, nutritional, and excretory needs of the embryo.

In the chicken embryo, four membranous sacs are formed by the growth and folding of the sheets of ectoderm, mesoderm, and endoderm at the edges of the blastodisc. These **extraembryonic** membranes are continuous with embryonic tissue, but lie outside (hence "extraembryonic") the embryo (Figure 19G-1a). The **amnion** is formed around the embryo and fills with fluid to serve a protective function. The vascularized **chorion** lies beneath the shell and serves a respiratory function by picking up oxygen that has diffused through the shell and transporting it to the embryo. The **allantois,** which invaginates from the hindgut, serves as a repository for wastes and eventually fuses with the chorion to aid in respiration. The **yolk sac** invaginates from the gut cavity and becomes highly vascularized by a network of vitelline vessels that pick up nutrients from the yolk and transport them to the embryo (Figure 19G-1b).



(a)



(b)

Figure 19G-1 *(a) Extraembryonic membranes of the chick. (b) The yolk sac vascularized by a network of vitelline vessels.*

Objectives

- ☐ List and give the functions of the four extraembryonic membranes.

Procedure

1. Work in pairs. Obtain an incubated 96-hour-old or 5- to 6-day-old chicken egg. Place the egg on a towel so that one side faces upward. The embryo will rotate to the top side after 3 to 5 minutes. Meanwhile, fill a small finger bowl with warm (37°C) chick Ringer's solution.
2. Gently crack the egg on the lower surface and allow the egg yolk and embryo to float out of the shell into the Ringer's solution. Do not jerk the two halves of the shell apart or separate them too quickly. It will take some time for the chorion to separate from the shell membranes of the older eggs.
3. Identify the yolk sac with its vessels. How far has the network of vitelline vessels extended. These vessels will eventually cover the entire surface of the yolk.
4. With a blunt instrument, push on the embryo. You will see that it is floating within a sac—the amnion.
5. Look for the allantois. It will appear as a small bubble emerging from the posterior end of the embryo.
6. With a pair of forceps, lift up the outermost tissue layer lying above the embryo. This is the chorion. How far does it appear to extend?

PART II IMMUNE RESPONSES

Consider that a mammalian organism can make more than 10 million different types of antibodies. However, there is not enough DNA in the organism's entire genome to code for all of these molecules. How can we account for the variety of antibodies, or **immunoglobulins,** that protect our bodies?

The answer lies in the observation that the DNA of eukaryotic chromosomes can rearrange itself, making new coding combinations from old sequences. Each antibody molecule is made up of two **heavy** (long) **chains** and two **light** (short) **chains.** A portion of each chain, the **variable region** (containing approximately 100 amino acids), is responsible for antibody diversity and function (Figure 19II-1).

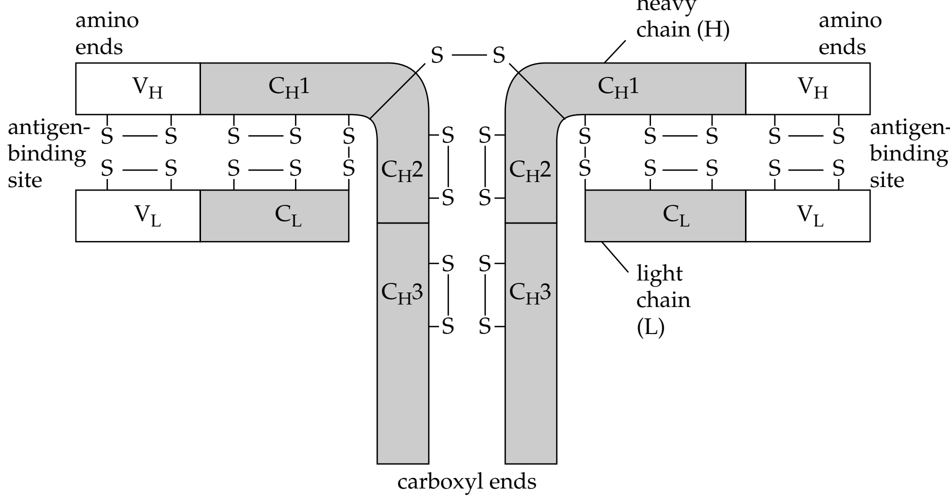


Figure 19II-1 *The immunoglobulin molecule. An antibody (immunoglobulin) molecule has two light (L) and two heavy (H) polypeptide chains, each of which has variable (V) and constant (C) regions. The heavy and light chains are connected to each other by disulfide bonds.*

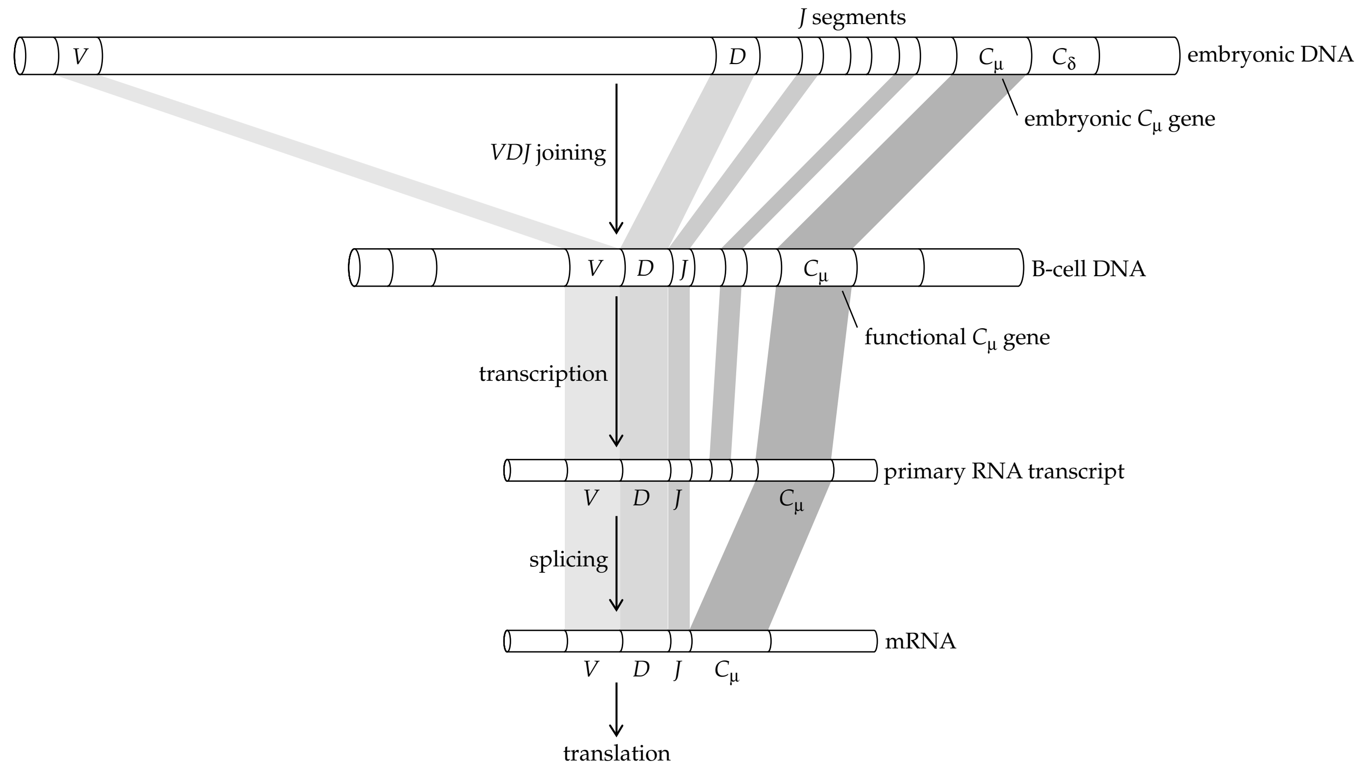


Figure 19II-2 *Rearrangement of the DNA segments of the genes for the heavy chains of immunoglobulins. The* V, D, J, *and* C *segments are joined to form the gene. After transcription, the primary RNA transcript is spliced to remove the transcripts of introns and extra* J *segments. The mature mRNA is then ready for translation.*

During production of the variable portions of heavy or light immunoglobulin chains, various combinations of DNA segments are put together by "selecting" and splicing together *V* (variable), *D* (diversity), and *J* (joining) segments of DNA. These are then combined with selected *C* (constant) regions for immunoglobulins I, M, A, D, and E. Introns are removed, as well as extra *J* segments, during splicing. The final product is a single and unique chain of *V*, *D*, *J*, and *C* segments (Figure 19II-2).

Understanding this genetic control of diversity among immunoglobulins has led us to a better understanding of their biochemical and physiological functions.

EXERCISE H | Demonstrating the Immune Response by the Precipitin Ring Test*

Immunology is the study of the mechanisms by which the body protects itself against disease-causing microbes. Any foreign substance (whether of microbial origin, including viruses, or from another species of animal) will trigger the immune response if that substance is sufficiently large (a molecular weight of at least 10,000), is sufficiently complex (such as a protein composed of at least 20 different amino acids), and is biodegradable—that is, can be broken down by naturally occurring biochemical interactions. Some large synthetic molecules, such as many plastics, are nonbiodegradable—tend not to interact with or be broken down by substances within the body—and therefore do not normally trigger an immune response. Foreign substances that cause an immune response are called **antigens.**

When antigens enter the body (for example, when you are injected with a flu vaccine), they set off a complex series of reactions which are demonstrable in a variety of ways. One of the easiest ways to demonstrate the immune response is to do a blood **serum titer analysis.** A titer is a measure of the amount of antibodies in the blood serum that can react with a specific antigen. (Serum is the liquid part of the blood remaining after cells and fibrin have been removed. Antibodies circulate in the blood serum.) If a series of measurements is taken over a period of several weeks after the antigen is injected, the time course for the development of antibodies looks like the graph in Figure 19H-1. The initial injection triggers the primary immune response; the effect of a "booster shot" is shown by the secondary immune

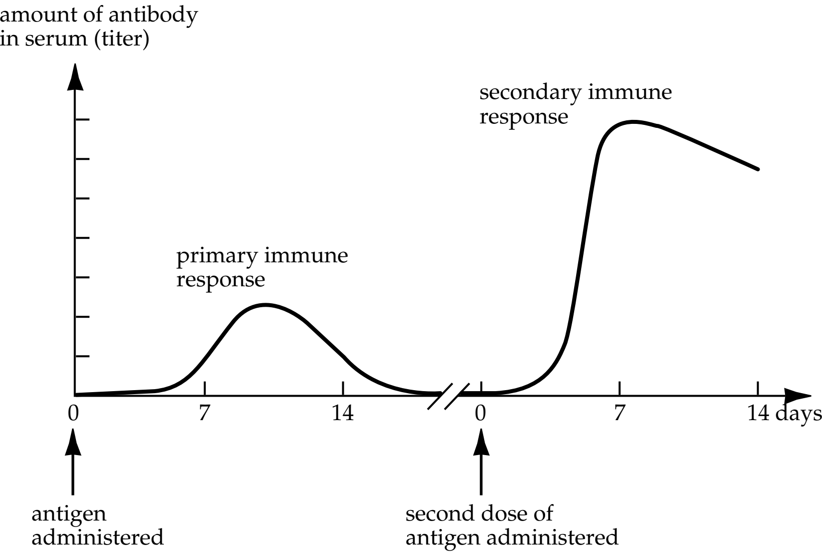


Figure 19H-1 *Changes in serum antibody levels in response to two injections of an antigen administered at different times.*

This exercise was developed by Dr. Fred Stutzenberger and Jzuen-Rong Tzeng, Clemson University, Clemson, South Carolina.

response—it is greater and longer-lasting than the primary response. This secondary response is specific for the challenging antigen—serum will not react this way with other antigens that the body has not yet experienced (a phenomenon known as **immunomemory**).

a. Why do some types of vaccines require a "booster shot"? ______________________________

____

b. Which cells are responsible for the secondary immune response?

____

c. Explain how this response occurs. ______________________________

____

Antibodies are **immunoglobulins** (globular-shaped proteins with an immune function) which can often be found in other body fluids, such as saliva, breast milk, and respiratory secretions, as well as in serum. Although immunoglobulins are found in several different forms, depending on their location in the body, all have similar structures (see Figure 19II-1). When serum antibodies combine with their specific antigen (Figure 19H-2), they form a complex **immunoprecipitate.** If this reaction is carried out in a test tube, the immunoprecipitate will form a ring at the interface where the antigen and antibody meet—hence the name "ring test."

The ring test is a useful qualitative method for the rapid detection of either antigen or antibody. It involves carefully overlaying a solution of antibody (antiserum) with a solution of antigen so that a sharp liquid interface is formed. Evidence of a positive reaction is the formation of a cloudy precipitate at the interface. Either antigen or antibody can be detected quickly in amounts as small as 1.0 μg of protein if care is taken in carrying out the test. However, the ring test is not a highly quantitative method.

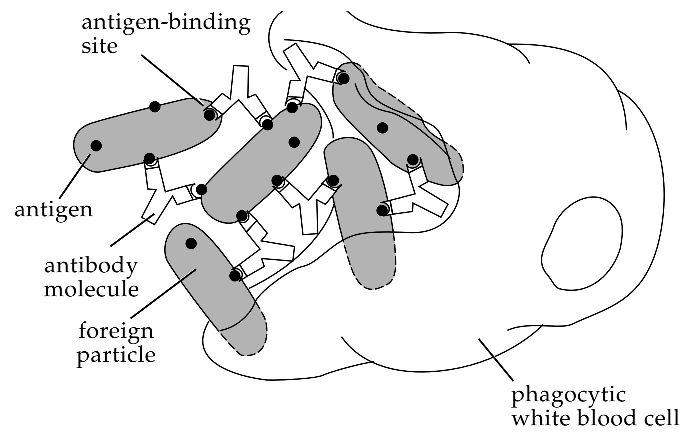


Figure 19H-2 *Binding of antibodies to antigens. Because each antibody molecule has two antigen-binding sites, a single antibody can bind to antigens on two different cells or particles, causing them to stick together (agglutinate). Phagocytic white blood cells then consume these larger masses of foreign particles and antibodies.*

Objectives

- ☐ Describe the relationship between antigen and antibody.
- ☐ Describe the primary and secondary immune responses.
- ☐ Use the precipitin ring test to demonstrate the immune response.

Procedure

1. Place five microculture tubes (numbered 1 through 5) in a rack. With a Pasteur pipette, introduce anti-BSA antiserum into tubes 1 and 5 to a height of about 10 mm. Be sure to note the approximate volume of the liquid in the Pasteur pipette. Using a separate pipette for each

solution, introduce a similar amount of buffer into tube 2 and of normal serum into tubes 3 and 4, as indicated in Table 19H-1.

2. Gently overlay each volume of solution with an equal volume of the buffer or antigen indicated in Table 19H-1. *Be careful not to mix these two layers of solution.* This can be avoided by slightly slanting the tube and carefully introducing the buffer or antigen solution along the wall of the tube near the surface of the bottom solution, then gradually pulling up the Pasteur pipette. Place the rack of tubes in front of the light source and observe after 10 to 15 minutes at room temperature.

Table 19H-1 Ring Test for Detection of an Antigen (BSA) or an Antibody (anti-BSA)

	Tube Number*				
	1	2	3	4	5
Top layer	B	Ag	B	Ag	Ag
Bottom layer	Ab	B	N	N	Ab
Reaction					

*B = buffer, Ag = antigen, Ab = antiserum, N = normal serum.

3. A positive reaction is indicated by formation of a milky white ring at the interface. Record reactions in Table 19H-1.

d. Explain why you had a positive reaction or no reaction in:

Tube 1 ______________________

Tube 2 ______________________

Tube 3 ______________________

Tube 4 ______________________

Tube 5 ______________________

e. How might the ring test be used to check whether an individual demonstrates immunity to a particular antigen, perhaps to a disease such as measles or chicken pox?

____

f. How is this reaction similar to the type of reaction that would occur if a person with type B blood received a transfusion of type A blood by mistake? ______________________

Laboratory Review Questions and Problems

1. Fill in the following table to summarize the major events of early development.

	Formation of the Blastula	Early Gastrula	Late Gastrula	Neurula
Processes occurring				
Type of structure formed				
Characteristics of structure				
Significance of stage				

2. Compare and contrast major developmental events in the sea urchin, frog, and chick by completing the following table.

	Sea Urchin	Frog	Chick
Type of egg			
Type/pattern of cleavage			
Distinguishing characteristics of blastula			
How gastrulation occurs			
Events of neurulation			
Distinguishing characteristics of later development			

3. What are cytoplasmic determinants? How do they affect early development, including cleavage of the zygote?

4. How can maternal DNA affect the development of an embryo when the maternal genes are not included in the egg produced as a result of meiosis?

5. What are homeotic genes? How do they control the development of segmented organisms?

6. How is the heavy chain of an immunoglobulin molecule of the M type constructed?

7. How can you explain the tremendous variability among immunoglobulins if DNA does not have enough genes to code for each molecule individually?

8. What are antigens and antibodies? What types of cells make antibodies?

9. Why would it be important to be able to determine whether a person had antibodies to a specific antigen? Can you think of a medical crisis in which this knowledge would be helpful?

LABORATORY 20

The Genetic Basis of Evolution

OVERVIEW

To the population geneticist, evolution means changes in the frequencies of the **alleles,** alternative forms of the same gene, or genotypes, in the gene pool. The potential of a population to experience evolutionary change as it adapts to its environment depends upon the amount of variation within the population's gene pool. **Natural selection,** the differential reproduction of phenotypes, is one of the chief agents of evolutionary change. Evolution depends on natural selection operating in a population having a variety of alleles that produce a variety of genotypes.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

✔ EXERCISE A Understanding Variation

It is important to understand that variation in phenotype is due not only to genetic variation but also to environmental effects. Variation may be either **continuous,** characterized by small gradations between individuals, or **discrete,** showing clear-cut differences. The continuous variations in human anatomical traits you will measure in this exercise are subject to both genetic and environmental influences. It is not known to what extent natural selection influences these traits.

Objectives

- ☐ Understand continuous variation through examination of some human traits.
- ☐ Make a quantitative analysis of continuous variation within a population.

✔ PART I Measuring Cephalic Index

The **cephalic index** is a ratio that relates the breadth of a skull to its length. This measure is used by anthropologists to compare head shape in human populations and by paleontologists to study fossilized skulls.

Procedure

1. Work in pairs. Use a set of calipers and a meter stick to measure the breadth and length of your partner's head in centimeters. Breadth is the maximum width of the head measured

above and behind the ears (Figure 20A-1a). Length is the distance from the bulge in the forehead (glabella), just above the nose, to the bony protrusion at the base of the skull (opisthocranium) (Figure 20A-1b). Round your measurements to the nearest whole number.

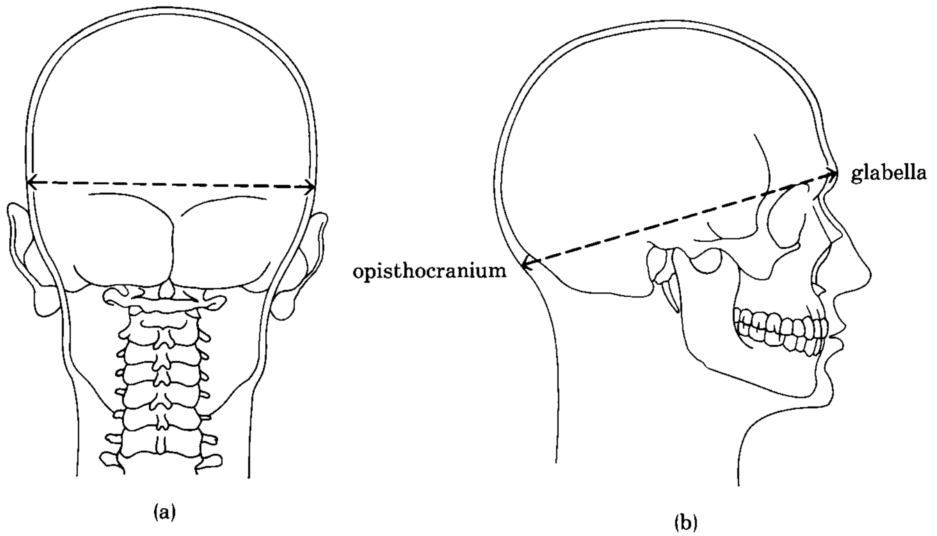


Figure 20A-1 *Measuring (a) head breadth and (b) head length.*

2. To obtain the cephalic index, divide the breadth of the skull by its length and multiply the quotient by 100. (Keep in mind that the cephalic index in humans is not well correlated with either brain size or intelligence.) Use the space below for your calculations.

3. Your partner should now calculate your cephalic index.
4. Write both cephalic indexes on the blackboard. Designate your sex by M or F.
5. On the bar graph in Figure 20A-2, mark cephalic index measurements by placing an **X** for each member of the class in the column directly above the appropriate cephalic index range on the horizontal axis. Use different colored pencils or pens to mark measurements for males and females.

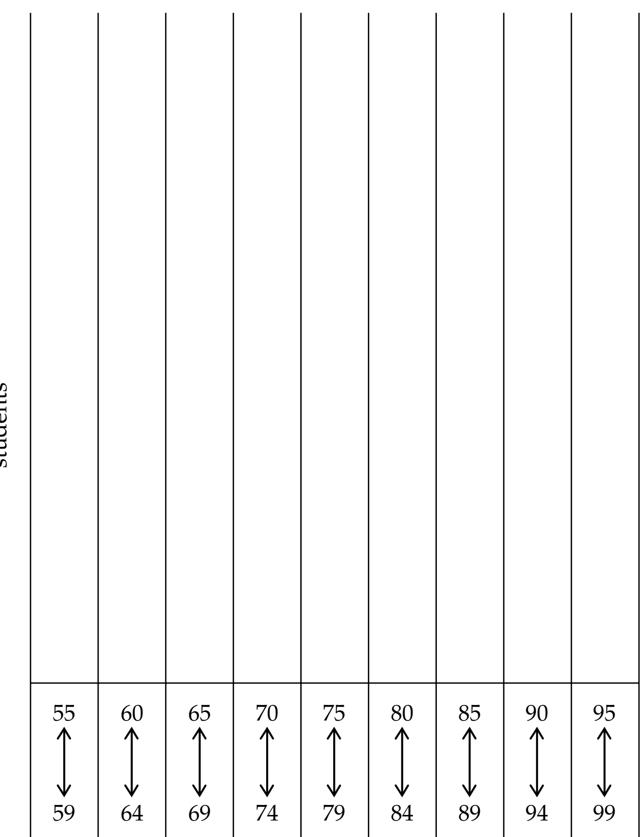
   a. What is the average cephalic index for the class? ______ *For males?* ______

   For females? ______

   b. For which sex is the range of measurements greater? (The range is the distance between the highest and the lowest values in a group of measurements.) ______________

   (If there is disagreement, your instructor may use a statistical calculation to decide the question.)

Figure 20A-2 *Bar graph for class cephalic index data.*



✔ PART 2 Measuring Relative Sitting Height

Another highly variable trait in humans is the length of the torso relative to overall stature.

Procedure

1. Work in pairs. Have your partner sit as erect as possible on the lab table while you measure the distance from the top of the head to the top of the lab table in centimeters.
2. Now have your partner stand against the wall of the classroom. Mark a point just above the top of the head, then measure from the floor to your mark.
3. To obtain relative sitting height, divide your partner's sitting height by his or her overall height and multiply the quotient by 100.
4. Now your partner should repeat these measurements and calculations for your relative sitting height.
5. Write both relative sitting heights on the blackboard. Designate your sex by M or F.
6. On a separate sheet of paper, construct a bar graph like the one in Figure 20A-2. The intervals of five along the horizontal axis should begin with the multiple of five that includes the lowest measurement in the class and proceed, in fives, up to the multiple of five that includes the highest measurement. Use different colored pencils to mark measurements for males and females in the appropriate sitting-height category. Add this graph to your laboratory manual.

   a. What is the average relative sitting height for the class? ______ *For males?* ______

   For females? ______

   b. For which sex is the range of measurements greater? ____________

c. Is there a greater range in relative sitting heights among the males or among the females in your class? ________________ *How did you arrive at your conclusion?* ________________

____

(Again, your instructor may verify this conclusion with a statistical calculation.)

d. Recall that in Laboratory 13 you examined the genetic trait of corn seed color, a discrete (discontinuous) phenotypic variation. Compare the variation in corn seed color with the variations you observed in this exercise. Describe the differences. ________________________________

____

e. What do these differences tell you about the genes involved in the inheritance of different traits?

____

____

EXERCISE B | Estimating Allelic Frequency from a Population Sample

Evolution can be defined as a change in allelic frequencies. **The allelic frequency of allele *A* in a population is the fraction of all the alleles at the *A*/*a* locus in the population's gene pool that are *A*.** For example, if all the organisms in the population have the genotype *Aa,* then the frequency of allele *A* is 0.5 (because *half the alleles* are A). Note that the allelic frequency is *not* the fraction of the organisms that have an *A* allele, or the fraction that have a particular genotype or phenotype.

As another example, say that the population is 10 percent *AA*, 60 percent *Aa*, and 30 percent *aa.* Then we would compute the frequency of *A* as follows:

All the alleles of the *AA* organisms are *A*, so the contribution of the *AA* organisms to the frequency of *A* is (1.0)(0.1).

Half of the alleles of the *Aa* organisms are *A*, so the contribution of the heterozygotes is (0.5)(0.6).

None of the alleles of the *aa* organisms is *A*, so the contribution of the *aa* organisms is (0)(0.3).

Thus, the frequency of $A = (1.0)(0.1) + (0.5)(0.6) + (0)(0.3) = 0.4$.

Note that if the population has only *A* and *a* alleles at the gene (*A*/*a*) locus, then any allele that is not *A* is *a.* The frequency of the *a* allele is 1− the frequency of the *A* allele. Therefore, in the population above, in which the frequency of *A* was 0.4, the frequency of *a* must be $1 - 0.4 = 0.6$.

In situations in which there are only two alleles at a locus and one is dominant and one is recessive, we define two variables, *p* and *q:*

p = the frequency of the dominant allele (0.4 in the example above)

q = The frequency of the recessive allele (0.6 in the example above)

Then,

$$p + q = 1$$

a. A population consists of 20 percent AA, *30 percent* Aa, *and 50 percent* aa *organisms. What is the frequency of the* A *allele in this population?* ________________________________

The **Hardy-Weinberg rule** predicts what will happen to allelic frequencies and the proportion of genotypes in the population under a very restrictive set of conditions:

1. There is no mutation of the alleles.
2. Mating is random: no genotype has a mating advantage, and every genotype is equally likely to mate with any other genotype.

3. The population is very large.
4. Immigration and emigration do not occur.
5. There is no selection: all genotypes have an equal chance of surviving and reproducing.

These conditions are almost never true in nature. However, if they were true, the population is said to be at **Hardy-Weinberg equilibrium.** In a population at Hardy-Weinberg equilibrium:

1. **Evolution does not occur, because allelic frequencies never change.**
2. **Genotypic frequencies can be predicted from allelic frequencies.**

We will be focusing on this second consequence of the Hardy-Weinberg equilibrium in this exercise.

A **genotypic frequency** is the fraction of the population that is a particular genotype. For example, if a population is 20 percent *AA*, 20 percent *Aa*, and 60 percent *aa*, the genotypic frequencies are 0.2 *AA*, 0.2 *Aa*, and 0.6 *aa*.

If we *cannot* assume Hardy-Weinberg equilibrium, there is no necessary relationship between allelic frequencies and genotypic frequencies. For example, the *allelic frequencies* of *A* and *a* are both 0.5 in all the populations listed in the table below.

	AA	*Aa*	*aa*
Population 1	0.00	1.00	0.00
Population 2	0.50	0.00	0.50
Population 3	0.20	0.60	0.20
Population 4	0.25	0.50	0.25
Population 5	0.40	0.20	0.40

However, if a population is at Hardy-Weinberg equilibrium, where the frequency of $A = p$ and the frequency of $a = q$, we will always see the following genotypic frequencies:

	AA	*Aa*	*aa*
Hardy-Weinberg equilibrium population	p^2	$2pq$	q^2

For example, the population described above, where $p = 0.5$ and $q = 0.5$, if the population is at Hardy-Weinberg equilibrium, we would expect to find the frequency $AA = (0.5)^2$ or 0.25; the frequency $Aa = 2\ (0.5)\ (0.5) = 0.50$; and the frequency $aa = (0.5)^2 = 0.25$.

b. Among the five populations listed in the table above, are there any that might be at Hardy-Weinberg equilibrium?

_______ *Which one(s)?* ______________________________

If there are only two possible alleles at a locus, the only possible genotypes are the two homozygotes and the heterozygote. Therefore, the frequencies of these genotypes must add up to 1.0. This gives us the **Hardy-Weinberg equation:**

$$p^2 + 2pq + q^2 = 1$$

If we know all of the genotypic frequencies in a population, we can determine if these *observed* genotypic frequencies agree with what would be *expected* if the population were at Hardy-Weinberg equilibrium. For example, if we are told that a population's genotyopic frequencies are 0.1 *AA*, 0.2 *Aa*, and 0.7 *aa*, we can determine whether or not the population is in Hardy-Weinberg equilibrium by a three-step procedure.

Step 1 Determine allelic frequencies from genotypic frequencies.

p (frequency of A) = (1.0)(0.1) + (0.5)(0.2) + (0.0)(0.7) = 0.2

q (frequency of a) = 1 − p = 1 − 0.2 = 0.8

Step 2 Determine *expected* genotypic frequencies if the population is at Hardy-Weinberg equilibrium.

Expected frequency of $AA = p^2 = 0.04$

Expected frequency of $Aa = 2pq = 0.32$

Expected frequency of $aa = q^2 = 0.64$

Step 3 Compare observed and expected (if in equilibrium) genotypic frequencies.

	AA	*Aa*	*aa*
Observed	0.10	0.20	0.70
Expected	0.04	0.32	0.64

There is a fairly serious disagreement between observed and expected here, so we would probably conclude that this population is *not* at Hardy-Weinberg equilibrium. If we wanted a more exact determination of agreement, we could do a statistical test with chi-square (Appendix I).

c. *A population has 10 percent* AA, *45 percent* Aa, *and 45 percent* aa *organisms. Does this population seem to be at Hardy-Weinberg equilibrium?* ______

One final application of the Hardy-Weinberg rule is using it to estimate allelic frequencies where the genotypic frequencies are partially unknown *but the Hardy-Weinberg equilibrium can be assumed.* For example, say *EE* and *Ee* individuals have normal earlobes, but *ee* individuals have a distinctive shape to their earlobes. We know that 16 percent of the North American population shows this latter phenotype (*ee*), and we are willing to assume Hardy-Weinberg equilibrium. We can use two steps to estimate the frequencies of the alleles and the genotypes in the population:

Step 1 Determine allelic frequencies.

q^2 (frequency of *ee*) = 0.16

$q = \sqrt{0.16} = 0.40$

$p = 1 - q = 0.60$

Step 2 Determine expected genotypic frequencies if the population is at Hardy-Weinberg equilibrium.

Expected frequency of $EE = p^2 = 0.36$

Expected frequency of $Ee = 2pq = 0.48$

Expected frequency of $ee = q^2 = 0.16$

Note that if we are unwilling to assume Hardy-Weinberg equilibrium, we cannot do any of this. Without Hardy-Weinberg equilibrium, the Hardy-Weinberg equation does not apply, and although we still know that 16 percent of the population is *ee*, the normal-earlobe phenotypes might be all *EE*, all *Ee*, or any mixture of *EE* and *Ee*. Therefore, it is important to realize when you can and cannot do certain computations.

- You can always calculate allelic frequencies from a complete list of genotypic frequencies, *whether or not* you assume Hardy-Weinberg equilibrium.
- You can compute genotypic frequencies from allelic frequencies *only* if you can assume Hardy-Weinberg equilibrium.
- You can estimate allelic and genotypic frequencies from the fraction of homozygous recessives in a population *only* if you can assume Hardy-Weinberg equilibrium.

In this exercise you will use the class as a sample population and will assume Hardy-Weinberg equilibrium. You will estimate the frequencies of alleles of a gene controlling the ability to taste the chemical phenylthiocarbamide (PTC). A bitter-taste reaction to PTC is evidence of the presence of a dominant allele, in either the homozygous condition (frequency = p^2) or the heterozygous condition (frequency = $2pq$). The inability to taste the chemical at all depends on the presence of a homozygous recessive genotype (frequency = q^2). To find the frequency of the PTC-tasting allele in the population, you must find p; to find the frequency of the non-PTC-tasting allele, you must find q.

Objectives

☐ Estimate allelic frequencies for a specific trait within a sample population and compare these with estimated frequencies for the entire population.

Procedure

1. Your instructor will provide you with PTC taste test papers. Tear off a short strip and press it to the tip of your tongue. A few PTC tasters report a salty, sweet, or sour taste rather than a bitter taste. These individuals are considered tasters for the purpose of this experiment.
2. Calculate a decimal number representing the proportion, or frequency, of tasters ($p^2 + 2pq$) by dividing the number of tasters in the class by the total number of students in the class. Obtain a decimal number representing the frequency of nontasters (q^2) by dividing the number of nontasters by the total number of students. In Table 20B-1, record these numbers under the appropriate headings.

   a. *Why is the term* 2pq *included in the taster phenotype?* ______________________

   ____

Table 20B-1 Phenotypic Proportions of Tasters and Nontasters of PTC and Frequencies of the Determining Alleles

	Phenotype		Allelic Frequency	
Sample	Tasters ($p^2 + 2pq$)	Nontasters (q^2)	p	q
Class population				
North American population	0.55			

3. Assuming that your class is in Hardy-Weinberg equilibrium, use the Hardy-Weinberg equation to determine the frequencies (p and q) of the two alleles. The frequency q can be calculated by

taking the square root of q^2. Since $p + q = 1$, you can calculate the frequency p of the taster allele. Record your data in Table 20B-1.

4. Use the data on the North American population given in the table to calculate the expected frequencies (p and q) of the two alleles. Record these in Table 20B-1.

   b. How do the sample frequencies for the class compare with the frequencies for the population at large?

   ____

   ____

Note: You may want to determine whether the difference between the allelic frequencies (p and q) for your class and the known frequencies for the North American population is significant. Use the chi-square test (Appendix I).

c. How do you explain any discrepancy in these frequencies? ______________________

____

✔ EXERCISE C | The Founder Effect as an Example of Genetic Drift

As indicated in Exercise B, the Hardy-Weinberg principle states that five conditions must be met for allelic frequencies to remain constant. Briefly stated, these are the conditions required: (1) no mutation, (2) random mating, (3) large population, (4) no migration, (5) no selection. Under these conditions, evolution will not occur. *Why not?* ____

____

These conditions are so restrictive that they are almost never met in nature, even for short periods. The consequence of this is that allelic frequencies are constantly changing. In Exercises C and D you will explore two cases in which allelic frequencies change. In this exercise, the change in allelic frequencies, genetic drift, is caused by small population size (the **founder effect**). In Exercise D you will discover how **migration** can also cause fluctuations in allelic frequencies.

Genetic drift is the change in allelic frequencies that results from the random outcome of matings. An analogy would be a coin toss. If 100 people each tossed coins at the same time, we would be very surprised if 50 percent of the coins turned up heads and 50 percent tails on toss after toss. The same chance deviations from predicted frequencies occur for combinations of alleles. For example, even if the frequency of both *B* and *b* is 50 percent in a population, and *BB, Bb,* and *bb* zygotes are all equally viable, in every "round" of mating, the percentage of zygotes with *BB, Bb,* and *bb* genotypes will undergo chance fluctuations from their calculated frequencies of 25 percent, 50 percent, and 25 percent.

Drift is exaggerated in small populations such as founder populations. A **founder population** is a small segment of a population that splinters off from a main population. Often a founder population, because of its small size, has a gene pool that differs from that of the parent stock; the incidence of a rare inherited condition may be greater in the founder group than in the parent group.

If we continue the coin-toss analogy, but with only two people (a founder population) tossing coins, the frequency of heads will fluctuate from 0 percent to 50 percent to 100 percent. Likewise, small populations experience larger chance fluctuations in allelic frequencies.

One last coin-toss analogy should help to confirm the importance of genetic drift. Say that we have a rule stating that *if all coins in the toss turn up heads, then all future coins will be made into heads on both sides, and tails will never be seen in the "population" again.* If there are 100 coins in the toss, there is little chance that all 100 coins will land heads up at the same time. But if there are only two, then within a very few tosses both coins will come up heads, and tails will vanish forever.

This is similar to the dynamics of genes in a population. If the population is *small,* there is a good chance that random fluctuations will, over many generations, result in the *loss* of an allele when its frequency randomly fluctuates to zero. We say that the allele has become "extinct" and that the alternative

allele has been "fixed" in the population. Unless migration or mutation brings the extinct allele back into the population, it has disappeared forever. Thus, even if all other conditions for the Hardy-Weinberg equilibrium hold, when the population is small, genetic drift can cause rapid and permanent changes in gene frequencies.

One practical consequence of drift is the damaging effects of such "genetic bottlenecks" in, for example, the small population of an endangered species. Drift may cause a loss of many alleles and a drastic reduction in genetic diversity. Even if the population recovers in numbers of individuals, its reduced ability to adapt to changing conditions may assure its eventual extinction.

Objectives

- ☐ Observe the founder effect using a simulation in which a founder population becomes isolated from its parent population.
- ☐ Define genetic drift and explain why it occurs when small populations become isolated from parent populations of organisms.
- ☐ Explain why drift produces more severe changes in allelic frequencies in smaller populations.

Procedure

1. Divide the class into groups of four students. Each group should obtain a jar containing beads of two colors. The two colors represent the two alleles (*A* and *a*) of a particular gene. Your instructor will give you the exact count for each color and tell you which color bead produces the recessive trait that you will follow in the founder population. [Beads representing the recessive (and in this case, rare) allele will be fewer in number than those representing the dominant allele.] Compute the frequency of each color (p and q) by dividing the number of beads of each color by the total number of beads. Record the allelic frequencies in the spaces below "Parent Generation" in Table 20C-1.

2. Each pair of individuals in your group should pick beads randomly from the jar (without looking), placing a total of 10 beads in each of 10 cups to represent founder populations of 5 individuals (10 beads; 2 alleles per individual). Count the number of rare alleles in each cup and record your data in Table 20C-1. Record class data in the same table, indicating for each group the number of cups that contain 0, 1, 2, 3, or more rare alleles.

3. Compute the percentage of populations (cups) containing 0, 1, 2, 3, or more rare alleles by dividing the total number of cups in each column by the total number of cups in the class. Record these percentages in Table 20C-1.

4. Examine the class data.

a. Are there any founder populations (cups) in which the rare alleles are absent? ________

b. In what percentage of all populations is the rare allele absent? ________

c. Are there any founder populations (cups) in which the rare allele appears in higher proportions than in the parent population? ________

d. In what percentage of all populations is the allelic frequency the same as in the parent population? ________

e. From your data, what do you observe about the allelic frequencies of small founder populations compared with those of parent populations? ____

____

5. From the class data, determine the range in allelic frequencies for the rare allele. (What are the highest and lowest allelic frequencies when all the cups in the class are considered?) Record your data in Table 20C-2 for founder populations of 5 individuals.

Table 20C-1 Data for Comparison of Allelic Frequencies in Parent Population and Founder Populations

Parent Generation $p =$ ____; $q =$ ____	Number of Founder Populations (Cups) Containing the Following Numbers (0–6) of Rare Alleles							
	0	1	2	3	4	5	6	
Group 1								
Group 2								
Group 3								
Group 4								
Group 5								
Group 6								
Group 7								
Group 8								
Group 9								
Group 10								
Group 11								
Group 12								
Group 13								
Group 14								
Group 15								
Group 16								
Total no. of cups with indicated no. of rare alleles								Total cups ____
Percent of all populations with indicated no. of rare alleles								

Table 20C-2 Range of Allelic Frequencies in Founder Populations

Population Size (number of individuals)	Lowest q	Highest q
5		
10		
25		
50		

6. Transfer your data for founder populations of 5 individuals that contain zero rare alleles (the column under “0” in Table 20C-1) to the first column in Table 20C-3.

Table 20C-3 Effect of the Size of Founder Populations (5, 10, or 25) on the Frequency of Rare Alleles

	Number of Founder Populations with 0 Rare Alleles for Populations of 5, 10, or 25 Individuals		
	5	10	25
Group 1			
Group 2			
Group 3			
Group 4			
Group 5			
Group 6			
Group 7			
Group 8			
Group 9			
Group 10			
Group 11			
Group 12			
Group 13			
Group 14			
Group 15			
Group 16			
Total no. of cups or groups of cups with zero rare alleles			
Percent of all populations with zero rare alleles			

7. Now you will increase the size of the founder population to 10. Randomly (so you may want students from another group to help) choose pairs of cups from your set of 10 cups. Each pair of cups represents a founder population of 10 individuals (20 beads; 2 alleles per individual).

f. Do you have any populations with rare alleles? ________

8. Collect class data for the number of populations with zero rare alleles. Record the data in Table 20C-3 in the column under "10."

g. What percentage of populations (of 10 individuals) in the class have zero rare alleles? ________ *Record this in Table 20C-3. Is this more or less than observed when founder populations were smaller (only 5 individuals)?* ________________

9. From the class data, determine the range in allelic frequency for the rare allele in populations of 10 individuals. Record your data in Table 20C-2.

10. Randomly group your 10 cups into two founder populations of 5 cups each to represent founder populations of 25 individuals (50 beads; 2 alleles per individual). Collect class

data for the number of populations with zero rare alleles. Record your data in Table 20C-3 under "25."

h. Now, what percentage of populations (of 25 individuals) in the class have zero rare alleles?

______ *Record this in Table 20C-3. Is this more or less than observed when populations were smaller?* ____________

11. From the class data, determine the range in allelic frequency for the rare allele in populations of 25 individuals. Record your data in Table 20C-2.

12. If you consider all 10 cups as a single population of 50 individuals, what are the allelic frequencies for *A* and *a* in the population?

$A(p) =$ ________

$a(q) =$ ________

13. From the class data, determine the range in allelic frequency for the rare allele in populations of 50 individuals. Record your data in Table 20C-2.

i. From your data and observations, what do you conclude about the relationship between the size of a founder population and the likelihood that alleles may be "lost" (become extinct) due to drift when founder populations become isolated from parent populations? ____________________________

____

j. From your data and observations, what do you conclude about the size of a founder population and the likelihood that it will resemble the parent population (will have similar allelic frequencies)?

____

____

k. What could be the consequences of drift for a small population? ____________________________

____

EXERCISE D | The Role of Gene Flow in Similarity Between Two Populations

Gene flow is the term used by population geneticists to describe the movement of alleles from the gene pool of one population to the gene pool of another. Gene flow occurs as **emigration** (individuals leaving the population) or **immigration** (individuals entering the population). When gene flow takes place randomly between two populations, that is, without respect to the phenotype of the immigrants and emigrants, then the frequencies of alleles in the two populations approach equality, and because of this the two populations become more genetically similar.

An analogy may help explain the role of gene flow in making two populations more similar. Imagine several small ponds, some of which are sheltered from the sun and are cold, and some of which are in the open and are warm. If all the ponds are interconnected by underground pipes and there is a constant exchange of water between them, the temperatures in each pond will become very similar. However, if the water exchange stops, each pond's temperature may vary greatly from that of the other ponds. Likewise, when populations have even a low level of gene flow due to interbreeding, their allelic frequencies will tend to be similar and fluctuations in allelic frequencies will be moderated. (Even in founder populations, separated but not totally isolated from parent populations, fluctuations in allelic and genotypic frequencies may be moderated if some interbreeding with the parent population is still going on.)

In all cases, however, if interbreeding stops, each isolated population will feel the full effects of drift; allelic frequencies may fluctuate drastically, and alleles may be lost. Thus, it is small *isolated* populations that usually give rise to *new species.*

Objectives

☐ Demonstrate, using a simulation, the effect of gene flow on the genetic similarity of two populations that exchange individuals.

Procedure

On the lab table are two trays, labeled 1 and 2, containing beads that represent pairs of alleles—*A*, the dominant allele, and *a*, the recessive allele—in the gene pools of two neighboring populations. Note that the proportions of the two colors of beads in the two trays are different, indicating that the two populations are genetically different.

1. Work in pairs. Count the number of beads of each color in tray 1 and calculate the frequencies (p_1 and q_1) of the two alleles represented by the two different colored beads. Enter these frequencies as p_1 and q_1 in Table 20D-1. Repeat this procedure for tray 2 and record the initial frequencies as p_2 and q_2.
2. Shake the two trays to distribute the beads randomly. Without looking at the beads, draw (one at a time) 10 beads from tray 1. At the same time your partner will draw 10 beads (also one at a time) from tray 2. Transfer your beads to tray 2 *at the same time* that your partner transfers beads to tray 1.

   a. Explain how these transfers of beads are related to immigration and emigration of individuals between two populations. ______________________

   ____

3. Count the beads in each tray, calculate the frequencies of the two alleles for the two trays, and record these frequencies for the first round of gene flow in Table 20D-1.

Table 20D-1 Changes in Allele Frequencies of Two Populations Experiencing Gene Flow

Round of Gene Flow	Tray 1		Tray 2		Differences Between Trays	
	p_1	q_1	p_2	q_2	$p_1 - p_2$	$q_1 - q_2$
One						
Two						
Three						
Four						
Five						

4. Repeat steps 2 and 3 four more times, each time recording the allele frequencies for both trays in Table 20D-1 for the appropriate round.
5. Complete the rest of the data table by subtracting corresponding allele frequencies ($p_1 - p_2$ and $q_1 - q_2$) for the two trays.

   b. How does the difference in the final frequency of allele A *(that is,* $p_1 - p_2$*) compare with the difference in the original frequency (first line of table)?* ______________________

   ____

c. On which round of gene flow did the allele frequencies in the trays show the greatest change?
_________ *Is this what you expected?* _______ *Why?* ____________________

____

d. On which round was the genetic makeup of the two gene pools most nearly the same? _________
How would you expect the two gene pools to compare at the end of 10 rounds of gene flow?

____

At the end of 20 rounds? ____________________

____

e. What does this simulation indicate about the effect of gene flow on the genetic makeup of neighboring populations? ____________________

____

f. In each of the following cases, explain how the results of the simulation would have been changed.
Only 4 beads, instead of 10, are transferred between trays on each round.

____

Beads are transferred from tray 1 to tray 2, but are not transferred from tray 2 to tray 1.

____

EXERCISE E | The Effect of Selection on the Loss of an Allele from a Population

Evolution is a change in frequencies of alleles and genotypes in populations over time. This change can be brought about by mutation, drift (see Exercise C), immigration or emigration (see Exercise D), and **selection.**

Selection refers to the differential rate of reproduction of different genotypes in a population. Each individual in a population, however, is the sum of *all* of its genotypes for *all* characteristics. Their cumulative expression gives rise to an individual's **phenotype:** the expression of many different genes, often working in a coordinated fashion. Those individuals whose phenotype is best adapated to their environment are more "fit"—they have a greater chance to survive and reproduce, contributing more offspring, and thus more alleles of certain types, to the next generation.

A single allele rarely determines **fitness** or a "winning" phenotype. If an African antelope has superior genes for every characteristic except watchfulness for lions, its phenotype will have reduced fitness if lions are very active in its habitat. All of its "superior" genes will not be passed on to the next generation because they are bound up in one package with its nearly lethal "predator watchfulness" characteristic. Thus, it is differential rates of reproduction of different phenotypes, resulting from interactions of organisms with their environments, which gives rise to changes in the relative frequency of alleles and genotypes in a population—an overall process that biologists call **natural selection.**

In a population, genotypes that reduce the fitness of the phenotype are said to be "selected against." Given the reduced reproductive potential of the individuals carrying these genotypes, deleterious alleles responsible for the genotypes should slowly decrease in frequency and eventually disappear from the gene pool of the population. This might raise the question of why any deleterious alleles are left after millions of years of natural selection. Several factors tend to slow or stop the allele extinction process:

1. Elimination of deleterious alleles is slow, perhaps requiring hundreds or even thousands of generations, *except* when the selective disadvantage of the genotype is severe.
2. Mutation or immigration might introduce new copies of deleterious alleles.
3. The "disadvantaged" genotype may be selected against only when it is abundant, and may even enjoy a selective advantage when it is rare (frequency-dependent selection).

4. Recessive deleterious alleles may not experience any selective disadvantage when they are masked by the dominant allele.
5. Heterozygotes may experience a selective advantage over either homozygote, and thus both alleles will persist.

In this exercise, you will explore recessiveness and heterozygote advantage as factors that tend to maintain deleterious alleles in a population.

PART I The Effects of Recessiveness on Deleterious Alleles

Deleterious alleles are those that have a detrimental effect on any organism that contains them as part of its genome. Most deleterious alleles exist as the recessive form of a gene at a particular locus. Let us say that the *B* gene exists as two alleles, *B* and *b*, and *b* is deleterious. The homozygous recessive genotype is most selected against because two copies of the maladaptive gene are present. In many cases, the homozygous recessive condition (*bb*) is lethal. Although the homozygous dominant genotype is unaffected, the heterozygote may also be affected, depending on how much the dominant allele is able to mask the effects of the deleterious recessive allele.

If the *Bb* heterozygote suffers no selective disadvantage (*B* masks *b*), then the *b* allele may persist for long periods in the population by "hiding out" in the *Bb* heterozygote. This heterozygote "refuge" will also become more and more effective as the *b* allele becomes increasingly rare. This occurs because the *b* allele will experience selective disadvantage only when a *bb* offspring occurs, and this will happen only when two heterozygotes mate. If the heterozygotes are affected (*B* does not mask *b*) and the heterozygotes become rare, then almost all matings of heterozygotes will be with *BB* organisms and the *b* allele will persist.

This part of the exercise will illustrate this principle. A population is isolated in an area where a particular allele (*b*) has become deleterious. First, you will determine the persistence of the deleterious *b* allele when *B* does not mask *b;* the *B* allele is only incompletely dominant over *b*, and *bb* and *Bb* experience selective disadvantage. Then you will do the same exercise for when *B* masks *b;* the *B* allele is completely dominant and only *bb* is at a disadvantage.

Objectives

- ☐ Determine the relationship between persistence of an allele in a population and its "fitness."
- ☐ Determine the effects of selection on the persistence of deleterious alleles in a population.

A Allele *B* Does Not Mask Allele *b*

In this simulation, all *bb* individuals die before reproducing, and there is a 50 percent chance that *Bb* individuals will die.

Procedure

1. Divide the class into groups of four students. For each group, students will work in pairs. Each pair of students should obtain a cup. Place into the cup 16 beads of one color representing the common (dominant) allele and 4 beads of a different color representing the rare (recessive) allele, for a total of 20 beads. Your instructor will tell you which colors represent the two kinds of alleles (*B* and *b*) of a particular gene. (If selected by pairs, each pair of alleles would represent an individual of a particular genotype.)
2. The sum of *all* beads (40) in the two cups of both pairs of students represents the total gene pool of zygotes making up the parent population. This parent population is composed of half males and half females; one pair of students represents the 10 females in the population while the other pair of students represents the 10 males.

   a. If there are 20 beads representing each sex, why are there only 10 individuals of each sex and not 20? ______________________________

You will consider this to be a *pre-reproductive* population since some zygotes representing particular genotypes (all *bb* and some *Bb*) may never mature to reproductive age (they die early). In the space below, compute the frequency of each allele (p and q) in the total pre-reproductive parent population by dividing the total number of beads of each color in both cups of the pre-reproductive parent population by the total number of beads in the population:

$$\text{Allele frequency} = \frac{\text{total number of one color in both cups}}{40}$$

Record the allelic frequencies of the pre-reproductive parent generation in Table 20E-1.

Table 20E-1 Effects of Recessiveness on the Persistence of a Deleterious Allele When *B* Does Not Mask *b*

Generation	Frequency of Alleles		Frequency of Genotypes		
	p (*B*)	q (*b*)	p^2 (*BB*)	$2pq$ (*Bb*)	q^2 (*bb*)
Parent					
F_1					
F_2					

3. Each pair of students should now pick pairs of beads from the cup randomly (close your eyes) and line up the 10 bead pairs on the laboratory table. One pair of students has 10 females and the other pair of students has 10 males. These represent the genotypes of the parent individuals. Compute the genotypic frequencies:

$$\text{Genotypic frequencies} = \frac{\text{number of beads of a particular genotype}}{20}$$

Record the observed genotypic frequencies for all 20 bead pairs representing individuals of the pre-reproductive parent population in Table 20E-1.

4. The parent individuals will now pair up and reproduce to form an F_1 population of offspring. First, however, you must eliminate all potential parents that do not reach reproductive maturity. Since all *bb* individuals die before reaching reproductive age, eliminate these individuals by placing the beads in a discard container. Since there is only a 50% chance that heterozygotes will live to reproductive maturity, for each heterozygote, flip a coin to determine if it will live to reproduce (heads = lives; tails = dies).

Rules for Fate of Individuals When *B* Does Not Mask *b*

Individual	Fate
BB	All live
Bb	Flip a coin to determine if individuals will live (heads = lives; tails = dies)
bb	All die

5. Rearrange the remaining "reproductive" parents in a row, left to right. A group of four students should now have two rows of bead pairs—a row of females and a row of males. If there are more individuals of one sex than the other, eliminate the excess bead

pairs at the right end of your row to even up the number of males and females (in this population, males and females pair-bond for life).

6. Now, pair each male (2 beads) with a female (2 beads). Determine the genotypes of the individuals in each mating pair. If the individual is *BB*, you know that he or she will donate a *B* allele to form an offspring. If an individual is *Bb*, flip a coin to determine which allele is contributed to the new offspring, *B* or *b* (heads = *B*; tails = *b*). Each mating pair should make *four* offspring in this manner, males and females donating one allele at a time in a gamete (it takes two gametes to form a zygote, thus each new individual has two alleles represented by a pair of beads). Obtain beads from the reserve containers on the table to make offspring. Line up the F_1 generation individuals on the table as they are created.
7. After all parent individuals have reproduced to form F_1 individuals, be sure to remove all parent individuals from the table, placing the beads in the discard container.
8. Count the number of beads of each color and the total number of all beads in the F_1 generation to calculate the allelic frequencies (p and q) for the F_1 generation. Record these values in Table 20E-1.

   b. How do these frequencies compare with the allelic frequencies of the parent generation?

   ____

9. Record the observed genotypic frequencies of the pre-reproductive F_1 generation.

   c. What trend do you see as you compare the genotypic frequencies of the parent and F_1 *generations?*

   ____

   ____

10. Repeat steps 5–8 using the F_1 generation to produce an F_2 generation. Record all data in Table 20E-1.

    d. What trend do you observe in allelic frequencies for the B *does not mask* b *experiment?*

    ____

    e. Why do you think this trend occurs? ______________________________________

    f. Are you data representative of the class data? ________

11. Put all beads that remain on the table into the discard container.

B Allele *B* Masks Allele *b*

In this simulation, *B* masks *b*, so all *BB* and *Bb* individuals live and are reproductively successful. Only *bb* individuals die.

Procedure

1. Return to step 1 in Part 1A, and start with a new population.
2. Repeat steps 1–10, but use the following rules for *B* masks *b* when determining which individuals will or will not live to reproductive maturity.

Rules for Fate of Individuals when *B* Masks *b*

Individual	Fate
BB	All live
Bb	All live
bb	All die

3. Record all data for parental, F_1, and F_2 allelic frequencies and genotypic frequencies in Table 20E-2.

Table 20E-2 Effects of Recessiveness on the Persistence of a Deleterious Allele when *B* Masks *b*

	Frequency of Alleles		Frequency of Genotypes		
Generation	*p* (*B*)	*q* (*b*)	p^2 (*BB*)	2*pq* (*Bb*)	q^2 (*bb*)
Parent					
F_1					
F_2					

g. What trend do you observe in allelic frequencies for the B *masks* b *experiment?*

____

h. Why do you think this trend occurs? ______________________________

____

i. Why do recessive deleterious alleles that are masked by a dominant allele persist longer in a population than alleles that cannot be masked by other alleles? ______________________________

____

4. Compare your observations for *B* masks *b* with those for *B* does not mask *b*.

j. Did you see the effect described in question h *in your data (*b *persisting longer when masked by* B*)?*

____

k. If recessiveness will help a deleterious allele persist longer in a population, would recessiveness speed or hinder the spread of a beneficial allele? ______________

Why? ______________________________

____

5. Put all beads that remain on the table into the discard container.

PART 2 Heterozygote Advantage

Another way in which deleterious alleles are preserved is if heterozygotes have a selective advantage over either of the homozygotes (assuming that only two alleles are possible at a locus). In the extreme case in which both homozygotes (say *bb* and *BB*) die before reproducing and only the heterozygote survives, both the *B* and *b* alleles will persist in the heterozygotes and both *bb* and *BB* individuals will continue to be produced from *Bb* × *Bb* matings, driving the allelic frequencies toward $p = 0.5$ and $q = 0.5$. *Why?*

____

A classic example of heterozygote advantage is the persistence of sickle-cell anemia in Africa. Sickle-cell anemia is a severe blood disease present in *ss* individuals. *Ss* individuals have some mild sickle-cell symptoms, but they are also resistant to malaria. Normal (*SS*), nonanemic individuals may suffer disability or death if they become infected with malaria. In areas of eastern Africa where malaria is prevalent, heterozygotes have a selective advantage of 26 percent over normal individuals, which seems to preserve the sickle-cell allele (*s*) in the gene pool. In some places, 45 percent of the population are heterozygotes. However, in areas where malaria is not common, *Ss* individuals have no advantage and the sickle-cell allele is eliminated. It is estimated that 300 to 350 years ago, 22 percent of the slaves in the

American South carried the sickle-cell allele; but by the early 1950s, only 9 percent of the black American population carried the allele.

In this simulation, you will demonstrate the preservation of a deleterious allele by heterozygote advantage. You will also observe the disappearance of a deleterious allele when the heterozygous condition is no longer advantageous.

Objectives

☐ Explain heterozygote advantage and how it may maintain alleles in a population.

Procedure

1. Follow the same procedures used in Exercise E, Part 1. Remember that all couples must produce *four* viable offspring. However, new rules for heterozygote advantage apply for reproduction.

Rules for Fate of Individuals in Heterozygote Advantage

Individual	Fate
BB	Flip a coin to determine if individuals will live (heads = lives; tails = dies)
Bb	All live
bb	All die

2. Repeat steps 1–10 of Part 1A. Record all data for parental, F_1, and F_2 allelic frequencies and genotypic frequencies in Table 20E-3.

Table 20E-3 Effects of Heterozygote Advantage on Allelic Frequencies

	Frequency of Alleles		Frequency of Genotypes		
Generation	p (*B*)	q (*b*)	p^2 (*BB*)	$2pq$ (*Bb*)	q^2 (*bb*)
Parent					
F_1					
F_2					

a. Compare the allelic frequencies of the parent population with those of the F_1 *and* F_2 *generations.*

____

____

b. Does the recessive allele ever disappear? ________ *Why or why not?* ______________________________

____

c. Toward what values of p *and* q *is the population in this simulation moving?*

____

Why? ______________________________

d. Could either p *or* q *be greater than 0.5 in this simulation?* ______

Why or why not? ______

e. Compare the genotypic frequencies of the parent population with those of the F_1 *and* F_2 *generations.*

______

______

f. Is your population typical of most of the populations in the class? ______

g. Explain how heterozygote advantage can maintain two or more alleles in a population.

______

______

h. Suggest why a deleterious allele that persists because of heterozygote advantage disappears once the heterozygote advantage is removed. ______

______

______

EXTENDING YOUR INVESTIGATION: SELECTION PRESSURE

Selection pressure is often placed on certain **prey** phenotypes by **predators.** Predators may be more successful at capturing prey with coloration or behavior that attracts attention and less successful at capturing those that blend into their habitat (cryptic coloration). For instance, variations in the color or markings of certain insects or their larvae affect predation by birds.

You will use yarn of different colors to simulate "wooly worms" of different phenotypes distributed on the grass at the test site. You will be given 10 minutes to collect as many pieces of yarn as you can. If collection of yarn is random, like flipping a coin, then the class should collect nearly equal numbers of all colors of yarn. If there are significant differences in the colors collected, it is possible that the differences can be explained by a process of selection. Formulate a hypothesis that can be used to explore the relationship between "wooly worm" phenotype and selection.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will happen to the "wooly worm" population being preyed upon in this experiment?

Identify the **independent variable.**

Identify the **dependent variable.**

Use the following procedure to test your hypothesis.

PROCEDURE:

1. Work in pairs. At the test site, collect as many pieces of yarn as possible in the 10 minutes allotted.
2. Return to your laboratory and count the number of pieces of each color of yarn collected. Record the numbers in column A of Table 20-I. Total all the numbers of column A and enter the sum at the bottom of the column.
3. The expected number for each color—that is, the number we would expect if the number collected were affected only by chance—can be determined as follows:

$$\frac{\text{Total number collected}}{\text{Number of colors used}} = \text{number expected for each color}$$

Enter this number in column B for every color. (It will be the same for each color.)

Table 20-I Chi-Square Calculations

Color	**A** No. Observed (collected)	**B** No. Expected (by chance)	**C** Observed − Expected	**D** (Observed − Expected)^2	**E** (Observed − Expected)^2/ Expected
1.					
2.					
3.					
4.					
5.					
6.					
7.					
8.					
9.					
10.					
11.					
12.					
13.					
14.					
15.					
Total number observed =		Sum of chi-square values =			

4. Use Table 20-I to determine the chi-square value for each color (see Appendix I for an explanation of the chi-square test).

a. Subtract the number in column B from the number in column A and enter the result in column C. Numbers may be positive or negative.

b. Square each number in column C and enter it in column D (negative numbers become positive).

c. Divide each number in column D by the number in column B.

d. Add all the numbers in column E to get the sum of chi-square values. Enter this number in the table.

RESULTS: You have completed the chi-square calculations. You can now check your results against the chi-square distribution (Table 20-II) to determine the probability that chance alone was responsible for the numbers of colored yarn pieces collected.

Degrees of Freedom. This will always be 1 less than the total events (colors) observed. For instance, if 10 colors of yarn were collected (observed), 9 degrees of freedom will be used.

Probability. The columns marked $p = 0.99$, 0.95, 0.50, 0.05, 0.01, and 0.001 refer to probabilities that your observations differ from those expected based on the actual numbers of yarn pieces of each color distributed. For instance, if 10 colors were used (9 degrees of freedom) and you obtained a chi-square value of 8.5, you could conclude that the numbers observed vary from expected numbers about 50 percent of the time. Thus, chance alone could cause such a variation and the null hypothesis is supported. However, if you obtain a chi-square value of 17 (16.919) or higher, which gives a probability of 0.05 (or less), this indicates that the numbers collected or observed would show such a variation *by chance alone* only about 5 percent of the time. In other words, there is only 1 chance in 20 that the variation you observed is by chance alone. The smaller the probability, the less likely it is that the results are due to chance. In general, researchers consider a 0.05 probability as a minimum for considering results to be due to factors other than chance. However, remember that even if you obtain a probability level of 0.05, there is still 1 chance in 20 that your results are due only to chance.

From your results, describe the effects of selection on your population of "wooly worms."

Do your results support your hypothesis?

Your null hypothesis?

Was your prediction correct?

What do you **conclude** about the effects of varying phenotypes on their selection?

Which colors of yarn were subjected to positive selection pressure and which to negative selection pressure?

Why?

If the environment remains constant over time, how will gene frequencies be affected in future generations?

How could you increase the level of certainty that the results you obtained are due to selection and not to chance? (*Hint:* Think about Mendel's experiments with peas, and especially about the experimental setup he used to obtain the 9:3:3:1 ratio.)

Table 20-II Chi-Square Distribution*

Degrees of Freedom	$p = 0.99$ (99 in 100)	$p = 0.95$ (95 in 100)	$p = 0.50$ (50 in 100)	$p = 0.05$ (5 in 100)	$p = 0.01$ (1 in 100)	$p = 0.001$ (1 in 1,000)
1	<0.001	0.004	0.455	3.841	6.635	10.827
2	0.020	0.103	1.386	5.991	9.210	13.815
3	0.115	0.352	2.366	7.815	11.345	16.286
4	0.297	0.711	3.357	9.488	13.277	18.465
5	0.554	1.145	4.351	11.070	15.086	20.517
6	0.872	1.635	5.348	12.592	16.812	22.457
7	1.239	2.167	6.346	14.067	18.475	24.322
8	1.646	2.733	7.344	15.507	20.090	26.125
9	2.088	3.325	8.343	16.919	21.666	27.877
10	2.558	3.940	9.342	18.307	23.206	29.588
11	3.053	4.575	10.341	19.675	24.725	31.264
12	3.571	5.226	11.340	21.026	26.217	32.909
13	4.107	5.892	12.340	22.362	27.688	34.528
14	4.660	6.571	13.339	23.685	29.141	36.123
15	5.229	7.261	14.339	24.996	30.578	37.697
	High probability that results are due to chance			Low probability that results are due to chance		

**p* is the probability that results could be due to chance alone. The numbers in parentheses below each value of *p* restate *p* in terms of chance. For example, at a *p* value of 0.01, chances are 1 in 100 that results are due to chance.

Laboratory Review Questions and Problems

1. What is an allelic frequency?

2. If you have a complete list of genotypic frequencies for a population, can you calculate allelic frequencies? Always? (Consider populations that *are* and *are not* in Hardy-Weinberg equilibrium.)

3. In a population that is 10 percent *AA,* 20 percent *Aa,* and 70 percent *aa,* what is the frequency of allele *A?* What is the frequency of *a?*

4. If *A* and *a* are the only alleles at the gene *A* locus, does the population in question 3 seem to be at Hardy-Weinberg equilibrium? If not, list some possible reasons why.

5. How would you tell if a population is at Hardy-Weinberg equilibrium?

6. A simple Mendelian trait has two alleles, *D* and *d*. If a population is in Hardy-Weinberg equilibrium and is 49 percent homozygous dominant, what percentage is heterozygous? What are the allelic frequencies in the population?

7. If you know the allelic frequencies for a particular gene (e.g., one that determines the degree of attachment of earlobes) in your class, can you calculate the genotypic frequencies for your class? Why or why not?

8. If you know allelic frequencies for a population, can you always calculate genotypic frequencies? Why or why not?

9. If you know the fraction of homozygous recessive individuals in a population, can you always estimate the allelic and genotypic frequencies for that population? What conditions must be met by the population in order to do this? (Compare your answer with that for question 6.)

10. In a population that is in Hardy-Weinberg equilibrium, the frequency of the *d* allele is 0.4. What fraction of the population is *DD?* Why is it important to mention that this population is in Hardy-Weinberg equilibrium?

11. What five conditions must be met for the Hardy-Weinberg equation to predict the genetic makeup of a population accurately? Why do we consider the Hardy-Weinberg prediction to be a "null" hypothesis?

12. When you relax (or violate) any of the conditions necessary for Hardy-Weinberg equilibrium, what happens to allelic frequencies in a population?

13. What is genetic drift? Why can drift usually be ignored in a large population, but never in a small population?

14. Explain why nonrandom mating and migration can disrupt Hardy-Weinberg equilibrium.

15. Can a population evolve solely due to genetic drift? Explain.

16. In Exercises B through E, which Hardy-Weinberg criteria did you violate? In each case, what happened to allelic frequencies?

Exercise B

Exercise C

Exercise D

Exercise E

17. In Exercise E, Parts 1A, 1B, and 2, which genotypes were favored? Why?

Part 1A

Part 1B

Part 2

18. To illustrate Hardy-Weinberg principles, a biology textbook describes the case of a homozygous recessive fatal disease that occurs in 4 percent of a population. Students are asked to use the Hardy-Weinberg equation to compute p and q for this population. Is this an appropriate use of the Hardy-Weinberg equation? Why or why not?

Genetic Basis of Evolution II—Diversity

LABORATORY

21

OVERVIEW

Evolution is one of the major unifying themes in biology. Organismal diversity (Laboratories 22 to 27) is a consequence of ongoing evolution.

The study of biological diversity is called **systematics.** Systematists reconstruct the evolutionary history or **phylogeny** of a group of related organisms, making it easier to classify the diversity of fossils as well as organisms alive today. Phylogenetic classification schemes group organisms into assemblages based on common ancestry. Traditionally, morphological, embryological, and fossil evidence have been compared to determine common lineage. Recently, molecular evidence from analysis of proteins, DNA, and RNA have also been gathered and used for building phylogenies.

In this laboratory, you will investigate the phylogenetic relationships among members of a group of ungulate mammals ("hoofed" herbivores). Electrophoretic analysis of structural differences in the protein lactate dehydrogenase (LDH) will provide evidence for determining evolutionary relationships among cows, goats, sheep, and horses.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency. Complete Exercise A before coming to the laboratory.

✓ EXERCISE A Understanding Evolutionary Classification

The goals of systematics, a branch of evolutionary biology, are to determine **relationships** among organisms, preferably based on genealogy or relationship by descent (phylogeny), and to develop useful **classifications** that reflect these relationships. The organization of species into **taxonomic groups** reflects a hierarchy of these phylogenetic relationships—from broad inclusive taxonomic groups to smaller and smaller branches of the phylogenetic tree. An understanding of relationships helps us ask relevant biological questions and understand the origins and adaptations of organisms making up our world. An understanding of classification helps us organize the diversity ("biodiversity") in our global environment and is necessary for the successful management of our total ecosystem.

Every organism is classified using a hierarchy of categories—kingdom, phylum or division, class, order, family, genus, and species (with some intermediate levels). Classification defines the position of a species within a broader phylogenetic spectrum of taxonomic groups.

A group of organisms at any level in the hierarchy (species, genus, family, etc.) is called a **taxon** (plural, taxa). A taxon is **monophyletic** if a single ancestor has given rise to all species within that group

and to no other species in any other group. (Note that monophyly is relative. In one sense, all birds may be regarded as monophyletic; but so may all vertebrates or all animals. The concept of monophyly is most useful within genera or families.) Ideally, all taxa *should* be monophyletic. A taxon is **polyphyletic** if its members are derived from more than one ancestor. (It is not always possible to separate species phylogenetically, particularly in less known taxa or in groups that do not reproduce sexually, because reproductive isolation is the criterion for separating biological species. Certain taxa may be "dumping grounds" or may contain species placed there provisionally pending further study.) A taxon is **paraphyletic** if it includes the group and its common ancestor but excludes all other groups that may also have come from the common ancestor. (We know that birds and mammals trace their ancestry to somewhat different groups of reptiles; monophyly would suggest that all three "classes" be placed together. Our common practice of separating reptiles, birds, and mammals, by criteria you will use in Exercise A, provides a useful classification in spite of being "paraphyletic.")

A major goal of systematics is to determine phylogenies and to arrange taxa into monophyletic groups related by descent.

Objectives

- ☐ Identify levels of phylogenetic classification.
- ☐ Distinguish among monophyletic, polyphyletic, and paraphyletic classifications.
- ☐ Understand approaches by systematists to determine relationships and construct classifications.

Procedures

1. Use information in your text to classify humans.

   Kingdom ____________________

   Phylum ____________________

   Subphylum ____________________

   Class ____________________

   Subclass ____________________

   Order ____________________

   Family ____________________

   Genus ____________________

   Species ____________________

   Be sure that the species name consists of *two* words—the genus name and the species (or "specific") epithet (an adjective)—providing a **binomial nomenclature** as first practiced consistently by Linnaeus. Note that the species epithet is an adjective and can *never* be used alone. Thus *Homo* is humans' generic name. The species name is *Homo sapiens.* (Where the complete binomial species name has been used and there is no possibility of confusing *Homo* with any other generic name, you may see the species name abbreviated *H. sapiens.* But never, under any circumstances, can humans be named using only the specific epithet "*sapiens.*")

2. In Figure 21A-1, circle all monophyletic groups and label them "M."
3. Circle all polyphyletic groups and label them "P."
4. Circle all paraphyletic groups and label them "A."
5. In the hypothetical phylogenetic tree of primates (Figure 21A-2), circle all monophyletic, polyphyletic, and paraphyletic groups and label them as in steps 2–4.
6. Within the Vertebrata (a subphylum of the Chordata), there are several classes of living organisms that are familiar to you. List the major traits or characteristics by which you would

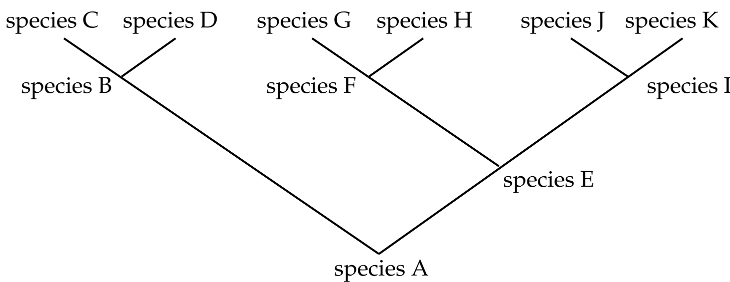


Figure 21A-1 *Hypothetical phylogeny of species groups.*

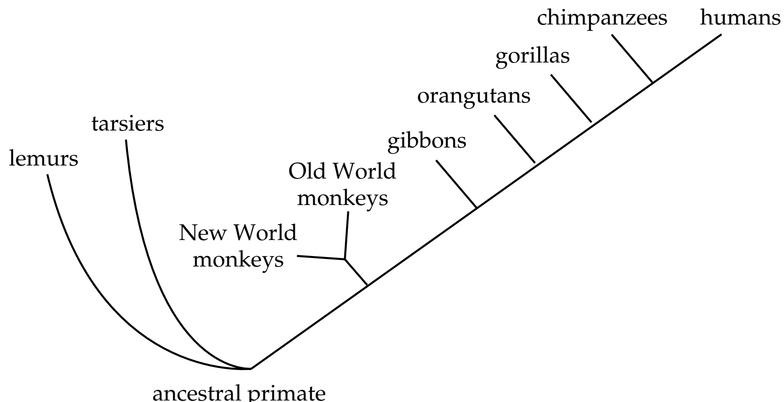


Figure 21A-2 *Hypothetical phylogeny of primates.*

recognize the following taxa. Use information from your text to group the listed organisms into their appropriate classes.

Cartilaginous fishes (sharks, skates, rays, chimaeras) ______________________________

____

Bony fishes (e.g., herring, trout, goldfish) ______________________________

____

Amphibians (frogs, salamanders) ______________________________

____

Reptiles ____

____

Birds ____

____

Mammals ____

____

a. *What features are common to all of these groups of vertebrates?* ______________________________

____

b. *What features distinguish among the groups and allow you to place* all *species in their appropriate classes?* ______________________________

____

c. *Which of these features do you suppose have a common genetic/developmental basis that verifies a true relationship?* ______________________________

d. Which of these features are similar but may not be related by ancestry?

____

____

The observations and inferences you have just made are very similar to those made by systematists in determining relationships and constructing classifications. However, it is important that **homologous traits**, those having a common genetic and developmental substrate or background, be distinguished from characteristics that resemble each other (**analogous traits**) or that provide a similar function (**homoplastic traits**). The forelimbs of fish (involved in swimming), birds (flying), and mammals (walking or running) are all homologous, even though they have different shapes and functions (and in the case of fishes, may have quite different structures). The wings of birds and beetles are, however, only analogous, even though in both cases they are involved in flying. Lungs and gills are homoplastic—both are involved in gas exchange, but they are unrelated structures.

7. Return to your list of vertebrates above and circle traits that you believe are homologous.

Early biologists classified organisms by similarities. After Darwin's publications and a general understanding of the concept of natural selection, attempts were made to refine classifications along phylogenetic lines. This trend toward a "new systematics" developed in the 1940s with publications by Dobzhansky, Mayr, Stebbins, Simpson, and many others. Systematists attempted to use a combination of traits to construct phylogenies, but the resolution of conflicts often depended on relatively arbitrary decisions (not necessarily wrong).

In the 1950s, with the advent of computer technology, a group of systematists employed numerical techniques to determine "similarities" among species—a number of characters were measured and "crunched" to construct a matrix that allowed species to be arranged by similarity (but not necessarily by relationship—no attempt is made to distinguish among homologies and analogies). Thus, the school of **phenetics** developed. With modern computers, numerical techniques have become important in all phases of systematics (particularly in the analysis of DNA and molecular data), but phenetic concepts have not satisfied our desire to understand phylogeny.

Another approach to determining relationships among organisms is based on "clades"—evolutionary branches—and the school of phylogenetic systematics developed by Hennig is commonly known as **cladistics.** Cladists determine branching of evolutionary lines by novel homologies unique to all descendants contained within that branch—so-called "synapomorphies" or shared derived characteristics. Cladists consider only branching, not the degree of evolutionary divergence among the organisms they study. This and some of the assumptions made by cladists may diminish the value of this approach and limit its ability to produce stable classifications.

Modern **evolutionary systematists** use a combination of numerical techniques and all of their understanding of the biology of a group of organisms (including both branching and divergence) in an attempt to construct realistic **phylograms** (phylogenetic trees) that can be used to produce a useful and stable classification. They recognize, for example, that modern birds as a group are uniquely defined by feathers and endothermy and have more in common with each other than with their reptilian ancestors. It is "useful" to recognize birds as a higher category even though birds and reptiles are related by descent.

8. Using the groups of vertebrates listed in step 6, draw a "cladogram" (dichotomous branches only) and a "phylogram" to express your understanding of the different approaches used by systematists.

Cladogram | Phylogram

Systematists have studied and named about 1.6 to 1.7 million species to date. Estimates of the total number of species present on the planet range from 5–6 million to 30 million or more.

e. Why is it vital for us to study and catalog this diversity immediately?

____

____

Each species contains a unique genetic library. *f. Why is it necessary for us to manage our environment and planet to preserve as much of this genetic diversity as possible?*

____

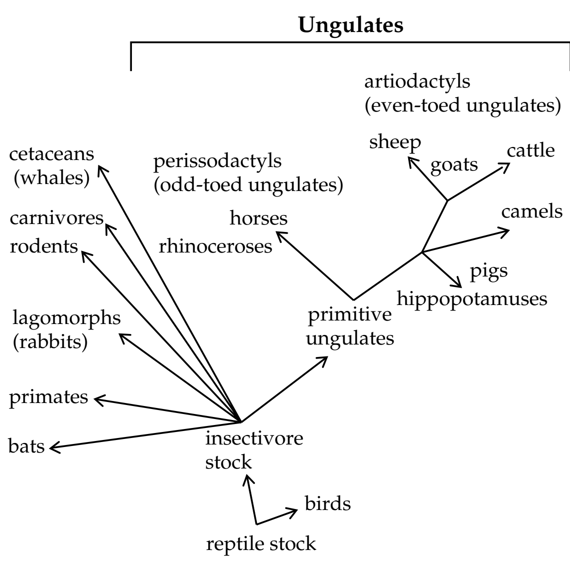
EXERCISE B | Electrophoretic Analysis of LDH in Ungulate Mammals*

As species evolve, subtle changes occur in macromolecules within organisms. These changes cause alterations in the structures of proteins, RNA, and DNA that can be studied using electrophoretic and other molecular techniques. In particular, enzyme proteins vary in composition in various organisms, giving rise to dissimilar banding patterns using electrophoresis. Systematists using molecular techniques expect differences among organisms to reflect the length of time that the lines have been distinct and the degree of divergence between those lines. They assume that small "neutral" mutations occur at a *constant* rate and that the greater the interspecies differences between chromosomal DNA, mitochondrial DNA, ribosomal RNA, and common, highly conserved proteins (such as the cytochromes), the longer the populations have been separated. Unfortunately, evolution does not always proceed at a constant rate, and so-called molecular clocks defined by this approach must be realistically calibrated. Molecular studies have been very useful and can be coupled with other systematic approaches, often with similar results.

The analysis of cytochrome *c* is probably the most noted application of these molecular techniques (molecular systematics). Human cytochrome *c* differs from the cytochrome *c* of yeast in 44 of the 104 amino acid residues. In contrast, the amino acid sequences of cytochrome *c* of humans and chimpanzees are identical. This evidence has helped confirm the ancestral link between humans and other primates.

In this exercise, you will compare the properties of a specific protein, lactate dehydrogenase, in different ungulate mammals. Ungulates are hoofed, herbivorous animals that can be divided into two major orders: the artiodactyls and the perissodactyls. Artiodactyls, having an even number of toes on each foot, include pigs, camels, cattle, sheep, and goats. Perissodactyls, with an odd number of toes, include rhinoceroses, present-day horses, and zebras. The evolution of ungulates is shown in Figure 21B-1.

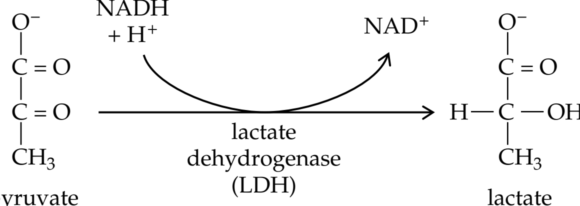
Figure 21B-1 *Mammalian phylogenetic tree.*



This exercise is adapted from John D. Anderson, Protein Fingerprinting: Teaching Modern Biology in the Laboratory (Modern Biology, Inc., 1994), Experiment 6 (106).

Lactate dehydrogenase (LDH) is an enzyme that catalyzes the conversion of pyruvic acid (pyruvate) to lactic acid (lactate) (Figure 21B-2). This protein is produced by most tissues but is especially prevalent in muscle. LDH is also found in blood serum, the noncellular portion of blood, as a result of normal cell death with release of cellular enzymes into the bloodstream.

Figure 21B-2 *Conversion of pyruvate to lactate. Pyruvate, the end product of glycolysis, can accept hydrogen electrons from NADH + H^+, reoxidizing the NADH + H^+ to NAD^+.*



Lactate dehydrogenase production is controlled by two genes. Each gene is monoallelic (it has only one form) and produces a single type of peptide (monomer). Monomeric peptides combine to produce tetramers. LDH is a tetramer, and in mammals it exists as five isoenzymes (different molecular forms of the same enzyme). If all of the LDH peptides are of one type, the enzyme exists as the LDH-1 isoenzyme or the LDH-5 isoenzyme, depending on which gene produced the peptide monomers. The remaining three isoenzymes result from different combinations of the peptides produced by the two genes. If we call the two genes and their peptide products **M** (for muscle) and **H** (for heart), the LDH isoenzymes are designated as follows:

MMMM	LDH-1
MMMH	LDH-2
MMHH	LDH-3
MHHH	LDH-4
HHHH	LDH-5

The order of listing the peptides is inconsequential; MMMH is the same as MHMM.

All five LDH isoenzymes catalyze the same reaction, but they differ in their amino acid composition and net charge. These differences make them easily separable by electrophoresis. LDH-1 is the lightest (lowest molecular weight) and will therefore travel the farthest on a gel. LDH-5 is the heaviest and will remain closer to the origin.

While all mammals are capable of producing all five isoenzymes, the amount of each isoenzyme produced is controlled by regulatory genes that control the activities of the two LDH-producing genes. Therefore, not all isoenzymes will be detectable on a gel even if they are present. (Some may occur in concentrations too low to react with the stain.) Furthermore, LDH isoenzymes composed of the same combination of monomers are not identical among different mammalian species. There are antigenic differences among mammals, indicating differences in amino acid composition. Therefore LDH from two different mammalian species will not necessarily band in the same place on the gel. It is these differences, combined with the number of different bands observed, that make it possible to determine evolutionary trends among the mammals tested.

In this exercise, you will separate the serum proteins from different ungulates (cow, sheep, goat, and horse) by electrophoresis, and then use a special staining technique to identify the LDH isoenzymes. The results from each serum sample will appear as a distinct gel fingerprint of LDH activity, with different isoenzymes showing different banding patterns. A comparison of the LDH fingerprints should enable you to determine the basic evolutionary relationships among the ungulates tested.

Objectives

- ☐ Use electrophoretic evidence to distinguish among related species of organisms.
- ☐ Determine the phylogenetic relationships among ungulate mammals.

Procedure

Formulate a hypothesis for this investigation.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will be the outcome of this investigation?

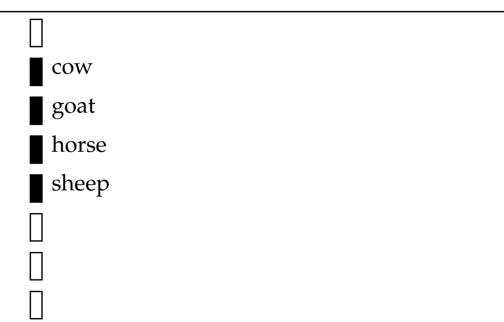
What is the **independent variable**?

What is the **dependent variable**?

Use the following procedure to test your hypothesis.

1. Work in pairs. Place the gel comb into the end slots of the gel carrier and seal off the ends with tape to make a gel box.
2. Pour 50 ml of 1.2% agarose into the gel box until an even layer of approximately one-eighth of an inch of agarose covers the bottom of the box.
3. Allow the gel to cool for approximately 15 minutes.
4. Remove the comb and tape from the gel box, and place the gel box into the electrophoresis unit so that the gel wells are at the negative electrode (black) end.
5. Pour enough tris-citrate buffer into the electrophoresis unit to cover the gel completely.
6. Load 15 μl of each type of serum into the sample wells as indicated in Figure 21B-3. The serum has been combined with a loading dye (bromphenol blue and glycerol).

Figure 21B-3 *Loading order for ungulate sera samples.*

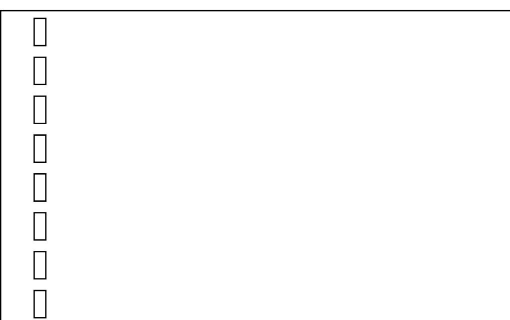


7. Conduct electrophoresis at 100 volts until the loading dye has migrated to within 2 cm of the positive electrode end of the gel.
8. *Just* before completion of the electrophoresis run, prepare a fresh stain solution by combining the solutions listed in Table 21B-1. This solution couples the LDH-catalyzed reaction (pyruvate $\longrightarrow$ lactate) to a second reaction that produces a formazan compound, which is brown in color and collects at the sites of LDH activity. Therefore, this stain selectively excludes all proteins in the serum except LDH, allowing the simple differentiation of LDH patterns among the samples tested. (You will have enough stain for only your own gel.)

Table 21B-1 Chemical Composition of the LDH-Staining Solution

Solution	Amount (ml)
Tris-HCl buffer	30
Lilactate	6
NAD	1.3
MTT	0.3
PMS	0.5

9. Remove the agarose gel from the electrophoresis unit and gel box, and place it into a staining dish. (Warning: Be certain that the power has been turned off and the electrodes removed from the power supply before attempting to remove the gel.)
10. Cover the gel with the staining solution you have prepared, and place the gel into a 37°C incubation chamber for 30 minutes. (The incubation chamber may be an incubator or a water bath equipped to support staining dishes.) *The staining reaction should occur in the dark,* so be sure to replace the lid on the chamber.
11. After incubation, pour the staining solution into the waste beaker. Cover the gel with the 10% acetic acid destain solution, and observe the banding patterns.
12. Sketch the LDH banding patterns on your gel.



a. Which organisms show the most similar banding patterns? ______________________

____

b. Does this agree with your predictions? ______

Do your results support your hypothesis? ______ *Your null hypothesis?* ______

From the results obtained in this exercise, what do you ***conclude*** *about the evolution of ungulates?*

____

____

c. Are the ungulates a monophyletic or polyphyletic taxon? ______________ *How do you know?*

____

Laboratory Review Questions and Problems

1. Define the following terms and give an example for each.

Phylogeny

Taxon

Binomial nomenclature

Monophyletic taxon

Polyphyletic taxon

Paraphyletic taxon

Cladistics

Evolutionary systematics

Homology

Analogy

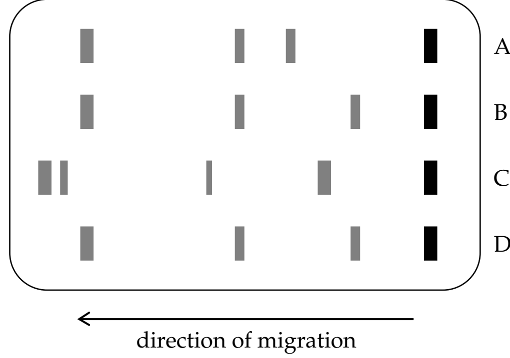
Homoplasy

Phylogeny

Classification

Systematics

2. The gel shown below represents the electrophoretic pattern of a protein from four different but related organisms.



a. Which two organisms are most closely related?

b. Which organism is least related to the others, although it shares a common ancestor?

c. In the space below, construct a possible phylogenetic tree showing the evolutionary relationships among organisms A, B, C, and D.

Diversity—Kingdoms Eubacteria, Archaebacteria, and Protista

LABORATORY 22

OVERVIEW

Biologists currently recognize three *domains* of living things: **Bacteria, Archaea,** and **Eukarya.** The domain Bacteria is comprised of one kingdom, **Eubacteria** (true bacteria). The domain Archaea is also composed of one kingdom, **Archaebacteria** (ancient bacteria). The domain Eukarya includes all other living organisms: kingdoms **Protista** (unicellular animals and plants, slime molds, and algae), **Fungi, Plantae** (multicellular plants), and **Animalia** (multicellular animals).*

Among the diverse organisms in these kingdoms you will find two major types. **Prokaryotes** are unicellular (single-celled) organisms characterized by the absence of a membrane-bound nucleus and membrane-bound organelles. In contrast, **eukaryotes** have both a well-formed nucleus and many other types of membrane-bound organelles.

Prokaryotes and eukaryotes differ also in the chemical composition of their cell walls (if present), the organization of their genetic material, and the structure of their flagella. The kingdoms **Eubacteria** and **Archaebacteria** are composed of prokaryotic organisms. Eukaryotic organisms are found in the other four kingdoms.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

PART I DOMAINS BACTERIA AND ARCHAEA

Prokaryotes represent the oldest and simplest living things. Prokaryotes are clearly different from eukaryotes, but ordering them taxonomically into subgroups of organisms according to anatomical and physiological affinities is difficult. Based on comparison of DNA and RNA sequences, it appears that prokaryotes diverged early in evolutionary history into two distinct lineages now recognized as domains, Bacteria and Archaea (Figure 22I-1).

Belonging to the domain Archaea, the kingdom Archaebacteria is composed of four phyla of organisms that live at environmental extremes: methanogens (bacteria that use H_2 to reduce CO_2 to

**Because scientists are continually learning more about the structure and origins of organisms, the assignment of organisms within a particular classification may change. Indeed, the number of phyla and even the number of kingdoms have changed during the past decade. It is possible that your instructor will adopt a different classification scheme than that used in the following exercises, but the principle of classification will remain the same—the grouping of organisms according to similarities that indicate evolutionary relationships.*

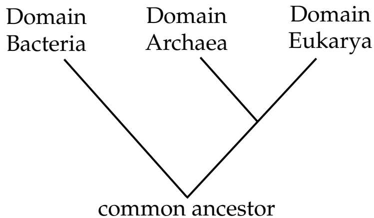


Figure 22I-1 *According to present thinking, the domains Eukarya and Archaea share a common ancestry and thus are more closely related to each other than to the domain Bacteria.*

methane), thermoacidophiles or hyperthermophiles (bacteria that live where it is both hot and acidic), extreme halophiles (salt-loving bacteria), and thermoplasma. Belonging to the domain Bacteria, the kingdom Eubacteria is composed of 12 phyla, among which are gram-negative, gram-positive, and photosynthetic bacteria. These include myxobacteria, rickettsias, desulfovibrio, purple nonsulfur bacteria, rhodopseudomonas, purple sulfur bacteria, spirochetes, actinomycetes, clostridia, and mycoplasmas.

Bacteria are virtually ubiquitous. Not only do bacteria surround you, they thrive within you. Most of these bacteria are nonpathogenic and actually keep your body free from more harmful bacteria through competition. However, conditions sometimes make the difference between a pathogen and a nonpathogen. For example, *Staphylococcus aureus*, a common bacterium, can cause severe infections if introduced into an open cut. The same bacterium is also thought to be an agent in toxic shock syndrome.

Other bacteria play a large role in the environment and in the economy. Some bacteria are important as decomposers, recycling dead material into components required by living organisms. In sewage treatment plants, they promote the breakdown of solid wastes. Many foods, such as vinegar, sour cream, yogurt, and cheeses, are made with the help of bacteria. Certain antibiotics are produced by bacteria. Some bacteria can even be used to clean up oil spills. Bacteria are also a main focus of genetic and biochemical research.

✓ EXERCISE A | Morphology of Bacteria

Most bacteria may be classified into one of three major morphological groups: rods (bacilli), spheres (cocci), or spirals (spirilla). You can observe the morphology of these groups both microscopically and macroscopically, by observing growth forms.

Objectives

☐ Recognize the different morphological forms of bacteria.

Procedure

1. Check the demonstration microscopes and observe the basic morphological forms of bacteria (Figure 22A-1). Draw the bacterial forms and identify each in the space that follows.

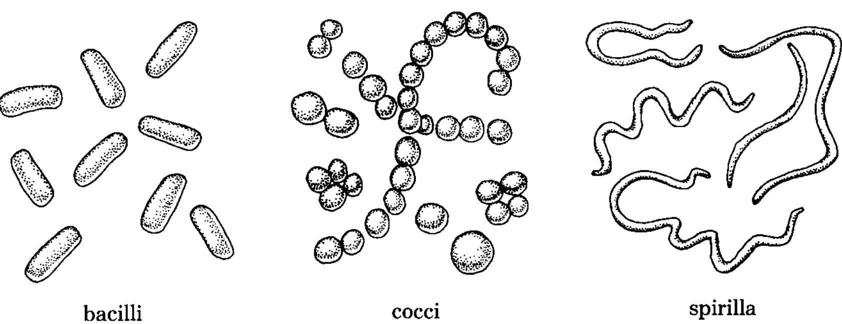


Figure 22A-1 *Morphology of bacteria.*

Demonstration A Demonstration B Demonstration C

Type ________________ Type ________________ Type ________________

a. Strep throat is caused by streptococcal bacteria. What shape would you expect these bacteria to have?

____

b. The prefix "strep" means strip or chain. How would you expect streptococcal bacteria to be arranged? ________________________

c. How are the bacterial cells arranged in the demonstration slides you have observed? As single cells? Clusters? Strands? A ____________ *B* ____________ *C* ____________

2. Gram stain is a specific stain (purple) used to test for gram-positive bacteria. In these bacteria, the cell wall contains a small amount of lipid combined with a thick layer of a peptide/sugar material (peptidoglycan). The peptidoglycan molecules trap Gram stain and the cell walls retain a purple color. The cell walls of gram-negative bacteria contain a great deal of lipid but little peptidoglycan and the purple Gram stain washes out. A second stain, safranin, stains the gram-negative bacteria pinkish-red. What color were the bacteria on the demonstration slides? Were they gram-positive or gram-negative?

Color	Gram + or Gram −
A	
B	
C	

d. Are all coccal bacteria gram-positive? ______ (Hint: *Review the slides on demonstration.)*

3. Streptococcal bacteria are gram-positive. Physicians use Gram stain to identify specific bacteria.

e. What color would Gram-stained streptococcus be? ____________

4. Penicillin is usually an effective antibiotic for treating infections caused by gram-positive bacteria. Penicillin affects the ability of bacterial cells to synthesize peptidoglycan.

f. Would penicillin be an effective way to treat strep throat? ____________

5. You were given an agar-filled Petri dish with instructions to expose it to a potential source of bacteria in your environment, for example, keys, money, shoes; air in various locations (inside the house, in a bathroom; outside, in moist or dry environments); your hands before washing, after washing with regular soap, after washing with an "antibacterial" hand soap; the foot of a

cat, dog, or bird. Examine your dish and those of your classmates. *Do not remove the lids.* Note the number of bacterial colonies in each plate. These will have the appearance of small, shiny masses. The "fuzzy" masses that may appear on some plates are fungi. Theoretically, each colony of bacteria or fungi arose from a single bacterium or fungal spore (though closely adjacent individual colonies may coalesce and appear as one). The appearance of a bacterial colony on agar is used in classification. Record your observations of the number of different colonies on each of four plates, and the shape, color, texture, and size of each type of colony.

Plate 1 ______________________________

Plate 2 ______________________________

Plate 3 ______________________________

Plate 4 ______________________________

EXERCISE B | Characteristics of Bacteria: Sensitivity to Antibiotics

Since the number of basic bacterial morphologies is limited, identification of species is largely dependent on biochemical and physiological characteristics. Composition of the cell wall, DNA or RNA sequencing, sensitivity to antibiotics, mode of metabolism, and mode of reproduction can all be used to distinguish among bacterial types. These characteristics are the topics of this exercise and Exercises C through D.

The bacterial cell wall and ribosomes represent two major sites for the action of antibacterial agents. As you have already learned, many of the common antibiotics treat bacterial infections by inhibiting the synthesis of the essential structural polymer, peptidoglycan, in the bacterial cell walls. Recall that penicillin is an example of one of these antibiotics. But how effective are other antibiotics against gram-positive bacteria? And what about gram-negative bacteria?

Objectives

- ☐ Determine the sensitivity of selected bacteria to several antibiotics.
- ☐ Describe the action of antibiotics.

There are two general types of antibiotics. **Narrow-spectrum antibiotics** target a limited number of bacterial species, while **broad-spectrum antibiotics** are effective against a wider spectrum of bacterial types. This exercise will show the relationship between bacterial cell-wall structure, as indicated by Gram stain, and susceptibility to different types of antibiotics.

Procedure

1. Examine the Petri dishes on display. In preparing these, the agar surfaces were first covered with a suspension of a known type of bacteria—one plate with gram-positive and the other with gram-negative. Paper disks impregnated with an antibiotic or antiseptic were then placed on top of the bacteria (Figure 22B-1). After an incubation period, bacterial growth will be

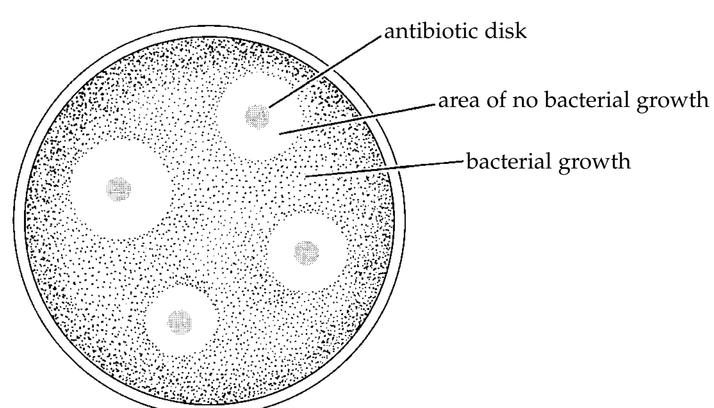


Figure 22B-1 *Bacteria growing on an agar plate demonstrate sensitivity to antibiotics. Clear areas indicate inability of bacteria to grow in the presence of an antibiotic. The diameter of the zone of inhibited growth depends on the relative diffusibility of the antibiotic and the sensitivity of the particular bacterium.*

visible unless inhibited by the substance in the disk. (This procedure is often used to determine which drug would be most effective against a particular bacterium.)

2. Record the results in Table 22B-1. List the antibiotics in the left-hand column, then record the effects of each antibiotic on each of the two types of bacteria. Use the following symbols to record your observations of the dishes: −, growth not inhibited; +, growth slightly inhibited; ++, growth greatly inhibited.

Table 22B-1 Effects of Antibiotics

	Effect on Growth	
Antibiotic	Plate 1: Gram −	Plate 2: Gram +
A.		
B.		
C.		
D.		
E.		

3. In Table 22B-2, list the antibiotics and the types of bacteria against which they are effective, and then decide whether each antibiotic has a broad or narrow range, or spectrum, of action.

Table 22B-2 Spectrum of Antibiotic Activity

Antibiotic	Bacteria Affected	Spectrum
A.		
B.		
C.		
D.		
E.		

a. *Under what circumstances might a doctor prescribe a narrow-spectrum antibiotic?*

____

A broad-spectrum antibiotic? ____

EXTENDING YOUR INVESTIGATION: HOW EFFECTIVE IS YOUR SOAP?

Why do you use the brand of soap that you do? Is your soap effective in clearing bacteria from your hands or face? Would water be just as effective? Is medicated or deodorant soap better than "just plain soap"? Is alcohol an effective disinfectant? What about commercial disinfectants such as Lysol or Mr. Clean? When antibacterial substances are used on living organisms, they are called **antiseptics.** Those used to clean surfaces are called **disinfectants.**

Formulate a hypothesis concerning the effectiveness of an antiseptic or disinfectant of your choice.

HYPOTHESIS:

NULL HYPOTHESIS:

What do you **predict** will be the effect of the soap or the antiseptic or disinfectant solutions you have chosen?

What is the **independent variable?**

What is the **dependent variable?**

The following materials will be available to you: an *S. aureus* (gram-positive) culture or plate, an *E. coli* (gram-negative) culture or plate, detergents, soaps, and disinfectants (or bring your own). Use these materials to design an experiment based on the general procedure that follows.

PROCEDURE: Your instructor may provide you with agar plates that have been inoculated with bacteria. If not, inoculate the plates as in steps 1–3. Then proceed to testing your selected detergents, soaps, or antiseptics (steps 4–10).

1. Wipe your laboratory area clean with a 10% Clorox solution.

2. Obtain an agar plate and a broth culture of *S. aureus* or *E. coli.*

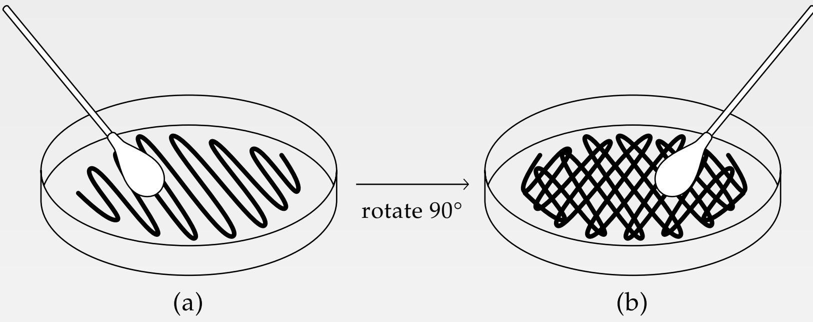
3. Prepare a lawn of bacteria as follows:

a. Lift the lid of the agar plate and hold it above the plate.

b. Use a sterile cotton swab to remove some bacterial culture from one of the flasks. Make sure the cotton is saturated, but not dripping.

c. Apply the bacteria to the agar plate in a zig-zag motion over the entire surface, as shown below (a).

d. Rotate the plate 90° and repeat this motion (b).



e. Dispose of the cotton swab in the container provided.

4. Pour small amounts of your selected liquid antiseptics or disinfectants into shallow dishes or other containers. If you are testing a solid material such as a bar of soap or a soap powder, make a liquid solution.

5. Using a pair of forceps, submerge a paper disk in a solution. Drain off excess liquid by touching one end of the disk to a paper towel.
6. Place the paper disk on your agar plate for 2 minutes.
7. While the disk is on the agar, mark the undersurface of the agar plate with a circle to show the placement of the disk and the name of the substance tested.
8. Repeat this procedure for three more disks impregnated with different solutions. Remember to mark and label the agar plate for each disk position. Remove the disks and dispose of them in the container provided.
9. Incubate the dishes, upside down, until the next laboratory period. Be sure that your name is on the culture plate.
10. After you complete your work, wash your work area with 10% Clorox. Also, wash your hands with soap and water!

RESULTS: A clear area around the position of a paper disk indicates that bacterial growth has been inhibited. Quantify your results by measuring the diameter of this region and noting it in the following table.

Test Substance	Zone of Inhibition (Diameter)

Do your results support your hypothesis?

Your null hypothesis?

What do you **conclude** about the effectiveness of the disinfectant, antiseptic, or soap solutions you tested?

✔ EXERCISE C Nitrogen-Fixing Bacteria

Although all organisms require nitrogen for the production of proteins and nucleic acids, eukaryotic organisms cannot use atmospheric nitrogen (N_2) directly. It must first be converted to a compound such as ammonia (NH_3). Only bacteria and cyanobacteria are capable of **nitrogen fixation,** the conversion of N_2 into NH_3. This capacity is the basis of a significant relationship between bacteria and eukaryotes.

Legumes, the family of plants to which peas and beans belong, form a symbiotic relationship with bacteria of the genus *Rhizobium. Rhizobium* live in the root nodules of the legumes and supply the plants with usable nitrogen by converting atmospheric N_2 into NH_3. The plants supply *Rhizobium* with sugar, the product of photosynthesis.

Objectives

☐ Recognize *Rhizobium* bacteria on the root nodules of legumes.

Procedure

1. Examine the two groups of soybean plants provided. One group was inoculated with *Rhizobium.*
   a. Which group of plants is more robust? ____________________________
   b. Examine the roots. Which group of plants has root nodules? ____________________________
   c. Which group of plants has a symbiotic relationship with Rhizobium*?*
   ____
2. Observe *Rhizobium* bacteria on a demonstration slide if available. Make a sketch in the space below.
   d. Morphologically, how would you classify Rhizobium *bacteria?* ____________________

✓ EXERCISE D | Bioluminescent Bacteria

Some marine bacteria are capable of producing light in the presence of oxygen. Several marine fishes are equipped with bizarre light organs inhabited by these bacteria. The fish provide the bacteria with nutrients and oxygen and use the light produced by the bacteria primarily to attract prey.

Bioluminescence in bacteria is the result of a reaction between a *luciferin* (an aldehyde compound), reduced flavin mononucleotide, or $FMNH_2$ (FMN is a component of the electron transport chain), and O_2 in the presence of the enzyme *luciferase.*

Objectives

☐ Observe the bioluminescent properties of bacteria.

Procedure

1. Your instructor has prepared a broth culture of the bioluminescent marine bacterium *Vibrio fisheri.* Obtain a tube of the culture and place a rubber stopper in the end of the tube.
2. In a darkened room or closet, shake the tube vigorously.
   a. What do you see? ____________________________ *What color does the light appear to be?*
   ____
   b. Why did you have to shake the tube? ____
   ____
3. Stop shaking the tube. *c. What happens?* ____
   ____
   d. Which part of the tube darkens first? ____________________________ *Why?*
   ____
   ____
   e. How is the supply of oxygen normally maintained in the seas? ______________________________
   ____

✔ EXERCISE E Diversity and Structure of Cyanobacteria

Traditionally, cyanobacteria have been called "blue-green algae." However, they are not algae, but prokaryotes: gram-negative photosynthetic bacteria. Cyanobacteria contain chlorophyll *a* (as do photosynthetic eukaryotic green plants and algae), but it is characteristically masked by blue, red, and purple pigments. These pigments (**phycobilins**) enhance light absorption by the cells and serve as nitrogen reservoirs. All cyanobacteria are unicellular, but individual cells are commonly attached to each other by a gelatinous sheath, thus producing filaments or colonies. Like some other bacteria, many cyanobacteria are able to fix atmospheric nitrogen.

Cyanobacteria share with other bacteria the ability to inhabit the most inhospitable locations on earth, such as hot springs and bare rocks. Cyanobacteria can be desiccated for many years yet resume growth when water is again present.

Objectives

- ☐ Recognize several types of cyanobacteria and compare these to other types of bacteria.
- ☐ Recognize several cell types common to cyanobacteria.

Procedure

Work in pairs. Prepare and examine material on a wet-mount slide and then exchange slides with your partner. Depending on the material available, examine one or more examples of cyanobacteria. Refer to Table 22E-1, and add further information to the table where necessary. Make sketches if instructed to do so, adding labels where possible.

Most cells of cyanobacteria are structurally undistinguished, but a few specialized cell types can be recognized. **Heterocysts** are round or oval, *clear* cells that allow cyanobacteria to fix atmospheric nitrogen. Many cyanobacteria can fix nitrogen when they are in an anaerobic environment, but heterocysts are

Table 22E-1 Characteristics of Cyanobacteria

Type	Morphology	Distinctive Features
Nostoc (akinete, heterocyst) (a)	Filaments of round cells; gelatinous sheath surrounds filament.	Can combine in large gelatinous balls containing hundreds of filaments. Reproduce by fission or fragmentation.
Cylindrospermum (akinete, heterocyst) (b)	Filaments of rectangular cells; length greater than width.	**Heterocysts** at the ends of filaments function in nitrogen fixation. Reproduce by fission or fragmentation. **Akinetes** are special sporelike reproductive cells resistant to adverse environmental conditions.
Oscillatoria (hormogonium, dead cell) (c)	Filaments of rectangular cells covered by a sheath; width greater than length.	Oscillate, seek specific conditions in water. Reproduce by fragmentation only. **Hormogonia** are short fragments between dead cells where fragmentation takes place.

(continued)

Table 22E-1 *(continued)*

Type	Morphology	Distinctive Features
Anabaena akinete heterocyst (d)	Barrel-shaped vegetative cells held in a gelatinous matrix.	Heterocysts are integral or terminal and function in nitrogen fixation. Reproduce by fission or fragmentation. Akinetes are dispersed among vegetative cells.
Gleocapsa (e)	Spherical cells; single or groups of 2 to 8; each cell surrounded by its own sheath; colony surrounded by sheath.	Can fix nitrogen despite absence of heterocysts. Reproduce by fission.
Sketches		

necessary for aerobic nitrogen fixation. **Akinetes** are generally larger, usually oval, densely packed, sporelike reproductive cells that are resistant to adverse conditions.

a. *What are the basic cyanobacterial cell shapes?* ______________ *How are these individual cells combined to form colonies and filaments?* ______________

b. *Which of the specialized cell types can you recognize in each of the types of cyanobacteria?* ______________ *What does the presence of these cells (heterocysts, akinetes) indicate about the environment of these organisms?* ______________

c. *Cyanobacteria grow prolifically in streams and lakes with low oxygen levels and high nutrient concentrations. How might the presence or absence of cyanobacteria be used as an index of pollution in lakes?*

____

d. *How can you determine, from your microscope observations, whether cyanobacteria are prokaryotes or eukaryotes?* ______________

Name three differences between prokaryotes and eukaryotes.

____

____

e. *Cyanobacteria have a peptidoglycan cell wall. Would you expect them to be sensitive to antibiotics? Explain.*

____

f. *Prokaryotes have membrane-bound organelles. Did the cyanobacteria that you examined contain chloroplasts?* ______ *Chlorophyll?* ______

PART II KINGDOM PROTISTA

With the kingdom Protista,* we begin our study of eukaryotes. The cells of eukaryotic organisms contain both a nucleus and membrane-bound organelles. All other eukaryotic organisms (including fungi, plants, and animals) probably originated from the primitive protists.

For our purposes, protists can be divided into three broad groups, usually based on modes of nutrition.

Protozoa Unicellular heterotrophs, typically animal-like.

Fungus-like protists (slime molds) Sometimes referred to as the "lower fungi" because they may be multinucleate, as are fungi, during some part of their life cycle. They are classified with protists because of their similarities to protozoans.

Algae Unicellular and multicellular plant-like organisms.

PROTOZOA

Protozoans are unicellular organisms. Most are motile. Protozoans can be found in free-living and parasitic forms and in freshwater or marine environments.

EXERCISE F Identifying Protozoans

There are many phyla of protozoans. Some of the most common forms are represented below. They can be distinguished by body form and mode of locomotion (Table 22F-1).

Objectives

- ☐ Identify and classify representative protozoans.

Procedure

Observe material using prepared slides or, if fresh material is available, make temporary wet-mount slides according to directions in Table 22F-1. Label structures and make notes or sketches of any identifying characteristics or behaviors.

Table 22F-1 Characteristics of Protozoans

Phylum/Representative	Method of Observation	Mode of Locomotion
Zoomastigophora undulating membrane flagellum nucleus *Trypanosoma* (a)	Study a prepared slide.	Flagellar movement. A single **flagellum** is united basally with the body of cell by an undulating membrane. Amoeboid extensions (**pseudopodia**) are also found in many flagellates. *Trypanosoma gambiense* is the causative agent of African sleeping sickness.

(continued)

**Originally, only unicellular organisms were assigned to the kingdom Protista, but in recent years it has been suggested that the kingdom be expanded to include some multicellular organisms—the multicellular algae and fungus-like organisms that lack some of the important characteristics of true fungi. The name Protoctista has been proposed for this "expanded kingdom."*

Table 22F-1 *(continued)*

Phylum/Representative	Method of Observation	Mode of Locomotion
Rhizopoda *Amoeba* (b)	Examine living amoebas. You can see the organism on the bottom or side of the culture dish. Remove an amoeba with a pipette and place it on a glass slide. Observe without a coverslip. Adjust light. If motion does not occur, add coverslip.	Amoeboid movement—pseudopodia. Cytoplasmic extensions change in size.
Ciliophora *Paramecium* (c)	Examine living paramecia. Place a small drop of culture medium on a clean glass slide. Mix in a drop of Protoslo (methylcellulose). Cover with a coverslip and observe. Is ciliary movement coordinated? ________ Can you see food vacuoles? ________ Do you see any contractile vacuoles? ________ How often do they fill and empty? ____________________	Ciliary movement. Cilia have the same internal structure as flagella, but are shorter.
Apicomplexa *Plasmodium vivax* (d)	Study a prepared slide. Locate sporozoites. See life cycle, Figure 22F-1.	Nonmotile phases predominate. Blood parasites.

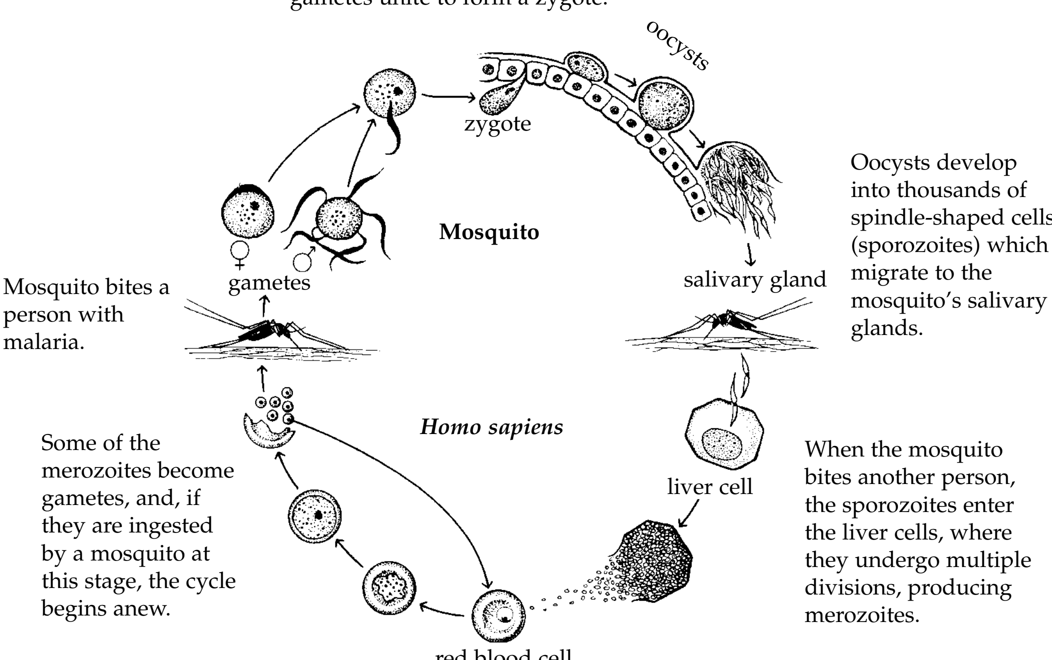
(continued)

Table 22F-1 *(continued)*

Phylum/Representative	Method of Observation	Mode of Locomotion
Actinopoda axopodia (e)	Not studied in this laboratory.	Slender pseudopodia called **axopodia** (reinforced by microtubules) help the organisms to float and feed. Phylum includes heliozoans and radiolarians.
Foraminifera (f)	Not studied in this laboratory.	Foraminiferans (forams) are named for their porous shells. Strands of cytoplasm extend from holes in the shell for swimming and feeding.

Figure 22F-1 *Life cycle of a* Plasmodium.

In the mosquito's digestive tract, gametes unite to form a zygote.



The merozoites enter the red blood cells and, again, divide repeatedly and break out at intervals of 48 or 72 hours.

How does Plasmodium's *lack of motility affect the mechanism required for infection in humans?* ______________________________

____

EXERCISE G | Symbiosis in the Termite: A Study of Flagellates

A very good source of protozoans is the gut of a termite. Termites are well known for their ability to eat their way through wooden buildings. However, these organisms cannot, on their own, digest the cellulose of wood, even though it is the major constituent of their diet. The guts of termites are well populated by flagellated protozoans, and at least some of these have the enzyme required to digest cellulose. The termite–flagellate relationship is an example of **symbiosis.**

Symbiosis is the close living relationship of two organisms. A symbiotic relationship that benefits both organisms, as is seen in the termites and flagellates, is called **mutualism.** For example, one organism may obtain nourishment from another and in turn provide a nutrient that is necessary to the host. In some mutualistic relationships, each organism becomes so dependent on substances or services provided by the other that neither can survive alone; this is *obligatory mutualism.*

In another type of symbiosis called **commensalism,** one organism provides something of value to the second, but is neither harmed nor helped by the relationship.

The most extreme form of symbiosis is **parasitism.** The parasite is detrimental to its host, sometimes to the point of causing the host's death.

Procedure

1. Use a dropper to place a drop of insect Ringers (a saline solution isotonic to the tissues of the termite) onto a glass slide.
2. Place a termite into the Ringers and observe using the dissecting microscope.
3. Use two dissecting needles or fine forceps to open the termite. Place one point at the posterior end and one at the anterior end and pull in opposite directions.
4. The intestine is long and tubular. Locate it and move the remaining material to one side.
5. Use the dissecting needles to tear open the intestine.
6. Cover your preparation with a coverslip and observe using the compound microscope at 4× and 10× .
   a. *What do you observe? Is more than one type of organism present?* _______ *Do you see* Trichonympha *(Figure 22G-1)?* _______ *How many flagella are present on each organism?* _______ *In which phylum would you place these organisms?* ________________
   b. *Describe their movements.* ____
   c. *Suggest the major function of* Trichonympha *in termites.* (Hint: *Consider the diet of the termite.)*
   ____
7. On a separate sheet of paper, sketch several flagellates. Include flagella and indicate the direction of movement.
8. Allow your slide to air dry into a thin film, then place it on a staining trough or other suitable container and flood the slide with a few drops of giemsa stain. After 15 minutes, rinse off the excess with distilled water and air dry. Add a coverslip, using glycerol, and examine under the microscope again. Estimate the number of different flagellates present. Compare these to Figure 22G-1 and make tentative identifications.
9. In the space below, record your speculations on how symbiotic relationships such as that between termites and protists might have evolved.

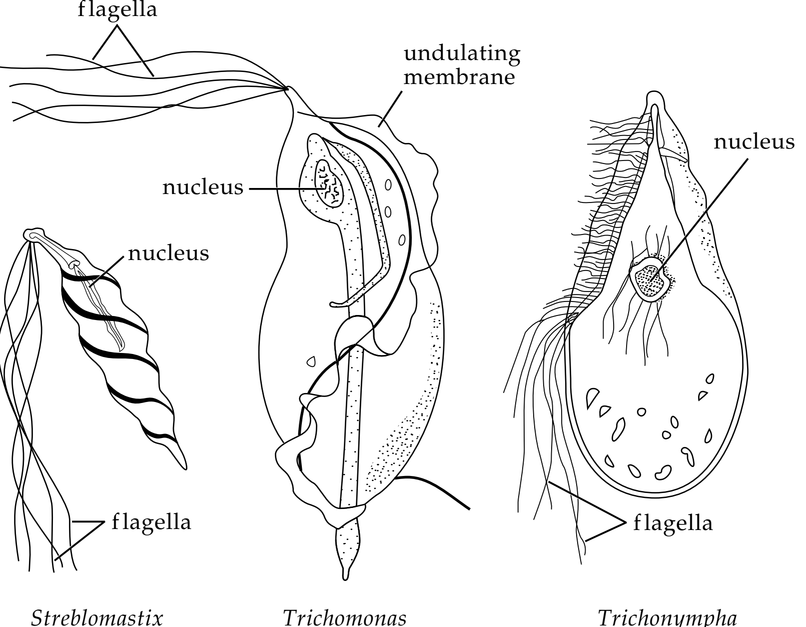


Figure 22G-1 *Some flagellated protists found in the termite gut.*

d. *What significant ecological role do termites and their symbionts perform (aside from destroying human-built wooden structures)?* ______________________________

____

e. *Is their feeding niche shared by other organisms?* ______ *Name some examples.*

____

FUNGUS-LIKE PROTISTS

✔ EXERCISE H Plasmodial Slime Molds

The slime molds are sometimes referred to as the "lower fungi" because they are multinucleate during part of their life cycle. Also, there are some ways in which slime molds resemble amoebas.

Some slime molds, known as **plasmodial slime molds** (phylum Myxomycota), are multinucleate masses of streaming protoplasm (Figure 22H-1). Others, the **cellular slime molds** (phylum Dictyostelida

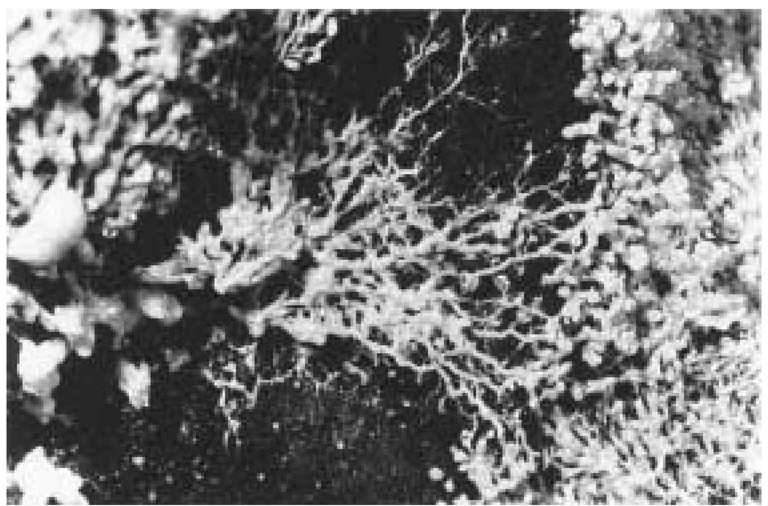


Figure 22H-1 *The plasmodial slime mold,* Physarum.

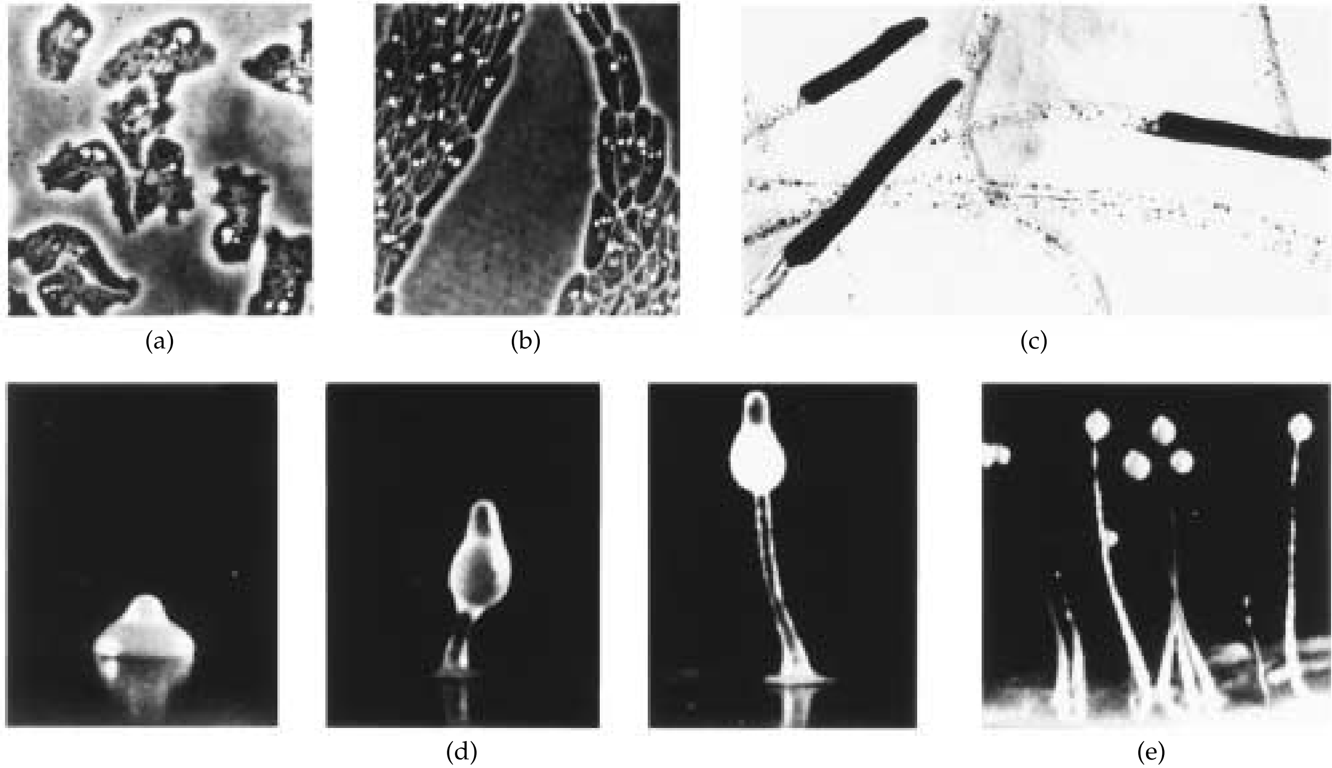


Figure 22H-2 *Life cycle of a cellular slime mold,* Dictyostelium discoideum. *(a) Haploid* (n) *amoebas in the feeding stage. (b) Amoebas aggregating. (c) Migrating slugs (pseudoplasmodia). (d) At the end of migration, each pseudoplasmodium* (2n) *gathers together and begins to rise vertically, differentiating into a stalk and fruiting body (e). Meiosis restores the haploid condition.*

and Acrasida), have bodies, or plasmodia, composed of aggregates of small cells called amoebas. These amoebas retain their identity as individual cells (Figure 22H-2).

Objectives

- ☐ Describe the structure of a typical plasmodial slime mold, *Physarum.*
- ☐ Determine behavioral responses of *Physarum* to heat, light, and overcrowding.

Procedure

1. Examine the well-developed body (**plasmodium**) of *Physarum* on demonstration. This multinucleate mass of protoplasm ingests food particles as it moves across the agar surface of the Petri dish. In the natural environment, plasmodia can be found on rotting stumps or fallen logs, usually underneath the bark.
2. Study the reproductive structures (**sporangia**) of those species on demonstration. The onset of unfavorable conditions—for example, the lack of water or food—triggers the formation of sporangia.

   a. Are these reproductive structures plant-like or animal-like? ____________________
3. Obtain a piece of filter paper containing dried plasmodial material. Wet the paper and place it on the agar in the middle of a Petri dish.

4. Place two or three oat flakes around the material.

5. Take this preparation home with you and observe what happens during the next week.

b. When the slime mold runs out of space, what happens? ______________________

____

c. Keep the slime mold in one place and observe the direction in which it moves. To what stimulus does it seem to be responding? ______________

d. Punch a hole in the plasmodium. What happens to it? ______________________

e. Slightly warm one-half of the dish. What happens to the slime mold?

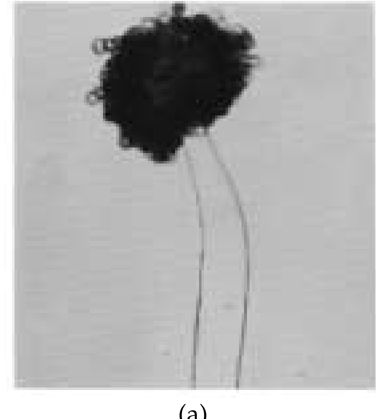
____

f. Illuminate one-half of the dish with your desk lamp and cover the other half with aluminum foil. Does Physarum *react to light? Why or why not?* ______________________

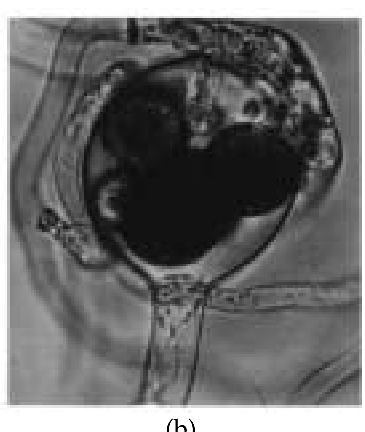
6. Summarize your experiments and their results on a separate sheet of paper. Be sure to include the hypotheses that determined the experimental procedures you used. Hand in your report, if assigned.

✓ EXERCISE I | Water Molds

Oomycetes, the "egg fungi" (phylum Oomycota), get their name from their sexual reproduction cycle in which large nonmotile eggs are produced inside a special structure called an **oogonium** (Figure 22I-1). Egg fungi are also called by several common names, including water molds, algae-like fungi, and downy mildews.



(a)



(b)

Figure 22I-1 *(a) Asexual reproduction in the water mold* Achlya ambisexualis *is characterized by production of motile flagellated zoospores from sporangia located on the tips of the hyphae. (b) Sexual reproduction is characterized by formation of large nonmotile eggs within an oogonium (a type of female gametangium) to which tubal outgrowths on the antheridia (male gametangia) fuse, allowing sperm to enter the oogonium and fertilize the eggs. Zygotes develop thick coats and are called oospores.*

Unlike other fungi, with cell walls composed of chitin, the cell walls of oomycetes are made up of cellulose. Another distinction is that the spores formed by oomycetes during asexual reproduction are flagellated, a distinctly protistan characteristic.

You are probably more familiar with the oomycetes than you realize. Since these molds can attack diseased or dying fish, you may have experienced a problem with *ick*—a disease caused by water molds—

in your aquarium. One oomycete, *Phytophthora,* was responsible for the potato famine in Ireland in the 1850s. Another, *Plasmopara,* almost destroyed the French wine industry.

Objectives

- ☐ Identify the structural characteristics typical of Oomycota, and distinguish between the structures of asexual and sexual reproductive phases.
- ☐ Explain the basis for the name "Oomycota."

Procedure

1. In the demonstration area, you will find water molds growing on dead seed. Note the cottony mass of filaments, or hyphae, which constitute the mycelium.

   a. Are these oomycetes parasitic or saprophytic? ______________________________

2. Examine (at 10× and 40×) a wet-mount slide of a portion of the water mold mycelium on demonstration. Look for denser areas at the tips of the hyphae. These individual cells, called **zoosporangia,** produce motile zoospores asexually.
3. Examine a prepared slide of the water mold *Saprolegnia.* Identify oogonia, antheridia, and zoosporangia. Use the space below to draw and label your observations.

ALGAE

A number of distinct lines of simple, photosynthetic eukaryotes, the **algae**, evolved more than 450 million years ago. All modern algae have chlorophyll *a* as their main photosynthetic pigment, and they also have accessory pigments. Distinctions are made among divisions of algae based on the type of accessory photosynthetic pigments; the nature of the stored food reserve; the composition of the cell wall, if present; and whether the body (**thallus**) is unicellular or multicellular.

✔ EXERCISE J | Studying and Classifying Algae

You will study six phyla of algae. Representatives range in size from microscopic to extremely large. Both freshwater and marine species exist.

Objectives

- ☐ Identify types of algae based on morphological characteristics.

Procedure

Obtain fresh material or prepared slides to study representatives of the six divisions of algae. Refer to Table 22J-1 for characteristics and methods of observation.

Table 22J-1 Characteristics of Algae

Phylum/Representative	Characteristics/Method of Observation
Euglenophyta* *Euglena* (a)	Unicellular. True eye-socket algae. **Flagellum** attached within reservoir, distinct orange-red eyespot adjacent to the flagellum. A flexible protein layer (pellicle) rather than a rigid cell wall allows the organism to change its shape. Many bright green chloroplasts. Locomotion: swimming, creeping, or floating. Method of Observation Make a wet mount of *Euglena.* Mix a drop of Protoslo with culture.
Chrysophyta *Diatoms* (b)	Diatoms only: Unicellular or chains of rod (**pennate**) or circular (**centric**) shapes. Cell walls of silica with numerous holes. Walls make two overlapping halves (**thecas**) that fit together like the halves of a Petri dish. Cells are brownish yellow. Locomotion: attached, gliding, or floating. Method of Observation Make a wet mount of diatomaceous earth, if available, or use material collected from stream rocks.
Pyrrophyta (Dinophyta) *Peridinium* *Gymnodinium* (c)	Unicellular. Spinning flagellates. All members are biflagellate and motile. One flagellum wraps around the middle of the cell and allows it to spin; another flagellum trails and pushes the cell along. Cell wall composed of many interlocking plates, giving an armored appearance. Brownish color. Locomotion: floating or swimming. Method of Observation Study prepared slide of *Peridinium.*
Phaeophyta (brown algae) *Fucus* *Laminaria* *Ectocarpus* (d)	Multicellular, small to massive. Organisms called rockweeds, kelps, and brown seaweeds. All marine. Body differentiated into **blade, stipe, holdfast,** and, in some, **air bladder floats.** Cell walls contain mucilage, algin, used commercially as additive to food and cosmetics and as a thickener for some ice cream. Almost all are attached to the bottom (**benthic**) in coastal marine environments. Method of Observation Study preserved specimens.

(continued on next page)

Table 22J-1 *(continued)*

Phylum/Representative	Characteristics/Method of Observation
Rhodophyta (red algae) *Corallopsis* *Dasya* *Gigartina* (e)	Multicellular filaments to medium-sized seaweeds. Red-colored. Predominantly marine. More branched than brown algae. Cell walls in certain species contain mucilage—carrageenan or agar used to give a smoother, thicker texture to many milk products and to make bacterial growth media Almost all are benthic and attached. Method of Observation Study preserved specimens.
Chlorophyta (green algae) *Chlamydomonas* *Spirogyra* *Gonium* *Volvox* *Zygnema* *Stigeoclonium* *Ulva* (see below)	Unicellular, colonial, filamentous. Green color, starch inside plastids. Many different forms adapted for attachment on benthic substrate or for swimming or floating in planktonic environments. Mostly freshwater types, some marine. Method of Observation Study fresh specimens or prepared slides as available.

*May be included among the flagellated protozoans.

✔ EXERCISE K | Diversity Among the Green Algae: Phylum Chlorophyta

It is thought that the ancestor of land plants was a green alga. Several evolutionary trends are obvious among the **Chlorophyta,** including:

- Increase in size accompanied by **cell differentiation.** Within a group of cells, certain cells have specific functions; individual cells do not act independently.
- Sexual reproduction. Among the algae are three types of sexual reproduction (listed from the most primitive to the most advanced):

  Isogamy Male and female gametes look exactly like (isogametes); both are motile.

  Anisogamy Also called heterogamy. Male and female gametes look alike except that the female gamete (egg) is larger; both are motile.

  Oogamy The male gamete (sperm) is small and motile. The female gamete (egg) is large and nonmotile.

Objectives

- ☐ Compare and contrast the representatives of the phylum Chlorophyta.
- ☐ Describe the progression in complexity of form observed among the green algae.

Procedure

Use prepared slides or, if fresh material is available, make temporary mounts of the following organisms. Observe the progression in size and complexity illustrated in the green algae. Sketch the organisms in the spaces provided.

Chlamydomonas (class Chlorophyceae)
Unicellular thallus.

Gonium (class Chlorophyceae)
Spherical colony made up of 4 to 32 cells, depending on the species.

Volvox (class Chlorophyceae)
Spherical colony made up of 500 to 50,000 cells, depending on the species.

Zygnema (class Chlorophyceae)
Simple, unbranched filament (cell division occurs in a single plane).

Stigeoclonium (class Chlorophyceae)
Branched filament (cell division occurs in two planes).

Oedogonium (class Chlorophyceae)
Simple unbranched filament with netlike chloroplast. Oogamous.

Ulva (class Ulvophyceae)
"Sheets" that are two cells thick (cell division occurs in three planes).

Ulothrix (class Ulvophyceae)
Simple unbranched filament with only the basal cell differentiated into a holdfast. Isogamous.

Spirogyra (class Charophyceae)
Unbranched green alga with one or more ribbonlike chloroplasts helically arranged. Isogamous.

Desmids (class Charophyceae)
Unicellular (some multicellular). Cell wall is in two sections with a narrow construction, the isthmus, between them.

✓ EXERCISE L | Recognizing Protists Among the Plankton

Plankton is a general term for small (mostly microscopic) aquatic organisms found in the upper levels of water where light is abundant. Plankton includes both plant-like photosynthetic forms (*phytoplankton*) and animal-like heterotrophic forms (*zooplankton*). A sample from enriched natural water, such as a fish pond, is an excellent source of algae and protozoans, as well as microscopic animals.

Objectives

☐ Identify diverse types of flagellates, ciliates, sarcodines, and algae.

Procedure

1. Place a small drop of the plankton sample on a slide and add a coverslip. Your instructor will provide some illustrations of types of organisms you are likely to see.
2. Identify as many organisms as possible. Various types of algae (diatoms, desmids, and, possibly, filamentous green algae) may be visible. Some of the flagellates you find may belong to the algal division Euglenophyta rather than Zoomastigophora, but they, too, illustrate the way in which flagellates, in general, move. Study this movement carefully.
   a. *Can you find any algae?* _______ *To which divisions might these algae belong?*
   ____

   How do you distinguish between algae of different divisions?
   ____

   b. *Are there any sarcodines?* _______ c. *Are there any ciliates?* _______
   d. *Can you find any multicellular rotifers? (These are members of the phylum Rotifera, one of the animal phyla.)* _______
3. Sketch representatives in the space provided below.

   e. *What might be the role of the plankton in the food chain of an ocean or lake?*
   ____

   f. *Both phytoplankton and zooplankton can migrate to various depths in a lake or ocean. What might cause these organisms to surface or to move to greater depths within a lake or ocean environment?*
   ____

Laboratory Review Questions and Problems

1. List three characteristics that distinguish prokaryotes from protists.

2. Why are the cyanobacteria included with the bacteria?

State the differences that distinguish cyanobacteria from green plants.

3. Fill in the table below, giving the distinguishing characteristics of each protozoan phylum and an example of a representative organism studied in the laboratory.

Protozoan Phylum	Distinguishing Characteristics	Example
Zoomastigophora		
Rhizopoda		
Ciliophora		
Sporozoa		

4. Slime molds and water molds are included among the Protista rather than in the kingdom Fungi. Why?

5. Fill in the table below, giving the distinguishing characteristics of each algal phylum and an example of a representative organism studied in the laboratory.

Algal Phylum	Distinguishing Characteristics	Example
Euglenophyta		
Chrysophyta		
Pyrrophyta		
Phaeophyta		
Rhodophyta		
Chlorophyta		

6. *Euglena* can be classified among the algae or among the flagellated protozoans. Explain why this organism is so difficult to classify.

7. A friend comments that you have more in common with an archaean than with a bacterium. Is this true? Why?

LABORATORY

Diversity—Fungi and the Nontracheophytes

23

OVERVIEW

Multicellularity allows for great increases in the size of organisms and for the specialization of their parts. The advantages of multicellularity have allowed organisms belonging to the kingdoms Fungi and Plantae to develop the specific structural modifications, life styles, and unique reproductive mechanisms required to adapt to and succeed in a variety of habitats.

During this laboratory period you will examine representatives of the **Fungi** and begin your study of the Plantae by examining the **nontracheophytes**—plants that lack special vascular tissues for the distribution of water, minerals, and photosynthetic products.

STUDENT PREPARATION

Prepare for this laboratory by reading the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

Last week you were given three plastic bags and a slice of bread. You exposed the pieces of bread to different environmental conditions (heat, cold, light, or dark) for approximately 6 days. You should also have formulated a hypothesis stating how you expected the experimental conditions you chose to affect fungal growth.

In Exercise A, Extending Your Investigation (p. 23-4), state your hypothesis and summarize your method of treatment (experimentation), results (observations), and conclusions supporting or refuting your supposition. Bring your experiment on fungal growth to the laboratory and place your materials on the demonstration table for other students to observe.

On campus or in a nearby woods, you may be able to find examples of fungi, lichens, and mosses. Bring them to class for identification. If you find something you do not recognize, bring it too. You and your classmates can try to identify unknown plant organisms during this laboratory period.

PART I KINGDOM FUNGI

Members of the kingdom Fungi were long classified among the plants, but they are so unlike any other plant group that taxonomists now assign them to a separate kingdom. Like other eukaryotes, fungi probably originated from an ancestral heterotrophic protist. Most fungi are multinucleate or multicellular, although some, including the yeasts, are uninucleate and unicellular. Unlike plants, the fungi lack chlorophyll. They have cell walls composed of cellulose or **chitin.**

Because fungi lack chlorophyll and therefore cannot manufacture their own food, they feed either on decomposing organic matter (**saprophytes**) or on living organisms (**parasites**). Fungi maximize their

contact with sources of nutrients by making branched or unbranched threadlike filaments called **hyphae,** which form a spreading mass called a **mycelium.**

Both asexual and sexual reproduction are common among the fungi and result in the production of **spores.** Elaborate mechanisms of spore production, including formation of many different, and sometimes bizarre, types of **fruiting structures,** protect fungal spores and promote their dissemination to new habitats.

Fungi can be grouped into four phyla based on their basic structure and patterns of reproduction, particularly sexual reproduction. The four phyla include: the Zygomycota, zygospore fungi; the Ascomycota, sac fungi; the Basidiomycota, club fungi; and the Chytridiomycota, chytrids. Because the imperfect fungi (deuteromycetes) are probably polyphyletic (all other phyla of fungi are monophyletic), they are not assigned phylum status.

EXERCISE A | Phylum Zygomycota

If you leave a piece of bread or other bakery product covered and at room temperature for a while, a fuzzy gray or black mold will appear. This fungus, *Rhizopus stolonifer,* a common bread mold, is representative of the **zygomycetes,** members of the phylum Zygomycota. This group of fungi is characterized by the formation of **zygospores**—special resting structures composed of a zygote surrounded by a thick protective wall. The zygospores germinate to form a fruiting structure, the **sporangium,** which produces spores by meiosis. Accumulations of these dark-colored sporangia give *Rhizopus* its gray-black color.

Objectives

- ☐ Identify the structural characteristics typical of zygomycetes, and distinguish between asexual and sexual reproductive structures.
- ☐ Explain the basis for the name "Zygomycota."

Procedure

1. Locate the following structures in the life-cycle diagram of *Rhizopus stolonifer* (Figure 23A-1) and familiarize yourself with the function or importance of each structure.

   Hypha Strands (hyphae) composing the fungal body are multinucleate (**coenocytic,** containing many haploid nuclei) in most zygomycetes. Some hyphae arch upward and are called **stolons.** These form hyphal **rhizoids** wherever they touch the substrate (surface on which the fungi are growing).

   Mycelium The fungal body composed of a mass of hyphae.

   Sporangium The structure responsible for producing spores, either asexually by mitosis from hyphal cells (**sporangiospores**) or sexually by meiosis from the **zygospore** (meiospores). A long stalk raises the sporangium above the surface, where air can disperse the spores.

   Gametangium Haploid cell formed at the tip of a hypha. Gametangia fuse to produce the zygote (Figure 23A-2a).

   Zygospore Zygote formed by the fusion (**syngamy**) of two gametangia and covered by a thick protective wall. Since the zygote is not derived from the fusion of true gametes (egg and sperm) but from the fusion of gametangia, it is called a zygospore. The zygospore germinates to form a sporangium which produces spores by meiosis (Figure 23A-2b).

2. *Without opening the dishes,* use a dissecting microscope to identify as many structures as possible in the available living material. Note the black "dots" on older parts of the mycelium.

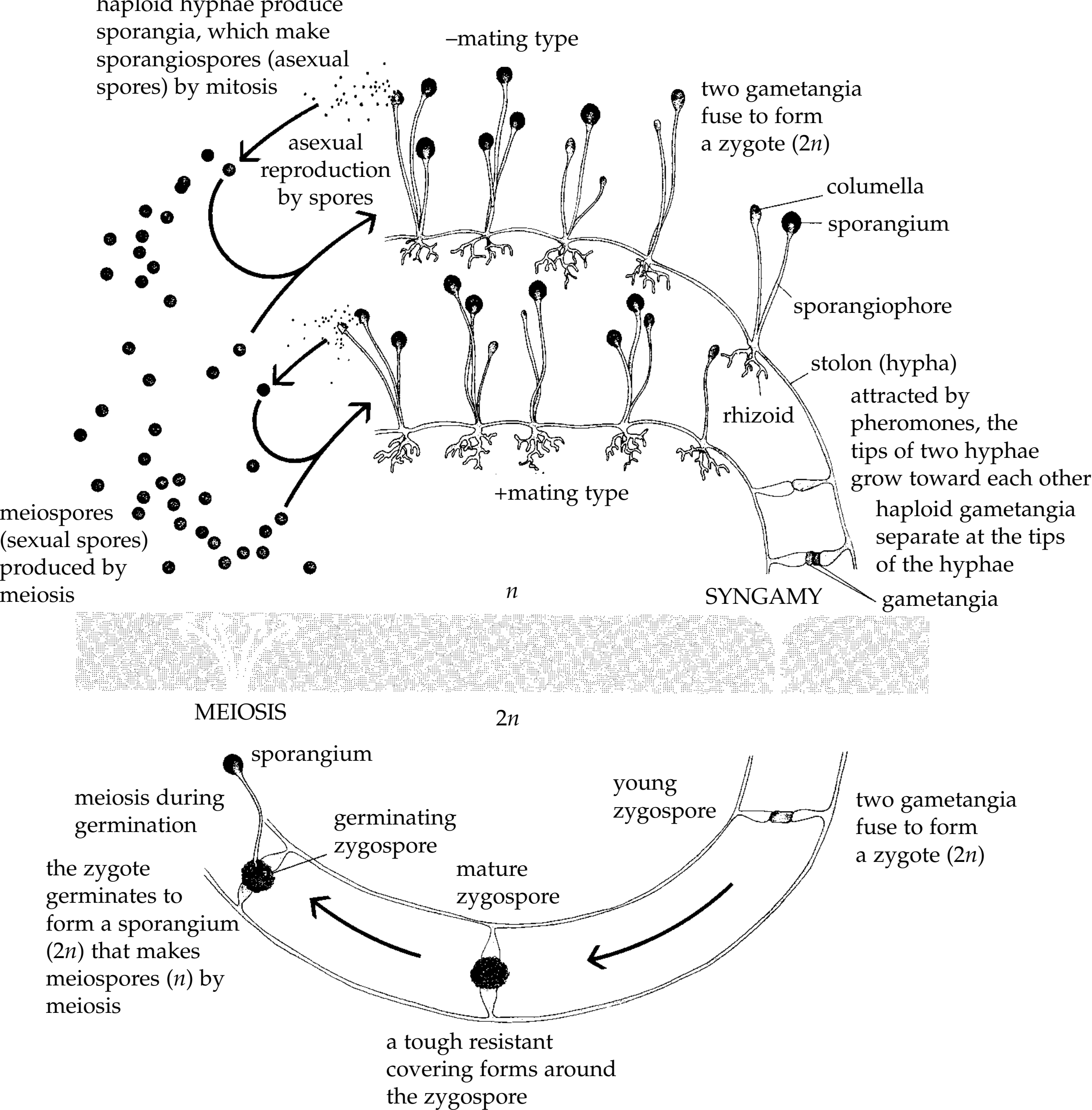
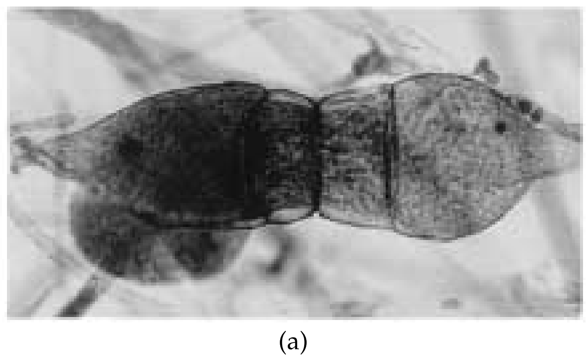
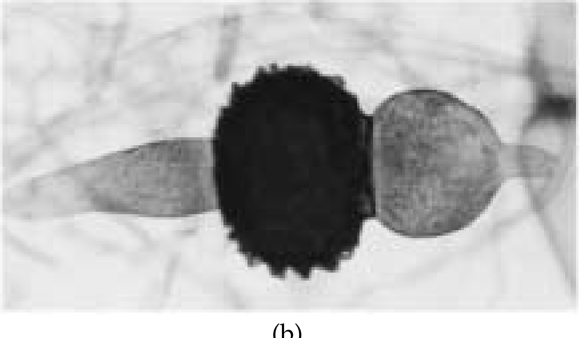


Figure 23A-1 *Life cycle of the zygomycete* Rhizopus stolonifer. *Sexual reproduction occurs between different mating strains, traditionally referred to as the + and − mating strains, even though they are morphologically alike. Asexual reproduction occurs by the mitotic production of spores within haploid sporangia.*

These are sporangia. Observe the upright stalks (**sporangiophores**) bearing the sporangia. Locate the stolons and rhizoids. Draw and label these structures in the space below.



(a)



(b)

Figure 23A-2 *(a)* Gametangia, *the gamete-producing structures of* Rhizopus stolonifer. *(b) A zygospore, or sexual resting spore (the dark mass in the center).*

3. Examine a prepared slide of *Rhizopus* showing stages of sexual reproduction. Locate the gametangia and zygospores. Draw and label these structures in the space below.

a. *Is the mycelium haploid* (n) *or diploid* (2n)*?* ________

b. *What types of products* (n *or* 2n) *are produced by mitosis?* ________ *By meiosis?* ________

c. *Are true gametes formed?* ________

d. *By what process are gametangia formed?* ______________________________

4. On Figure 23A-1, circle and label the portion of the life cycle involving sexual reproduction; now do the same for the portion of the life cycle involving asexual reproduction.

EXTENDING YOUR INVESTIGATION: CONDITIONS FOR FUNGAL GROWTH

Last week you were asked to choose experimental conditions for growing fungus on bread and to formulate a hypothesis about how these conditions would affect fungal growth. What was your hypothesis?

HYPOTHESIS:

NULL HYPOTHESIS:

What did you **predict** would happen under the conditions that you chose?

What was the **independent variable?**

What was the **dependent variable?**

What experimental procedure did you use to conduct your investigation? Describe it in the space below.

PROCEDURE:

OBSERVATIONS AND RESULTS:

What type of mold did you find growing on the bread? Was *Rhizopus* present? Examine the slices of bread brought to the laboratory by your classmates. Each person treated the bread in a different experimental manner. In the chart that follows, indicate the conditions tested by your classmates and state the results, growth or no growth.

Condition Tested	Growth or No Growth

Based on these data, what conditions favor the growth of mold or fungi?

From your observations, why do you suppose that molds are so common?

Do your results and those of your classmates support your hypothesis?

Your null hypothesis?

EXERCISE B Phylum Ascomycota

Ascomycetes, or sac fungi, are often referred to (along with the basidiomycetes) as "higher fungi" because their hyphae are made up of uninucleate cells partitioned by cell walls (septate), whereas the hyphae of Zygomycota are coenocytic (multinucleate).

Ascomycetes include some familiar fungi such as morels, truffles, *Sordaria*, *Neurospora*, and yeasts. One member of this division, *Claviceps*, produces a mycelium known as ergot on grasses such as rye grass. Perhaps one of the most well known ergot-derived products is LSD. Yeasts produce ethyl alcohol by the

process of fermentation and thus are important in the production of beer and wine. Chestnut blight, Dutch elm disease, apple scab, brown rot in fruit, and powdery mildew infections are serious plant diseases caused by the ascomycetes. There are approximately 30,000 known species of ascomycetes.

Asexual reproduction in the ascomycetes occurs by the mitotic production of haploid **conidiospores,** which are produced in long chains at the end of specialized hyphae called **conidophores.**

Sexual reproduction in most ascomycetes results in the development of an **ascus** (derived from the Latin word for "sac") containing four to eight **ascospores** (four are produced by meiosis; additional spores are the result of mitosis). A group of asci are usually found together in a fruiting body known as an **ascocarp** (Figure 23B-1). The ascocarp occurs in a variety of shapes, by which different ascomycetes can be identified. Species that have an ascocarp are collectively called *euascomycetes* (true ascomycetes); those without ascocarps are called *hemiascomycetes* (half ascomycetes).

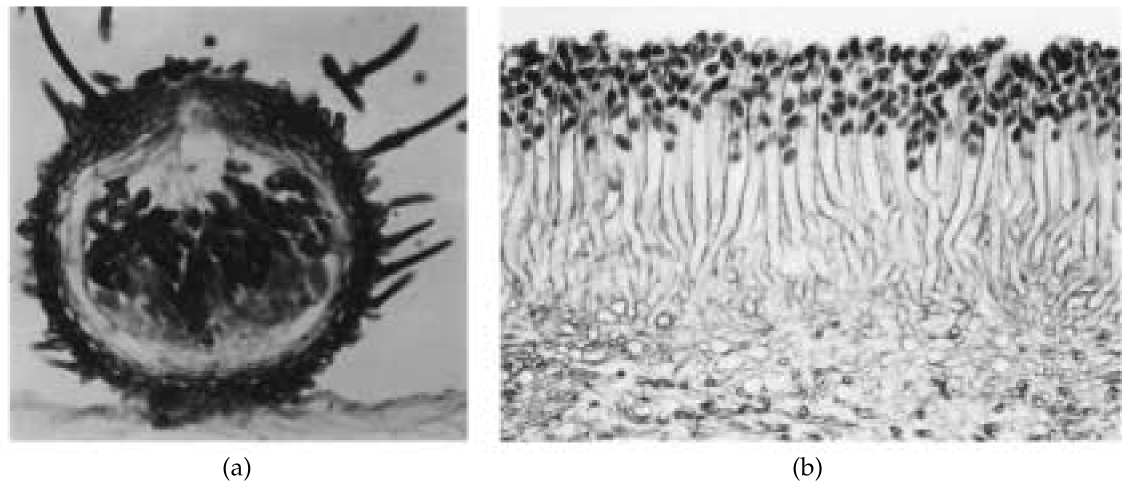


Figure 23B-1 *(a) Ascocarp of* Chaetomium erraticum *showing enclosed asci and ascospores. Note the small pore at the top. (b) A section through a layer of asci on the inner surface of an ascocarp of* Morchella.

Objectives

- ☐ Identify the structures typical of ascomycetes.
- ☐ Distinguish between mechanisms of asexual and sexual reproduction.
- ☐ Explain the basis for the name "Ascomycota."

Procedure

1. Use a dissecting microscope to examine living material from *Sordaria,* or observe prepared slides using the 40× objective on your microscope. In the space below, sketch and label the fruiting bodies (ascocarps), asci, and ascospores of the ascomycete *Sordaria.*

2. Remove several of the small, black, round fruiting bodies from a culture of *Sordaria* and prepare a wet-mount slide. Press on the coverslip with a small cork to squash the ascocarps and release the asci (Figure 23B-2). Study the slide at high power (40× objective). In the space next to Figure 23B-2, draw and label your observations.

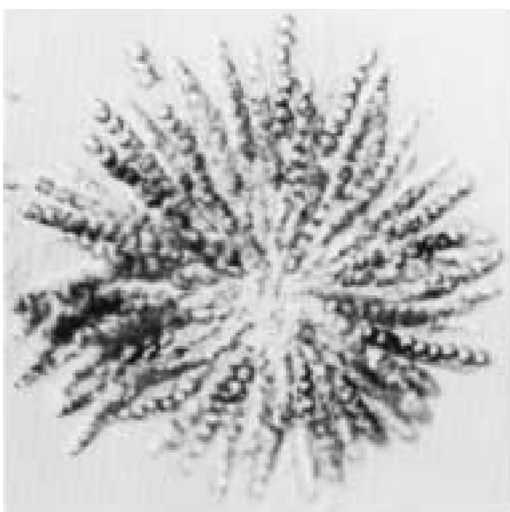


Figure 23B-2 Sordaria *ascospores within asci.*

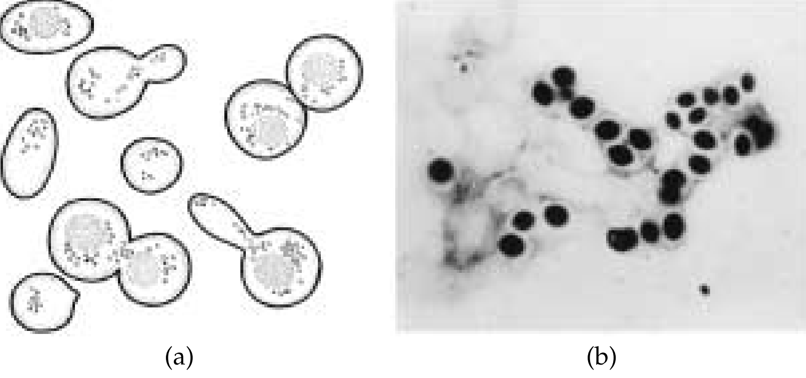
a. How many ascospores are present in each ascus? ______

b. By what division process (or processes) were the ascospores produced?

____

3. Obtain a drop of yeast culture and make a wet-mount slide. Notice the buds on some of the cells. Instead of having a hyphal filament, yeasts are unicellular and can reproduce asexually by budding or sexually by the production of asci (Figure 23B-3a, b).

Figure 23B-3 *Yeasts. (a) Budding cells of bread yeast,* Saccharomyces cerevisiae. *(b) Asci with ascospores of* Schizosaccharomyces octosporus.



4. Examine a prepared slide of *Schizosaccharomyces* on demonstration. Identify an ascus containing ascospores (Figure 23B-3b).

c. How many ascospores are present in each ascus? ______

✔

EXERCISE C | Phylum Basidiomycota

The **basidiomycetes,** or club fungi, have a septate mycelium, as do the ascomycetes, but they differ from the ascomycetes in having sexual spores (**basidiospores**) borne *externally* on a club-shaped structure, the **basidium,** instead of within a sac.

The fruiting body of the basidiomycetes is the **basidiocarp.** The mature basidiocarp develops a large number of pores or gills on its underside. These gills contain numerous club-shaped basidia, single cells that produce basidiospores (Figure 23C-1). Basidiocarps come in many different sizes, shapes, and colors. Some, such as the mushrooms with which you are familiar, are edible.

Rusts, smuts, puffballs, toadstools, and shelf fungi are also members of the phylum Basidiomycota. Many species form **mycorrhizal** associations with plants, in which case a symbiotic relationship develops between fungal hyphae and plant roots, providing both the plants and fungi with important nutritional elements.

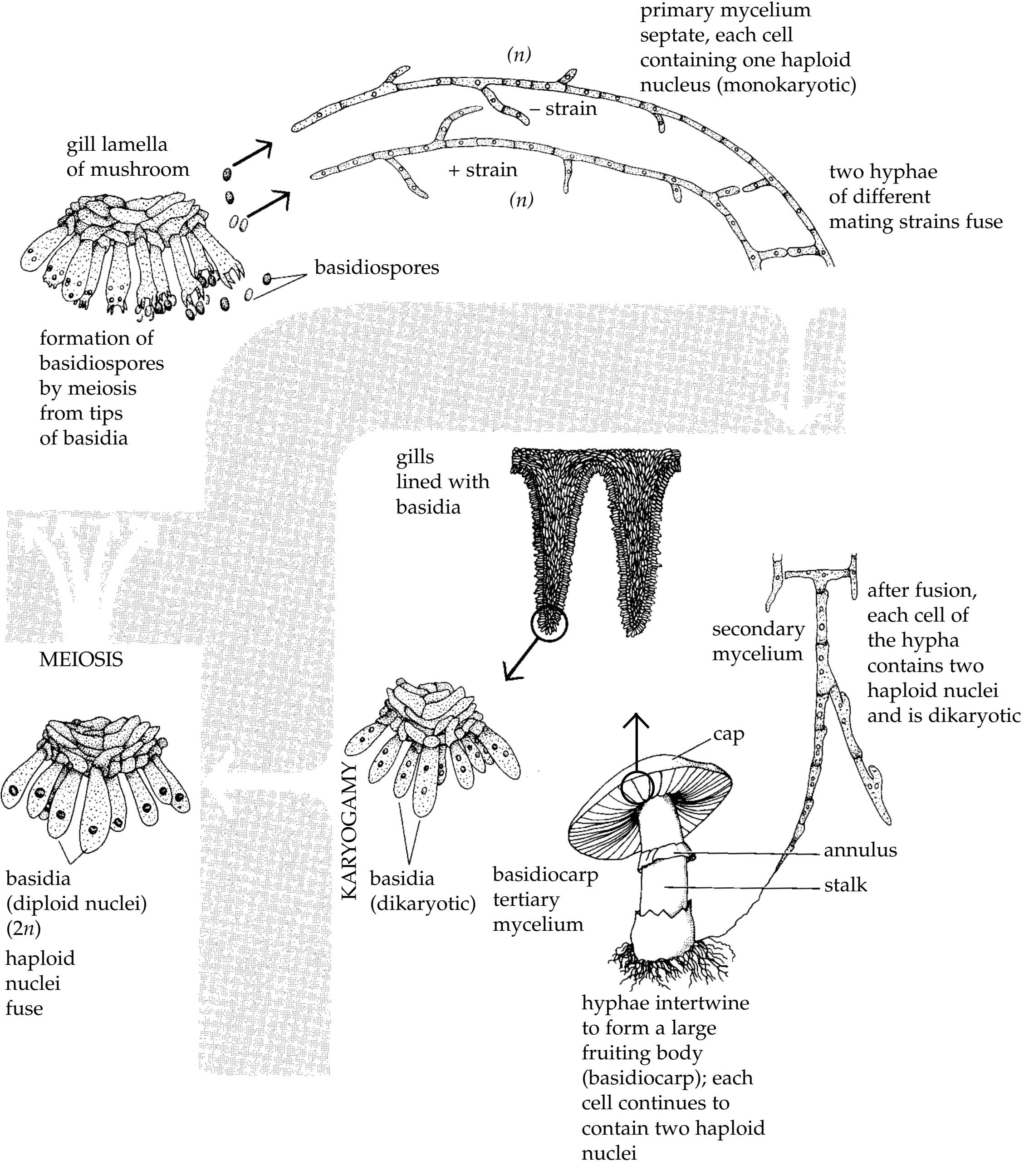


Figure 23C-1 *Generalized life cycle of a basidiomycete.*

Objectives

- ☐ Identify the structures typical of basidiomycetes.
- ☐ Describe how a "mushroom" is formed.

Procedure

1. Obtain a fresh edible mushroom, *Agaricus*, and examine it carefully. Identify the parts described below and locate these on the life-cycle diagram (Figure 23C-1).

   Cap The umbrella-shaped portion of the fruiting body (basidiocarp).

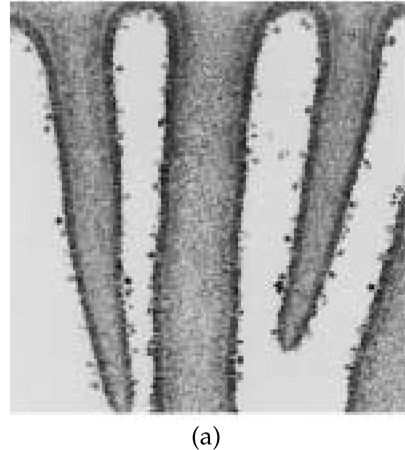
   Gills Radiating strips of tissue (**lamellae**) on the undersurface of the cap; basidia form on the surface of the gills.

   Basidia Club-shaped, spore-producing structures on the surface of the gills (Figure 23C-2a).

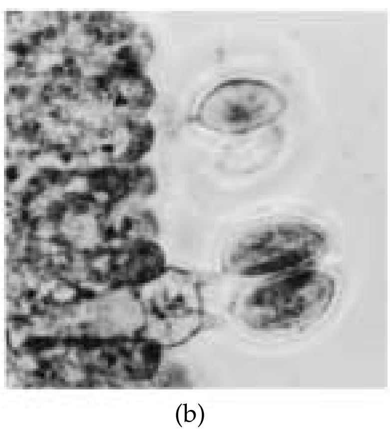
   Basidiospore A spore produced by meiosis on the outside of a basidium (Figure 23C-2b).

   Stalk The upright portion of the fruiting body that supports the cap—a mycelium composed of many intertwined hyphae.

   Ring (or **annulus**) A membrane surrounding the stalk of the fruiting body at the point where the unexpanded cap was attached to the stalk.



(a)



(b)

Figure 23C-2 *(a) Section through the gills of* Coprinus. *The dark margins constitute the layer of developing basidia. (b) Mature basidiospores attached to a basidium.*

2. Remove a small portion of several gills. While holding them together, cut a very thin cross section with a razor blade. Put sections in a drop of water on a slide and cover this with a coverslip. You should be able to see basidia on the surface of the gills. Study your slide at high power (40× objective).

   a. *Can you see the hyphae making up the thickness of the gills?* _______ *Are they septate?* _______

   Are the basidia club-shaped? _______

3. For a clearer view of the reproductive structures of basidiomycetes, examine a prepared slide of a cross section through the cap of the basidiomycete *Coprinus.* Find basidia and basidiospores (Figure 23C-2a, b). In the space below, draw and label your observations.

4. Note the diversity of structures in other examples of basidiomycete fruiting bodies on demonstration. Draw several of these in the space below.

EXERCISE D | Phylum Chytridiomycota

Chytridiomycota is the most ancient of the fungal phyla (Figure 23D-1). Chytrids are often classified among the protists, along with the Oomycetes and other water molds. This is because they have flagellated spores and gametes—the only flagella present in the kingdom Fungi. Molecular evidence indicates, however, that they are monophyletic with the fungi. They were probably the first to diverge from a common fungal ancestor. All fungi in the other branch lost their flagella.

Chytrids are either parasitic or saprobic. Most live in fresh water or in damp soil, but some are marine. Some are unicellular, while others take the form of branched chains. They can reproduce sexually or asexually. *Allomyces,* a common chytrid, displays alternation of generations. See Figure 23D-2.

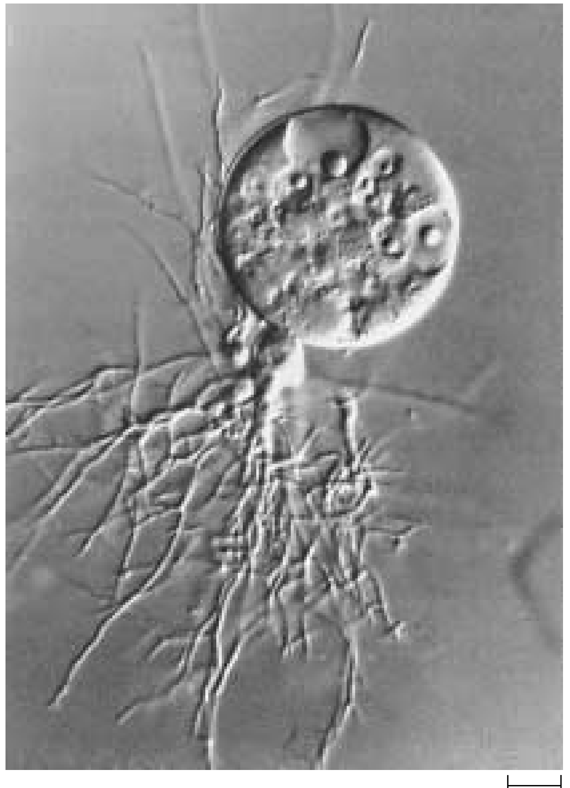


Figure 23D-1 Chytridium confervae, *a common chytrid. You can see the slender rhizoids extending downward.*

Objectives

- ☐ Observe life-cycle stages of chytrids.

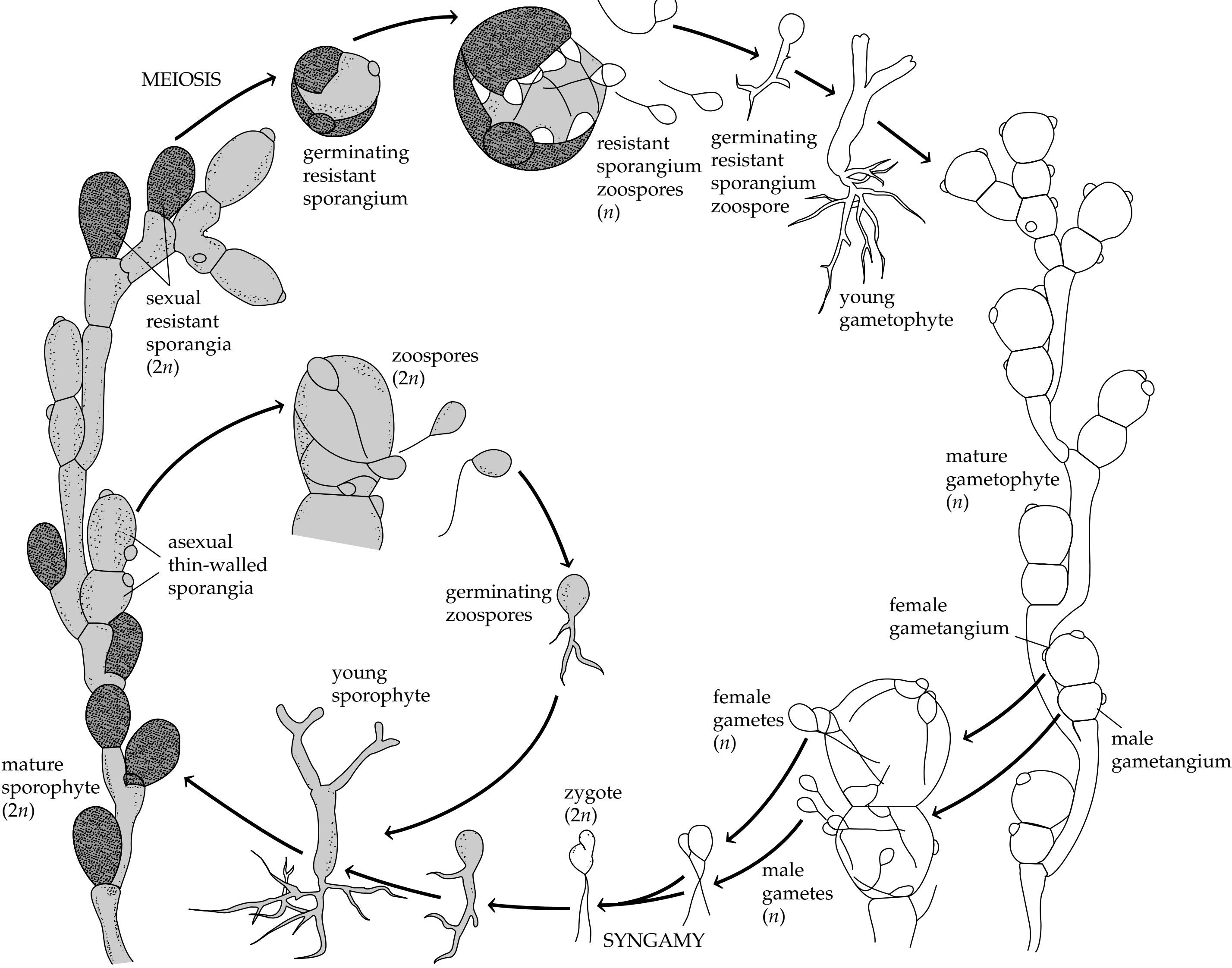


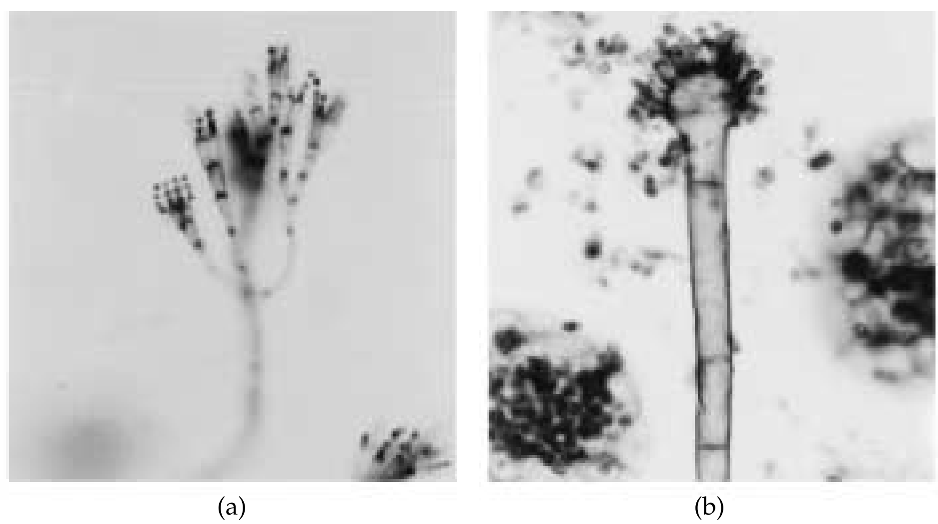
Figure 23D-2 *Life cycle of the chytrid* Allomyces arbusculus. *In* Allomyces, *haploid zoospores germinate on the seeds or dead plant material available. They form male and female gametangia, which produce haploid gametes by mitosis. Both types of gametes have flagella. The female gametes produce a chemical that attracts the male gametes, and they fuse to form a diploid zygote. Cell divisions give rise to a small diploid organism that can then produce diploid flagellated zoospores, which germinate to form more diploid organisms. Eventually the diploid organisms form a thick wall around themselves and become resting zygospores. Meiosis within the resting zygospores produces haploid zoospores, and the cycle begins again.*

Procedure

1. Examine living cultures of *Allomyces* on demonstration. Identify the resting spores.
2. Remove a sample of the lake water and examine it under the microscope (40×).
   a. Do you see any flagellated zoospores? ______

✓ **EXERCISE E** | **The Deuteromycetes**

Deuteromycetes, the "imperfect" fungi, are those fungi in which the sexual stages are not known to exist. This may be because these fungi have not been completely studied, or because the sexual stages have truly been lost during the course of evolution. Reproduction is asexual by **conidia** (Figure 23E-1). Unlike spores, conidia are produced at the tips or sides of haploid hyphae rather than within sporangia. Examples in this group include blue molds and green molds, some of which are important sources of antibiotics; others are used to add flavor or odor to certain cheeses. The genus **Trichophyton** causes athlete's foot.



(a) (b)

Figure 23E-1 *The conidiophores of (a)* Penicillium; *(b)* Aspergillus.

Objectives

☐ Explain why the deuteromycetes are considered to be "imperfect" fungi.

Procedure

Examine a prepared slide of *Aspergillus* or *Penicillium.* Study the slide using the compound microscope (40× objective) and identify conida. Notice that most of the conidia dispersed as the slide was made. Air currents perform this task in nature. In the space below, make a labeled sketch of your observations.

a. Since the imperfect fungi are not known to have a sexual cycle, are conidiospores produced by mitosis or meiosis?

____

b. Of what economic importance is the mold Penicillium *to the medical industry?*

____

To the food industry? ____

EXERCISE F Identification of Collected Fungi

Objectives

☐ Classify a variety of local fungal specimens by phylum or type.

Procedure

1. Now that you have completed your study of the fungi, try to determine the type of fungus you collected. Give the reasons for your decision.
2. On a 3" × 5" card, write your name, the date, the type of fungus collected, and the place where it was found, and describe the substrate upon which the fungus was growing. Place the card and your material in the demonstration area. Examine the other specimens and decide whether or not you agree with your classmates' identifications. If you disagree with a particular identification, locate the "collector" and see if you can come to an agreement.
   a. *List several ways in which fungi are beneficial to humans.* ______________________

   ____

   b. *List several ways in which fungi are harmful to humans.* ______________________

   ____

EXERCISE G Diversity Among the Lichens

Lichens are distinct organisms that are actually two organisms in one. The body is made up of certain genera of green algae or cyanobacteria that embed themselves in the mycelium of a fungus (usually an ascomycete or a basidiomycete) and live symbiotically with it. The fungus is the dominant (most prominent) of the two organisms. Thus lichens are usually studied with the kingdom Fungi.

Lichens are found on tree trunks, rocks, and arctic mountain tops, to name just a few locations. Often lichens are the first colonists on bare, rocky areas.

Objectives

☐ Recognize lichens in nature and identify their growth forms.

Procedure

1. Note the growth forms of lichens on demonstration. See if you can identify the following three types (Figure 23G-1a–c): **crustose,** closely encrusting bodies; **foliose,** leafy bodies; **fruticose,** shrubby, branching bodies.
2. Examine a prepared slide of lichen thallus showing algal cells surrounded by fungal hyphae (Figure 23G-1d). In the space below, draw and label your observations.
3. Did you or others in your class collect any lichens?
   a. *Do your lichen collections (or lack or them) reflect the air quality in your community or are they simply indicative of the habitats surveyed for the collections?* ______________________

   ____

   b. *In which Kingdom would you classify lichens: with the algae?* ____________*with the fungi?*

   ____

   c. *Which lichen characteristics would you use for classification?* ______________________

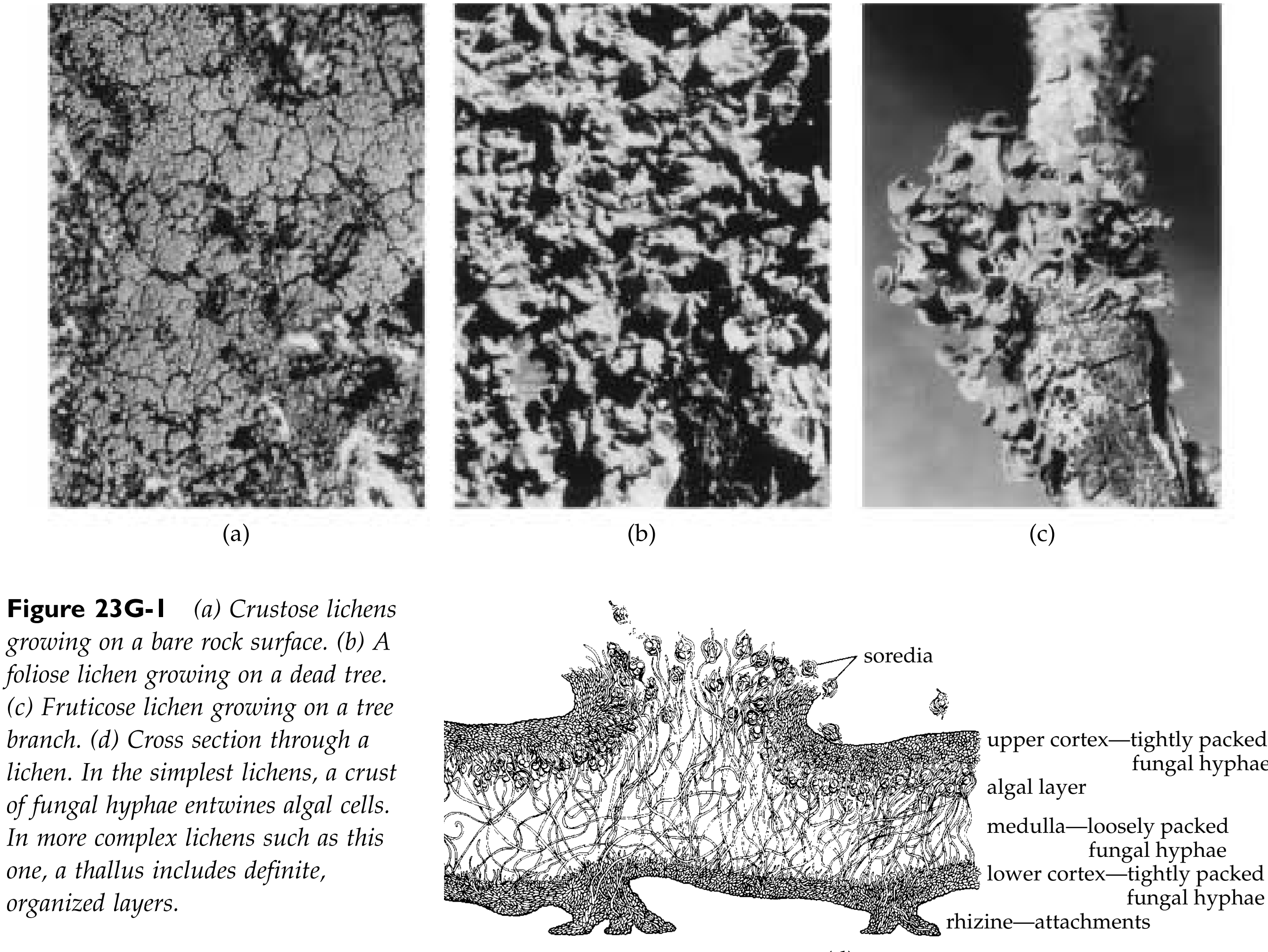


Figure 23G-1 *(a) Crustose lichens growing on a bare rock surface. (b) A foliose lichen growing on a dead tree. (c) Fruticose lichen growing on a tree branch. (d) Cross section through a lichen. In the simplest lichens, a crust of fungal hyphae entwines algal cells. In more complex lichens such as this one, a thallus includes definite, organized layers.*

PART II KINGDOM PLANTAE

NONTRACHEOPHYTES

Members of the kingdom Plantae are photosynthetic autotrophs. Those in existence today can be grouped into 12 phyla. Most are land plants that developed the following adaptations to make the transition from an aquatic to a terrestrial environment:

- A waxy coating or cork layer that retards water loss on plant parts located above ground.
- Pores (called **stomata**) in the aboveground parts for gas exchange.
- Multicellular reproductive organs (**gametangia** and **sporangia**).
- Retention of the fertilized egg within the female gametangium so that the young sporophyte plant is protected.

Not long after the transition to land, plants diverged into two lines—one gave rise to the **nontracheophyte plants** (including mosses, hornworts, and liverworts) and the other to the **tracheophyte plants** (including ferns, gymnosperms, and angiosperms). (*Note:* The term "nonvascular plants" has long been used to describe the mosses, liverworts, and hornworts, but it is misleading because some mosses, unlike liverworts and hornworts, have limited amounts of vascular tissue. The term "bryophytes" has also been used to describe these three phyla of nontracheophytes, but this term is now used to refer to the mosses only.)

In general, tracheophytes (vascular plants) have specialized vascular tissues: **xylem** for the transport of water and minerals upward to the plant body and **phloem** for the distribution of photosynthetic products.

Nontracheophytes absorb moisture mainly through aboveground structures and depend on diffusion for transport.

All land plants have the same type of life cycle involving an alternation of generations. A haploid **gametophyte** plant produces haploid (n) gametes by mitosis. These gametes fuse to form a diploid ($2n$) **zygote,** which then grows into a diploid plant, the **sporophyte,** which produces spores (n) by meiosis. The haploid spores then develop into haploid gametophytes and the cycle begins again (Figure 23II-1).

Among the nonvascular plants, the gametophyte generation is the most conspicuous and occupies the dominant part of the life cycle.

Before you continue, make sure you understand the importance of the last two paragraphs. Check your understanding by answering the following questions.

a. In plants, what process produces gametes? ____________________

b. What is the process that produces gametes in animals such as humans? ____________________ *Is this the same process that produces gametes in plants?* ________

c. Why is it beneficial to plants to produce spores in addition to gametes?

____

d. What process produces spores? ____________________

e. Why don't humans produce spores? ____

f. What is the dominant part of the life cycle in the nontracheophytes?

____

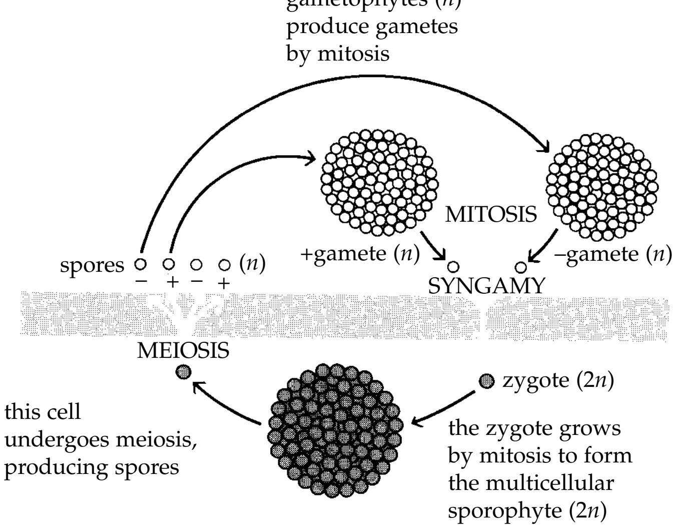


Figure 23II-1 *Generalized life cycle, known as alternation of generations, characteristic of land plants.*

EXERCISE H Nontracheophytes—Mosses, Liverworts, and Hornworts

Although basically terrestrial, nontracheophyte plants are restricted to moist habitats such as creek banks and moist woods. Some are even aquatic, though none are marine. Members of this group are small plants that have structures resembling roots, stems, and leaves, but because most nontracheophytes lack the vascular tissues typical of most land plants (phloem and xylem), they do not, strictly speaking, have "true" roots, stems, or leaves.

A distinct alternation of generations occurs. Both gametophyte and sporophyte are multicellular and visible to the eye, with the gametophyte being more prominent.

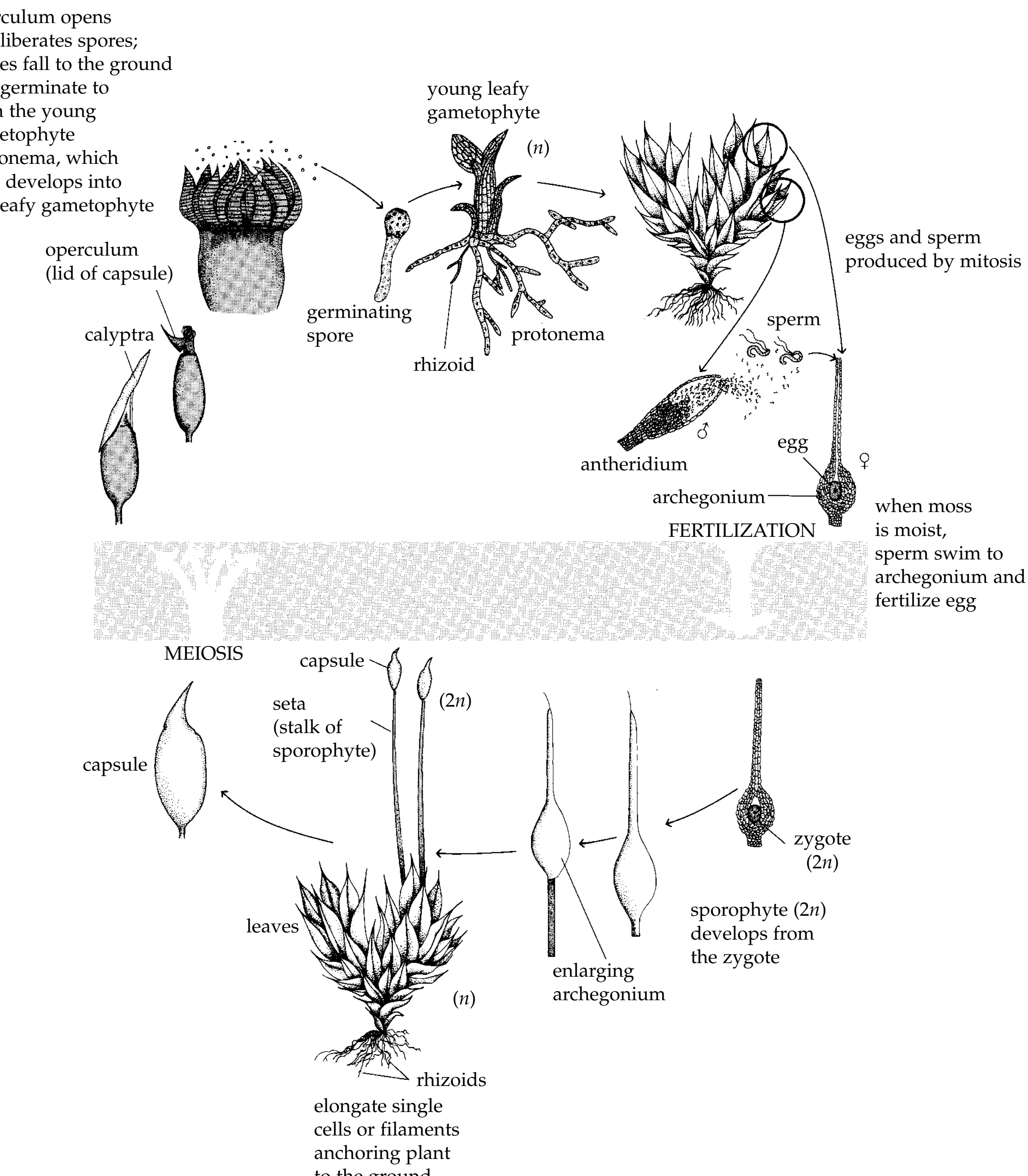


Figure 23H-1 *Life cycle of the moss. The gametophyte generation is the soft, green carpet you walk on in the woods. Gametes are produced by mitosis within specialized gametophyte structures called archegonia (produce eggs) and antheridia (produce free-swimming biflagellated sperm).*

If you examine moss closely at certain times of the year, you will see the sporophyte generation—small hairlike projections topped by capsules (sporangia)—rising above the green leaves of the gametophytes. Spores produced within the sporangia by meiosis fall to the ground and germinate, giving rise to a filamentous or platelike protonema—the new gametophyte generation.

Mosses (phylum **Bryophyta**), liverworts (phylum **Hepatophyta**), and hornworts (phylum **Anthocerophyta**) are the three phyla of nontracheophyte plants.

Objectives

- ☐ Name the major structures of a moss.
- ☐ Describe the life cycle of a typical bryophyte.

Procedure

1. Examine fresh moss (phylum Bryophyta) sporophyte and gametophyte material and identify the following structures. Refer to the moss life-cycle diagram (Figure 23H-1) and be sure that you understand the relative importance and function of each structure.

Gametophyte The leafy green plant of the haploid generation.

"Leaves" Bladelike structures spirally or alternately arranged around the axis of the moss gametophyte.

Rhizoids Rootlike structures anchoring the gametophyte.

Protonema Haploid structure produced by the germinating spore, which gives rise to the gametophyte.

Sporophyte The body of the diploid generation, consisting of a foot, stalk (**seta**), and capsule (Figure 23H-2).

Capsule (sporangium) The top portion of the moss sporophyte within which spores are produced. Spores are released through the lid (**operculum**) of the capsule.

Spores Haploid reproductive structures responsible for the asexual portion of the moss life cycle.

a. Why do mosses need a moist environment to reproduce sexually? (Refer to the moss life cycle, Figure 23H-1.) ______________________________

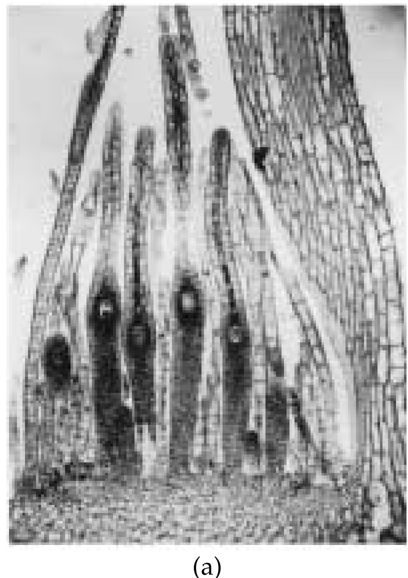
____

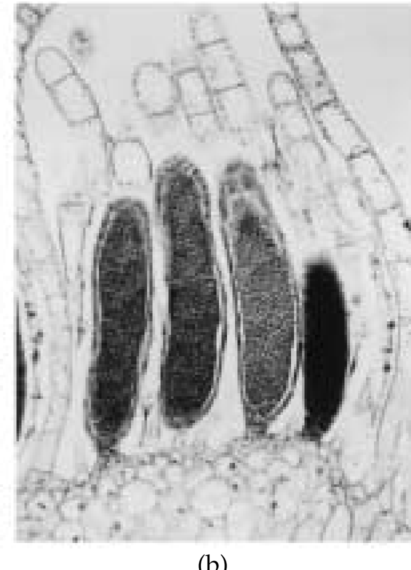
(a)

(b)

Figure 23H-2 *Spore-bearing setae of the hairy moss,* Pogonatum brachyphyllum. *(b) Capsule of the sporophyte of a moss, with the calyptra (the enlarged archegonium) totally removed, revealing the lid, or operculum, of the capsule.*

Figure 23H-3 *Gametangia of a moss,* Mnium. *(a) Longitudinal section through female gametangia (archegonia) with eggs. (b) Longitudinal section through male gametangia (antheridia) containing male gametes, sperm.*


(a)


(b)

b. How (or when) do mosses growing in dry environments reproduce?

____

____

2. Examine prepared slides of moss antheridia and archegonia (Figure 23H-3) and identify the following structures. (Refer to the moss life-cycle diagram to be sure that you understand their relationship to other gametophyte and sporophyte structures.) Draw and label your observations in the space below.

Antheridium The male reproductive organ in which sperm develop.

Sperm The motile (flagellated) male gamete produced in an antheridium.

Archegonium Female gametangium in which the egg develops.

Egg Nonmotile female gamete produced in an archegonium.

c. Is water necessary for fertilization? _______

d. The archegonium produces ____________________.

e. The antheridium produces ____________________.

f. The archegonium and antheridium are part of the ____________________ *generation.*

g. Is the gametophyte generation haploid or diploid? ____________________

h. The sporophyte structure produces ____________________.

i. Are spores haploid or diploid? ____________________

j. The process that produces spores is ____________________.

k. What do you notice about the shapes of the antheridia and archegonia?

____

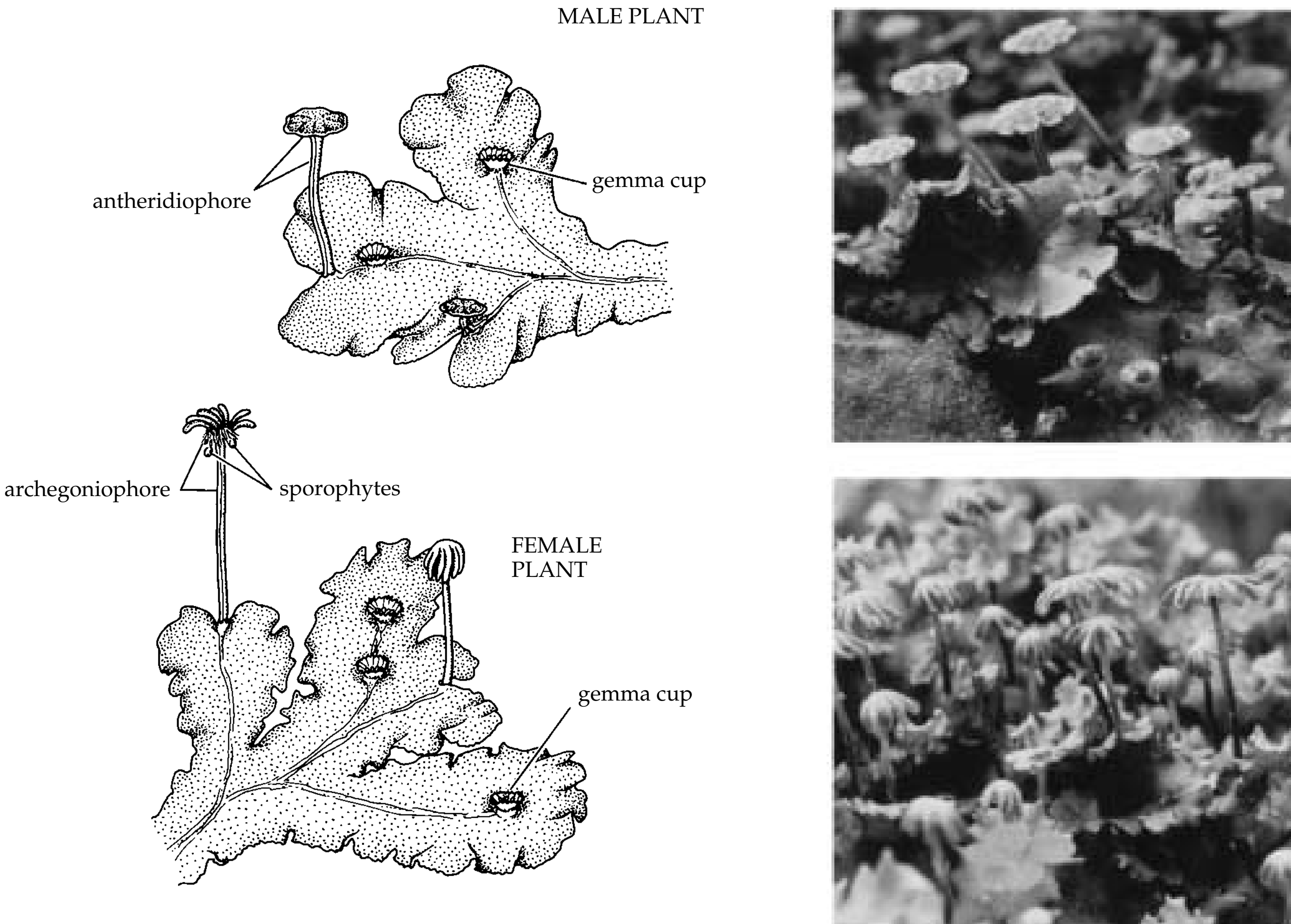


Figure 23H-4 *Gametophyte and sporophyte of the leafy liverwort* Marchantia. *The male and female gametophyte plants bear raised gametangia. Antheridia, which produce sperm, appear on the male plant; archegonia, which produce eggs, appear on the female plant.*

l. How would you describe the relationship between their shapes and their functions?

____

3. If liverworts (phylum Hepatophyta) are available, examine them carefully (Figure 23H-4). At first glance, a liverwort will probably not resemble any other kind of plant you have seen before. Notice that the plant body is not differentiated into recognizable roots, stems, or leaves. The type of structure you see is called a **thallus.** Liverworts assume one of two different forms: **thallose,** in which the plant is flat, ribbonlike, and dichotomously branched; or **leafy,** in which the thallus is lobed and leaflike in appearance (Figure 23H-4). More liverworts are leafy than thallose, but the species that you will see is a type of thallose liverwort.

4. Obtain a living liverwort, *Marchantia,* and examine it with a dissecting microscope. The plant body is the gametophyte—as in the moss, the gametophyte generation in the liverwort is the dominant one. The gamete-producing structures, archegonia and antheridia, appear as small palm-tree-like structures on the thallus of male and female plants. On the lower surface of the thallus, you will find rootlike structures (**rhizoids**) with which the liverwort adheres to its growth site and obtains nutrients. Also, look for **cupules** on the upper surface of the thallus.

These cups contain specialized reproductive structures called **gemmae** used for asexual reproduction (Figure 23H-4).

5. Using your knowledge of the typical life cycle of a moss and your observations of *Marchantia,* draw a typical life cycle for the liverwort in the space below.

6. If hornworts (phylum Anthocerophyta) are available in your laboratory, examine their structure and identify the parts belonging to the sporophyte and gametophyte generations (Figure 23H-5).

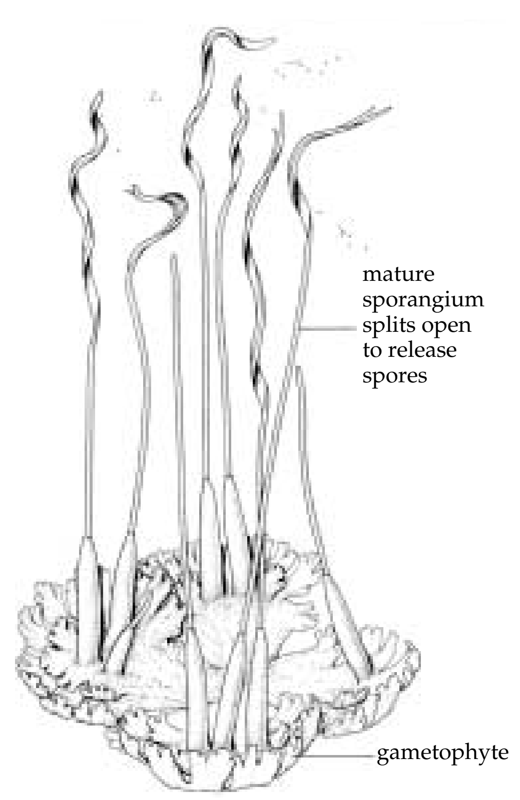


Figure 23H-5 *Gametophyte of* Anthoceros, *a hornwort, showing attached sporophytes.*

Laboratory Review Questions and Problems

1. Fill in the following table to summarize the differences among phyla in the kingdom Fungi.

Phylum	Distinguishing Characteristics	Type of Hyphae	Type of Reproductive Structures
Zygomycota			
Ascomycota			
Basidiomycota			
Chytridiomycota			

2. Why are the deuteromycetes ("fungi imperfecti") not included among the phyla of fungi?

3. Why are the chytrids (phylum Chytridiomycota) unusual among the fungi?

4. Lichens are usually studied with the kingdom Fungi. Is there another kingdom with which they might be studied? Explain.

5. Why can lichens be used as an example of symbiosis?

6. Why is it not correct to refer to mosses as "nonvascular plants"? How are the following terms related: bryophytes, nontracheophytes, nonvascular plants?

7. Draw a typical life cycle for a moss. Which generation is the conspicuous generation?

8. Define the following terms and indicate whether each term can be used to describe mosses, liverworts, or both.

Rhizoids

Sporangium

Thallus

Gemmae

Archegonium

Antheridium

Spores

LABORATORY 24

Diversity—The Tracheophytes (Vascular Land Plants)

OVERVIEW

Despite the variety of their forms and the diversity of the environments to which they have become adapted, all vascular land plants, or tracheophytes, share some characteristics that reveal a common evolutionary organ.

Alternation of generations is a characteristic common to all land plants. As you examine the evolutionary progression of land plants, from nonvascular to vascular forms, you will notice a continuous reduction in the size and complexity of the gametophyte until it ultimately becomes greatly reduced and nutritionally dependent upon a much larger, more complex sporophyte.

Also associated with the evolutionary trend toward sporophyte dominance is a change from the **homosporous** condition (the simplest vascular plants produce only one type of spore) to the **heterosporous** condition (more advanced vascular plants produce two different types of spores—megaspores, which develop into female gametophytes, and microspores, which develop into male gametophytes). Another factor essential to the success of tracheophytes on dry land is the change from unprotected zygotes to the protection of the embryo sporophyte within a seed. In addition, modern tracheophytes contain networks of vascular strands (xylem and phloem). Leaves in the most primitive vascular plants have only single vascular strands.

During this laboratory period, you will study representatives of the many types of vascular plants, both with and without seeds.

STUDENT PREPARATION

To prepare for this laboratory, read the text pages indicated by your instructor. Familiarizing yourself in advance with the information and procedures covered in this laboratory will give you a better understanding of the material and improve your efficiency.

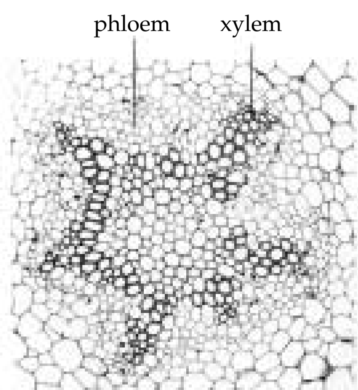
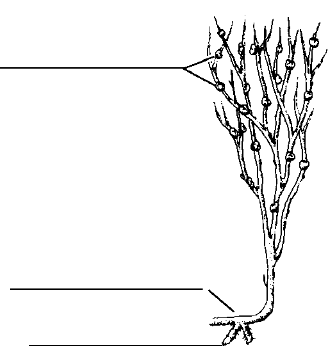
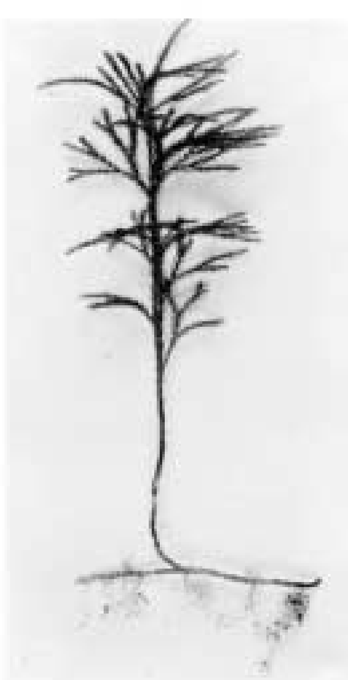
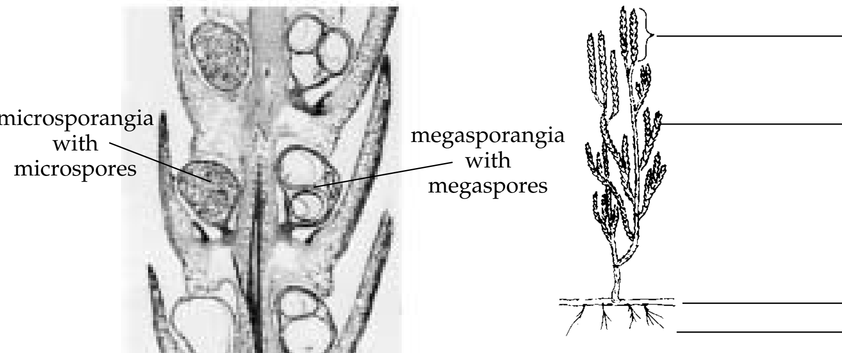
PART I TRACHEOPHYTES (VASCULAR PLANTS) WITHOUT SEEDS

EXERCISE A Examining Seedless Tracheophytes

The tracheophytes without seeds include the following phyla: the Psilophyta, whisk ferns; the Lycophyta, club mosses; the Sphenophyta, horsetails; and the Pterophyta, ferns. Most of the members of the first three phyla are extinct; their characteristics are summed up in Table 24A-1.

Extinct Lycophyta once loomed as giants in the forests of the Carboniferous period. All living lycopods, however, are small. *Lycopodium* species are commonly called ground pine or club moss. An entire patch of *Lycopodium* may be connected by a single **rhizome,** a characteristic that once made this plant a favorite for wreath-making (collection is now prohibited in most states).

Table 24A-1 Vascular Plants, Divisions Psilotophyta, Lycophyta, and Sphenophyta

Phylum	Characteristics	Method of Observation
Psilotophyta Rep. *Psilotum* (whisk fern) 	Sporophyte lacks both leaves and roots. Dichotomously branched triangular stem with small scalelike appendages in place of leaves. **Rhizomes** and **rhizoids** in place of roots. Vascular tissue present (see below). **Spores** produced in **trilobed sporangia** (spore-producing organs). Homosporous. 	Study preserved and living material on demonstration. Label sporangia, rhizomes, and rhizoids on the diagram below. Observe vascular tissue (phloem and xylem) in a cross section of a *Psilotum* stem, if available. 
Lycophyta Rep. *Lycopodium* (club moss) 	Leaves considered to be true leaves since they contain vascular tissue. Sporangia produced in the **axils** of leaves called **sporophylls;** these form conelike structures called **strobili.** Roots and rhizomes are present. *Lycopodium* is homosporous. *Selaginella* is heterosporous. In the *Selaginella* strobilus below, note the microsporangia and megasporangia. 	Identify specimens of *Lycopodium* and *Selaginella* on demonstration. Identify microphyllous leaves (sporophylls), strobili, roots, and rhizomes. Label these structures on the diagram below.

(continued)

Table 24A-1 *(continued)*

Phylum	Characteristics	Method of Observation
Sphenophyta Rep. *Equisetum* (horsetail)	Branched, ribbed stem with well-defined **nodes** and **internodes.** **Microphyllous leaves** occur regularly at each node. **Sporangia** produced within the **strobilus.** Homosporous.	Observe specimens on demonstration. Label the node, internodes, microphyllous leaves, and strobilus on the diagram below.

There is only one living genus of Sphenophyta—*Equisetum,* commonly called horsetail or scouring rush. Its ribbed, photosynthetic stems contain silica, providing an abrasive quality useful for scrubbing cookware while camping.

Alternation of generations is characteristic of all seedless vascular plants. In all four phyla the sporophyte is the conspicuous phase and the gametophyte is small and subterranean. Both sporophyte and gametophyte remain nutritionally independent of one another.

Seedless vascular plants require water for the sperm to reach the nonmotile egg. Therefore, one of the key events in the early invasion of land by these plants was the development of durable spores that could be dispersed over areas of dry land by wind. When all of the spores produced by a plant look alike, the plant is said to be **homosporous.** If two kinds of spores are produced, the plant is **heterosporous.** It was among certain seedless vascular plants that the heterosporous condition first appeared.

Objectives

- ☐ Distinguish between roots, rhizoids, and rhizomes.
- ☐ Describe the life cycle of a fern; distinguish between the gametophyte and sporophyte generations.

PHYLA PSILOPHYTA, LYCOPHYTA, AND SPHENOPHYTA

The presence of primitive conducting tissues (**vascular tissues**) consisting of xylem and phloem allows us to classify the whisk ferns, club mosses, horsetails, and ferns as tracheophytes. Refer to Table 24A-1 and examine the representatives of the Phyla Psilophyta, Lycophyta, and Sphenophyta that are available in the laboratory. *a) Which plants have photosynthetic leaves?* ________ *b) Where does photosynthesis occur in the Psilophyta?* ________ *c) Which plants have vascularized leaves?* ________

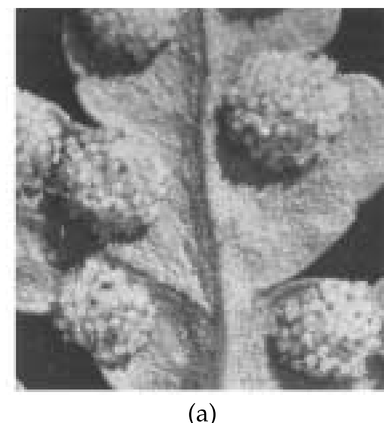
PHYLUM PTEROPHYTA

The obvious parts of ferns are leaves that may be large and are usually delicate and deeply divided. Stems usually grow either horizontally along the soil surface or underground (**rhizomes**), although some "tree ferns" with vertical stems are found in the tropics. Although ferns are commonly found in moist areas, a few are thick-leaved and adapted to living in hot, dry environments.

The fern life cycle is characterized by an alternation of generations in which the sporophyte generation is more conspicuous than the gametophyte generation. The large green leaves we recognize as ferns are the sporophyte generation. (*What was the conspicuous generation in the moss?*) The sporophyte generation is diploid (2*n*). *a. What does this generation produce?* ________________ *By what process?*

____

Terrestrial ferns are homosporous, producing only one kind of spore. Fern leaves bear **sporangia** that produce haploid (*n*) spores by meiosis. Sporangia usually appear in clusters as rusty-looking patches called **sori** on the underside of a leaf or in large aggregations on a modified leaf (Figure 24A-1).



(a)

(b)

Figure 24A-1 *Types of sori. (a) Bare sori with spores exposed in* Polypodium virginianum. *(b) Sori are located along the margins of the leaf blades, which are rolled back over them in* Pellaea glabella.

The haploid gametophyte develops from a germinating spore and is inconspicuous. The young gametophyte is a heart-shaped structure (**prothallus**) bearing **archegonia** (containing eggs) and **antheridia** (producing sperm). The nonmotile egg remains in the archegonium, and sperm swim to it. Water (rain or dew) is necessary for fertilization. The diploid sporophyte then develops from the fertilized egg or zygote (2*n*). The sporophyte and gametophyte are nutritionally independent (Figure 24A-2).

Procedure

1. Study the herbarium sheets and living fern sporophytes on demonstration. Identify the following parts of the mature sporophyte and study their function as part of the fern life cycle (Figure 24A-2).

   Leaf Fronds or sporophylls bearing sori—the most conspicuous part of the sporophyte.

   Sorus Cluster of **sporangia** that produce haploid spores by meiosis.

   Rhizome Underground horizontal stem of the sporophyte.

   Adventitious roots Roots extending from the rhizome.

2. Obtain a portion of a fern frond or leaf with sori on the underside. Heat the sporangia composing the sorus by placing a lamp near the frond. A single sporangium has a row of specialized cells (known as the **annulus**) which runs two-thirds of the way around its edge. When dry conditions cause water to evaporate from the annulus, changes in the size of its cells cause it to spring back sharply, releasing some spores, and then rapidly snap back, catapulting the remaining spores. Watch for the annulus to move violently as it disperses spores.

   b. What does this mechanism of spore release indicate about spore dissemination?

   ____

   ____

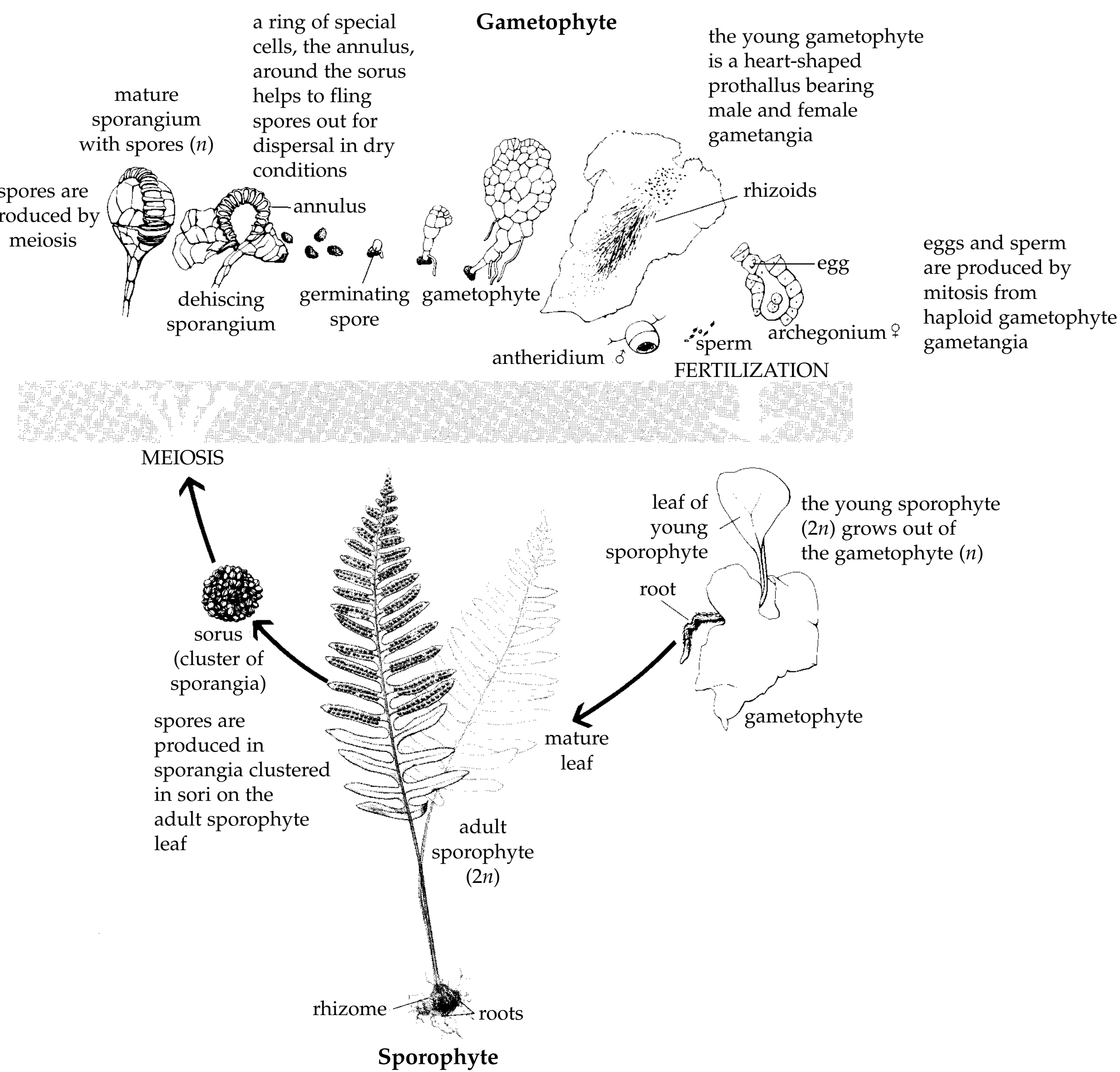


Figure 24A-2 *The life cycle of the fern.*

3. Prepare a wet mount of a living heart-shaped fern gametophyte (prothallus) or obtain a prepared slide of a young fern gametophyte (whole mount). Observe the specimen using the 10× and 40× objectives of your microscope. Identify the following parts and study their function as part of the fern life cycle (Figure 24A-2).

Rhizoids Specialized cellular filaments extending from the lower surface of the gametophyte into the substrate.

Antheridium Sperm are produced within the antheridia located on the lower surface of the gametophyte among rhizoids. When an adequate supply of water is present, mature antheridia burst and release sperm (Figure 24A-3a).

Sperm Flagellated male gametes produced in antheridia.

Archegonium This flask-shaped organ contains the female gamete (egg) and is located on the lower surface of the gametophyte (Figure 24A-3b). Sperm swim down the neck of the

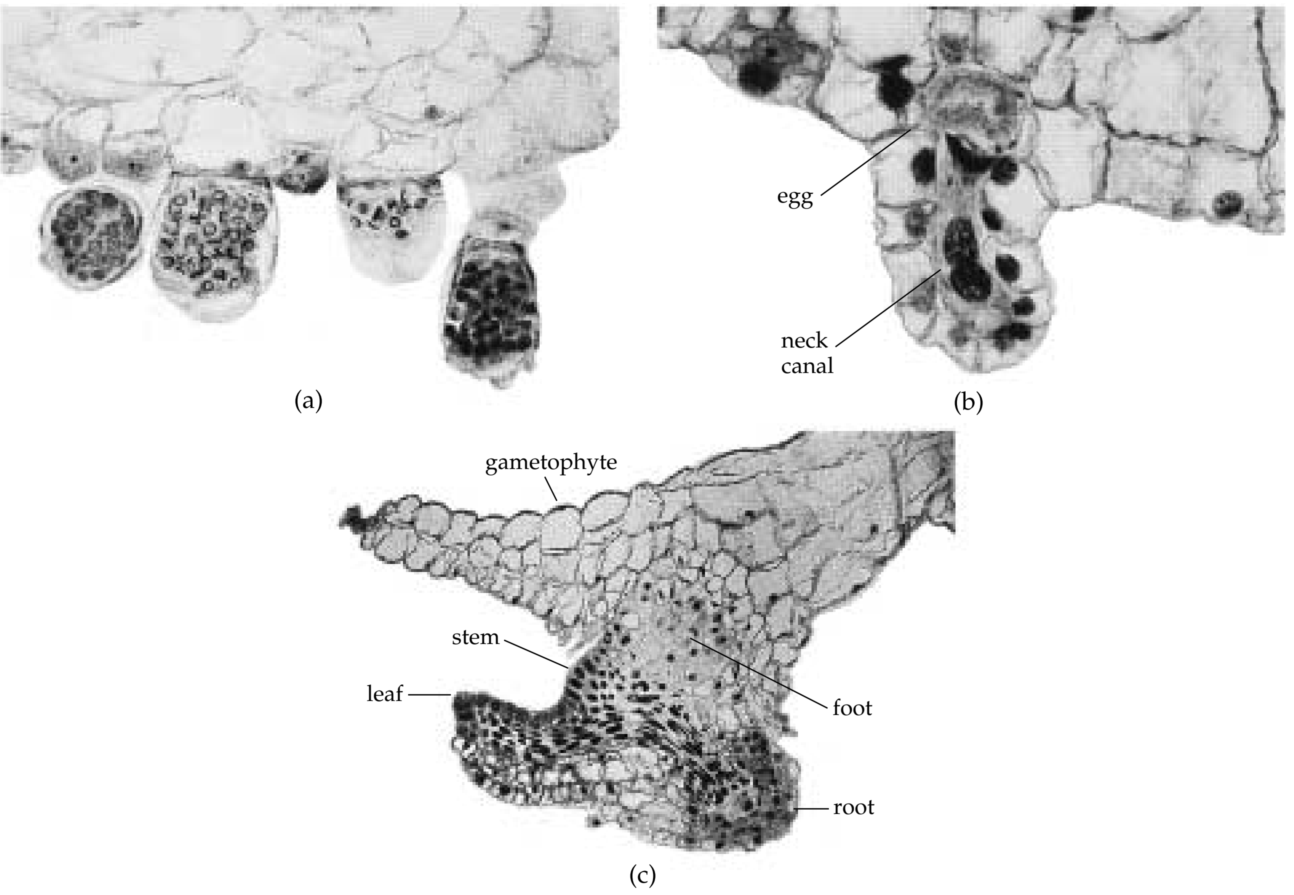


Figure 24A-3 *(a) Antheridia with nearly mature archegonia on the prothallus of* Osmunda. *(b) Archegonia. (c) Young sporophyte growing out of the gametophyte.*

archegonium to fertilize the egg, forming the zygote, which develops directly into the young embryo sporophyte.

Eggs Female gametes produced in archegonia.

c. How many cell layers are there in the young prothallus? ______

4. Obtain a prepared slide of a gametophyte with an attached sporophyte, or use living material (Figure 24A-3c). Fertilization has occurred several days earlier. The resulting sporophyte consists of one or more leaves and a *root.*

   d. Is the new plant haploid or diploid? ______

   e. What will be the fate of the gametophyte? ______

PART II TRACHEOPHYTES (VASCULAR PLANTS) WITH SEEDS—GYMNOSPERMS AND ANGIOSPERMS

What is the ecological advantage of a seed? Seeds provide a mechanism for a plant to survive during adverse environmental conditions, such as lack of water and extreme cold. Seed plants achieved prominence during a time when the earth's climate became colder and drier following the warm and humid Carboniferous period.

Gymnosperms are plants with "naked" or unprotected seeds, and **angiosperms** are plants with enclosed, protected seeds. In gymnosperms, seeds develop directly on the surface of the scales of the female cone; in angiosperms, seeds are enclosed in a fruit.

The evolutionary trend toward **sporophyte dominance** is most strikingly apparent among gymnosperms and angiosperms. In angiosperms, the mature female gametophyte is often as small as seven cells (one of which is the egg) and the mature male gametophyte may consist of only three cells (two of which are sperm). The inconspicuous gametophyte generation is completely dependent upon the sporophyte generation for nutrition.

Understanding the life cycles of heterosporous seed plants is made easier by first studying a generalized life cycle (Figure 24II-1). Keep in mind that the particulars may differ for individual gymnosperms and angiosperms.

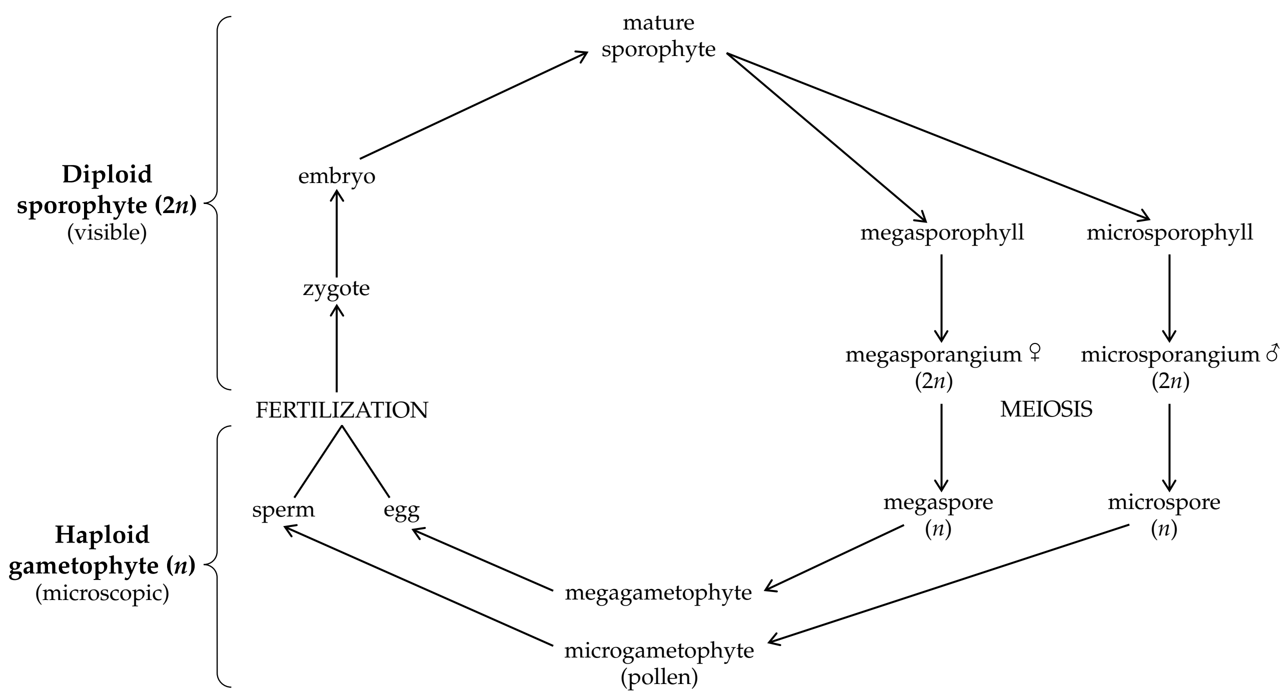


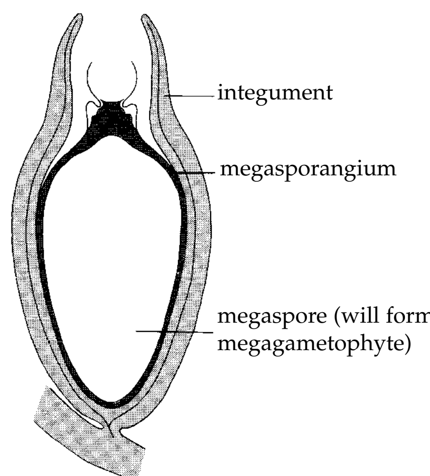
Figure 24II-1 *Generalized life cycle of the vascular plants. This life cycle is characterized by an alternation of sporophyte and gametophyte generations.*

The types of spores produced by heterosporous seed plants, called **megaspores** (n) and **microspores** (n), are produced by meiosis from **megasporangia** ($2n$) and **microsporangia** ($2n$). Within the megasporangium (*nucellus*), one megaspore divides many times to develop into the multicellular female gametophyte (*megagametophyte*) containing, as a rule, two archegonia. Inside each archegonium, an egg develops.

Microspores develop into the male gametophyte (**microgametophyte**). The mature microgametophyte is the **pollen grain,** which can be dispersed by the wind. After landing on an ovule, the pollen grain produces a special structure called a **pollen tube** that transports the sperm produced within the pollen grain into the vicinity of the egg. (In contrast to the nontracheophytes and seedless vascular plants, the heterosporous land plants do not require water for fertilization; thus, these plants thrive in a greater diversity of environmental conditions.)

The egg, developed from the megaspore, remains inside the female megasporangium (Figure 24II-2), often borne on a leaflike **megasporophyll** of the sporophyte plant. The fertilized egg develops into the young embryo within the megasporangium, which, in seed plants, is covered by one or two layers of

Figure 24II-2 *Sectional view of the ovule of the ancient seed plant* Eurystoma angulare, *showing the spatial relationship of the integument, the megasporangium, and the megaspore.*



protective tissue called **integuments.** The entire structure (megaspore within the megasporangium covered by integuments) is the **ovule.** The ovule develops into the **seed** with the integuments serving as the **seed coat.**

The seed, with its specialized structures for the nutrition, protection, and dispersal of the young sporophyte, is one of the most important innovations to arise among the land plants and is probably the major reason for their dominance.

✔ EXERCISE B | Gymnosperms

There are four phyla of **gymnosperms** with living representatives: the phylum Coniferophyta, conifers; phylum Cycadophyta, cycads; phylum Ginkgophyta, ginkgos; phylum Gnetophyta, gnetophytes.

Objectives

- ☐ Describe the relationship between microsporophyll, microsporangium, microspore, microgametophyte, and pollen.
- ☐ Describe the relationship between ovule, megasporangium, megaspore, and seed.

Procedure

1. Examine the branches and cones from different types of conifers on demonstration in your laboratory. Most conifers are large evergreen trees or shrubs with needlelike or scalelike leaves. Confiers can be identified by the length, appearance, and number of modified leaves held together in a single group attached to the stem. For each of the conifers you study, write a brief description that distinguishes it from the others (Table 24B-1). (*Note:* Your instructor may provide you with a dichotomous key to help you identify the conifers.

Table 24B-1 Types of Conifers on Demonstration

Conifer	Characteristics

2. Locate a male cone (staminate or pollen cone) from one of the conifers on demonstration.

a. How does it differ from the typical (female) pine cone? ______________________________

Carefully remove one of the scales (**microsporophylls**) that aggregate to form the cone. Observe with a dissecting microscope. Note the two attached microsporangia (Figure 24B-1).

3. Place the microsporophyll in a drop of water on a slide and carefully crush it to release the **microspores** from the microsporangium. A mature microgametophyte is known as a pollen grain (Figure 24B-2).

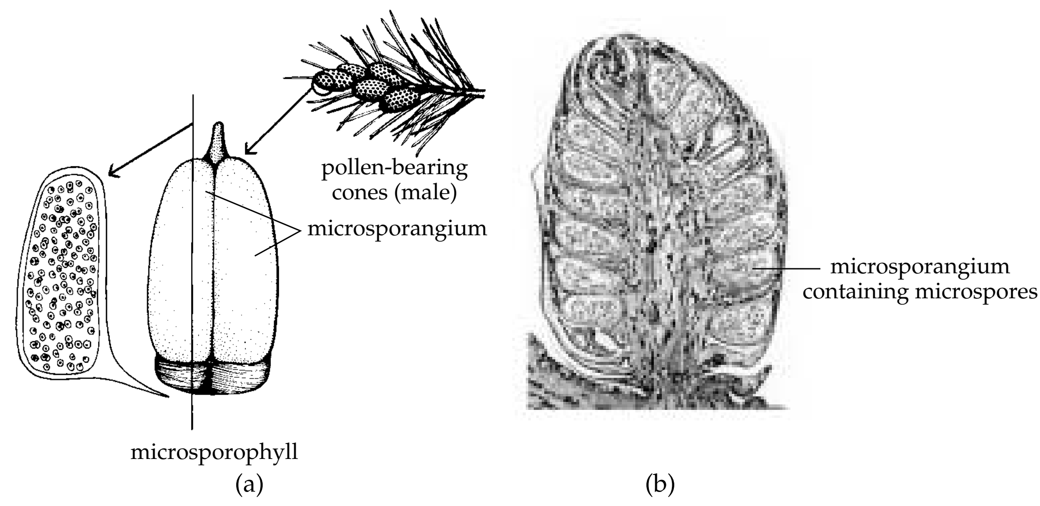


Figure 24B-1 *(a) Microsporophyll with attached microsporangia containing developing microspores. (b) Longitudinal view of a pollen-producing cone, showing microsporophylls and microsporangia containing mature pollen grains.*

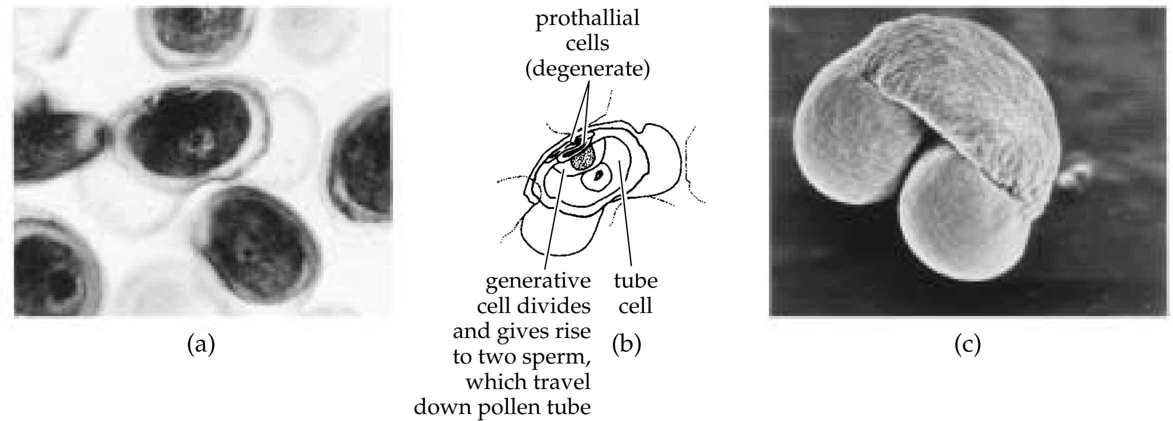


Figure 24B-2 *(a) Immature pollen grain. (b) The pollen grain consists of several cells; the generative cell will eventually divide to give rise to sperm cells. (c) Scanning electron micrograph of a pine pollen grain. When the pollen grain germinates, the pollen tube emerges from the lower end of the grain, between the wings.*

4. View a prepared slide of a longitudinal section of a staminate cone and look for microsporophylls, microsporangia, and microspores.
5. Examine a female cone (ovulate or seed cone) and locate the ovules (Figure 24B-3). The seeds of gymnosperms are said to be naked because they are produced on the surface of scales constituting the ovulate cone. At maturity, each scale bears two seeds or ovules containing embryos. Locate and examine a seed.

   b. How are conifer seeds dispersed? ______________________________

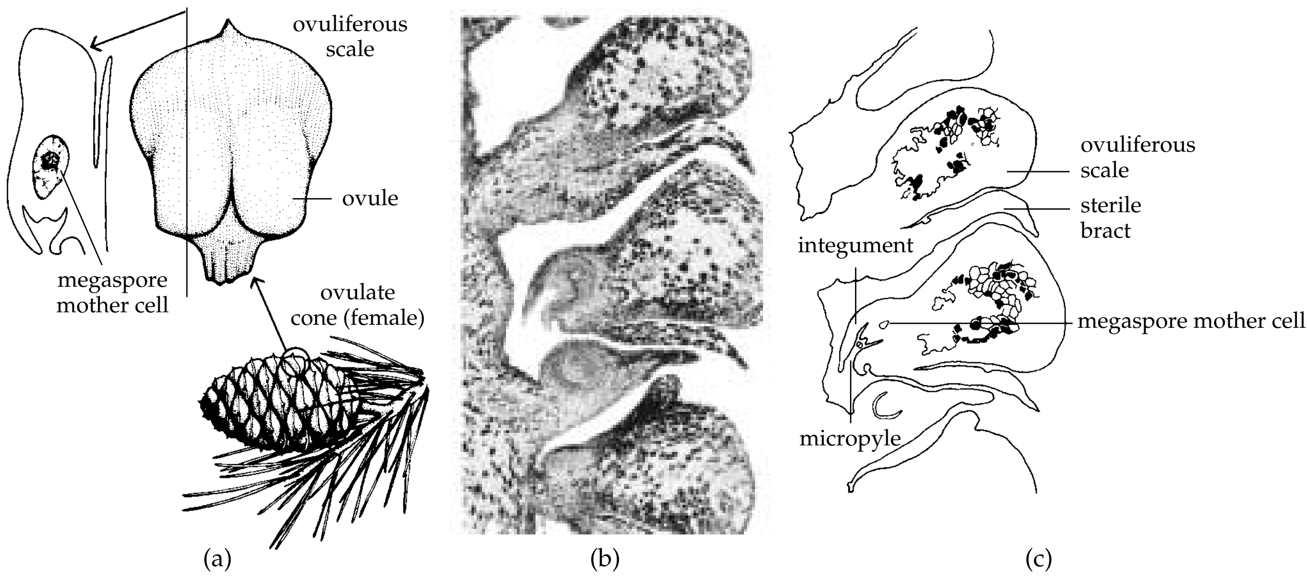


Figure 24B-3 *(a) Ovules on the scale of an ovulate cone. (b) Cross section of ovuliferous scales. (c) The megaspore mother cell divides to form the megaspores. The micropyle opening allows the pollen tube to enter so that fertilization can occur later.*

6. View a prepared slide of a longitudinal section of an ovulate cone. The **ovule** consists of **integuments** containing the **megasporangium** (Figure 24B-3). The **megaspore mother cell** develops within the megasporangium and gives rise to **megaspores** by meiosis. Locate these structures on your slide.
7. *Optional.* If assigned by your instructor, review the life cycle of pine in Figure 24B-4. Write the letter that follows each structure described in the caption in the appropriate box in the life-cycle diagram.
8. Examine living and preserved cycads (phylum Cycadophyta) and ginkgos (phylum Ginkgophyta) on demonstration in the laboratory. Phylum Ginkgophyta is represented by only a single living species, *Ginkgo biloba.* This tree was once a favored landscape planting for city sidewalks. However, the seeds have a fleshy outer layer that contains a foul-smelling compound, butyric acid. The putrid, slimy messes (not to mention the smell) created by the prolific production and dropping of seeds onto city sidewalks soon discouraged the planting of female *Ginkgo* trees. You will find the most recently planted *Ginkgo* trees are male.

   Fewer than a dozen living genera of Cycadophyta exist today; they occur naturally only in the tropics or subtropics. Note the fernlike leaves and short, thick stem. Also study the cones if present. Cycads and ginkgos are *dioecious*—pollen and seed cones are found on different plants. Look for ovules in female cones and microsporangia in male cones.

   c. How do representatives of these other gymnosperm phyla resemble the conifers?

   ____

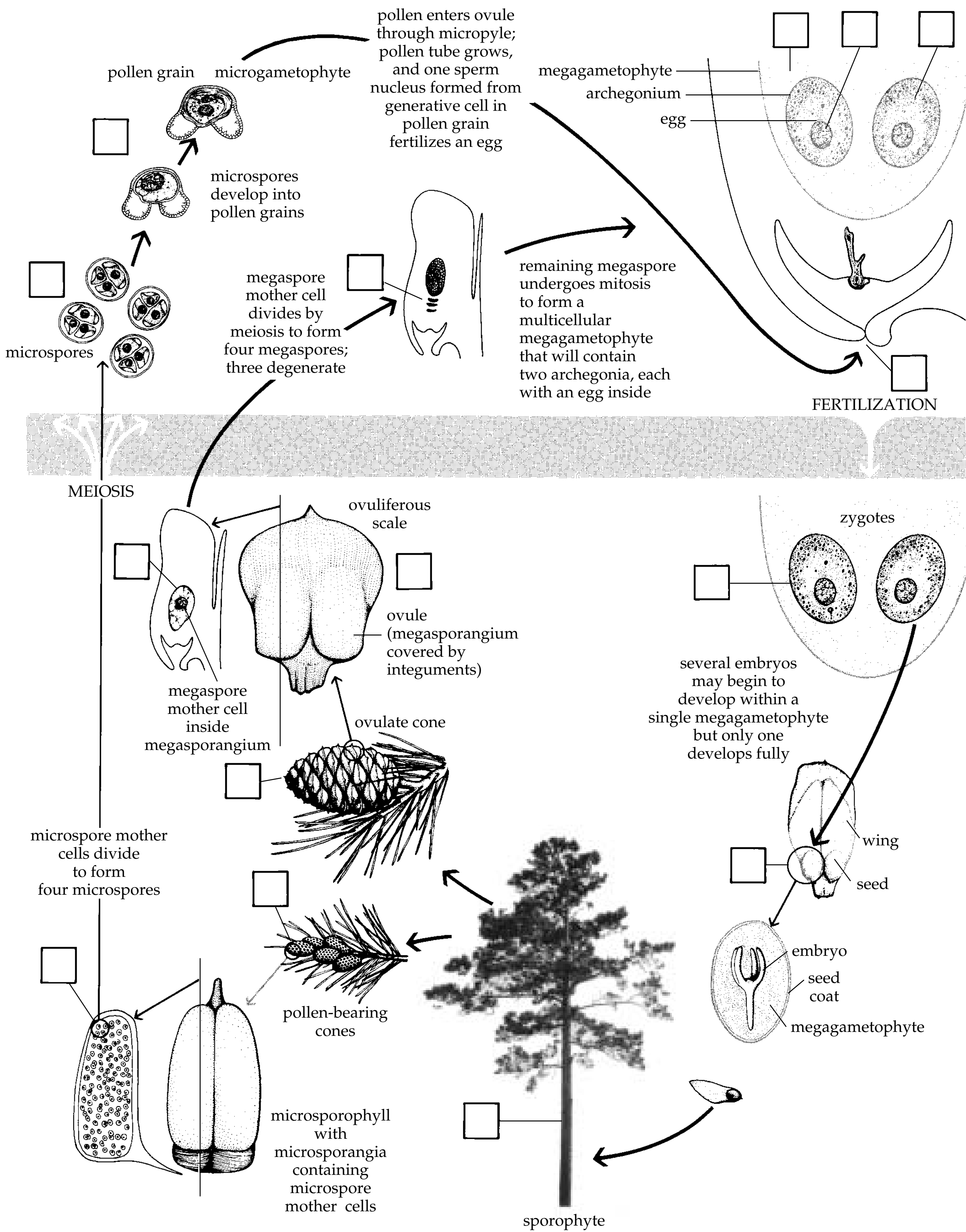


Figure 24B-4 *Beginning at the bottom of the diagram: The* ***immature*** *sporophyte (gymnosperm seedling) develops into the* ***mature sporophyte***

*(**a**). **Male cones** (**b**) are usually produced in clusters of up to 50 or more at the tips of low branches. The male cones produce pollen within the **microsporangia** (**c**) that occur in pairs on the microsporophylls (leaves) of the cone. Microspore mother cells within the microsporangia produce **haploid microspores** (**d**) that develop into four-celled immature **pollen grains** (**e**). At this stage, the pollen grains are shed.*

*After landing in the vicinity of a micropyle of a female cone, one of the cells of the pollen grain, the tube cell, will develop into a pollen tube during the germination of the pollen grain. Another of the cells will divide to form two sperm cells as the pollen grain matures. A mature pollen grain is the mature male microgametophyte. **Female cones** (**f**) are generally larger than male cones and consist of woody scales containing paired **ovules** (**g**). The ovule is a megasporangium covered by integuments. Inside each megasporangium is a **megaspore mother cell** (**h**), which produces four haploid megaspores by meiosis. Three of these cells degenerate (**i**) and the remaining one divides mitotically to produce a multicellular **megagametophyte** (**j**) that will contain two **archegonia** (**k**), each containing an egg (**l**). Pollen enters the ovule through the micropyle (**m**) opening in the integument. One sperm nucleus unites with the egg to fertilize it. Generally, all eggs are fertilized and several embryos (**n**) begin to develop within a single megagametophyte, but only one develops fully. Each scale bears only two seeds (**o**), which separate and are dispersed by the wind.*

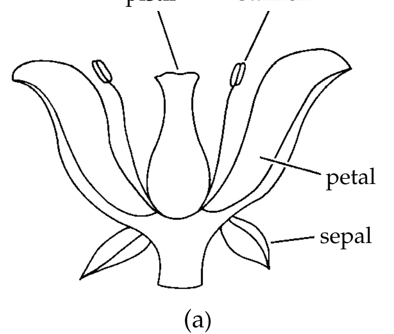
✓ EXERCISE C | Angiosperms (Phylum Angiospermae)

Angiosperm means "covered seed." Following fertilization, seeds produced by angiosperms develop from ovules and are covered by a structure called the **ovary** located within the **flower.** The ovary eventually develops into a **fruit** containing one or more seeds. Enclosed seeds, flowers, and fruits are all unique to angiosperms, which are, at present, the dominant plants on earth.

Angiosperms can be divided into two classes: the **dicots** (dicotyledons) and **monocots** (monocotyledons). These names refer to the fact that the dicot embryo has two seed leaves or **cotyledons** (leaflike parts of the embryo), while monocot embryos have only one.

Among monocots are the familiar grasses, lilies, irises, orchids, cattails, and palms. Dicots include many herbs and almost all shrubs and trees (other than conifers). The many distinctions between monocots and dicots will be discussed during later laboratories, which examine the structure of the leaves, stems, and roots of angiosperm plants. Here, you will turn your attention to the flower.

A **flower** can be considered a highly modified and specialized shoot: a stem tip where modified leaves, the petals and sepals, occur "bunched together." Floral parts and leaves have many similarities. Working from the outside toward the inside, similar parts are grouped as whorls (Figure 24C-1). Monocots usually have flower parts arranged in threes (or multiples of three). Dicots usually have flower parts arranged in fours or fives (or multiples of four and five).



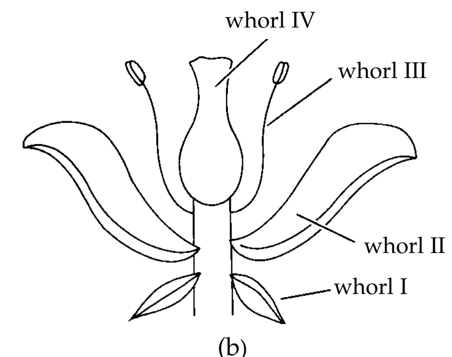


Figure 24C-1 *(a) Generalized flower parts. (b) Similar parts are grouped in whorls. In monocots, whorled structures occur in groups of three. In dicots, they occur in groups of four or five.*

Objectives

- ☐ Distinguish between monocot and dicot flower types.
- ☐ Describe the structures of the male and female parts of a flower.
- ☐ Differentiate between pollination and fertilization.

Procedure

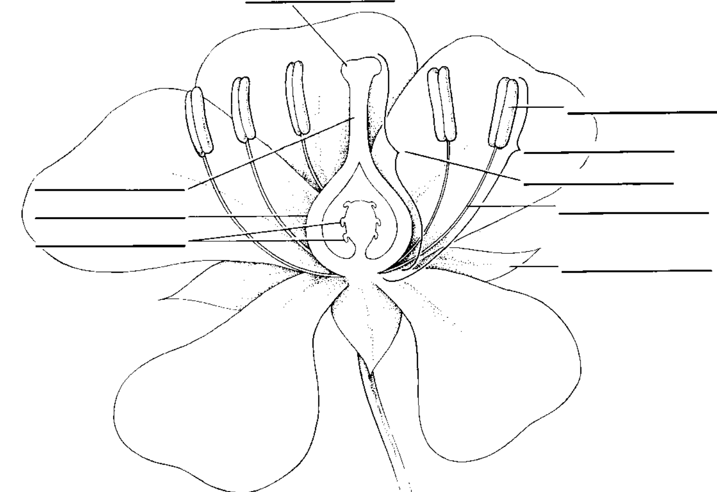
1. Obtain a flower from those provided. *a. Is your flower a monocot or dicot?* ____________________

2. Identify the parts of the flower in the following list. Locate as many parts as you can by simply looking at the flower. Then, using forceps and a dissecting microscope, start at the first whorl of the flower and work inward, locating each of the parts. Label these structures on the flower diagram (Figure 24C-2). Be sure that you understand the function of each part.

Peduncle Narrow stalk that bears a flower or group of flowers (**inflorescence**). The stalk of an individual flower in an inflorescence is called a **pedicel** (Figure 24C-3).

Receptacle Part of the flower stalk to which floral parts are attached.

Figure 24C-2 *Parts of a flower. Note: The receptacle is located at the base of the sepals and is not visible in this figure.*



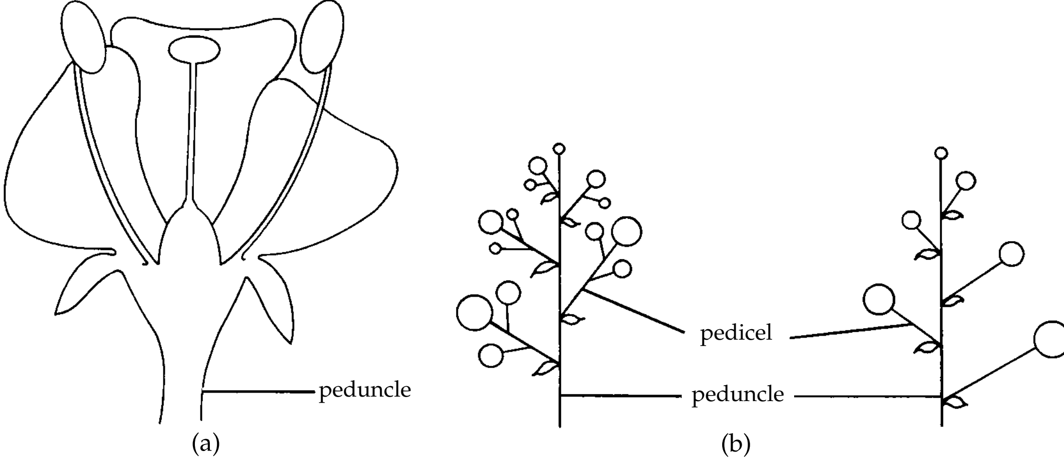


Figure 24C-3 *(a) A single flower attached at the peduncle. (b) Inflorescences supported by peduncles; each flower is attached to the peduncle by a pedicel.*

Sepals Outer leafy parts of the flower collectively called the **calyx.** Sepals usually enclose the outer flower parts, protect the bud, and later surround the ovary.

Petals Often brightly colored, forming the conspicuous inner whorl of flower parts. Collectively, petals are called the **corolla.**

Stamens Produce pollen grains, the male gametophytes. The gametes, sperm, are not produced until after pollination, as in gymnosperms. Stamens are actually microsporophylls consisting of a **filament** and a two-lobed **anther** containing the microsporangia.

Carpels Carpels may be individual (free) or fused. The pistil (composed of one or more carpels) is differentiated into a lower part, the **ovary,** and an upper part, or **stigma,** which receives pollen. The **style** connects the ovary to the stigma. The portion of the ovary to which the **ovules** are attached is called the **placenta** (Figure 24C-4).

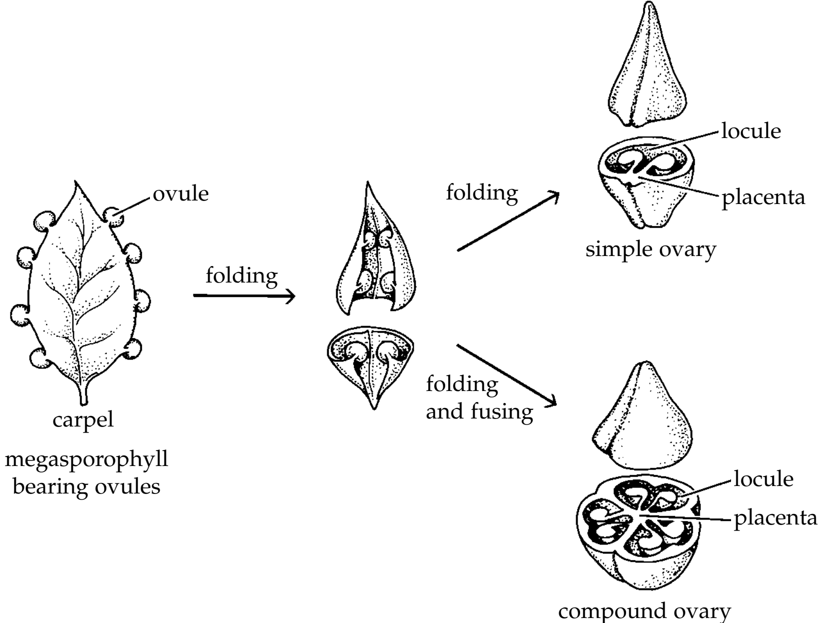


Figure 24C-4 *Presumed evolutionary development of simple and compound ovaries. A leaf-shaped carpel with ovules along its edge (left) folded in on itself, and the edges fused to form a simple ovary. Compound ovaries were formed by the fusing of separate infolded carpels.*

In angiosperms, pollination results in **double fertilization:** one sperm nucleus fuses with the egg cell to form a $2n$ **zygote** and the other sperm nucleus fuses with the two polar nuclei to form a polyploid (usually $3n$ or $5n$) **endosperm.** Endosperm provides a source of stored energy for the developing embryo. The fertilized ovule then develops into a seed. In monocots, endosperm persists, forming a food reserve to be used during germination. Most dicots absorb all of the endosperm during embryo development, storing nutrients in fleshy cotyledons for use during germination.

3. Examine pollen grains by tapping the anther of a flower over a drop of sucrose solution on a microscope slide. Add a coverslip and examine your preparation, first using low power (10× objective) and then high power (40× objective). Keep this slide until the end of the laboratory period (do not let it dry out). Before you leave, check to see if any of the pollen grains have produced pollen tubes.

   b. Why did you mount the pollen in sucrose solution rather than in water alone?

   ____

4. *Optional.* If assigned by your instructor, study the life-cycle diagram for angiosperms in Figure 24C-5. Write the letter that follows each structure or process described in the caption in the appropriate box in the diagram.

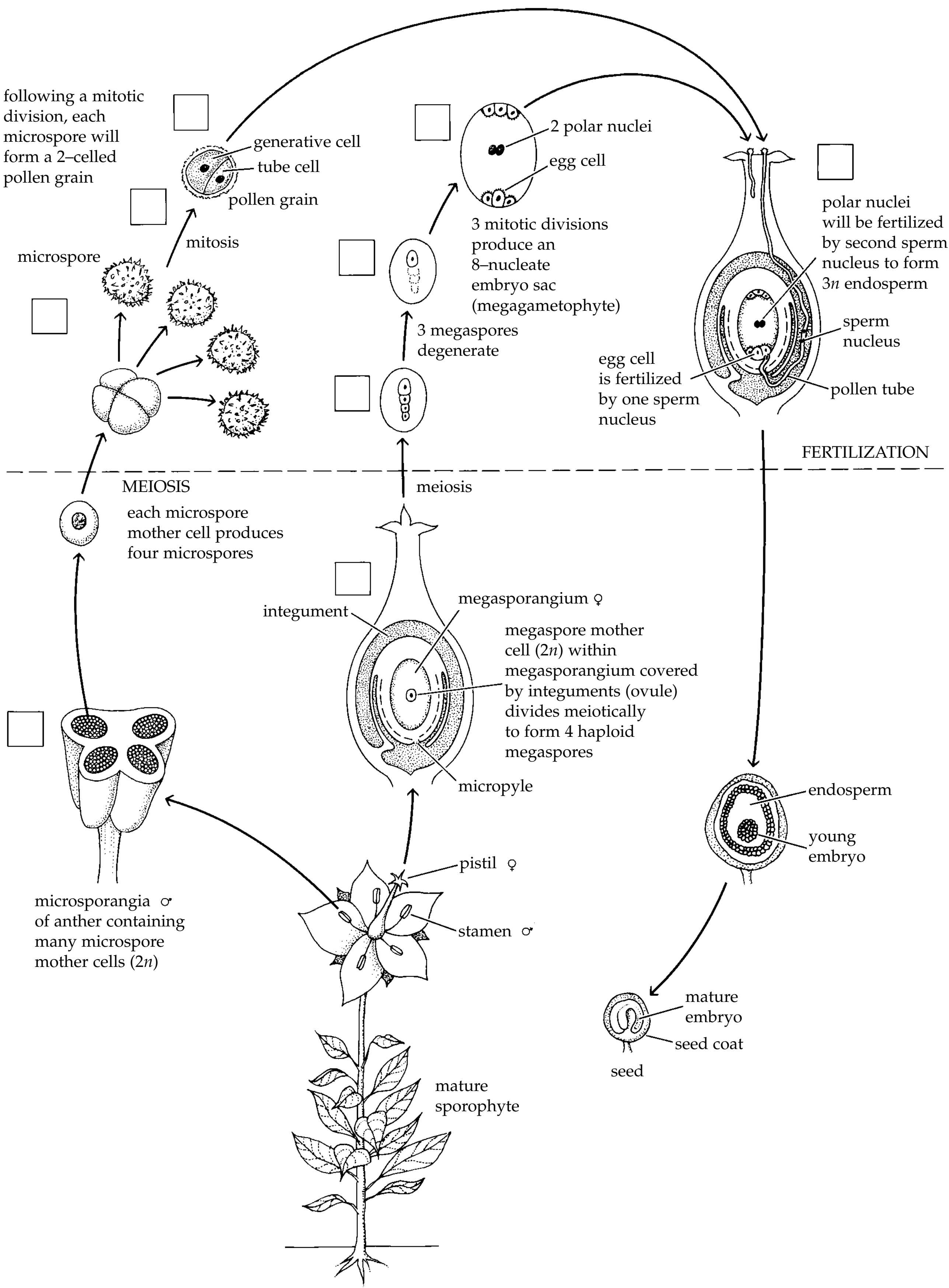

following a mitotic division, each microspore will form a 2–celled pollen grain
generative cell
tube cell
pollen grain
microspore
mitosis
MEIOSIS
each microspore mother cell produces four microspores
microsporangia ♂ of anther containing many microspore mother cells (2n)
2 polar nuclei
egg cell
3 mitotic divisions produce an 8–nucleate embryo sac (megagametophyte)
3 megaspores degenerate
meiosis
integument
megasporangium ♀
megaspore mother cell (2n) within megasporangium covered by integuments (ovule) divides meiotically to form 4 haploid megaspores
micropyle
pistil ♀
stamen ♂
mature sporophyte
polar nuclei will be fertilized by second sperm nucleus to form 3n endosperm
sperm nucleus
egg cell is fertilized by one sperm nucleus
pollen tube
FERTILIZATION
endosperm
young embryo
mature embryo
seed coat
seed

Figure 24C-5 *(page 24-15) Beginning in the center of the diagram: Within the **ovary** of the flower pistil, one or several **ovules** are attached to the ovary wall. Each of these ovules contains a megasporangium covered by **integuments** (**a**). Inside the megasporangium is the **megaspore mother cell** which produces four **megaspores** by meiosis (**b**), three of which degenerate (**c**). The remaining megaspore enlarges and undergoes three mitotic divisions to form eight nuclei. Of these, one nucleus develops a cell wall and becomes the egg; upon fertilization, two other nuclei (polar nuclei) will develop into a food source for the embryo (**d**). This structure represents the mature female gametophyte—the microscopic gametophyte generation in the sporophyte-dominated life cycle of seed plants.*

*Microsporogenesis takes place within the **anther** of the flower. Microspores are produced by meiosis within the pollen sacs (microsporangia) of the anther (**e**). Each microspore develops a resistant outer wall and the specialized structures common to **pollen grains** of that species (**f**). The microspore also divides mitotically (**g**) to form two cells, the **tube cell** and the **generative cell** (**h**). Most pollen grains are at this stage when released. A mature pollen grain containing these two cells represents the male **microgametophyte**—one of the only visible parts of the gametophyte generation in the sporophyte-dominated life cycle of angiosperms.*

***Pollination** occurs when wind or insects carry the pollen to the stigma of the flower. After pollination, the tube nucleus forms a **pollen tube**. The pollen tube grows down through the style of the pistil and enters the ovary and then the ovule through an opening in the integuments (**micropyle**) (**i**). Fertilization can then occur.*

Laboratory Review Questions and Problems

1. Compare the life cycle of a moss (Laboratory 23) with that of a fern. Which is the visible generation of each?

2. How do the tracheophytes (vascular land plants) differ from the nontracheophytes?

3. Do whisk ferns, club mosses, horsetails, and ferns have vascular tissue? Seeds? Based on your answer, how do we group these phyla in terms of a general category?

4. In what group of land plants do we first observe true roots, stems, and leaves?

5. What three major advances are obvious among the vascular plants with seeds?

6. Are ferns heterosporous or homosporous? ____________ Gymnosperms? ____________ Angiosperms? ____________

7. In the table below, indicate whether each structure is haploid (*n*) or diplois (2*n*), gametophyte or sporophyte.

Structure	*n* or 2*n*	Gametophyte or Sporophyte
Fern archegonium		
Moss antheridium		
Fern leaf		
Moss "leaflet"		
Pollen grain of pine		
Megagametophyte of angiosperm		
Microsporangium of angiosperm		
Pine tree		
Flower		

8. Flowers play an essential role in sexual reproduction of angiosperms. What types of insects or animals pollinate flowers? ____________ What do these flowers look like? ____________ What types of plants are pollinated by wind? ____________