7

Separating Organic Compounds

Column Chromatography, Paper Chromatography, and Gel Electrophoresis

Objectives

By the end of this exercise you should be able to:

1. Describe the basis for column chromatography, paper chromatography, and gel electrophoresis.
2. Use column chromatography, paper chromatography, and gel electrophoresis to separate organic compounds from mixtures.

Cells are a mixture of the types of organic compounds that you studied in Exercise 6: carbohydrates, proteins, lipids, and nucleic acids. Biologists characterize and study these compounds to understand how organisms function. This requires that biologists separate the compounds, such as amino acids and nucleotides, from mixtures. Three separation techniques that biologists use are column chromatography, paper chromatography, and gel electrophoresis.

In today's exercise you will use these common techniques to separate compounds from mixtures. The procedures are simple and model how these techniques are used by biologists in their research.

COLUMN CHROMATOGRAPHY

Column chromatography separates molecules according to their size and shape. The procedure is simple and involves placing a sample onto a column of beads having tiny pores. There are two ways that molecules can move through the column of beads: a fast route between the beads or a slower route through the tiny pores of the beads. Molecules too big to fit into the beads' pores move through the column quickly, whereas smaller molecules enter the beads' pores and move through the column more slowly (fig. 7.1). Movement of the molecules is analogous to going through or walking around a maze: It takes more time to walk through a maze than to walk around it.

The apparatus used for column chromatography is shown in figure 7.2 and consists of a chromatography column, a matrix, and a buffer.

- The **chromatography column** is a tube having a frit and a spout at its bottom. The frit is a membrane or porous disk that supports and keeps the matrix in the column but allows water and solutes to pass.
- The **matrix** is the material in the column that fractionates, or separates, the chemicals mixed in the sample. The matrix consists of beads having tiny pores and internal channels. The size of the beads' pores determines the matrix's **fractionation range,** the range of molecular weights the matrix can separate. These molecular weights are measured in units called daltons; 1 dalton $\approx$ 1 g mole^{-1}. Different kinds of matrices have different fractionation ranges. In today's exercise you'll use a matrix having a fractionation range of 1000 to 5000 daltons. As they move through the matrix, small molecules spend much time in the maze of channels and pores in the matrix. Large molecules do not.
- The **buffer** helps control the pH of the sample (see Exercise 5). A buffer is a solution with a known pH that resists changes in pH if other chemicals are added. The pH of a buffer remains relatively constant. This is important because the shapes of molecules such as proteins often vary according to their pH. The buffer carries the sample through the matrix, which separates the chemicals mixed in the sample.

Column chromatography can also separate compounds having the same molecular weight but different shapes. Compact, spherical molecules penetrate the pores and channels of the matrix more readily than do rod-shaped molecules. Thus, spherical molecules move through a column more slowly than do rod-shaped molecules.

During column chromatography, the buffer containing the sample mixture of chemicals moves through the column and is collected sequentially in test tubes from the bottom of the column. Biologists then assay the content of the tubes to determine which tubes contain the compounds in which they are interested.

Question 1

In today's exercise you'll isolate colored compounds from mixtures. However, most biological samples are colorless. How would you determine the contents of the test tubes if all of the samples were transparent?

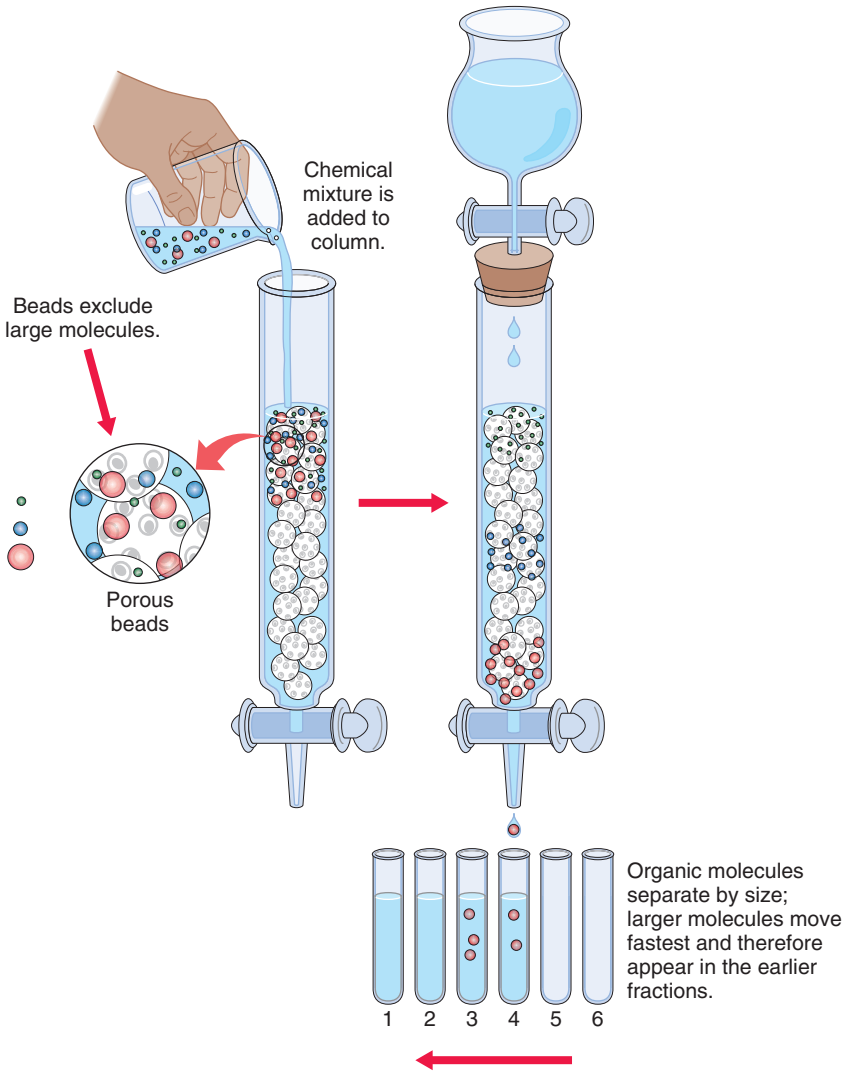


Figure 7.1

Separation of organic molecules by column chromatography. As the solution flows through the column, the smaller molecules are slowed down as they pass through the pores of the beads. Medium-sized molecules will pass through a bead with pores less frequently, and the largest molecules will quickly flow around all the beads. The exiting fluid is collected in fractions. The first fractions collected will contain the largest molecules.

SAFETY FIRST Before coming to lab, you were asked to read this exercise so you would know what to do and be aware of safety issues. In the space below, briefly list the safety issues associated with today's procedures. If you have questions about these issues, contact your laboratory assistant before starting work.

Procedure 7.1

Separate compounds by column chromatography

1. Label nine microtubes 1–9.
2. Obtain an apparatus for column chromatography and carefully remove all of the buffer from above the beads with a transfer pipet. Do not remove any of the matrix.
3. Obtain a sample to be separated. The sample is a mixture of Orange G (molecular weight = 452 g $mole^{-1}$) and a rodlike polymer of glucose stained blue and having a molecular weight of about 2,000,000 g $mole^{-1}$.
4. Use a transfer pipet to slowly load 0.2 mL of the sample onto the top of the beads. Drip the sample down the inside walls of the column.

Figure 7.2
Apparatus for column chromatography. A fraction is being collected in the beaker.

5. Place a beaker under the column.
6. Slowly open the valve. This will cause the sample to enter the beads. Close the valve after the sample has completely entered the beads (i.e., when the top of the beads is exposed to air).
7. Use a transfer pipet to slowly cover the beads with buffer. Add buffer until the reservoir is almost full.
8. Hold microtube 1 under the tube and open the valve until you've collected about 1.0 mL of liquid.
9. Repeat step 8 for tubes 2–9. The sample will separate in the column.
10. Identify the tubes containing (1) the most orange dye, and (2) the most blue dye that eluted from the column.
11. Refill the reservoir with buffer and cover the reservoir with Parafilm.

Question 2

a. Was the color separation distinctive? Would you expect a longer column to more clearly separate the compounds? Why or why not?

b. Suppose your sample had consisted of a mixture of compounds having molecular weights of 50,000, 100,000, and 1,000,000 g mole^{-1}. What type of results would you predict? Explain your answer.

PAPER CHROMATOGRAPHY

Biologists often analyze the amino acid content of samples to determine protein sequences and enzyme structures. Amino acids can be separated by partitioning them between the stationary and mobile phases of paper chromatography. The **stationary phase** is the paper fibers, and the **mobile phase** is an organic solvent that moves along the paper.

Separation by paper chromatography begins by applying a liquid sample to a small spot on an origin line at one end of a piece of chromatography paper. The edge of the paper is then placed in a solvent. As the solvent moves up the paper, any sample molecules that are soluble in the solvent will move with the solvent. However, some molecules move faster than others based on their solubility in the mobile phase and their attraction to the stationary phase. These competing factors are different for different molecular structures, so each type of molecule moves at a different speed and occurs at a different position on the finished chromatogram.

Amino acids in solution have no color but react readily with molecules of ninhydrin to form a colored product. A completed chromatogram is sprayed with a ninhydrin solution and heated to detect the amino acids. The distance of these spots from the origin is measured and used to quantify the movement of a sample. The resulting R_f value (retardation factor) characterizes a known molecule in a known solvent under known conditions, and is calculated as follows:

$$R_f = \frac{\text{Distance moved by sample}}{\text{Distance from origin to solvent front}}$$

Procedure 7.2

Separate amino acids and identify unknowns by paper chromatography

1. Obtain a piece of chromatography paper 15 cm square. Avoid touching the paper with your fingers.

Table 7.1

Chromatography Data for Determining Amino Acid Unknowns

Tick Mark Number	Amino Acid or Sample Number	Distance to Solvent Front	Distance Traveled by Sample	R_f	Identity of Unknown
1					
2					
3					
4					
5					

Use gloves, tissue, or some other means to handle the paper because oils from your skin will alter the migration of the molecules on the paper.

2. Lay the paper on a clean paper towel. Then use a pencil to draw a light line 2 cm from the bottom edge of the paper.
3. Draw five tick marks at 2.5 cm intervals from the left end of the line. Lightly label the marks 1–5 below the line.
4. Locate the five solutions available for the chromatography procedure. Three of the solutions are known amino acids. One solution is an unknown. The last solution is a plant extract or another unknown.
5. Use a wooden or glass applicator stick to "spot" one of the solutions on mark #1. To do this, dip the stick in the solution and touch it to the paper to apply a small drop (2–3 mm in diameter). Let the spot dry; then make three to five more applications on the same spot. Dry between each application. Record in table 7.1 the name of the solution next to the appropriate mark number.
6. Repeat step 5 for each of the other solutions.
7. Staple or paper clip the edges of the paper to form a cylinder with the spots on the outside and at the bottom.
8. Obtain a quart jar containing the chromatography solvent. The solvent should be 1 cm or less deep. The solvent consists of butanol, acetic acid, and water (2:1:1).
9. Place the cylinder upright in the jar (fig. 7.3). *The solvent must be below the pencil line and marks*. Close the lid to seal the jar.
10. Keep the jar out of direct light and heat. Allow the solvent to move up the paper for 2 hours (h) but not all the way to the top.
11. Open the jar and remove the chromatogram. Unclip and flatten the paper. Dry it with a fan or hair dryer. Work under a hood if possible to avoid breathing the solvent vapors.
12. Spray the chromatogram with ninhydrin. Carefully dry the chromatogram with warm air.
13. Circle with a pencil each of the spots. Measure the distance each of the spots has traveled and calculate the R_f for each spot. Record the values in table 7.1.
14. Determine the contents of the unknown solutions by comparing R_f values. Record the results in table 7.1.

GEL ELECTROPHORESIS

Gel electrophoresis separates molecules according to their charge, shape, and size (fig. 7.4). Buffered samples (mixtures of organic chemicals) are loaded into a Jello-like gel, after which an electrical current is placed across the gel. This current moves the charged molecules toward either the cathode or anode of the electrophoresis apparatus. The speed, direction, and distance that each molecule moves are related to its charge, shape, and size.

The apparatus for gel electrophoresis is shown in figure 7.5 and consists of an electrophoresis chamber, gel, buffer, samples, and a power supply.

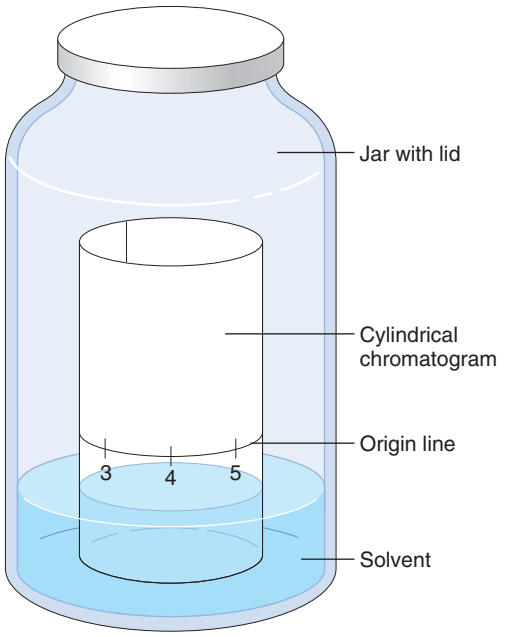


Figure 7.3

Apparatus for paper chromatography. Numbers on the chromatogram indicate the positions of multiple samples applied to the chromatogram. The samples will move up the chromatogram along with the solvent.

- The gel is made by dissolving agarose powder (a derivative of agar) in hot buffer. When the solution cools, it solidifies into a gel having many pores that function as a molecular sieve. The gel is submerged in a buffer-filled chamber containing electrodes.
- The buffer conducts electricity and helps control the pH. The pH affects the stability and charge of the samples.
- The samples are mixtures of chemicals loaded into wells in the gel. These samples move in the gel during electrophoresis. Samples are often mixed with glycerol or sucrose to make them denser than the buffer so that they will not mix with the buffer.
- The power supply provides a direct current across the gel. Charged molecules respond to the current by moving from the sample wells into the gel. Negatively charged molecules move through the gel toward the positive electrode (anode), whereas positively charged molecules move through the gel toward the negative electrode (cathode). The greater the voltage, the faster the molecules move.

The sieve properties of the gel affect the rate of movement of a sample through the gel. Small molecules move easier through the pores than do larger molecules. Consequently, small, compact (e.g., spherical) molecules move faster than do large, rodlike molecules. If molecules have similar shapes and molecular weights, the particles having the greatest charge move fastest and, therefore, the farthest.

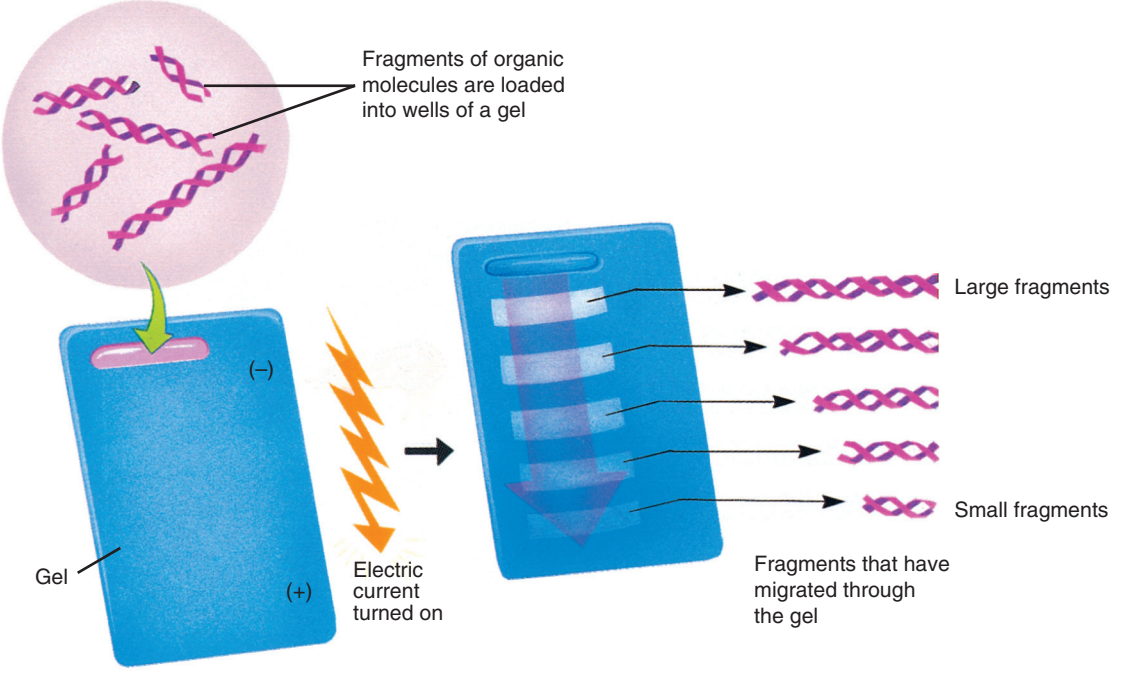
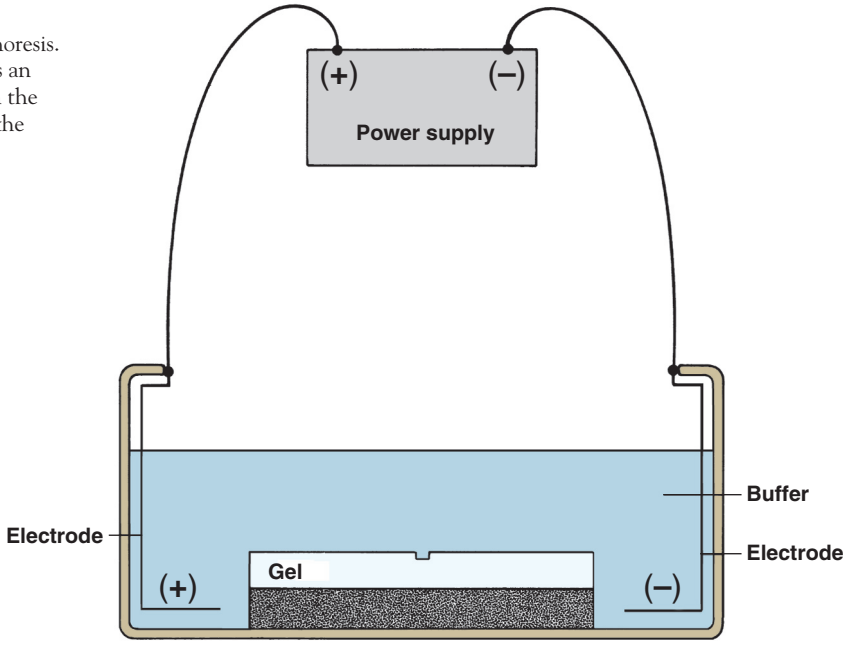


Figure 7.4

Gel electrophoresis. This process separates DNA fragments, protein fragments, and other organic compounds by causing them to move through an electrically charged gel. The fragments move according to their size, shape, and electrical charge; some fragments move slowly and some move quickly. When their migration is complete the fragments can be stained and visualized easily. In the example shown here, the DNA fragments were separated by size.

Figure 7.5
Apparatus for gel electrophoresis. The power supply produces an electrical gradient between the + and − poles and across the gel.



Procedure 7.3

Separate organic molecules by gel electrophoresis

1. Obtain an electrophoresis chamber. Cover the ends of the bed as shown in figure 7.6 and demonstrated by your instructor.
2. Place a six-tooth comb in or near the middle set of notches of the gel-cast bed. There should be a small space between the bottom of the teeth and the bed.
3. Mix a 0.8% (weight by volume) mixture of agarose powder in a sufficient volume of buffer to fill the gel chamber. Heat the mixture until the agarose dissolves.
4. When the hot agarose solution has cooled to 50°C, pour the agarose solution into the gel-cast bed (fig. 7.7).
5. After the gel has solidified, gently remove the comb by pulling it straight up (fig. 7.8). Use of a plastic spatula may help prevent tearing the gel. Use the sketch in figure 7.9 to label the wells formed in the gel by the comb.
6. Submerge the gel under the buffer in the electrophoresis chamber.
7. You will study six samples:

   Sample 1: Bromophenol blue (molecular weight = 670 g $mole^{-1}$)

   Sample 2: Methylene blue (molecular weight = 320 g $mole^{-1}$)

   Sample 3: Orange G (molecular weight = 452 g $mole^{-1}$)

   Sample 4: Xylene cyanol (molecular weight = 555 g $mole^{-1}$)

   Samples 5 and 6: Unknowns

Figure 7.6
Cover the ends of the removable gel bed with rubber end-caps or tape.

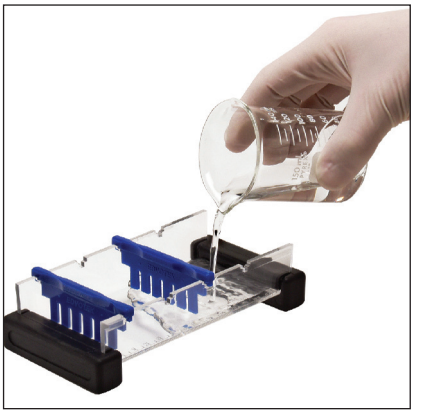


Figure 7.7
Place the comb near the center set of notches of the gel bed. Prepare the agarose solution and pour the gel.

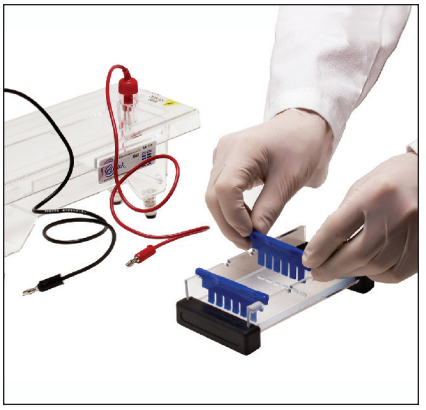


Figure 7.8
After the gel solidifies, gently remove the rubber end-caps (or tape) and pull the combs straight up from the gel.

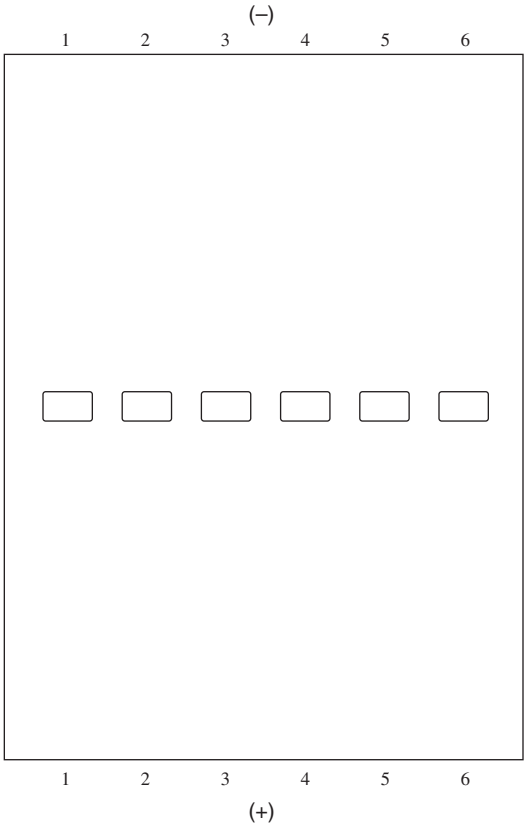


Figure 7.9
Sketch of the wells formed in the gel by the comb as viewed from above.

Use a micropipettor or a simple pipet and bulb to load the samples into the wells of the gel. If you use a micropipettor, your instructor will demonstrate its use. If you use a simple pipet and bulb, gently squeeze the pipet bulb to draw Sample 1 into the pipet. Be sure that the sample is in the lower part of the pipet. If the sample becomes lodged in the bulb, tap the pipet until the sample moves into the lower part.

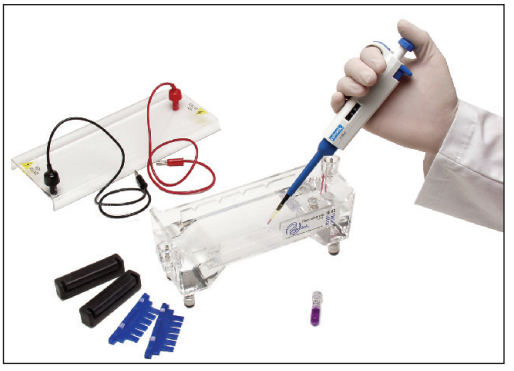


Figure 7.10
Submerge the gel in the buffer-filled electrophoresis chamber and load the samples into the wells of the gel.

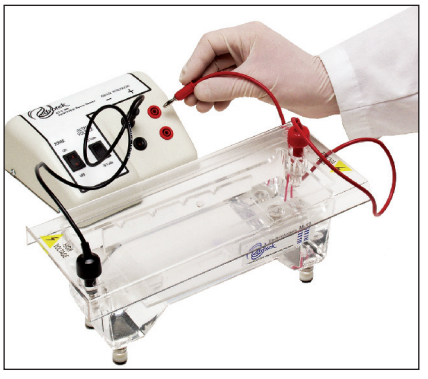


Figure 7.11
Attach the safety cover, connect the power source, and run the electrophoresis.

8. To eliminate excess air hold the pipet above the sample tube and slowly squeeze the bulb until the sample is near the pipet's opening.
9. Place the pipet tip into the electrophoresis buffer so it is barely inside sample well 1 (fig. 7.10). Do not touch the bottom of the sample well. Maintain pressure on the pipet bulb to avoid pulling buffer into the pipet.
10. Slowly inject the sample into the sample well. Stop squeezing the pipet when the well is full. Do not release the pressure on the bulb. Remove the pipet from the well.
11. Thoroughly rinse the pipet with distilled water.
12. Load the remaining five samples into the gel by repeating steps 6–10 (fig. 7.10). Load Sample 2 into the second well, Sample 3 into the third well, etc.
13. Carefully snap on the cover of the electrophoresis chamber (fig. 7.11). The red plug in the cover should be placed on the terminal indicated by the red dot. The black plug in the cover should be placed on the terminal indicated by the black dot.
14. Insert the plug of the black wire into the black (negative) input of the power supply. Insert the plug of

the red wire into the red (positive) input of the power supply.

15. Turn on the power and set the voltage at 90 V. You'll soon see bubbles forming on the electrodes. Examine the gel every 10 min.

16. After 30 min, turn off the power and disconnect the leads from the power source. Gently remove the cover from the chamber and sketch your results in figure 7.9.

Question 3

a. Bromophenol blue, Orange G, and xylene cyanol each have a negative charge at neutral pH, whereas methylene blue has a positive charge at neutral pH. How does this information relate to your results?

b. Did Orange G, bromophenol blue, and xylene cyanol move the same distance in the gel? Why or why not?

c. What compounds do you suspect are in Samples 5 and 6? Explain your answer.

INTERPRETING A DNA-SEQUENCING GEL

Examine figure 7.12, which includes a photograph of a gel used to determine the order, or sequence, of nucleotides in a strand of DNA. To prepare the sample for electrophoresis, samples of the DNA being investigated were put into each

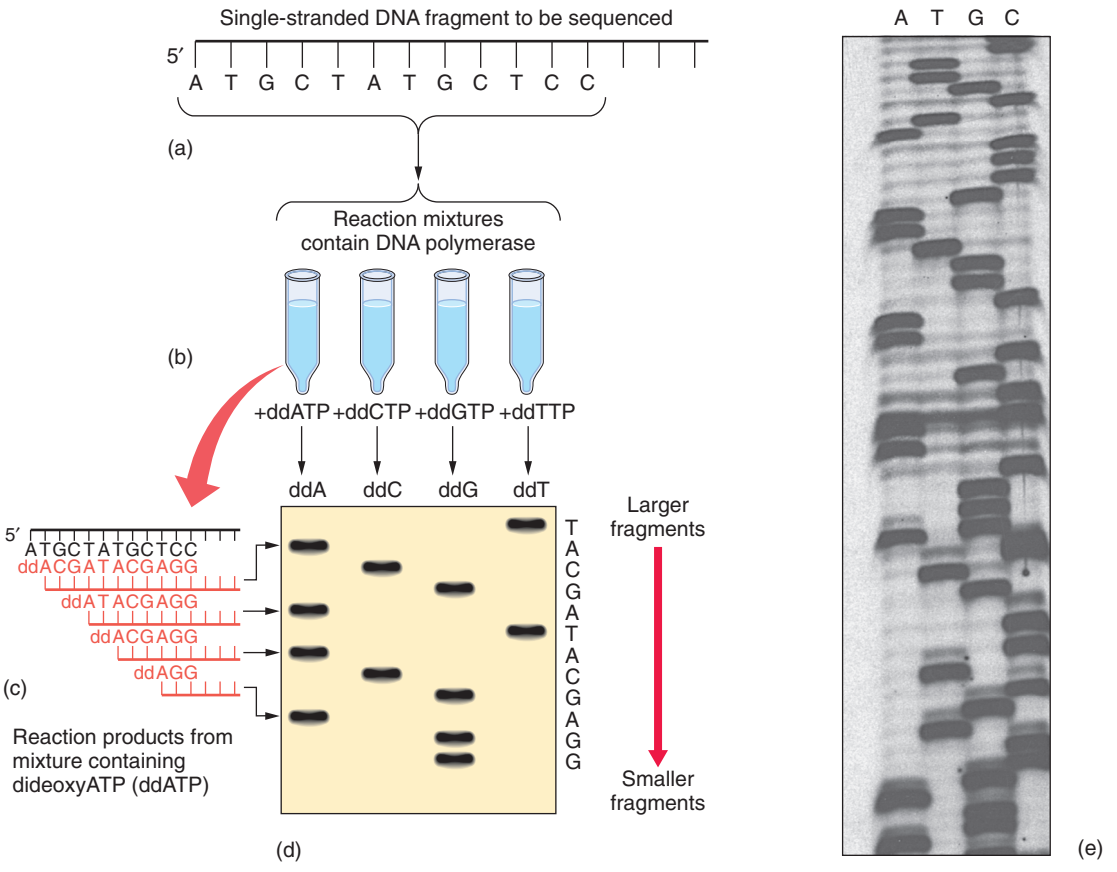


Figure 7.12

Determining the sequence of nucleotides in DNA. (*a*) Treating DNA with sodium hydroxide (NaOH) denatures double-stranded DNA into single-stranded DNA. One of the single strands of DNA to be sequenced is placed in each of four tubes. (*b*) The enzyme DNA polymerase is added to each tube along with a specific nucleotide-terminator. As polymerase replicates the DNA, the terminators are incorporated and will terminate various lengths of fragments of DNA. For example, the terminator ddATP will halt the reaction wherever adenosine occurs. The terminator ddATP (dideoxy adenosine triphosphate) will terminate a growing strand because it lacks a 3′ hydroxyl group and therefore cannot bond with the next deoxynucleotide. (*c*) Each tube will contain a sample of all possible replicated fragment lengths corresponding to the positions of that specific nucleotide. The sequences in red are the complement strands. (*d*) During electrophoresis, the fragments migrate at different rates according to their length. (*e*) The lanes of the resulting gel are labeled according to their base: A, adenine; T, thymine; G, guanine; and C, cytosine. This technique is usually referred to as "Sanger" sequencing in honor of Fred Sanger, a Nobel laureate who, in 1977, first sequenced a piece of DNA.

of four tubes and induced to replicate. Also, into the first tube, an adenine-terminator was added in addition to all the other nucleotides. As the complementary strand was being constructed the terminators were occasionally incorporated wherever an adenine nucleotide was used. This random incorporation resulted in all possible lengths of DNA pieces that had an adenine on the end. The same process was conducted in the other tubes with thymine-, guanine-, and cytosine-terminators; one treatment for each of the four lanes in the gel. Electrophoresis separated the replicated pieces of DNA by size. Staining the gel revealed which lengths of the complementary DNA were terminated by which nucleotide-terminators. Examine figure 7.12d.

The gel consists of four "lanes," labeled A, T, G, and C, indicating either adenine-, thymine-, guanine-, or cytosine-terminated pieces of DNA. By "reading" down the gel, you can determine the sequence of nucleotides in the DNA. For example, the uppermost band of the gel is in the T (thymine) lane. Therefore, the first base of the piece of DNA is thymine. Similarly, the next bands are in the A, C, G, and A lanes. Thus, the first five bases of the complementary strand DNA are T-A-C-G-A. List the next seven nucleotides of the DNA as indicated by the gel. Also list the sequence of the first 12 nucleotides in the original DNA being investigated.

Question 4

a. How did the sequence of nucleotides revealed on the gel differ from the sequence of the original strand of DNA?

b. Assume that the gel shown in Figure 7.12d is from blood collected at a murder scene. This blood does not match that of the victim. You have collected DNA from five people suspected of murder. Gels comparable to the one shown in Figure 7.12*d* read as follows for each of the suspects:

Suspect #1: T-A-C-G-A-T-A-C-G-A-C
Suspect #2: T-A-C-G-A-T-A-C-G-A-C
Suspect #3: T-A-C-G-A-C-A-C-G-C-G
Suspect #4: T-A-C-G-A-T-G-C-G-A-C
Suspect #5: T-A-C-G-A-T-C-C-G-T-C

What do you conclude from this evidence?

INVESTIGATION I

Refining the Paper Chromatography Procedure

Carefully planned and refined procedures are critical for laboratory techniques such as paper chromatography. The sensitivity of these techniques depends on a variety of factors, including the many parameters associated with timing, chemicals, measurements, and temperatures. In procedure 7.2 you were given a rather standardized protocol, but it can always be improved for specific experiments. For example, how would you modify the paper chromatography procedure to better resolve two amino acids having approximately the same R_f values? What parameter(s) of the experimental design might be tweaked to increase the technique's resolving power? We suggest that you begin your investigation in the following way:

a. List the parameters involved in paper chromatography. Think carefully; many factors are involved.

b. Choose one or two parameters that you can test for their impact on the chromatography results. Why did you choose these?

c. Choose two amino acids for experimentation. Why did you choose these two?

d. Choose your treatment levels for each parameter, and then do your experiment.

e. What did you conclude?

INVESTIGATION II

The Importance of the Length of the Column in Column Chromatography

Observation: Column chromatography is a common means of separating molecules according to their size and shape. The movement of molecules through a column is affected by several factors, including the column's matrix and the column's length.

Question: How does the length of a column affect the separation of molecules via column chromatography?

a. Establish a working lab group and obtain Investigation Worksheet 7 from your instructor.

b. Discuss with your group well-defined questions relevant to the preceding observation and question. Choose and record your group's best question for investigation.

c. Translate your question into a testable hypothesis and record it.

d. Outline on Worksheet 7 your experimental design and supplies needed to test your hypothesis. Ask your instructor to review your proposed investigation.

e. Conduct your procedures, record your data, answer your question, and make relevant comments.

f. Discuss with your instructor any revisions to your questions, hypothesis, or procedures. Repeat your work as needed.

Questions for Further Thought and Study

1. How are column chromatography, paper chromatography, and gel electrophoresis different? How are they similar?

2. How would the results of electrophoresis vary if the voltage was increased? If the agarose was made more dense? Or if the migration was allowed to run twice as long?

3. How could knowing the nucleotide base sequence of a piece of DNA be important to a biologist?

4. How could knowing the nucleotide base sequence of a piece of DNA be important to someone trying to solve a crime?

5. How could knowing the nucleotide base sequence of a piece of DNA be important for someone studying a hereditary disease?

6. How could knowing the nucleotide base sequence of a piece of DNA be important for someone wanting to improve the yield of a crop such as corn?

WRITING TO LEARN BIOLOGY

Which of the methods discussed in this exercise would best quantify the relative amounts of the molecules being separated? Why?