

# **Advanced Placement Biology Series**

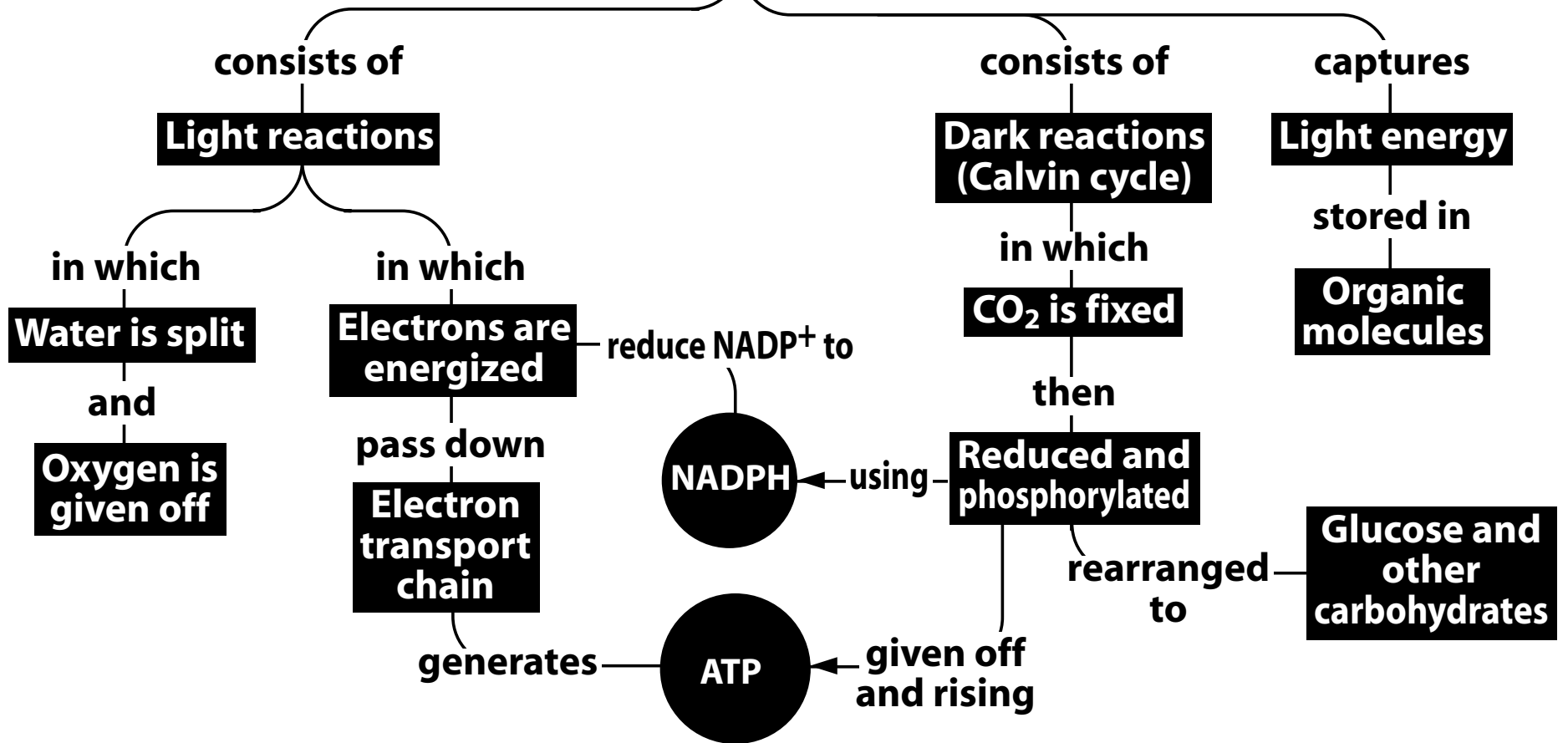
## **Laboratory Four**

### **Plant Pigments and Photosynthesis**

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# Photosynthesis



## Introduction

For many centuries, people believed that the increase in the size of plants was caused by the intake of material from the soil. It was not until a Belgian physician, Jan Baptista van Helmont {part bio} (circa 1577-1644), performed an experiment that demonstrated conclusively what we accept today: the increase in the size of a plant is not due simply to the plant obtaining a mystery substance from the soil; plants gain what they require through the process of photosynthesis.

Photosynthesis uses energy from light captured by photosynthetic pigments. Water molecules are split in the process; the plants fix carbon from carbon dioxide into glucose and fructose chains and oxygen, a byproduct, is released. In many plants the sugars then combine to form long chains known as starches. Many plants store their photosynthetic products this way.

Most plants produce their own organic molecules, without taking them from another organism, through photosynthesis; these plants are autotrophs. Not all plants are able to carry out photosynthesis, however. Many parasitic plants, without photosynthetic pigments, rely entirely on other, host species for nourishment; these plants are heterotrophs.

Examples of photosynthetic pigments are chlorophyll *a*, chlorophyll *b*, and carotene. Green plants usually have a high chlorophyll content; in a typical plant, approximately three-quarters of the chlorophyll is chlorophyll *a* and the rest is chlorophyll *b*. The presence of other pigments becomes apparent in some plants in the fall when the chlorophyll no longer masks their presence. Some plants are high in pigments that mask the chlorophylls during the whole growing season; e.g., red cabbage remains red because of the presence of anthocyanin. Each pigment absorbs light of a specific range of wavelengths. Chlorophyll *a*, the primary pigment used in photosynthesis, absorbs blue and red light. Chlorophyll *b* absorbs light in the blue-green and orange-red portions of the spectrum. Carotenoids absorb light in the blue and blue-green regions.

Plants lose water in two ways: from inside the leaves through transpiration, and from the surface of the leaves or from the soil through evaporation. The total loss of water from both sources is called evapotranspiration. To reduce the water lost through transpiration, leaves can close their pores, or stomata, using special cells called guard cells. However, this also limits the exchange of air and other gases, which enter and exit the leaves through the stomata, so photosynthesis might also be limited.

Plants may adapt to this by capturing sunlight's energy during the day, storing the energy until night, and exchanging gases at that time when water loss is lower due to the lower temperatures. This is known as the "light phase" of photosynthesis. The "dark phase" of photosynthesis can occur during the light or the dark, while the light phase can occur only when wavelengths of light that will stimulate photosynthetic pigments are present.

The first step in the conversion of light to chemical energy is the absorption of light by a pigment system. In all photosynthetic cells, except photosynthetic bacteria, the pigment system includes . Chlorophyll *a* occurs in all photosynthetic eukaryotes and in prokaryotic blue-green algae. In vascular plants, bryophytes, green algae, and euglenoid algae, chlorophyll *b*, an accessory pigment, is also found. In the leaves of green plants, chlorophyll *b* generally constitutes about one-fourth the total chlorophyll content. Chlorophyll *b* absorbs light wavelengths different from chlorophyll *a*, extending the range of light that can be used for photosynthesis. It shares with chlorophyll *a* the ability to absorb light energy and produce an excited state in the molecule. The excited molecule of chlorophyll *b* transfers its energy to a molecule of chlorophyll *a*, which then transforms it into chemical energy. Chlorophyll *c* or chlorophyll *d* takes the place of chlorophyll *b* in other groups of plants.

Carotenoids are also accessory pigments involved in the capture of light energy in photosynthesis. Carotenoids are red, orange, or yellow fat-soluble pigments found in all chloroplasts and also, in association with chlorophyll *a*, in the prokaryotic blue-green algae. There are two classes of carotenoids: those that do not contain oxygen are called carotenes, and those that do contain oxygen are called xanthophylls. In green leaves, the color of the carotenoids is masked by the much more abundant chlorophylls; in some tissues, such as those of a ripe tomato or the petals of an orange flower, the carotenoids predominate. During autumn, chlorophyll begins to break down as the leaf begins to senesce, allowing the carotenes and xanthophylls to display the brilliant colors we associate with fall.

Carotenoids, which are not water soluble, are not found free in the cytoplasm, but like the chlorophylls are bound to proteins within the plastids. Only certain carotenoids serve as accessory pigments, but these are important for the overall process of photosynthesis in the green plant.

To measure the percentage of each wavelength of light absorbed by a pigment (the absorption spectrum), a spectrophotometer is used. A spectrophotometer directs a beam of light of a specific wavelength at the object to be analyzed, and records what percentage of the light of each wavelength is absorbed by the pigment or pigment system. The absorption spectrum is different from the action spectrum, which graphs the efficiency of different wavelengths of light in promoting a given photoresponse, as in photosynthesis or phototropism.

A spectrophotometer can also be employed to measure the rate of photosynthesis. In the dye-reduction technique, the compound DPIP (2,6-dichlorophenol-indophenol), is substituted for NADP (nicotinamide adenine dinucleotide phosphate), the primary electron-accepting compound of photosynthesis. As DPIP is reduced by chloroplasts in the presence of light, it changes form blue to colorless. The spectrophotometer measures the increase in light transmittance over time, and thus indicates the rate of photosynthesis.

Chromatography is a technique for analyzing or separating mixtures of gases, liquids, or dissolved substances such as chlorophyll pigments. There are many types of chromatography, including column, paper, thin-layer, gas-liquid, ion exchange, and gel filtration. In general, all types of chromatography involve two distinct phases: the stationary phase and the moving phase. The separation depends on competition for molecules of sample between the moving phase and the stationary phase.

Column, paper, and thin-layer chromatography can be used to separate extracted plant and algal pigments. In paper chromatography, the separation takes place through absorption and capillary action. A drop of the mixture to be separated is placed at the bottom of a strip of chromatography paper, which holds the substance by absorption. The chromatography paper and developer are then placed in a chamber. The paper acts as a wick, drawing the developer upward by capillary action and dissolving the mixture as it passes over it. The components of the spotted mixture move upward at differing rates, determined by both the solubility of the pigments in the solvent and their relative attractions to the cellulose of the chromatography paper, resulting in the different pigments in the mixture showing up as colored streaks or bands. The pattern formed on the paper is called a chromatogram.

To establish the relative rate of migration for each pigment, the  $R_f$  value of each pigment is calculated. The  $R_f$  value represents the ratio of the distance a pigment moved on the chromatogram relative to the distance the solvent front moved. It is calculated using the following formula:

$$R_f = \frac{\text{Distance Substances (Pigments) Traveled}}{\text{Distance Solvent Traveled}}$$

Any molecule in a given solvent matrix has a uniquely consistent  $R_f$ . The  $R_f$  value is used by scientists to identify molecules.

## **Jan Baptista van Helmont (1580-1644)**

Jan Baptista van Helmont was a Belgian physician and chemist who pioneered experimentation and an early form of biochemistry. He was the first scientist to distinguish between gases and air and believed that the basic elements of the universe are air and water.

He believed that plants are composed only of water and claimed to have proven this theory by planting a willow of known weight in soil of known weight and weighing the willow and the soil five years later. Although only water was added to a potted willow, it gained nearly 75 kg (165), whereas the soil it stood in lost only about 60 g (about 2 oz) of weight over a period of the time. This demonstrated that the soil contributes very little to the increase in the weight of plants.

## **The Electromagnetic Spectrum**

The range of wavelengths or spectrum is a series of colors in the order violet, blue, green, yellow, orange, and red produced by splitting white light into its component colors. This can be produced by passing sunlight through a glass prism as first explained by the English mathematician and physicist Sir Isaac Newton in 1666.

When a ray of light passes from one transparent medium, such as air, into another, such as glass or water, it is bent; upon reemerging into the air, it is bent again. This bending is called refraction, and the amount of refraction depends on the wavelength of the light. Violet light, for example, is bent more than red light in passing from air to glass or from glass to air.

During the 19th century, scientists discovered that beyond the violet end of the spectrum, radiations could be detected that were invisible to the human eye but that had marked photochemical action. These were called ultraviolet. Beyond the red end of the spectrum, infrared radiations were detected that, although invisible, transmitted energy, as shown by their ability to raise the temperature of a thermometer.

Today's spectrum includes these invisible radiations, and has since been extended to include radio waves beyond the infrared, and X-rays and gamma rays beyond the ultraviolet.

## **Chromatography**

Chromatography is a technique used for the chemical separation of mixtures and substances. The technique depends on the principle of selective adsorption, a type of adhesion. Chromatography was first introduced in 1906, but was not widely used until the 1930s. In this procedure plant pigments were separated by pouring petroleum-ether extract of green leaves over a column of powdered calcium carbonate in a vertical glass tube. As the solution percolated through the column the individual components of the mixture migrated downward at different rates of speed, so that the column became marked with horizontal bands of colors, called a chromatogram. Each band corresponded to a different pigment.

Column chromatography now uses a wide range of adsorbent solids, including silica, alumina, and silica gel. Liquids may also be adsorbed on these solids and in turn serve as adsorbents - a process called partition chromatography.

In paper chromatography, a liquid sample flows down a vertical strip of adsorbent paper, on which the components are deposited in specific locations. Another technique, known as gas-liquid chromatography, permits separation of mixtures of gas compounds or substances that can be vaporized by heat. The vaporized mixture is forced by an inert gas along a narrow, coiled tube packed with a material through which the components flow at different rates and are detected at the end of the tube.

In ion chromatography, a gas may be broken down into ions by passing it through a hydrogen flame, bombarding it with X rays or radioactive material, or using adsorbent substances that exchange ions with the material being analyzed. Gel permeation chromatography is another method, based on the filtering action of an adsorbent with pores of uniform size. Molecules of high molecular weight are separated and detected by this method.

Chromatography is essential to the separation of pure substances from complex mixtures and is widely used in the analysis of foods, drugs, blood, petroleum products, and radioactive-fission products.

## Learning Objectives

Students will use paper chromatography to separate plant pigments and use a dye reduction reaction to measure the rate of photosynthesis in isolated chloroplasts.

## Time Requirements

Two 45-minute lab periods

## Materials Requirements

### Materials Included in the Kit

For 8 Lab Groups (36 W 7103)	For 2 Lab Groups (36 W 7114)	
		Phosphate Buffer, 60ml
		Sucrose 0.5M, 120ml
		Indophenol Solution, 500ml
50	50	Chromatography Paper Strips
		Chromatography Solvent, 120ml
		Parafilm
1	1	Syringe, 5ml
15	15	Pipets
8	2	Vials
16	4	Cups, 1 oz.
		Cheesecloth
		Aluminum Foil

### Materials Needed but Not Provided

Spinach	Spectrophotometer
Erlenmeyer Flask, 4L	Floodlight, 100W
Beaker, 250ml	Blender
Ring Stand	Balance
Test Tube	Ice Bucket
Test Tube Clamp	Hotplate
Test Tube Rack	Coin
Cuvettes	Gloves
Lens Tissue	Apron
Distilled Water	Goggles

### Materials Needed per Lab Group (Chromatography of Plant Pigments)

1	Vial
1	Chromatography Paper Strip
	Chromatography Solvent, 10ml
	Coin
	Spinach

### Materials Needed per Lab Group (Photosynthesis)

	Indophenol Solution, 3ml	Spectrophotometer
	Phosphate Buffer, 4ml	Floodlight, 100W
	Prepared Boiled Chloroplast	Aluminum Foil
	Prepared Unboiled Chloroplast	Pipets
	Parafilm	
4	Cuvettes	
	Test Tube Clamp	
	Test Tube Rack	

## Plant Pigments and Photosynthesis

## Pre-Lab Preparation

1. Prepare DPIP solution: Dissolve 0.036g of DPIP in 500ml distilled water. Mix thoroughly and place solution in a labeled bottle.
2. Prepare chloroplast suspension: Incubate the fresh spinach leaves by placing them under a light for several hours. Do not allow the leaves to overheat. Pour enough 0.5M sucrose solution into a blender to cover the blades. Add incubated spinach leaves to a level of one inch above the blades. Blend the spinach leaves in three 10-second intervals. Filter the spinach through two layers of cheesecloth into a beaker on ice. Transfer the suspension to an amber bottle or vial. Cap the bottle or vial and store refrigerated or on ice.
3. Prepare boiled chloroplast suspension: Place chloroplast suspension-2ml for each lab group-in a test tube. Place test tube in a boiling waterbath for approximately five minutes. Transfer the boiled extract to an amber bottle or vial. Cap the bottle or vial and store refrigerated or on ice.

## Chromatography of Plant Pigments

## Experiment

**Safety:** Avoid inhaling the chromatography solvent. Wear protective eyewear, gloves, and apron or smock when working with flammables. Avoid open flames.

1. With a pencil, draw a line 1.5cm from the bottom of the paper.  
**Note:** Touch the paper as little as possible; the oils from your fingers will interfere with the chromatogram.
2. Place a piece of spinach over the line. Rub the ribbed edge of a quarter over the spinach leaf to extract the pigments. Repeat eight or 10 times, making sure you are rubbing the coin over the pencil line.
3. Pour 10ml chromatography solvent into a glass vial. Place the chromatography paper in the vial so that the pigment end of the paper is barely immersed in the solvent. Cap the vial and leave it undisturbed until the solvent reaches approximately 1cm from the top of the strip.
4. Remove the paper and immediately mark the location of the solvent front; it will evaporate very quickly.
5. Mark the location of each of the four bands and record your data in Table 1.

## Photosynthesis

## Experiment

1. Turn on the spectrophotometer to warm it up. Adjust the wavelength control knob to 605nm.
2. Set up an incubation area with a floodlight, a flask of water for a heat sink, and a test tube rack.
3. With a glass marking pen, label four cuvettes at the very top rim-1, 2, 3, and 4. Wipe down the outside of each cuvette with lens paper to remove any fingerprints and oils.
4. Wrap the outside of cuvette 2 with foil and make a foil cap for the top to keep the chloroplast solution in complete darkness. It will be used as the experiment control.
5. Add the following:
  - 1ml phosphate buffer to all four cuvettes
  - 4ml distilled water to cuvette 1
  - 3ml distilled water to cuvettes 2, 3, and 4
  - 1ml DPIP to cuvettes 2, 3, and 4
6. Zero the spectrophotometer by adjusting the amplifier control knob until the meter reads 0% transmittance.
7. Obtain 2ml of boiled chloroplast and 2ml of unboiled chloroplast. Transfer three drops of unboiled chloroplasts to cuvette 1. Cover the top of the cuvette with parafilm and invert to mix.

8. Place cuvette 1 in the sample holder of the spectrophotometer. Be sure the cuvette is wiped clean and is inserted into the sample holder in the same direction every time to ensure consistent readings. Adjust the light control knob until the meter reads 100% transmittance.  
**Note:** *Cuvette 1 will be used to recalibrate the spectrophotometer between readings.*
9. Transfer three drops of unboiled chloroplasts into cuvette 2 with a pipette. Immediately cover the cuvette with parafilm and invert to mix.
10. Remove the foil sleeve and foil top, and place the cuvette in the sample holder. Read the percent transmittance and record it as Time 0 in Table 2.
11. Place the cuvette back into its foil sleeve and cover it with the foil top. Place the cuvette in the test tube rack between percent transmittance readings.
12. Repeat readings at 5, 10, and 15 minutes. Be sure to cover and mix cuvette before each reading. Read the percent transmittance and record the data in Table 2.  
**Note:** *Be sure to use cuvette 1 to check and recalibrate the spectrophotometer periodically to ensure consistent results.*
13. Transfer three drops of the unboiled chloroplasts into cuvette 3. Immediately cover the cuvette with parafilm and invert to mix.
14. Place the cuvette in the sample holder. Read the percent transmittance and record it as Time 0 in Table 2.
15. Place the cuvette in the test tube rack between percent transmittance readings.
16. Repeat readings at 5, 10, and 15 minutes. Be sure to cover and mix cuvette before each reading. Read the percent transmittance and record the data in Table 2.  
**Note:** *Be sure to use cuvette 1 to check and recalibrate the spectrophotometer periodically to ensure consistent results.*
17. Transfer three drops of the boiled chloroplasts into cuvette 4. Immediately cover the cuvette with parafilm and invert to mix.
18. Place the cuvette in the sample holder. Read the percent transmittance and record it as Time 0 in Table 2.
19. Place the cuvette in the test tube rack between percent transmittance readings.
20. Repeat readings at 5, 10, and 15 minutes. Be sure to cover and mix cuvette before each reading. Read the percent transmittance and record the data in Table 2.

# Data Table One

## Chromatography of Plant Pigments

Band Number	Pigment	Migration Distance (mm)	R <sub>f</sub> Value
1 (top)	Carotene		
2	Xanthophyll		
3	Chlorophyll a		
4	Chlorophyll b		
—	Solvent		

# Data Table Two

Cuvette	Time			
	0 min.	5 min.	10 min.	15 min.
2 (Dark)				
3 (Unboiled)				
4 (Boiled)				

## Questions

1. Which pigment migrated the farthest and why?
2. Which of the two forms of chlorophyll is more soluble?
3. Why do leaves change color in autumn?
4. What is the function of the chlorophylls in photosynthesis?
5. What are the accessory pigments and what are their functions?
6. What are some other types of chromatography?
7. What does the  $R_f$  value represent?

8. What is the absorption spectrum?

9. In what way is the spectrophotometer used to measure the rate of photosynthesis?

## Glossary

- Absorption Spectrum** — A graph that shows the absorption of light at different wavelengths by chlorophyll *a*, chlorophyll *b*, and beta carotene dissolved in ether; measured by a spectrophotometer.
- Action Spectrum** — Discovered by T.W. Englemann in 1882; reveals the rate of photosynthesis in a filamentous alga using the rate of oxygen production. Englemann used bacteria that are attracted by oxygen and a spectrum projected on a microscope slide. The oxygen-seeking bacteria, when placed under the microscope, congregated where the violet and red wavelengths fell on the algal filament. The results paralleled the absorption spectrum of chlorophyll; Englemann concluded that photosynthesis depends on the light absorbed by chlorophyll.
- Blue-Green Algae** — Prokaryotic organisms occurring in all aquatic habitats, unicellular but sometimes joined in colonies or filaments by a sheath of mucilage. Formerly classified as algae, now termed cyanobacteria. Also referred to as blue-green bacteria.
- Bryophytes** — (Bryophyta) A division of plants including the mosses (Musci) and liverworts (Hepaticae). Bryophytes differ from algae in that the multicellular gametangium is surrounded by a protective jacket of sterile cells.
- Carotenes** — Group consists of *a*-carotene, *b*-carotene, *y*-carotene, and lycopene.
- Carotenoids** — Beta-carotene and xanthophylls, such as zeaxanthin, constructed from isoprene. Zeaxanthin is the pigment responsible for the yellow color of corn kernels. "A" vitamins are produced by carotenoid molecules obtained from plants splitting in the bodies of the animals that require them. The carotenoids have a light absorption peak between 450 and 480nm.
- Chlorophyll** — The green pigment in plants that functions in photosynthesis by absorbing radiant energy from the sun, predominantly from the blue (435-438nm) and red (670-680nm) regions of the spectrum. The principal chlorophyll variants are chlorophylls *a* and *b* in land plants, and *c* and *d* in marine algae.
- Chromatography** — Originally invented by Russian Botanist Mikhail Tsvet in 1906. A vertical glass tube is packed with an absorbent material, such as alumina. The sample is poured into the column and eluted-continuously washed through with a solvent. Different components of the sample are absorbed to different extents and move down the column at different rates. The usual method is to collect the elute as it passes out of the column in fractions. Tsvet used plant pigments, which separated into colored bands when passing down the column; hence the name chromatography.
- Euglenoid Algae** — (Euglenophyta) A division of unicellular protists-sometimes regarded as algae, sometimes as protozoa-characterized by a single flagellum, the paramylum as a storage product, chlorophylls *a* and *b*, and the absence of sexual reproduction.
- Gas-Liquid Chromatography** — Used for separating or analyzing mixtures of gases. The sample, often a volatile liquid such as fatty acids, is vaporized and swept through a column containing a non-volatile liquid, such as hydrocarbon oil, by a carrier gas such as hydrogen. The components pass through the column at different rates and are detected as they leave, either by measuring the thermal conductivity of the gas or by flame detector.
- Gel Filtration** — A type of column chromatography in which mixtures of liquids pass through a column

containing a gel. Small molecules in the mixture enter pores in the gel and move slowly down the column; large molecules, which cannot enter the pores, move more quickly.

**Ion-Exchange Chromatography** — Exploits the differences in net charges of the amino acids at a given pH. A cylindrical column is filled with an insoluble matrix—usually granules of synthetic resin—to which charged groups have been attached. A matrix with groups bearing negative charges is called a cation exchange resin because it retards the flow of cations through the column.

**Thin-Layer Chromatography** — A thin layer of an absorbing solid is spread on a glass plate and dried in an oven. The mixture to be analyzed is placed near one edge of the plate, which is then stood upright in a solvent. The solvent rises through the layer by capillary action, carrying the components up the plate at different rates, depending on the extent to which they are absorbed by the solid. After a given time, the plate is dried and the location of spots noted. It is possible to identify constituents of the mixture by the distance moved in the given time.

**Xanthophylls** — Group consists of lutein, violaxanthin, fucoxanthin, neoxanthin, astaxanthin, diatoxanthin, diadinoxanthin, zeaxanthin, peridinin, dinoxanthin, antheraxanthin, taraxanthin, myxoxanthin, myxoxanthophyll, oscilloxanthin, and echinenone.

## Further Investigations

- To learn more about chromatography, experiment separating pigments found in substances such as fruit juices, ink, and dyes, using various polar and nonpolar solvents such as water, vinegar, alcohol, salt solution, etc. Determine which solvents are more capable of bringing about a separation and calculate the  $R_f$  values of the separated pigments.

## Related Materials

36W0062	Chromatography of Simulated Plant Pigments
36W5738	Photosynthesis: The Hill Reaction
36W3035	Photosynthesis: Synthesis of Starch by Phosphorylase
38W6822	Chlorophyll Chromatogram and Wavelength Kit
36W5737	Photosynthesis Made Easy
14W8312	Photosynthesis Demonstration Apparatus
193W6451	Chloroplast Structure & Photosynthesis, VHS
193W0314	Photosynthesis Videos, Set of 6
36W7103	Plant Pigments & Photosynthesis, APBioLab 4, 8 groups
36W7124	Plant Pigments & Photosynthesis, APBioLab 4, 8 groups refill
36W7114	Plant Pigments & Photosynthesis, APBioLab 4, 2 groups
36W7136	Combination Multimedia Lab CD-ROM & kit
36W0062	Chromatography of Simulated Plant Pigments
36W5738	Photosynthesis: The Hill Reaction
36W3035	Photosynthesis: Synthesis of Starch by Phosphorylase
38W6822	Chlorophyll Chromatogram and Wavelength Kit
36W5737	Photosynthesis Made Easy
14W8312	Photosynthesis Demonstration Apparatus
193W6451	Chloroplast Structure & Photosynthesis, VHS
193W0314	Photosynthesis Videos, Set of 6
36W7103	Plant Pigments & Photosynthesis, APBioLab 4, 8 groups
36W7124	Plant Pigments & Photosynthesis, APBioLab 4, 8 groups refill
36W7114	Plant Pigments & Photosynthesis, APBioLab 4, 2 groups
36W7136	Combination Multimedia Lab CD-ROM & kit