Chapter 5

Experimental Studies of Permeability in Red Blood Cells

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Ruth Von Blum did her undergraduate work at Smith College, graduating with honors in 1965. She obtained a Master's degree in Botany at the University of Hawaii in 1967. After teaching general biology for two years at Norfolk State College, she returned to graduate school at the University of California, Berkeley, where she received a Ph.D. degree in Science/Math Education in 1972. She held a joint appointment in the Graduate Group in Science and Mathematics Education and in the department of Biology at Berkeley from 1973–1978, teaching undergraduate courses in biology and graduate courses in science education. During this time she was assistant director of SABLE, a curriculum development project funded by the National Science Foundation, that produced a series of self-instructional units using the computer to introduce students to scientific reasoning in biology. She was a program manager in the Development in Science Education Program at the National Science Foundation during 1978–79. Currently she is acting as a consultant for the Educational Technology Center at the University of California, Irvine.

Introduction—For the Instructor

This laboratory was developed as one of a series of guided laboratory experiments by Project SABLE* at the Lawrence Hall of Science, University of California, Berkeley. These experiments were primarily designed for (and were tested in) an introductory level college course for biology majors. Since the written tutorials carefully explain each of the fundamental concepts involved in the experiment, and since neither the experimental manipulations nor the calculations are difficult, the laboratory could probably be used in a course for non-majors, or even in an advanced high school class, without significant modification.

The objectives of this (and all other) SABLE laboratory units are twofold; 1) to teach basic biological concepts, and 2) to engage students in real scientific experimentation. To insure that the students understand the principles of diffusion across biological membranes, a written tutorial (Tutorial A) is handed out to be completed before coming to the lab. The laboratory itself is specifically designed to provide students with a simple, yet powerful tool for exploring the properties of red blood cell membranes, and to provide freedom for them to perform experiments of their own design with this new technique. Students are guided through observations of red blood cells, and through two experiments in which specific hypotheses are tested concerning membrane properties. This guidance is provided by a written tutorial (Tutorial B) which students follow as they do the laboratory work. In this part of the lab they learn to use the slit-lamp technique to measure hemolysis time. The second part of the laboratory consists of a student-designed independent investigation of cell permeability using the concepts and procedures just learned.

The laboratory is most effective when two lab periods are set aside for it. Students read Tutorial A at home. Then, the first lab period is spent working through Tutorial B and the tutorial on the spectrophotometer, and for learning the procedures. The second lab period is for the student independent investigation. If time constraints are such that only one laboratory period is available, however, the laboratory can still be used. In this case, it is important to allow enough time for students to perform a small experiment of their own at the end of the period.

Perhaps the most important aspect of this laboratory is the form in which it appears. The specific laboratory activities undertaken are not at all extraordinary, but the use of guided tutorials and the placing of each experiment within a framework of *Experimental Question* followed by *Hypothesis* followed by *Experiment,* is unusual. So too is the inclusion of student independent investigations as a central component of the lab activity. With some effort, many otherwise cut and dried laboratory exercises can be transformed in this way into exciting experiences in scientific thinking.

*Systematic Approaches to Biological Laboratory Explorations, a project funded in part by grants from the National Science Foundation

TUTORIAL A—CELL PERMEABILITY

Diffusion, Osmosis, and Biological Membranes

This tutorial should be completed AT HOME BEFORE you come into the laboratory.

Objectives

When you have finished this tutorial, you should be able to:

1. State in what respects diffusion and osmosis are similar phenomena and in what respects they differ.
2. Given two solutions of different concentrations separated by a differentially permeable membrane,
   (a) predict in which direction there will be net movement of H_2O.
   (b) define the relationship between the solutions as hyper-osmotic, hypo-osmotic, or iso-osmotic.
3. Given a cell—the contents of which are of known concentration—suspended in one of the above solutions,
   (a) predict in which direction there will be net movement of H_2O.
   (b) define the relationship of the solution to the cell as hyper-osmotic, hypo-osmotic, or iso-osmotic.
   (c) define the relationship between the solution and the cell as hypotonic, hypertonic, or isotonic.
4. Explain how the forces of osmosis, facilitated diffusion, and active transport work.
5. Describe how a membrane may be differentially permeable to various molecules.
6. Describe why surface area affects the rate of solute penetration.

Note: Tutorial A and B were written by Ruth Von Blum, Ann Norberg, and Suzanne Pfeffer. The tutorial on the spectrophotometer was written by Lynn Carter.

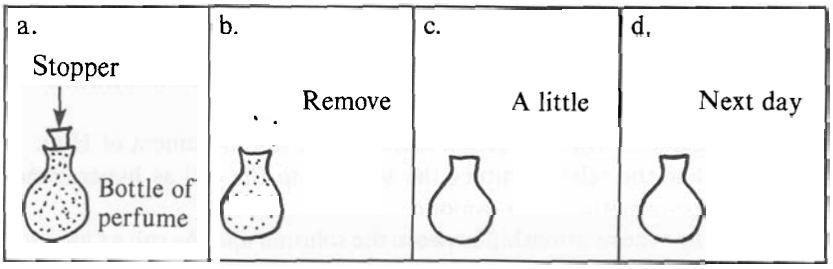
INANIMATE SYSTEMS

1

Diffusion

DIFFUSION may be defined as the movement of molecules from a region of higher concentration to one of lower concentration. This is brought about by the random motion of the molecules. Therefore, diffusion is a temperature dependent process.

▶ Fill in the two drawings (c, d) showing what would happen to the molecules of perfume with the passage of time.



▶ This process is called _____

2

Osmosis

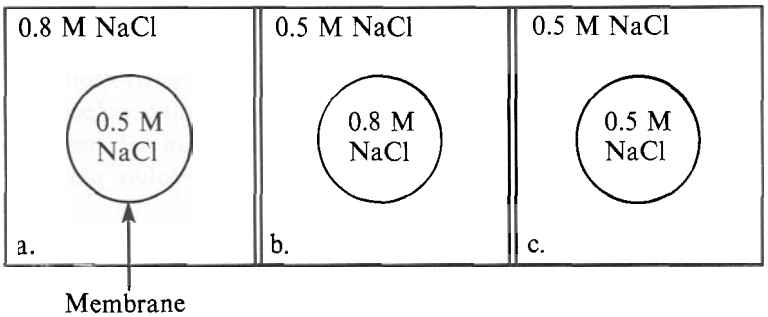
OSMOSIS is merely a special form of diffusion. It is primarily the diffusion of WATER across a differentially permeable membrane.*

▶ a. In which, if any, of the following examples would osmosis occur?

_____

b. Draw arrows indicating the direction in which there would be a NET movement of water.

*A differentially permeable membrane is one which will let some molecules pass while restricting the passage of other molecules.



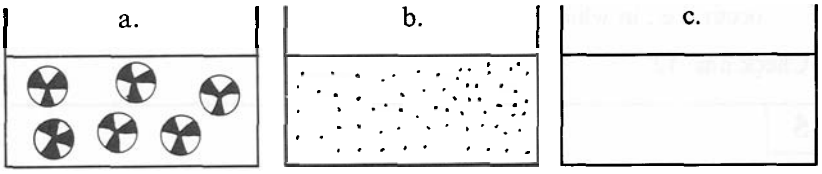
Membrane impermeable to charged ions

Check ans. 11

3

In this and subsequent examples, concentrations are given in moles.* This allows us to make direct comparisons in concentrations. One mole of any substance contains the same number of molecules as one mole of any other substance. Working with percent, while it may seem simpler, does not allow us to make such comparisons.

For example:



"Solution" of 45% beach balls $\sim 8 \times 10^{-12}$ M

"Solution" of 45% sand particles $\sim 8 \times 10^{-9}$ M

Solution of 45% NaCl ~ 8 M

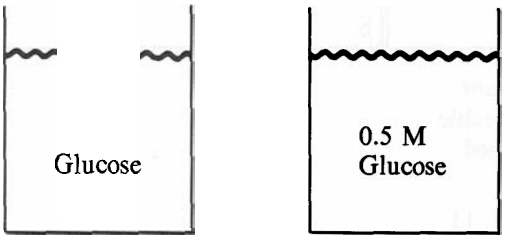
If these three "solutions", all at 45% concentration, were separated by a differentially permeable membrane, in which direction would the H_2O flow? _____

Check ans. 1

*A one molar solution has one mole (6×10^{23} particles) of solute dissolved per one liter of solution.

4

Since osmosis primarily concerns the movement of water molecules, we are especially interested in the concentration of water molecules in the solution. When we use molar concentrations, we can determine the relative concentration of water molecules as well as of solute particles.



A. B.

For example, container A has 6×10^{23} particles dissolved in one liter of solution, while container B has only 3×10^{23} particles dissolved per one liter of solution. The concentration of glucose particles is greater in A than in B. The concentration of WATER MOLECULES, however, is greater in B than in A!

If the contents of containers A and B were separated by a differentially permeable membrane, in which direction would osmosis occur, i.e., in which direction would the water move?

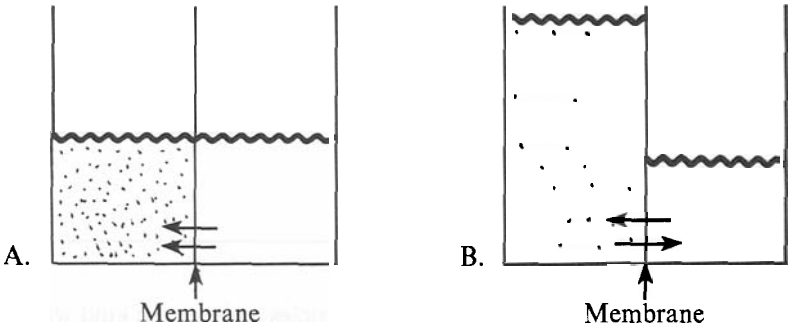
Check ans. 12

5

Osmotic Pressure

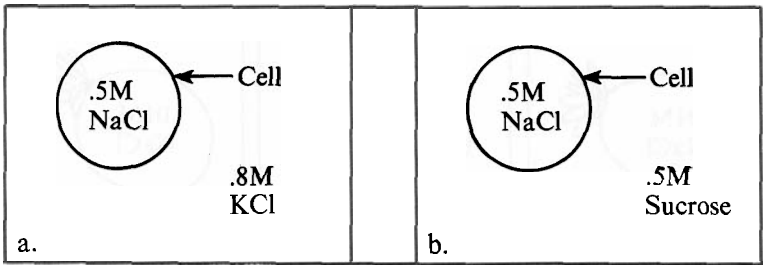
The greater the difference in concentration between the two solutions, the greater the tendency of the water to move. This difference in concentration is caused by the NUMBER of particles and not by the KIND.

This difference may be measured and is called the OSMOTIC PRESSURE of the solution. In the following diagram, the water molecules in the right chamber tend to pass through the membrane and attempt to equalize the water concentration on both sides.



When the weight of the water in the left column exerts a pressure just equal to that resulting from the tendency of water to move in, there is no further net change and water moves back and forth with equal speed. The pressure of the column of water is termed the osmotic pressure of the solution. It may be defined as the pressure which exists in a solution because of the tendency of water molecules to move into that solution in response to an osmotic gradient.

The solution on the left half of container A has a greater osmotic pressure than the solution in the right half of container A since water molecules tend to move from the right to the left.



_____

In which direction would water move? _____

b. In case b, which has the greater osmotic pressure, the cell or the surrounding medium? _____

In which direction would water move? _____

Check ans. 8 If right, go to Frame 7.
If wrong, go to Frame 6.

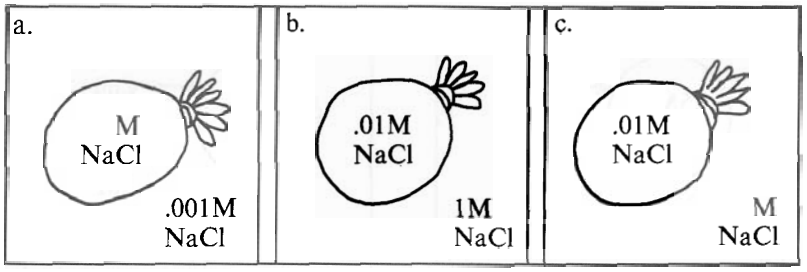
6

Remember: It is the NUMBER of particles and not the kind which determines the osmotic properties of the solution. A 0.5 M solution of a molecule which dissociates (like KCl or NaCl) produces twice the number of particles as does a 0.5 M solution of a molecule that does not dissociate (like glucose).

7

Osmotic Relationships

SOLUTIONS may be described in terms of their OSMOTIC RELATIONSHIPS WITH OTHER SOLUTIONS. Let us suppose that we have a differentially permeable membrane bag filled with a salt solution. We drop it into three solutions:



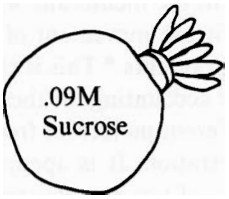
Draw an arrow in each case indicating the direction in which there would be a net movement of water. The answers are below.

In case (a), the water would tend to move from the outside solution into the bag. In a case like this, the outside solution is said to be *hypo-osmotic* to the contents of the bag, i.e., the concentration of water molecules outside the bag is greater than the concentration of water molecules inside the bag.

In case (b), the water would tend to move from the bag to the outside solution. Here, the outside solution is said to be *hyper-osmotic* to the contents of the bag. [The concentration of H_2O molecules outside the bag is less than the concentration of H_2O molecules inside the bag.]

In case (c), there would be no net movement of water because the concentration of H_2O molecules is the same inside and outside the bag. This solution is said to be *iso-osmotic* to the contents of the bag.

Label the following solutions as hypo-osmotic, hyper-osmotic, or *iso-osmotic* to this membrane-bound solution.



a.	b.	c.	d.	e.
.75M Sucrose	.003M Glucose	.045M NaCl	.09M KCl	1.38M Sucrose
_____	_____	_____	_____	_____

Check ans. 4

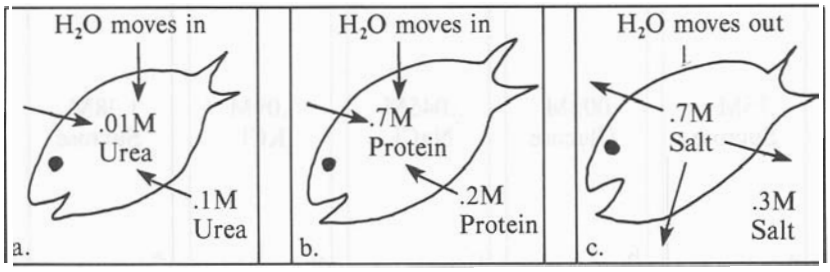
LIVING SYSTEMS: THREE FACTORS INFLUENCING TRANSPORT OF MATERIALS ACROSS A MEMBRANE

8

Permeability of the Membrane

Membranes of living cells are differentially permeable. To a certain extent, the capacity of the membrane to restrict the passage of certain molecules can be described in terms of structural features, e.g., protein-lipid composition, "pores" in the membrane with varying charge distribution. These features limit the movement of water and solutes along concentration or electrical gradients.* This is the case in simple osmosis, described above. Even after accounting for these factors, however, slight chemical differences in different molecules frequently show up as large differences in ease of penetration. It is apparent that no single factor determines the permeability of any membrane to a given substance.

Which of the following can NOT be explained in terms of simple osmosis?



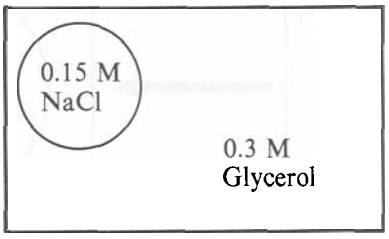
Check Ans. 7

*Charged particles will tend to move in a direction to equalize charge differences just as other particles move to equalize concentration differences.

9

Lets stop for a few seconds. So far we have discussed ONLY THE MOVEMENT OF *WATER* THROUGH A MEMBRANE. The *SUBSTANCES* we have been considering are unable to pass through the membrane. Now we shall also be dealing with COMPOUNDS or PARTICLES THAT CAN PENETRATE THE MEMBRANE. Diagrams a and c in Frame 8 illustrated examples of PARTICLE AND WATER MOVEMENT through a membrane.

Let us suspend a cell in a solution of glycerol which is *iso-osmotic.*



If the glycerol could not permeate the cell, would you expect a net movement of water? **[Check ans. 2]**

But, the cell is permeable to glycerol and the glycerol begins to diffuse into the cell under the influence of a concentration gradient (i.e., outside the cell, the glycerol concentration is ~0.3 M and inside the cell it is zero). Each glycerol molecule that enters the cell contributes to the total number of solute particles in the cell. This will have the following effect(s):

[Check the correct choice(s).]

_____ a. Osmotic pressure of the cell increases.

_____ b. Osmotic pressure of the cell decreases.

_____ c. Osmotic pressure of the cell remains constant regardless of the influx of glycerol.

_____ d. Water enters the cell.

_____ e. Water leaves the cell.

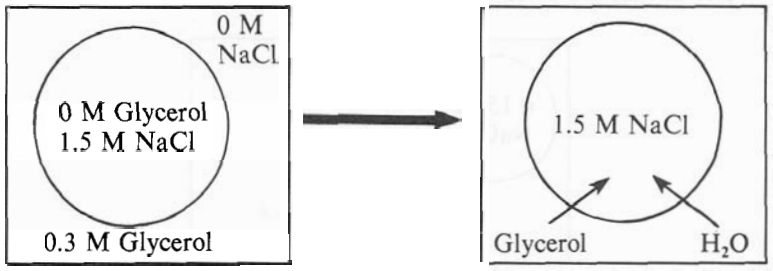
_____ f. There is not net movement of water.

Check ans. 2

10

Tonicity

A cell may swell or shrink even when placed in an iso-osmotic solution (i.e., a solution with ORIGINALLY the SAME NUMBER of particles and water molecules as found in the cell). This occurs when the membrane is permeable to the particles in the suspending solution. The particles will move along a concentration gradient into the cells (as illustrated below).



NaCl cannot get out. Glycerol goes in; therefore H_2O goes in.

As the particles (i.e., glycerol) enter the cell and the internal concentration of total particles (both glycerol and NaCl) increases, the water flows in immediately to compensate.*

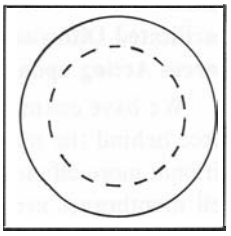
In addition to talking about *hypo-osmotic, hyper-osmotic,* or *iso-osmotic* solutions which deal with the concentration of water and its movement along a concentration gradient, we need another term to describe the BEHAVIOR OF THE CELL IN VARIOUS SOLUTIONS. To do this, we use the term TONICITY. TONICITY describes the behavior of the cell in these various solutions.

a. A *hypertonic* solution is one which will cause a cell to shrink. **What is actually causing the shrinkage?**

Check ans. 6a

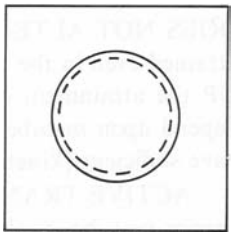
*The rate-limiting step in the process is the rate of glycerol penetration. This step consequently determines the rate of water penetration and thus swelling of the cell. [You will determine this in the laboratory. See Tutorial B]

b. A *hypotonic* solution is one which will cause a cell to swell. What is actually causing the swelling?



Check ans. 6b

c. An *isotonic* solution is one which will cause no change in the volume of the cells.
Why is there no change in volume?



Check ans. 6c

11

Lets stop again for a few seconds.
In summary, remember that:

—OSMOTIC: refers to the movement of water molecules based upon the initial number of particles in the solution.

—TONIC: refers to the behavior of the cell in various solutions.

Label the following solutions hypotonic, hypertonic, or isotonic to these hypothetical "cells".

a.

0.3 M Glycerol
0.0 M NaCl

b.

0.2 M Glycerol
0.0 M NaCl

c.

0.0 M Glycerol
0.6 M NaCl

Now, label each solution as hypo-osmotic, hyper-osmotic, or iso-osmotic.

Check ans. 9

12

Facilitated Diffusion and Active Transport: Forces Acting upon the Substance

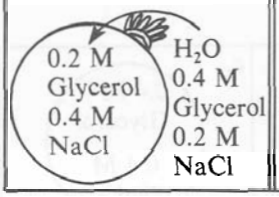
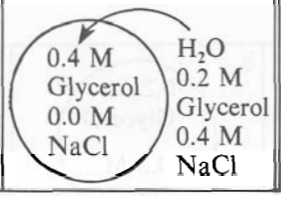
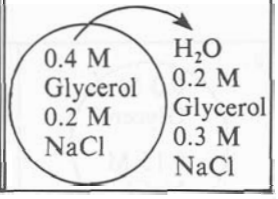
We have examined what occurs when simple osmosis is the driving force behind the movement of substances into and out of the cell. Additional more efficient transport systems involving "carrier" proteins on cell membranes are known to exist. These carriers pick up materials from the inner or outer membrane surfaces and carry them across at a faster rate than would occur through simple osmosis.

FACILITATED DIFFUSION is one such type of movement. It DOES NOT ALTER THE FINAL EQUILIBRIUM which could be attained even in the absence of a "carrier system", but it does SPEED UP the attainment of the equilibrium. Facilitated diffusion does not depend upon metabolic energy, but the solute carrier-complexes must have sufficient [kinetic] energy to be able to penetrate the membrane.

ACTIVE TRANSPORT is another type of movement in which a carrier may be involved. However, it is distinguished from facilitated diffusion since the passage of the solute through the membrane occurs AGAINST APPARENT CONCENTRATION AND ELECTROCHEMICAL GRADIENTS at the EXPENSE OF METABOLIC ENERGY*, such as ATP.

The cellular mechanisms of facilitated diffusion and active transport are not clearly understood.

▶ Label each of the following as simple osmosis or active transport.

a. Membrane bag	b. Red blood cell	c. Red blood cell
		

▶ d. How can you distinguish simple osmosis from facilitated diffusion?

Check ans. 5

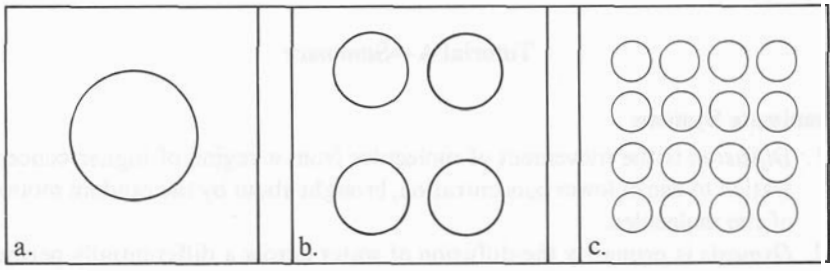
*Active transport is affected by metabolic poisons, whereas facilitated diffusion is not.

13

Surface Area

Another factor to consider when dealing with membrane permeability is a simple question of surface area. Whatever process is occurring (osmosis, facilitated diffusion, or active transport), it will be limited by the area of the membrane which is available to penetration.

In which of the following membrane-bound figures of the same total volume would you expect the most rapid osmosis? Why?



Check ans. 10

14

Summary of Transport in Living Systems

When you are concerned with membrane transport, you must then consider these three factors:

1. The *differential permeability* of the membrane determines which substances can pass through and which are excluded. This may vary with the physiological condition of the membrane, from cell to cell, and from organism to organism.
2. The *force* which drives the substance—osmotic (chemical concentration and electrical gradients), facilitated diffusion, or active transport.
3. The *surface area* of exposed membrane which is available for penetration.

Little can be inferred unless all factors are accounted for. In next week's laboratory, you will examine more closely some of the ways in which membrane permeability phenomena can be studied. Be sure that you see just how each experimental observation provides information. As you do the exercises in the laboratory, try to think of alternative questions which might be asked or procedures which might be followed.

Food for Thought

You and a friend are broiling steak for dinner. Your friend salts his (her) steak and pops it into the broiler. After browning it on both sides, he (she) broils it to medium rare.

You brown your steak on both sides, then salt and broil it to medium rare.

Can you predict a difference in the juiciness of the two steaks?

[If unsure, check Frame 10.]

Tutorial A—Summary

Inanimate Systems

1. *Diffusion* is the movement of molecules from a region of higher concentration to one of lower concentration, brought about by the random motion of the molecules.
2. *Osmosis* is primarily the diffusion of water across a differentially permeable membrane.
3. *Osmotic pressure* is the pressure in a solution which exists because of the tendency of water molecules to move into that solution in response to an osmotic gradient.
4. Solutions may be described in terms of their osmotic relationship with other solutions:

   Hypo-osmotic is a term to describe a situation where the osmotic pressure outside of the membrane is less than the osmotic pressure inside of the membrane, i.e., there is a greater concentration of H_2O molecules outside than inside.

   Hyper-osmotic is a term to describe a situation where the external osmotic pressure is greater than the internal osmotic pressure, i.e., there is a greater concentration of H_2O molecules inside than on the outside.

   Iso-osmotic is a term to describe a situation where the osmotic pressure of the internal and external solutions are identical.

Living Systems—Three Factors Influence Transport of Materials Across a Membrane

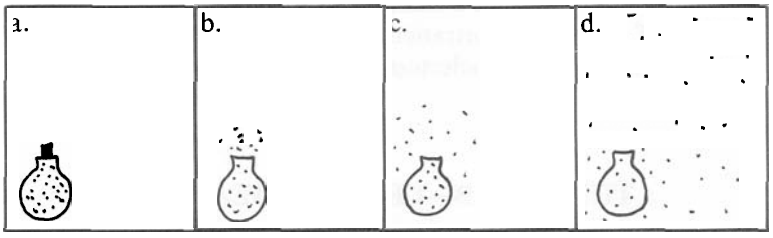
1. Permeability of the membrane is dependent upon membrane structure (pores, lipid composition, charge distribution).

   Tonicity is a term used to describe the behavior of a cell in a given solution.

   a. hypotonic—cell swells
   b. hypertonic—cell shrinks
   c. isotonic—no change in cell volume
2. Forces acting on the substance:
   a. osmosis—movement of water molecules based upon the initial number of particles in the solution and in accordance with concentration gradients.
   b. facilitated diffusion—movement in accordance with concentration gradients but more rapid.
   c. active transport—movement against concentration and electrochemical gradients. Requires metabolic energy.
3. Surface area—rate of penetration dependent upon surface area of the membrane through which penetration occurs.

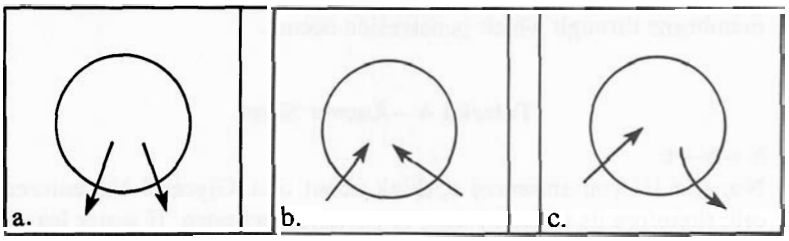
Tutorial A—Answer Sheet

1. a→b→c
2. No; a, d (If you answered e, think about this. Glycerol has entered the cell; therefore its concentration of particles has risen. If water leaves the cell, its concentration of particles will go even higher.)
3. Diffusion



4. a. hyper-osmotic b. hypo-osmotic
   c. iso-osmotic (remember—2X as many particles in NaCl)
   d. hyper-osmotic e. hyper-osmotic
5. a. simple osmosis b. active transport
   c. simple osmosis (Glycerol is leaving the cell in response to a concentration gradient and water follows. See Frame 10 again if you need help.)
   d. An equilibrium state would be reached faster through facilitated diffusion.
6. a. Cell shrinks in a hypertonic solution because water leaves.
   b. Cell swells in a hypotonic solution because water enters.
   c. Cell remains the same in an isotonic solution since the osmotic pressure of the internal and external solutions are identical. No net movement of water.

7. a, c
8. a. surrounding medium, H_2O will move out of the cell.
   b. cell, H_2O will move into the cell (If wrong, see Frame 6.)
9. a. hypotonic (cell swells); iso-osmotic (original concentration of H_2O **molecules the same inside as outside).**
   b. **hypotonic (cell swells); hypo-osmotic** (original concentration of H_2O **molecules greater outside than inside).**
   c. **hypertonic (cell shrinks); iso-osmotic** (original concentration of H_2O molecules the same inside as outside).
10. c, more surface area in c than in a or b.
11. Osmosis occurs in a, b, c. However, there is no NET movement in c.



Explanation. H_2O molecules constantly move in both directions through a differentially permeable membrane. NET movement will always be from an area of greater concentration of water molecules to an area of lesser concentration of water molecules, i.e., along a concentration gradient.

12. from b to a.

TUTORIAL B—CELL PERMEABILITY

Experimental Section

This tutorial will act as a guide during this laboratory. You will learn a technique for measuring permeability phenomena, and you will use this technique to examine some of the variables which affect membrane permeability.

Turn on the spectrophotometer (left front knob).

1

The Red Blood Cell

If we are interested in studying membrane properties which affect permeability, we need an easily obtainable, functional, membrane-bound system which will allow us to readily determine microscopic events involving the transport of materials through the membrane. The red blood cell (erythrocyte) is ideal for such studies.

The mammalian red blood cell (RBC) is a membrane-bound, circular, biconcave disc which at maturity has no nucleus. (See Fig. 1.)

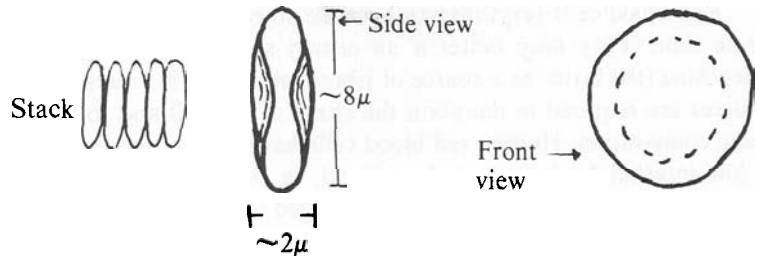


Figure 1

The disc shape seems to be a good arrangement for permitting rapid gas exchange to support the O_2 and CO_2 carrying capacity of the blood.

▶ Why would this shape be superior to a simple sphere?

Check ans. 8

2

A single membrane 75–80 A in thickness (a lipid and protein structure) surrounds the red blood cell. The cell interior is largely composed of hemoglobin,* which accounts for 34% of the total weight and occupies about 25% of the cell volume. Hemoglobin is the chief oxygen carrier in the organism. Red blood cells have no respiratory system (mitochondria). The only energy generating systems they possess are glycolysis and the oxidative pentose pathway. You will learn about both of these systems later in lecture.

▶ How might the lack of a nucleus and aerobic respiratory system (mitochondria) be an advantage to such a cell?

Check ans. 5

*Hemoglobin is a protein (globin) combined with a porphyrin ring structure (heme) containing iron. The heme structure of hemoglobin is similar to the magnesium containing porphyrin group of chlorophyll.

3

Red blood cells (erythrocytes) can be stored in the refrigerator for some time. They keep better if an energy source such as glucose or adenosine (the latter as a source of ribose, not ATP) is present. Energy sources are required to maintain the shape of the cell and to maintain ionic composition. Human red blood cells have a high internal K^+ and a low internal Na^+ content. In general, in the absence of an energy source, red cells are impermeable to charged ions.

Small non-electrolytes (uncharged molecules) and lipid-soluble molecules tend to penetrate into the cells (e.g., molecules like urea and glycerol). Larger molecules either do not enter or permeate slowly.

Should red blood cells be able to carry on active transport? (Hint: Recall Tutorial A, Frame 12.)

Check ans. 10

4

Normal whole blood consists of enormous numbers of these erythrocytes suspended in a solution called PLASMA. The density of these cells is greater than plasma, and they will settle to the bottom of an undisturbed beaker of blood. Thus, the cells can be easily isolated from the plasma.

If isolated erythrocytes are suspended in distilled water, they will swell very rapidly. Why?

See next Frame.

5

Hemolysis

The distilled water is hypotonic to the contents of the erythrocytes. The cell membrane is permeable to water, and the water flows into the erythrocytes causing them to swell. (See Tutorial A, Frame 9 if you would like a review of tonicity.)

As the water continues to flow into the erythrocytes and the membrane is stretched, "holes" develop in the membrane which allow the cell contents including hemoglobin to leak out. HEMOLYSIS, the liberation of hemoglobin into the surrounding solution, is the term used for this process. Hemolysis is not a true lysis since the cell membranes are NOT lysed or broken during the process. You will observe this microscopically in Frame 6.

Macroscopically you can detect hemolysis easily. A normal SUSPENSION of erythrocytes or RBC appears opaque. When hemolysis occurs, the original suspension suddenly become transparent.

Why do you suppose that the hemolyzed suspension is transparent and the non-hemolyzed suspension is opaque?

Check ans. 2

The length of time it takes for hemolysis to occur gives us an easily observable measurement of the rate at which substances enter the cell.

If hemolysis results from an influx of water only, how can it be used to measure the time taken for solutes to enter the cell? (If you need a hint, go to Tutorial A, Frame 9.)

Check ans. 1

6

ACTIVITY FOR ALL STUDENTS: *Microscopic Observation of Normal, Hemolyzed, and Lysed RBC.*

1. With a pasteur pipette place a small drop of beef blood on a microscope slide and smear the blood with a cover slip to spread it out evenly. (See Fig. 2.) Place a cover slip on this smear.

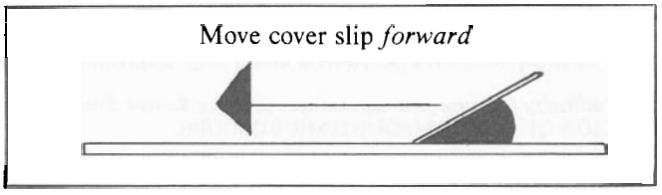


Figure 2

2. Position the slide on the stage of the microscope under the low power (10X) objective and focus on the red blood cells.* At 100X magnification you will barely be able to distinguish individual erythrocytes.

   Now switch to the 45X objective.

3. *Normal RBC.* Compare and contrast the RBC you see with those shown in Fig. 1.

4. *Hemolysis* is the liberation of hemoglobin from the RBC. Try adding a drop of water to an edge of the cover slip (without lifting the cover slip). If your side has become dry, prepare a fresh one.

   To help distinguish the cells, add one drop of methylene blue to the same edge of the cover slip (again without lifting the cover slip). You can help draw the liquid through the blood smear by touching a Kimwipe to the opposite side of the cover slip.

   Watch what happens to the shape of the cells over the next few minutes. By looking closely, you should be able to see the collapsed cell membranes or "ghosts".

▶ Sketch some of the normal RBCs.

▶ Sketch some of the hemolyzed RBCs.

Check ans. 7

5. *Lysis* is the partial breakage or total dissolution of the cell membrane. A high concentration of the nonionic synthetic detergent, Triton X-100, or of various household detergents will drastically disrupt the molecular configuration of the membrane, which then irreversibly fragments releasing the cell contents.

   Prepare a side with a fresh drop of blood (steps 1 & 2) and a drop of 10% Triton X-100. MIX the drops THOROUGHLY with the tip of a pipette, and position a cover slip appropriately.

*If you have difficulty focusing your microscope, use your Review Sheet from Tutorial B—OPERATION OF THE COMPOUND MICROSCOPE.

Focus the slide at low power; switch to high power. What has happened to the RBC? Perhaps methylene blue will help you distinguish what you see.

Is hemolysis the same as lysis of the cell membrane? Explain.

Check ans. 4

7

Slit-Lamp Technique

As stated previously, we would like to have a membrane system with which we can readily determine events dealing with the transport of materials through the cell membrane. The phenomenon of hemolysis in red blood cells, which can be timed accurately, allows us to make such determinations. First, let us become familiar with the slit-lamp technique for measuring hemolysis.

When hemolysis occurs, the suspension of red blood cells turns from opaque (turbid) to transparent (clear).

ACTIVITY FOR ALL STUDENTS: *Determining Hemolysis by the Slit-Lamp Technique* [WORK IN PAIRS]

1. The "slit" is merely a piece of cardboard in which a hole has been cut. A black thread is suspended in the opening. Place the cardboard in front of a light source. (See Fig. 3.) If the light intensity of the lamp is too high, place a Kimwipe or something similar across the slit to reduce the illumination. In determining hemolysis time, you will hold a test tube of blood mixed with the appropriate solution next to the slit and look for the appearance of the black thread. At the very first instant that you can barely see the thread, roughly 75% of the cells are hemolyzed. This will happen *very quickly* in distilled water. The most accurate readings may be obtained by timing the rates of permeability on a moving stopwatch. Have one student look at the stopwatch while the other, holding the solution, calls out the end point.

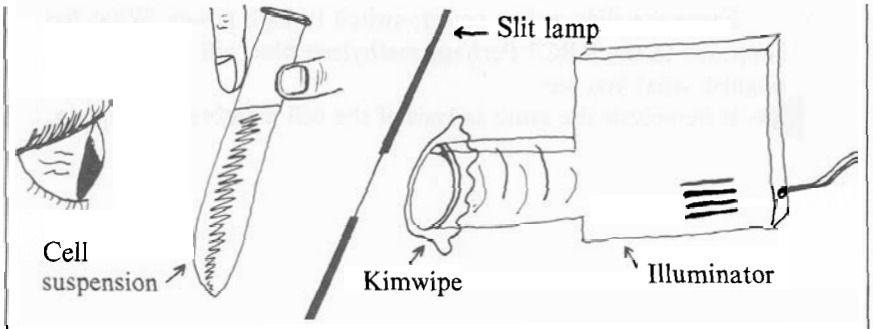


Figure 3

2. Rinse out the test tube with water and shake it to remove excess water. You will need a COMPLETELY DRY test tube. This is facilitated by rinsing the test tube again, this time with acetone. Shake it dry to remove all droplets.
3. Next deliver 2 drops of blood with a *pasteur pipette* to the bottom of the test tube. To insure reproducibility, hold the pipette in the same position each time you allow the drops to fall into the test tube.
4. Place the test tube which contains the 2 drops of blood in front of the slit, as shown in Fig. 3. While one person holds the test tube, the other person should squirt 5.0 ml of a test solution into it. (Blow the solution from the *graduated pipette.*) This will mix the solution. Start the stopwatch immediately, timing the reaction until the first instant you can see the black thread.
5. Reproducible observations will be obtained from repeated trials, if the procedure used is reliable. Practice this technique until you have mastered it. Many trials may be required to gain reproducible results. Are the time values you recorded fairly close?

   The same results should be obtained using the same procedure, whether the experiment is done by yourself or by another person. Compare your results with those of another pair of students.

8

Now, let us use this technique to attempt to answer some of the questions which came to mind in studying membrane permeability. The procedure we shall follow here is one which scientists usually use in attacking a problem. First, we have observed the phenomenon, in this case, that some things can enter the cell more easily than others. Now, we shall attempt to explain this observation. We shall try to isolate some of the relevant variables and deal with each one independently. Each

experiment is based upon a hypothesis. "If this is true, then it follows that the following should occur." Each experiment is designed in such a way that:

1. It accounts for all the relevant variables.
2. It is reproducible by any other scientist, who will obtain the same results by following the same procedure.
3. It tests the hypothesis proposed.

9

EXPERIMENTAL QUESTION 1: What is the influence of molecular size on permeability?

HYPOTHESIS 1: If the membrane is composed of many small pores, then the size of a molecule alone would be an important factor in ease of permeability.

If we want to investigate only the effects of molecular size, then we must make sure that the diffusing molecules which we choose do not have any of the following characteristics. (Choose 1 or more.)

a. Lipid (fat) soluble.
b. Different molecular weights.
c. Charged groups present in molecule.

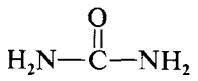
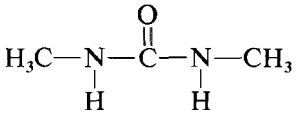
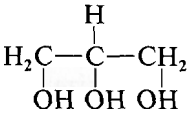
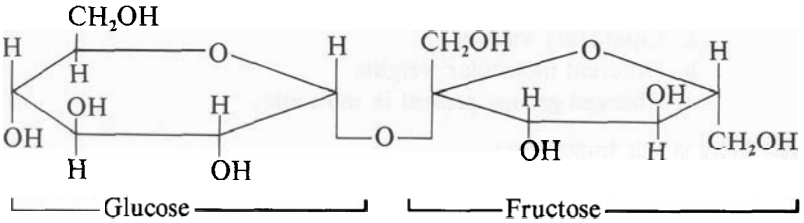
Why is this important?

Check ans. 6

EXPERIMENT 1:

Procedure: To narrow the problem down, we have selected a series of compounds, all of which are only slightly soluble in ether (a lipid solvent), are relatively inert as far as the membrane is concerned, but do have different molecular sizes.

1. Make 3 determinations of the hemolysis time in distilled water.
2. Make 3 determinations of the hemolysis time in 0.3 M solutions of the following compounds, listed on page 5:

Compounds	*Molecular Weight*	*Molecular Structure*
Urea	60	
Dimethyl urea	88	
Glycerol	92	
Sucrose	342	

Data presentation:

Hemolysis time

	1	2	3	Average
Distilled water				
Urea				
Dimethyl urea				
Glycerol				
Sucrose				

Discussion:

▶ Do your data indicate a relationship between molecular size and permeability?

▶ Does the relationship seem to be direct?
(i.e., if you double one factor, is the other factor doubled?)

▶ What other factors might be involved?

10

EXPERIMENTAL QUESTION 2: Do individual cell membranes of the same type from a single organism all have the same properties?

HYPOTHESIS 2: If there is a difference in membrane permeability among cells in a given cell population, then they will not all hemolyze in a given hypotonic solution.

We can suspend the cells in hypotonic solutions of different osmotic pressures and determine the percentage of cells which have hemolyzed. The percentage of hemolyzed cells may be estimated by measuring the amount of hemoglobin released into the solution relative to the total hemoglobin present in all of the cells.

EXPERIMENT 2:

Procedure:

1. We can determine the concentration of hemolyzed cells by using a Bausch and Lomb spectrophotometer to measure the amount of red pigment (hemoglobin) released. Before you proceed with this exercise, learn the operation of the spectrophotometer using the programmed guide entitled, THE SPECTROPHOTOMETER.
2. Using a felt-tipped marker pen, number six dry test tubes *1/2" below the rim.*
3. Fill the number 1 test tube with water. This will be your water blank to be used when setting the spectrophotometer at 100% transmittance (0 absorbance).
4. Drop 2 drops of blood in each of the other tubes (numbers 2–6) as you did before, making sure the drops are the same size.

Into each test tube squirt 5.0 ml of the appropriate solution—H_2O, 0.05 M NaCl, 0.07 M NaCl, 0.08 M NaCl, 0.1 M NaCl.

5. Place the tubes in the clinical centrifuge—check to see that the tubes are balanced opposite each other. Turn the centrifuge speed up slowly and *centrifuge 3 minutes at top speed.* Then turn the centrifuge down and let it *come to rest.* DO NOT SHAKE THE TUBE AFTER CENTRIFUGATION. The hemolyzed and non-hemolyzed cells will have been centrifuged to the bottom. The liquid above the cells (supernatant fraction) will have a faint red color.
6. Readings on the spectrophotometer will be made on the lower scale of the meter which is *Absorbance.* After the spectrophotometer has warmed up for several minutes, set the wavelength at 540 mμ. Using the left knob, adjust the indicator needle until it points to infinite (∞) absorbance. Place tube #1 (distilled H_2O blank) in the same chamber and replace the lid. Using the right knob, adjust the indicator needle to read zero (0) absorbance. Put tube #2 (complete hemolysis) into the holder and record the absorbance. Next record the absorbance of the first unknown. Repeat the two zeroing steps (left knob to ∞, right knob to zero with blank) after each unknown to help keep the machine from wandering.

Data Presentation:

Tube	Solution	Absorbance	% Hemolysis
1	Distilled water (blank)		
2	Distilled water		
3	0.05 M NaCl		
4	0.07 M NaCl		
5	0.08 M NaCl		
5	0.1 M NaCl		

7. Calculation: $\frac{\text{Absorbance unknown}}{\text{Absorbance tube \#2 (completely hemolyzed)}} \times 100 = \%$ hemolysis

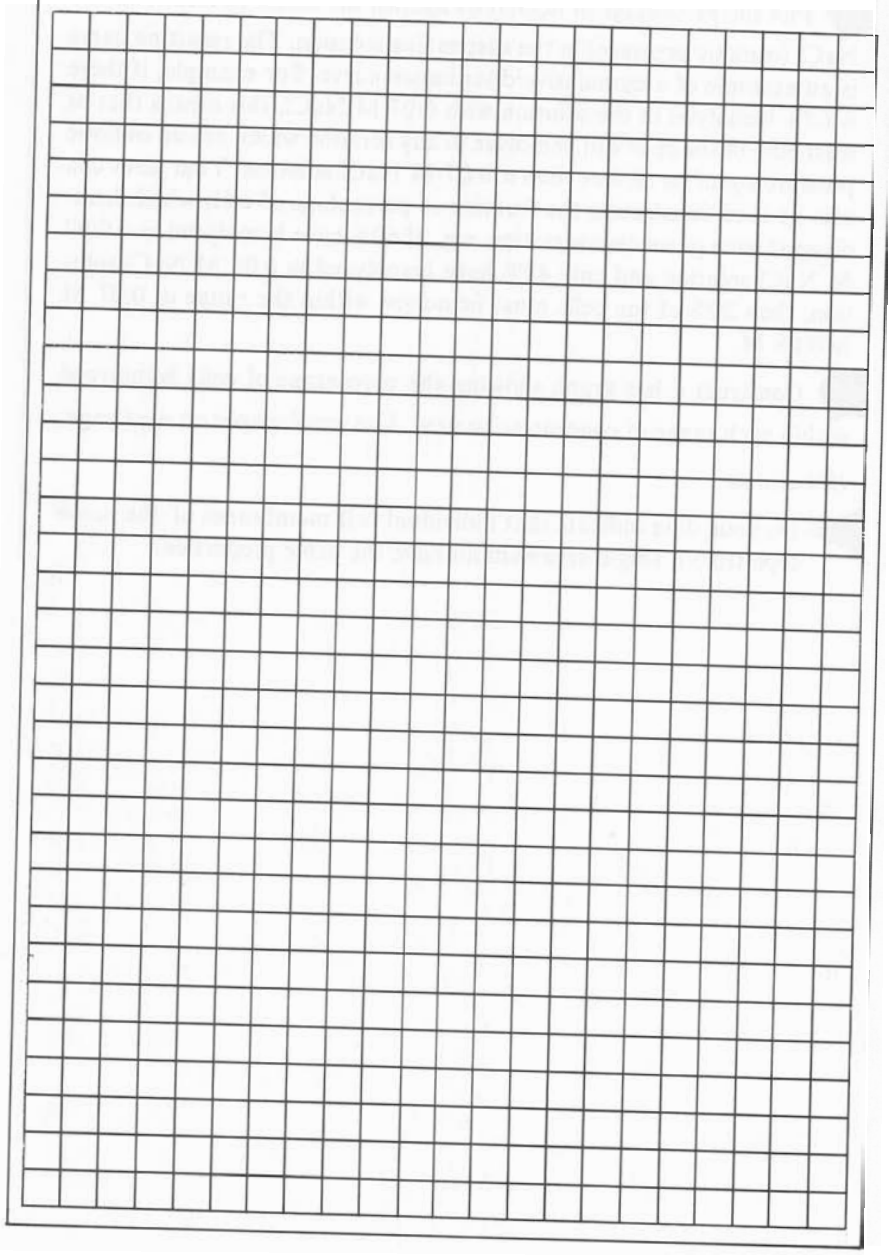
Plot the percentage of hemolysis against the molar concentration of NaCl (osmotic pressure) in the suspending solution. The resulting curve is an example of a cumulative distribution curve. For example, if there is 60% hemolysis in the solution with 0.07 M NaCl, this means that at least 60% of the cells will hemolyze in any solution which has an osmotic pressure *equal to or less than* a 0.07 M NaCl solution. Your data can also be used to estimate the fraction or percentage of cells which hemolyze at each given concentration, e.g., if 60% have hemolyzed in a 0.07 M NaCl solution and only 40% have hemolyzed in 0.08 M NaCl solution, then 20% of the cells must hemolyze within the range of 0.07 M to 0.08 M.

Construct a bar graph showing the percentage of cells hemolyzed within each range of concentration used. Use graph paper on next page.

Discussion.

Do your data indicate that individual cell membranes of the same type from a single organism all have the same properties?

What other factors might account for the observed results?



STOP!

Now that you have some idea of how rates of hemolysis can be used to study membrane permeability phenomena, you can choose what you wish to do for the rest of this laboratory.

1. You may continue and follow experimental question 3—What is the relationship between the size of the osmotic gradient and the rate of H_2O penetration?—and/or experimental question 4—What is the influence of lipid solubility upon permeability?
2. You may return to experiments 1 and 2 and try to improve the reliability of your results or to investigate anomalous results.
3. You may formulate your own experimental question and hypothesis. Using one of the techniques you learned today and any of the equipment and chemicals available for the four experiments (plus glucose and other types of blood cells), you may conduct an experiment of your own design.

11

EXPERIMENTAL QUESTION 3: What is the relationship between the size of the osmotic gradient* and the rate of water penetration?

HYPOTHESIS 3: Because biological membranes respond to osmotic phenomena, the number of particles in the solution will influence the rate at which water enters the cell.

The water flows in response to osmotic gradients which we can control by making up various concentrations of NaCl. Cell membranes are not readily permeable to NaCl. By suspending the cells in solutions of different NaCl concentrations and measuring the rate of swelling (e.g., by the time required for hemolysis), we should be able to get a rough idea of the relationship between the size of the osmotic gradient and the rate of water penetration.

▶ If we suspend erythrocytes in a solution of hypotonic NaCl, in which direction will the water flow? What about the NaCl?

Check ans. 3

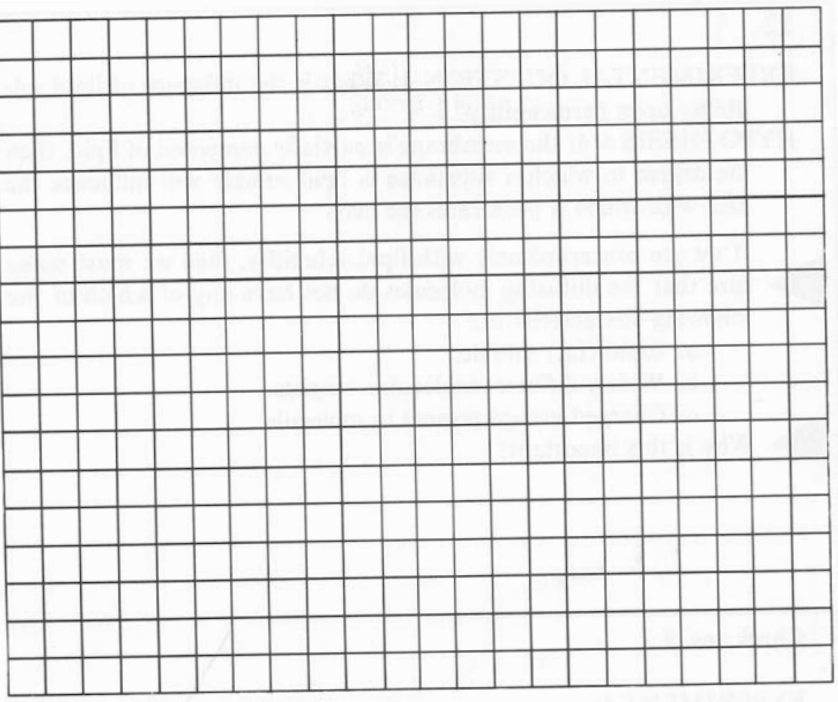
*Here gradient refers only to a difference in concentration.

EXPERIMENT 3:

Procedure:

1. Make 3 determinations of the hemolysis time in water.
2. Make 3 determinations of the hemolysis time in each of the four graded concentrations of NaCl (0.05 M–0.1 M).

	Hemolysis Time			
	1	2	3	average
Distilled H_2O				
0.05 M NaCl				
0.07 M NaCl				
0.08 M NaCl				
0.1 M NaCl				



Data Presentation: Calculate the average reading for each solution and plot the hemolysis time against the concentration.

Discussion:

Do your data indicate a relationship between the size of the osmotic gradient and the rate of water penetration? Is the relationship direct?

What other factors besides simple osmotic gradients may be involved?

12

EXPERIMENTAL QUESTION 4: What is the influence of lipid solubility upon permeability?

HYPOTHESIS 4: If the membrane is partially composed of lipid, then the degree to which a substance is lipid soluble will influence the ease with which it penetrates the cell.

If we are concerned only with lipid solubility, then we must make sure that the diffusing molecules do not have any of which of the following characteristics?

a. Lipid (fat) soluble.
b. Widely different molecular weights.
c. Charged groups present in molecule.

Why is this important?

Check ans. 9

EXPERIMENT 4:

Procedure: **To** narrow the problem down, we have chosen a few compounds. These are roughly the same size but have different solubilities in lipids. The ratio of the solubility of a compound in oil to its solubility in water is called its partition coefficient. Use the following compounds to find the correlation between permeability and lipid solubility by measuring hemolysis times.

Compound	*Partition Coefficient*
Methyl alcohol	.0097
Ethyl alcohol	.0357
Propyl alcohol	.156

Which of these three compounds is most lipid soluble?

Check ans. 11

	1	2	3	Average
Methyl alcohol				
Ethyl alcohol				
Propyl alcohol				

Discussion:

Do your data indicate a relation between permeability and lipid solubility?

Does the relationship seem direct?

What other factors may be involved?

Tutorial B—Summary

1. The mammalian red blood cell (erythrocyte) is a membrane bound, biconcave disc which at maturity has no nucleus. It has no mitochondria and generates energy through glycolysis and the oxidative pentose pathway. Hemoglobin is the chief component of the cell.

ACTIVITY: 1. Observation of hemolysis is made under the microscope. Comparison is drawn between hemolysis and lysis. (RBCs lyse in 10% Triton, a detergent.)

2. Determination of hemolysis is made using the slit-lamp procedure. This procedure is practiced until students feel confident that they can take the readings accurately and quickly.

2. The erythrocyte is ideal for use in membrane permeability studies because:
   a. it is easily obtained and isolated.
   b. it is a functioning, membrane-bound system.
   c. it undergoes hemolysis, a phenomenon which can be used to indicate rate of solute penetration.
3. Hemolysis occurs when the erythrocyte swells due to the influx of water. After the membrane swells to a certain critical point, it allows the hemoglobin to escape into the solution because "holes" have developed in the membrane. This causes the solution to go from opaque → transparent. The time required for this to occur can be measured.
4. The slit-lamp technique of determining hemolysis is used to answer a series of experimental questions:
   a. EXPERIMENTAL QUESTION 1: What is the influence of molecular size on permeability?

      HYPOTHESIS 1: If the membrane is composed of many small pores, then the molecular size alone will be an important factor in ease of permeability.

      EXPERIMENT 1: Measure hemolysis time using four different solutes, their molecular weights range from 60 to 342.

   b. EXPERIMENTAL QUESTION 2: Do individual cell membranes of the same type from a single organism all have the same properties?

      HYPOTHESIS 2: If there is a difference in membrane permeability among cells in a given cell population, then they will not all hemolyze in a given hypotonic solution.

      EXPERIMENT 2: Measure actual percentage hemolysis using the spectrophotometer in a graded NaCl series. Plot NaCl concentration vs. percentage hemolysis. Construct a bar graph showing the percentage of cells hemolyzed within each range of concentration.

   c. EXPERIMENTAL QUESTION 3: What is the relationship between the size of the osmotic gradient and the rate of water penetration?

      HYPOTHESIS 3: Because biological membranes respond to osmotic phenomena, the number of particles in the solution will affect the rate at which water enters the cell.

      EXPERIMENT 3: Measure hemolysis time in a graded series of NaCl solutions. Plot NaCl gradient vs. hemolysis time.

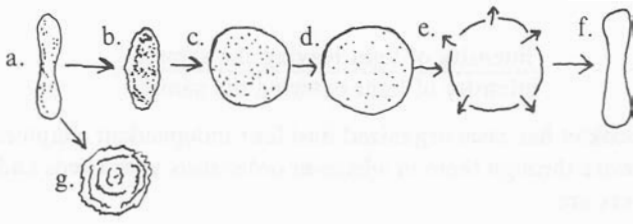
   d. QUESTION 4: What is the influence of lipid solubility upon permeability?

      HYPOTHESIS 4: If the membrane is partially composed of lipid, then the degree to which a substance is lipid soluble will influence the ease with which it enters the cell.

EXPERIMENT 4: Measure hemolysis time using three compounds with different partition coefficients.

Tutorial B—Answer Sheet

1. Water enters the cell in response to increased osmotic pressure from solutes entering the cell, but it penetrates more readily than most solutes. The rate at which water enters reflects the rate at which solutes enter.
2. The hemoglobin is packed tightly within the erythrocytes. These cells are suspended in the plasma, which makes the blood opaque. When the hemoglobin is released, it dissolves in the plasma. The membranous ghosts are empty and the blood appears transparent.
3. The water will flow into the cells. The NaCl will not enter the cell.
4. No. The cell membranes are not broken during hemolysis—the intact membranes (ghosts) remain. During lysis (such as is caused by detergents) the membrane disintegrates.
5. A nucleus and numerous mitochondria would take up considerable space in a cell. Since the sole function of the red blood cell is transport, the most efficient system is one which provides the maximum space for hemoglobin. Also, the absence of mitochondria eliminates the need for large amounts of oxygen in metabolism, thus making the bound oxygen available for release at the proper destination.
6. a, c [Both factors might affect permeability, and you must be careful to isolate only one variable at a time.]
7. Hemolysis:



a. original discoid cell; b. the concavities become convex; c. when the volume has increased by 30%, the cell is a "rough sphere"; d. the cell continues to swell as a sphere; e. when the volume is increased by about 100% and the surface (area) is the same as in a, hemoglobin leaves the cell; f. the resulting ghost may revert to the discoid shape; g. hemolysis without swelling results in a rough sphere with much reduced surface area.

8. The biconcave disc shape provides more surface area for diffusion of gases than would be possible with a simple sphere. There is a greater surface area/volume ratio in a biconcave disc than in a simple sphere.

9. b, c [Both factors might affect permeability, and you must be careful to isolate only one variable at a time.]
10. Yes. They still have an energy producing system (glycolysis and the oxidative pentose pathway) which can provide energy for active transport.
11. Propyl alcohol.

THE SPECTROPHOTOMETER

This booklet is an introduction to an instrument you will use in the lab during the next week. Please read it at home and bring it to the laboratory.

A spectrophotometer is an instrument that measures the amount of light of a selected wavelength that passes through colored or turbid (cloudy) solutions. The spectrophotometer is usually used to achieve one of the following goals.

1. Determining the concentration of a solution. This is possible because concentration is related to the amount of light absorbed or scattered by the solution.
2. Determining the identity of unknown substance(s) in the solution by observing which wavelengths of light are absorbed by the solution.

The principle of spectrophotometry is briefly as follows. You select light of a certain wavelength, and the spectrophotometer sends it through the sample solution. Substances in the sample absorb light, scatter light, or both, so that less light leaves the sample than entered it. The spectrophotometer detects the outgoing, or transmitted light and displays on a meter the value of the ratio:

$$\frac{\text{intensity of light leaving the sample}}{\text{intensity of light entering the sample}}$$

This booklet has been organized into four independent chapters. You are invited to work through them in whatever order suits your needs and interests. The chapters are:

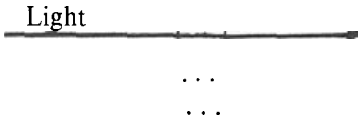
Summary of operating procedure 116

Summary of operating procedure 116

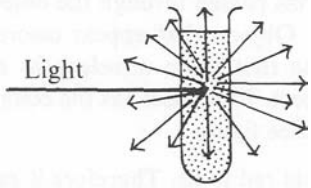
A. Interactions between Light and Matter: Transmission, Scattering, Absorption

What might happen when we put a solution in the path of a light beam? Among the possible outcomes are the following.

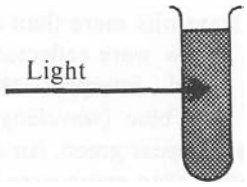
1. *Transmission.* Light passes unchanged through the solution.



2. *Scattering.* Light encounters particles of matter that scatter it in all directions.



3. *Absorption.* Light encounters particles of matter and is absorbed.* Not all wavelengths** of light are absorbed by a given substance, but rather each substance has a unique pattern of wavelengths that it absorbs. (This pattern is responsible for the color of a given substance. See the optional section on color if you are interested in it.



Footnotes: read only if interested.

* Details of absorption. Electrons in the matter absorb the light, which is a form of energy, thus jumping to a higher energy state. A very short time later the electron gives off the energy it had absorbed and falls back to its original state. However, the energy is given off, not in the form of light, but rather as thermal, or heat energy (the molecules of the absorbing material begin to move faster.)

** Wavelength is a property of light that we interpret as color, e.g., light having a wavelength of about 500–550 millimicrons or nanometers (nm) appears green to us, and light having a wavelength of about 550–570 nm appears yellow. White light, such as sunlight or light from an incandescent bulb is a mixture of all wavelengths.

Any one or any combination of these three processes can occur when a solution interacts with matter.

▶ Which process or processes reduce the amount of light that passes through the solution? _____

Check ans. 4

If you want to use the spectrophotometer to study a substance that only transmits light, it is often possible to add reagents that convert the clear, colorless substance to a colored solution, which will absorb light, or to a precipitate, which will scatter light.

Optional Section: Color

We see objects by perceiving light that has been reflected (a special form of scattering) or has passed through the object (been transmitted) on its way to our eyes. Objects that appear colored have absorbed one or more wavelengths so that these wavelengths are missing from the light that leaves the object. The object has the color of the wavelength(s) that are *not* absorbed. See figure 1.

▶ Hemoglobin appears red to us. Therefore it probably

_____ absorbs red light.

_____ does not absorb red light.

Check ans. 3

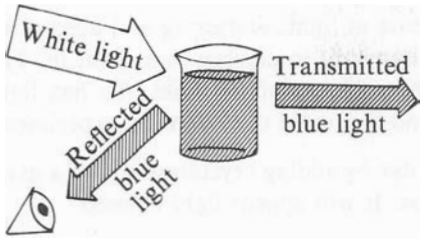
The subject of color becomes a little more complicated when the colored object reflects or transmits more than a narrow range of wavelengths. For example, if yellow were reflected and transmitted along with blue, our eyes would interpret the mixture of yellow (wavelength = 560 nm) and blue (wavelength = 470 nm) light as green, and the object would appear green. An object would also appear green if all wavelengths other than green were absorbed and pure green light (wavelength = 520 nm) were reflected or transmitted to our eyes.

▶ Consider a suspension of bacteria that does not appear to be any particular color (i.e. is white) but does appear slightly cloudy when held up to a source of light. What interaction(s) is/are occurring between light and the suspension? (Review page 101, if you need to.)

_____

Check ans. 1

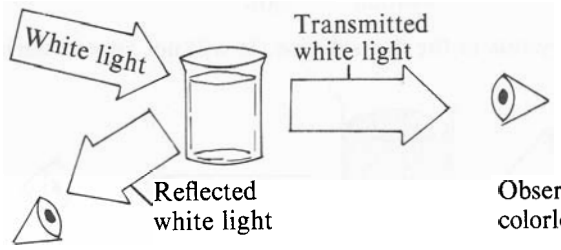
Blue solution: absorbs all wavelengths but blue



Observer sees
blue solution

Observer sees
blue solution

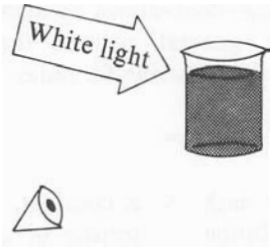
Colorless solution: absorbs no wavelengths



Observer sees
colorless solution

Observer sees
colorless solution

Black solution: absorbs all wavelengths



Observer sees
black solution

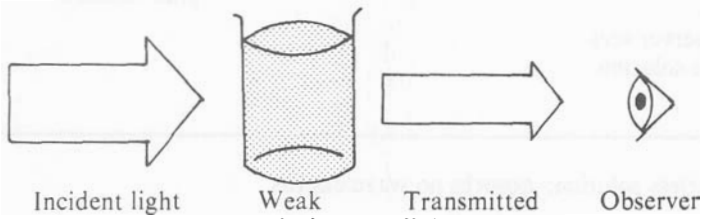
Observer sees
black solution

Figure 1

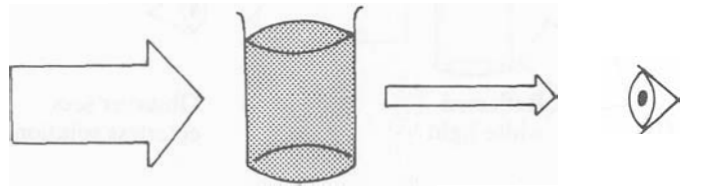
B. Relationship between Concentration and the Amount of Light Passing through the Solution

When matter intercepts a beam of light, scattering and absorption (described in Chapter A) cause less light to emerge than went in. The more matter that is placed in the pathway of the light, the less light that will emerge, as you would find if you did the following experiment.

1. Prepare a weak solution of a dye by adding crystals of dye to a glass of water. Observe the solution. It will appear light colored.



2. Add more crystals to the dye solution. It will not appear darker.



There is an exact quantitative relationship, which holds for most substances, between the concentration of the intercepting solution and the intensity of the light that comes through the solution. This relationship was found experimentally, by making observations on many concentrations and then discovering the general pattern that the observations followed. It is expressed by the following formula:

$$-\log\left(\frac{\text{intensity of light emerging from sample}}{\text{intensity of incident light}}\right) =$$

concentration of the solution (in moles) $\times$ length of light path through the solution (in cm) $\times$ a constant, characteristic of the substance in the soln.

or, more briefly,

$$-\log\left(\frac{I}{I^o}\right) = c\ p\ z.$$

The spectrophotometer measures the quantity called percent transmittance, which is

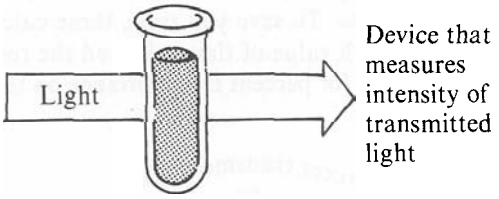
$$\frac{I}{I_o} \times 100\%$$

where I = intensity of light emerging from sample, and
I_o = intensity of incident light.

If you knew the percent transmittance of a sample, could you calculate the concentration of the sample? If not, what else would you need to know?

Check ans. 2

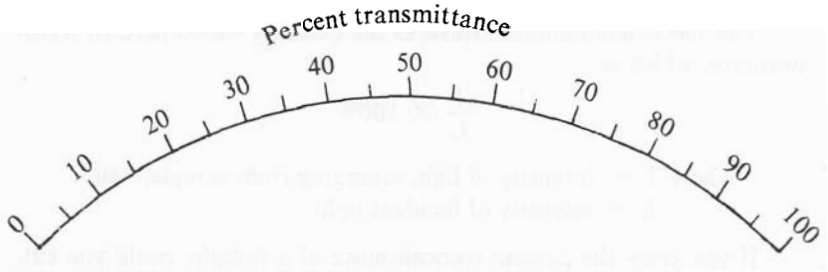
When you make a measurement with a spectrophotometer, you put the sample in a test tube and insert the test tube into a compartment in the instrument:



What physical measurement would give you the value of p in the formula?

Check ans. 5

On the spectrophotometer, values of percent transmittance are marked on a scale, and a needle points to the value it has measured for your sample:



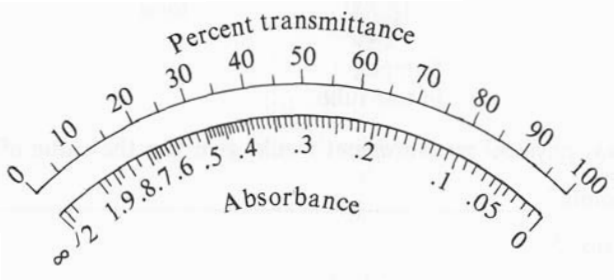
Imagine two solutions of $KMnO_4$, a violet colored compound.
Solutin *A* is .10 molar $KMnO_4$.
Solution *B* is .50 molar $KMnO_4$.
Which solution would have a higher percent transmittance?

Check ans. 6

To plug percent transmittance ($I/I_o \times 100\%$) into the formula

$$-\log\left(\frac{I}{I_o}\right) = c\ p\ z$$

you would have to divide by 100, find the log in a table, and change the sign, all of which is tedious. To save you time, these calculations have already been made for each value of the scale, and the result is printed in red just below the scale for percent transmittance on the spectrophotometer:



These values, $-\log I/I_o$, are given the name absorbance.*

True or false? Absorbance = c p z. _____

Check ans. 8

* Absorbance is also called optical density, or O.D.

The value of z is the final thing you need to know before you can calculate concentration from the formula, Absorbance = c p z.

If you have an idea of how the value of z could be found for a substance, write it here and check it against the description that follows. If not, continue directly with this frame.

One way to find the value of z would be to take some of the substance you are studying and prepare a solution of known concentration, put it in a test tube of known diameter, then measure the absorbance. For the formula

$$\text{Absorbance} = c\ p\ z,$$

you will know the values of absorbance, c and p, so you can calculate the value of z. Now you could measure the absorbances of many different solutions of this substance and, by plugging this value of z into the formula, calculate the concentration of each.

An even simpler procedure for calculating concentration of an unknown involves the same procedure of preparing a solution of known concentration (called the standard) and measuring its absorbance (A). You know that

$A_{sample} = c_{sample} \times$ _____
(fill in the blank)

and that

$A_{standard} =$ _____
(fill in the blank)

If you divide the first formula by the second, what do you have?

Check ans. 7

The values of p and x are the same for the sample and the standard, so they cancel out, giving

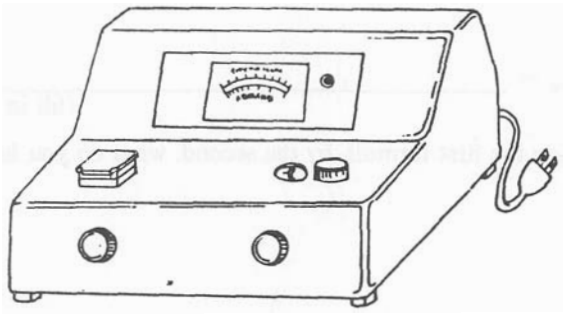
$$\frac{A_{sample}}{A_{standard}} = \frac{C_{sample}}{C_{standard}}.$$

C_{sample} is the only unknown in the above formula. What does C_{sample} equal? (Rearrange the formula to find out.)

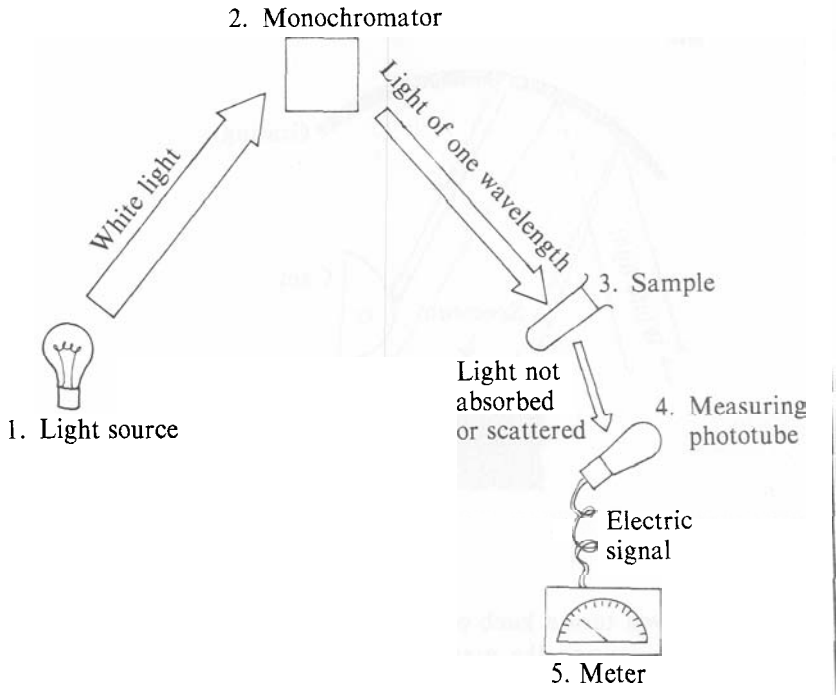
Check ans. 9

C. The Components of the Spectrophotometer and What They Do

The spectrophotometer you will use in Bio 1 is the Bausch and Lomb Spectronic 20. The outside of the spectronic 20 looks like this:



The internal components look like this:



1. Light source. A tungsten lamp that emits white light (a mixture of all wavelengths).
2. Monochromator. A device that isolates light of one* wavelength from the array of wavelengths that are present in white light.

   OPTIONAL SECTION: *How the monochromator works.*

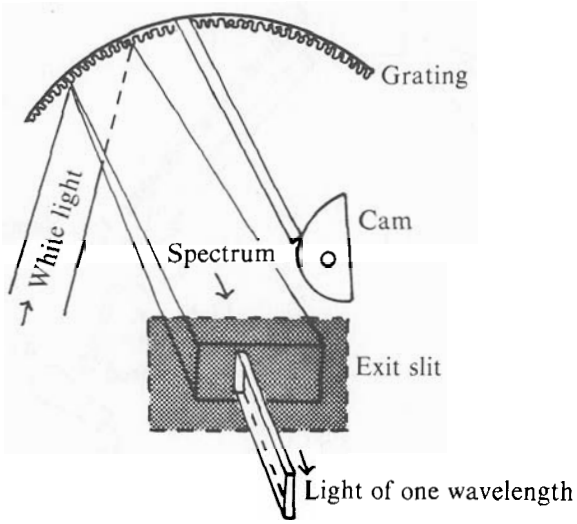
   The monochromator consists of

   a. A dispersing element, which spreads out the white light into its component wavelengths, or in other words, spreads the beam of white light into a spectrum. In some spectrophotometers the dispersing element is a prism; in others it is a grating. The Spectronic 20 employs a reflection grating. This is a mirror in which are engraved many parallel grooves (600 grooves/mm).

   b. An exit slit, which permits only a narrow portion of the spectrum to leave the monochromator.

* Actually the light is not purely of one wavelength. Some contamination by light of adjacent wavelengths occurs.

c. A cam, which moves the grating relative to the exit slit so that any desired part of the spectrum can be made to fall on the exit slit.



As you turn a knob on the Spectronic, the cam rotates on its axis, causing the grating to move. Consequently, a different wavelength band of the spectrum falls on the exit slit and emerges from the monochromator.

END OF OPTIONAL SECTION.

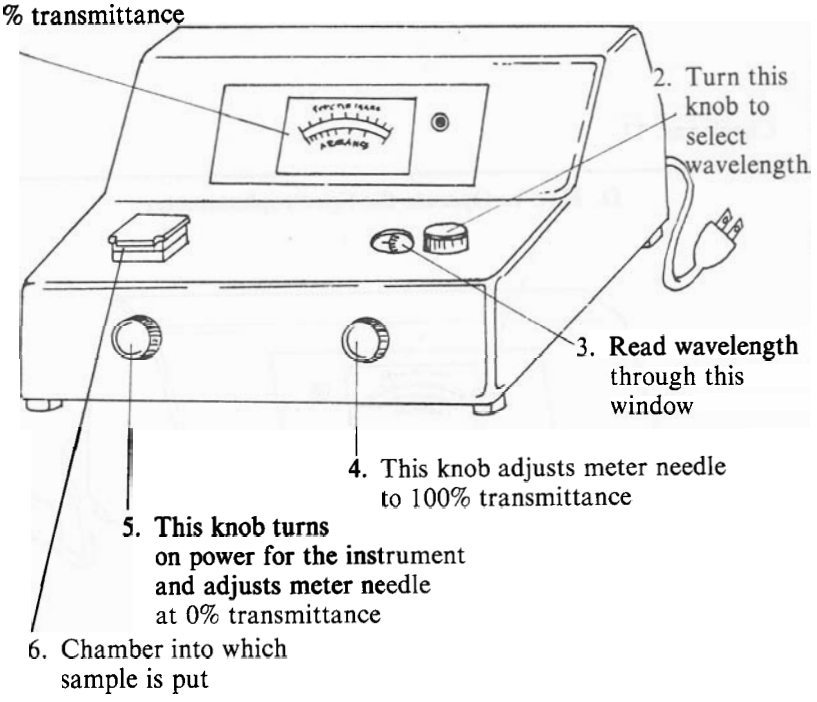
3. Sample of the substance being studied, in a test tube. Light of the selected wavelength falls on the sample (See Chapter A for interactions of light with matter.)
4. Measuring phototube. This is a light-sensitive vacuum tube that converts the energy of light that falls on it to an electric current.
5. Meter, which registers the strength of the electric current developed by the measuring tube and hence the amount of light* that emerged from the sample. (See Chapter B for relationship between concentration of the sample and the amount of light passing through the solution.)

* More precisely, when using the spectrophotometer, the operator adjusts the meter so that it measures the ratio:

$$\frac{\text{intensity of light emerging from the sample}}{\text{intensity of incident light}} \times 100\%.$$

This ratio is called "percent transmittance."

This is what the Spectronic 20 looks like from the outside.



▶ Which external parts are probably connected to the monochromator?

Check ans. 10

By the time the original light beam reaches part 6, it is in the form of _____ .

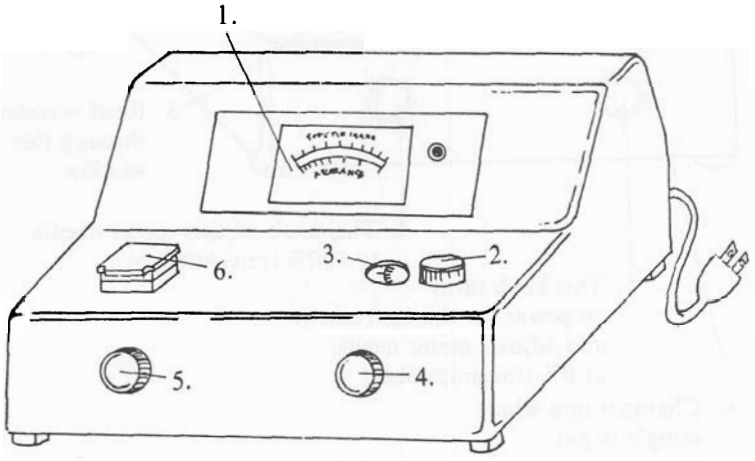
a. white light.
b. light of approximately one wavelength.
c. transmitted light.
d. absorbed light.
e. electricity.

Check ans. 12

What does the phototube do?

Check ans. 11

D. How to Operate the Spectrophotometer



NOTE: This is a detailed list of steps with explanations. A brief summary of steps is found on page 116.

1. Check to make sure the instrument is plugged in.
2. Turn the Spectronic 20 on by twisting the on-off knob (5) clockwise until you hear a click. At this point, the small red light to the right of the meter will go on. Wait at least 30 minutes for the instrument to warm up.
3. Set the desired wavelength by turning knob 2 until the wavelength you want appears next to the indicator in the small window (3).
4. In step 4 we want to adjust the meter needle so that it points to zero percent transmittance when no light is reaching the phototube. (This operation is analogous to setting the marker on a bathroom scales to zero before stepping on the scales to weigh oneself.) When the sample chamber contains no test tube and the lid is closed, a device inside the instrument blocks off the beam of light so that no light reaches the phototube.

Directions: Be sure the sample chamber is empty and the lid to the sample chamber is closed. Turn the on-off knob (5) until the needle on the meter points to "0 percent transmittance."

5. The electric current that reaches the meter is the measure of the intensity of light that passed through the sample. However, to find concentration, we want to know the ratio

$$\frac{\text{intensity of light passing through sample}}{\text{intensity of incident light}}$$

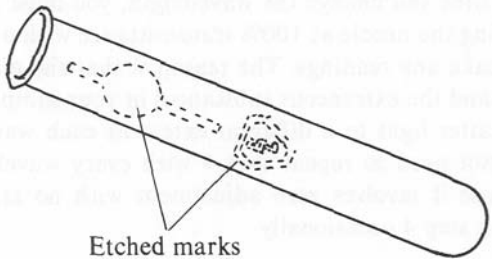
In this step we want to adjust the needle at the other end of the scale so that the meter readings actually give us that ratio. In other words, we want to set the needle at 100% transmittance while the meter is receiving electric current equivalent to the intensity of the incident light.

To arrange this, we prepare a "blank." The blank is a test tube of liquid identical to the sample in every way *except* that it doesn't contain the unknown, the substance whose transmittance we want to measure. For example,

If the sample is	*. . . the blank is*
Hemoglobin in distilled water	Distilled water
Yeast in 10 ml water + 3 ml vitamin solution + .5 grams glucose	10 ml water + 3 ml vitamin solution + .5 grams glucose

The amount of light that emerges from this blank is the amount of light *not* absorbed or scattered by the walls of the test tube or by extraneous substances in the solution. This is the amount of light that is available for the unknown to absorb or scatter, or in other words, the intensity of the incident light.

Directions: Use only Spectronic 20 tubes:



These tubes are of high optical quality, and they have been carefully made so that they are of uniform thickness and diameter. Using only Spectronic 20 tubes reduces the danger that irregularities in test tubes rather than differences in the concentration of the unknown are responsible for different transmittance or absorbance readings. In other words, it is a way to control for gross variability in the container that holds the sample.

Wipe the tube carefully with a Kimwipe before inserting it in the sample chamber (so you won't be measuring the light transmitted through your fingerprints.) Insert the blank into the sample chamber and always *line up the tube with the etched mark* toward you. (This procedure gets the tube in the same position from one reading to the next and insures that the same amount of light is transmitted through the glass tube for each reading.)

Close the lid of the sample chamber and adjust knob 4 until the needle on the meter points to 100% transmittance. Remove the blank from the sample chamber.

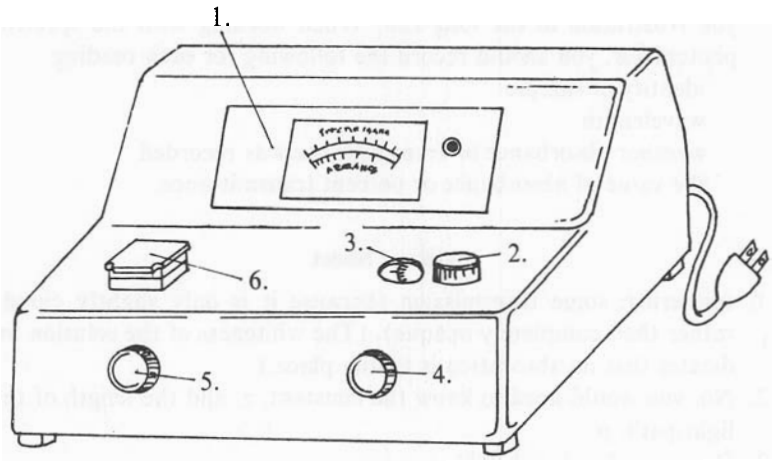
6. Making the reading. Clean the sample tube with a Kimwipe. Insert the sample tube into the sample chamber, rotate the tube until the etched mark is toward you, and close the lid. Read the absorbance or the percent transmittance (whichever you are interested in) off the dial. Note the mirror between the two scales on the meter. You will get the most accurate readings if you close one eye and move your head slightly until the needle appears lined up directly above its reflection in the mirror, then read off the value.
7. Making further readings.
   A. Readings at the same wavelength.
      If all readings are to be made at the same wavelength, simply repeat step 6. Steps 4 and 5 should be repeated from time to time to make sure the meter is not drifting.
   B. Readings at various wavelengths.
      Any time you change the wavelength, you must do step 5 (setting the needle at 100% transmittance with a blank) before you take any readings. The reason is that the glass of the test tube and the extraneous substances in your sample may absorb or scatter light to a different extent at each wavelength. You will not need to repeat step 4 with every wavelength change because it involves zero adjustment with no sample. Simply repeat step 4 occasionally.

8. Data recording.
   Complete and legible recording of data can save you time and spare you frustration in the long run. When working with the spectrophotometer, you should record the following for each reading:
   identity of sample
   wavelength
   whether absorbance or transmittance was recorded
   the value of absorbance or percent transmittance.

Answer Sheet

1. scattering; some transmission (because it is only slightly cloudy rather than completely opaque). (The whiteness of the solution indicates that no absorption is taking place.)
2. No, you would need to know the constant, *z*, and the length of the light path, *p*.
3. Does not absorb red light.
4. scattering and absorption.
5. the inside diameter of the test tube.
6. A
7. a. $p\,z$
   b. $c_{standard}\ p\ z$
   c. $$\frac{A_{sample}}{A_{standard}} = \frac{c_{sample}\ p\ z}{C_{standard}\ p\ z}$$
8. true
9. $$c_{standard} \times \frac{A_{sample}}{A_{standard}}$$
10. 2 and 3
11. detect light that has passed through the sample (by converting light energy to an electric current, the strength of which registers on the meter).
12. b

Summary of Operating Procedure



1. Make sure the instrument is plugged in.
2. Turn on instrument. Wait 30 minutes for it to warm up.
3. Select wavelength with knob #2.
4. With sample chamber empty and lid closed, turn the on-off knob (5) until meter needle reads 0 percent transmission.
5. *Clean tube* and insert blank into sample chamber, *etched side toward you;* close lid of sample chamber; adjust knob 4 until meter needle reads 100 percent transmittance.
6. Replace blank with sample, close lid, read percent transmittance or absorbance off meter.
7. Further readings:
   A. At same wavelength.
      Repeat step 6. From time to time, repeat steps 4 and 5.
   B. **At different wavelength.**
      Repeat step 5 each time you change the wavelength, then repeat step 6. Repeat step 4 from time to time.

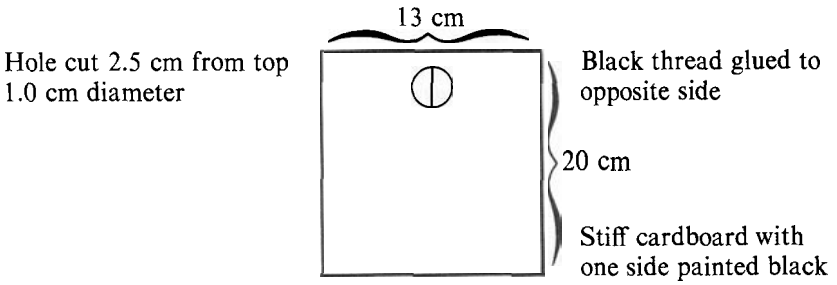
Instructor's Materials

Basically, this is an easy laboratory to prepare. In trials at the University of California, Berkeley, the students worked through Tutorial A at home. It took about an hour to complete. The lab periods were offered on an open basis, with students coming in and spending as much time as they needed. The laboratory has also been offered in a scheduled, four-hour time slot. While there are obvious advantages to the unscheduled system, we found that the students can complete the lab in four hours.

Equipment, Materials, and Supplies

The following quantities are for a lab of 20 students, working in pairs.

1. Compound microscopes (with 10× and 45× objectives)—10
2. High intensity illuminators (needed even if microscopes have built in illuminators for use with slit-lamps)—10
3. Slides and cover slips—1 box each
4. Spectrophotometers (with blue filters to read under 600mμ)—3
5. Glass tubes that can be used in both the spectrophotometer and the centrifuge—30
6. Centrifuge—2
7. Slit-lamps (see diagram)—10



8. Pasteur pipettes—1 box
9. Graduated pipettes—1ml—10
10. Graduated pipettes—5ml—10
11. Kimwipes (or similar tissues)—1 box
12. Standard size glass test tubes—20
13. Blood—50 ml
    We used beef blood from a local slaughterhouse. We brought in test tubes containing 120 ml of 2% aqueous sodium citrate (an anti-coagulant) and mixed it with 420 ml of fresh, warm beef blood. The mixture was shaken vigorously and stored in the refrigerator for up to one week. (You can also use outdated human blood from a blood bank.)
14. Methylene blue (0.1% aqueous)—5 dropping bottles
15. Triton—X detergent (10% aqueous)—1 dropping bottle

16. Solutions—100 ml of each (See Appendix I for preparation)
    Urea—0.3 M
    Dimethyl urea—0.3 M
    Glycerol—0.3 M
    Sucrose—0.3 M
    Dimethyl Alcohol
    Ethyl Alcohol } Full Strength
    Propyl Alcohol
    Sodium Chloride—.05 M; .07 M; .08 M; .1 M
    Distilled water
17. A chart of various types of blood cells—red and white—is good to have up on the wall for reference. Such a chart can be purchased from any major biological supply house, or you can make one yourself using your own drawings.
18. For the independent investigation part of the exercise, you can supply other types of blood and other solutions (like glucose) as the students request.

References

Many general biology texts present background material on the cell membrane phenomena examined. The student materials in this laboratory exercise themselves provide considerable informational background. As stated previously, however, learning about membranes is only one part of the exercise; the other is learning about doing science. I have therefore included in this bibliography references to investigative laboratories as well as to cell membranes.

1. Avers, C. J. *Cell Biology*. New York: D. Van Nostrand Co.; 1976.
   This is a good general cell biology text. Chapter 4 (pp 97–122) presents a clear description of membrane structure and function with good schematic diagrams.
2. Dyson, R. D. *Cell Biology: A Molecular Approach*. Boston, MA: Allyn and Bacon, Inc.; 1974.
   This cell biology text is more sophisticated than the one by Avers. Chapter 7 (pp 284–330) offers an excellent presentation of several theories of membrane structure and the regulation of transport across membranes. The material may be too quantitative for most introductory students, but would prove useful for students with better chemistry and math backgrounds.
3. Harrison, R.; Lunt, G. *Biological Membranes*. Glasgow, Scotland: Blackie and Son, Limited; 1975.
   This small book is clearly written with many good illustrations. It describes membrane morphology, organization, and transport systems. The chapter on membrane systems (pp 173–201) is especially useful.
4. Holt, C. E.; Abramoff, P.; Wilcox, R. V. Jr.; Abell, D. L. Investigative laboratory programs in biology. A position paper of the Commission on Undergraduate Education in the Biological Sciences. *BioScience* 19(12):1104; 1969.
   This is the "classic" article that led to the development of numerous experiments in investigative laboratories throughout the country. The ideas presented are as valid now as they were in 1969.

are as valid now as they were in 1969.

5. Keeton, W. T. *Biological Science.* 3d ed. New York: W. W. Norton and Co.; 1980.

This widely used and respected general biology book provides a good section in its chapter on the cell (chapter 3, pp 81–101) on the structure and function of cell membranes.

6. Von Blum, R. C. Individualizing instruction in large undergraduate biology laboratories. I. Development of the model. *American Biology Teacher* 37(8): 467; 1975.

This is an article describing in detail the development of the approach to the biology laboratory taken in this cell permeability laboratory.

7. Von Blum, R. C.; Hursh, T. M. Mastering genetics, with a little help from GENIE. *American Biology Teacher* 39(8): 468; 1977.

This paper describes a further expansion of the approach taken by SABLE, including the development of a computer-simulated laboratory in genetics.

Appendix I

Preparation of Solutions

To prepare the solutions needed in the cell permeability experiments, put each of the following chemicals into a graduated cylinder and add distilled water to make one liter.

.3 M Urea—18.02 g
.3 M 1–3 Dimethyl Urea—24.43 g
.3 M Glycerol—27.63 g (weigh the liquid)
.3 M Sucrose—102.65 g
.05 M Sodium Chloride—2.92 g
.07 M Sodium Chloride—4.09 g
.08 M Sodium Chloride—4.68 g
.1 M Sodium Chloride—5.85 g

A good source of the above chemicals is:

SIGMA Chemical Co.
P.O. Box 14508
St. Louis, MO 63178
(314) 771–5750
Customer Service (314) 771–5765
TELEX (910) 761–0593