Chapter 7

Diffusion Across a Sheep Red Blood Cell Membrane

Linda A. Scott

Department of Biology
Hartwick College
Oneonta, New York 13820
(607) 431-4749

Linda Scott is an Associate Professor of Biology at Hartwick College. She received her B.S. in Biology and M.S. in Physiology from the University of Missouri at Kansas City, and Ph.D. in Physiology from the University of Kansas. Her research interests include reproductive physiology and women's health issues. At Hartwick College she teaches Principles of Biology and several physiology courses, including Animal Physiology and Comparative Physiology, Physiological Biochemistry, and Biology of Reproduction.

Reprinted from: Scott, L. A. 1993. Diffusion Across a Sheep Red Blood Cell Membrane. Pages 115-140, *in* Tested studies for laboratory teaching, Volume 14 (C. A. Goldman, Editor). Proceedings of the 14th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 240 pages.

Contents

Introduction....116
Materials....117
Student Outline....118
Introduction to Physiological Principles and Techniques....118
Experiment 1: Determination of Isotonic and Hemolytic Molar Concentrations of Electrolytes and Nonelectrolytes and Degree of Electrolyte Dissociation....122
Experiment 2: Determination of Diffusion Rate of Molecules of Varying Size and Lipid Solubility Across the Cell Membrane....124
Experiment 3: Determination of the Relationship of Molecular Size, Number of Hydroxyl Groups, and Partition Coefficient to Diffusion Rate of Molecules Across the Cell Membrane....126
Questions for Investigative Experiments....128
Literature Cited....129
Laboratory Report....129
Notes for the Instructor....130
Literature Cited....135
Appendix A: Sample Tables and Graphs for Introductory Laboratory....136
Appendix B: Information Tables for Investigative Laboratory....137

Introduction

Laboratories in Animal Physiology are divided into five investigative laboratory blocks consisting of 2–3 weeks per block. These blocks include: (1) cell membrane physiology; (2) neurophysiology; (3) muscle physiology; (4) exercise physiology and human electrophysiology; and (5) hormonal physiology. The introduction to techniques and preliminary collection of data occurs during the introductory laboratory within each laboratory block. During the subsequent investigative laboratory session(s), student research teams (composed of three to four students) use the acquired techniques to design, perform, and analyze an investigative project and then report results to other research teams. The sequence of presentation of physiological techniques is designed to be cumulative, so that the techniques acquired in the first laboratory block are assumed in subsequent laboratory blocks.

This cell membrane physiology laboratory was originally designed to be the first investigative laboratory block in Animal Physiology, but it can also be used in a first year introductory biology course either in its entirely or as separate experiments. The complete introductory laboratory takes about 3 hours to complete (including an introduction), but time can be reduced if molar solutions are made prior to laboratory time or if individual student research teams perform the different experiments and share results.

The objectives of this cell membrane physiology laboratory are to introduce the techniques to determine isotonic and hemolytic molar concentrations of electrolytes and nonelectrolytes and the degree of electrolyte dissociation; and to determine diffusion rate of penetrating molecules across the sheep red blood cell (RBC) membrane. Other techniques acquired during this laboratory include preparation of varying molar solutions, operation of the spectrophotometer, and graphing using a computer. Student research teams then design, perform, graph and analyze an investigative

experiment to determine the degree of dissociation of different electrolytes, or to determine the effect of molecular weight, lipid solubility, age of the cell membrane, species variability, or the physiological state of the cell on diffusion rate across the RBC membrane. The research teams then report their results to the class to complete the understanding of cell membrane physiology.

Materials

Introductory Laboratory

Equipment

Computers with graphing program (2–3)
Electronic balances (2–3)
Spectrophotometer (Spec 20) (1/group)
Electric pipettor (1/group)
Stopwatch (1/group)
Spectrophotometer cuvets (10/group)

Materials

Pipets, 5 ml (1 box)
Pipets, 1 ml (1 box)
Test tube rack (1/group)
Flasks, 100 ml (10/group)
Spatulas (2/group)
Graduated cylinders, 100 ml (1/group)
White tape and marker pen (1/group)

Solutions

Dye for concentration series (optional): 10 ml of 12 N HCl to 1 liter water, then add cupric nitrate crystals till saturated. (1 liter)
Sheep red blood cell suspension: mix 75 ml 0.16 M NaCl and 10 ml of *well mixed* sheep red blood cells (0.1 ml of RBC suspension in 3 ml of 0.16 M NaCl should have an absorbance of 0.5–0.7). (100 ml/group)
NaCl and glucose (dextrose) (3–4 bottles)
Weigh the following and fill flask to 100 ml with distilled water (100 ml/group):
- Glucose, 0.3 M (5.4 g)
- Sucrose, 0.3 M (10.26 g)
- Urea, 0.3 M (1.8 g)
- Glycerol, 0.3 M (2.76 g)
- Ethanol, 0.3 M (1.38 g)
- Ethylene glycol, 0.3 M (1.86 g)
- Glycerol, 0.3 M (2.76 g)
- Erythritol, 0.3 M (3.66 g)

Distilled water (1 liter/group)
NaCl, 0.16 M: 9.36 g NaCl, fill to 1 liter with distilled water. (1 liter/group)

Live Materials

Fresh heparinized sheep blood (Cleveland Scientific, P.O. Box 300, Bath, OH 44210, 216-666-7676). (100 ml)

Investigative Laboratory

All of the equipment and materials used in the Introductory Laboratory, plus solutions and live material below (quantity dependent on independent projects chosen by research groups):

Equipment

Warm water baths
Ice in a bucket for water baths (1)

Solutions

Substances in amide series, urea series, alcohol series, and sugar series (refer to Appendix B)
Variety of electrolytes, and cyanide, azide, dinitrophenol, ouabain, digitalis (solutions to be made by students)
Buffered solutions with range of pHs (100 ml of each)
RBC suspension (as in Introductory Laboratory) (100 ml/group)
Distilled water (1 liter/group)
NaCl, 0.16 M (1 liter/group)

Live Materials

Aged heparinized sheep blood (3–4 months old) (100 ml bottle)
Fresh heparinized blood from different species (Cleveland Scientific, local meat packers, or lab animals; use 0.3 mg heparin per 1 ml fresh blood) (100 ml bottle)

Student Outline:
Introduction to Physiological Principles and Techniques

Cell Membrane Diffusion

The red blood cell (RBC) is one of the most studied membrane systems and is therefore used as a model to describe many membrane-solvent-solute interactions. A red blood cell placed into a hypotonic solution of nonpenetrating molecules (i.e., a solution with lower concentration of solute and a higher concentration of solvent than the cell, for example, water) will rapidly swell and hemolyse, as the water molecules influx by osmosis from higher to lower concentration. Conversely, a red blood cell placed into a hypertonic solution of nonpenetrating molecules (i.e., a solution with higher concentration of solute and lower concentration of solvent than the cell, for example, salt water) will rapidly shrink and crenate, as water molecules efflux by osmosis from a higher to a lower concentration. Further, a red blood cell placed into an isotonic solution of nonpenetrating molecules (i.e., a solution with the same concentration of solute and solvent as the cell, for example, saline solution) will neither swell or shrink because the osmotic influx and efflux of water is in equilibrium in the absence of a concentration gradient. The amount of time that it takes for hemolysis or crenation to occur is directly related to the rate of osmosis across the cell

membrane. Therefore, hemolysis can be used to determine the hypertonic, hypotonic and isotonic concentrations of particular nonpenetrating solutes and the hemolysis time can be used as an index of the rate of osmosis (Strand, 1983).

Hemolysis time can also be used as an index as to the rate of diffusion of penetrating molecules into the cell. For example, red blood cells can be suspended in hypertonic solutions of differing solutes. If the solute cannot penetrate through the cell membrane and pull water with it, no hemolysis will occur. However, if the solute can penetrate through the cell membrane, it does so because of its greater concentration outside of the cell and increases the concentration of osmotically active molecules inside the cell. The water is pulled into the cell due to the newly established osmotic gradient and hemolysis occurs. Therefore, the rate of hemolysis can also be used as an indicator of the diffusion rate for particular penetrating solutes (Bakko, 1985; Giese, 1963).

Hemolysis of red blood cells is accompanied by changes in light absorbance of the cell suspensions. As hemolysis occurs, the RBC membrane bursts open, releases its hemoglobin, then settles to the bottom of the tube, causing the solution to clear. This clearing can be measured by determining the light absorbance of a solution, through the use of a spectrophotometer. The spectrophotometer set at 600 nm (where absorption due to suspended hemoglobin is small) maximizes the difference between the cloudy solution of suspended red blood cells and the clear solution due to hemolysed red blood cells.

Spectrophotometry

The fact that a substance appears to be a particular color in white light (which is composed of all visible wavelengths of light) implies that certain wavelengths of light have been absorbed by the substance, and that certain wavelengths of light have been transmitted according to the property of that particular molecule. A spectrophotometer directly measures the amount of light of a particular wavelength transmitted by a substance, and therefore indirectly measures the amount of light of a particular wavelength absorbed by a substance. Further, the amount of light which is absorbed or transmitted is usually proportional to the concentration of the particular molecule in solution (Beer's Law). The amount of light absorbed or transmitted, measured by the spectrophotometer, is therefore used to calculate an unknown concentration of the substance when compared to absorbances of known concentrations of that same substance (Abramoff and Thomson, 1986).

Various kinds of spectrophotometers have in common two major component parts: (1) the light source which transmits light, and (2) the photoelectric tube (or photocell) which detects transmitted light (see Figure 7.1). The light is transmitted from a tungsten source (or a UV source) through a refracting prism, which splits the light into its component wavelengths. The wavelength of light desired from the spectrum can be selected by adjustment of an exit slit (by the wavelength control), so that the selected wavelength is directed through the cuvet containing the solution being examined. This selected wavelength of light then transverses the solution and is directed onto the photoelectric tube (or photocell). The photoelectric tube detects the amount of light going through the solution and generates an electric current proportional to the intensity of the light detected. The electric current is sent to the galvanometer and recorded on a scale as absorbance (log scale 0–2.0) or as percent transmittance on a scale of 0%–100% (Abramoff and Thomson, 1986).

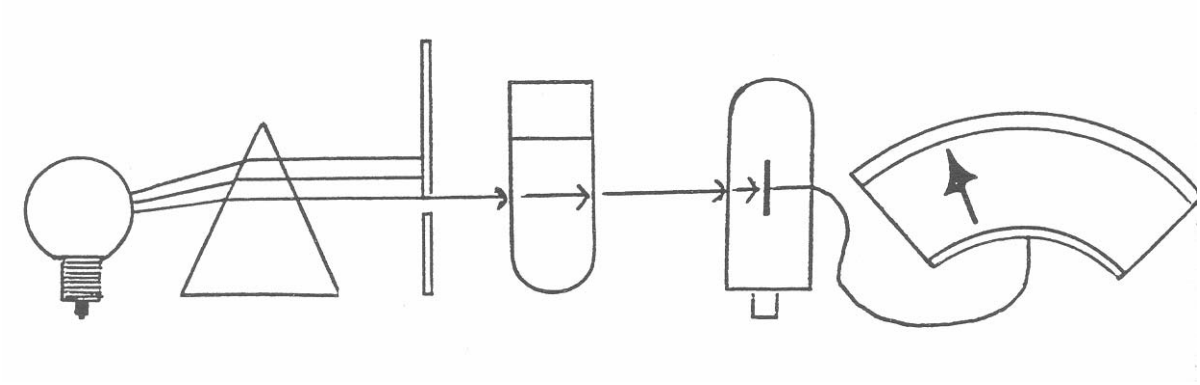

Figure 7.1. Path of light in a photoelectric spectrophotometer. Adapted from Abramoff and Thomson (1986).

Because most biological molecules are dissolved in a solvent before measurement, a source of error can be due to the absorption of light by the solvent itself. To assure that the spectrophotometric measurement will reflect only the light absorption of the molecules being studied, a mechanism for "subtracting" the absorbance of the solvent is necessary. To subtract the solvent absorption, a "blank" (the solvent without the solute being tested) is first entered into the chamber and the absorbance is set at 0 (or 100% transmittance). The unknown sample containing the solvent plus the solute to be measured is then entered into the chamber, and the absorbance is read. Since the solvent absorbance was subtracted with the "blank," the final absorbance reading only reflects the absorbance of the solute being tested (Abramoff and Thomson, 1986).

A solute concentration series is prepared by accurately measuring the different calculated amounts of the same solute and suspending the solute in the equal volumes of the same solvent. The absorbance (Å) of this concentration series can then be read and graphed on the *y*-axis against the known concentrations on the *x*-axis to produce a straight line. Unknown concentrations can be determined by reading the absorbance of the unknown concentration using the spectrophotometer and then reading the concentration from the graph (Abramoff and Thomson, 1986).

The concentrations of unknowns can also be calculated from the absorbance of the unknown compared to the absorbance of a standard of known concentration using the following formula (Abramoff and Thomson, 1986):

$$Concentration\ of\ unknown = \frac{Absorbance\ of\ unknown}{Absorbance\ of\ standard}\ x\ Concentration\ of\ standard$$

Molar Solutions

A 1 molar (M) solution contains 1 mole of solute in l liter of solution. One mole is equal to the molecular weight (MW) of the solute in grams, and contains 6.024×10^{23} molecules (Avogadro's number). Thus, solutions of equal molarity have the same number of molecules in solution, even though their molecular weights may be different.

For example, the MW of glucose is 180. To prepare a 1 molar (M) solution of glucose, weigh 180 g of glucose, place the glucose in 1 liter flask, and then fill the flask with distilled water to a total volume of 1 liter. Eighteen grams of glucose placed in a 100 ml flask, which is filled with distilled water to 100 ml would also be a 1 molar (M) concentration. These examples indicate that decreasing the amount of solute and solution by the same proportion does not change the concentration of the solution. These 1 molar (M) solutions are also termed 18% solutions, since a percent solution equals the grams of solute per milliliter volume of solution multiplied by 100; that is, percent solution = (g solute/ml solution) × 100. Following the same procedure, to make a 1 molar (M) solution of NaCl (MW = 58.5), place 58.5 g of NaCl in a 1 liter flask, and fill with distilled water to a total volume of 1 liter. This would be the same as 5.85 g of NaCl filled to a volume of 100 ml, and both solutions are 5.85% solutions.

Because of the low concentrations of solutes in body fluids, physiological techniques often require millimolar (mM) concentration. If 180 mg of glucose is dissolved to a total volume of 1 liter, a 1 mM concentration is produced. Similarly, 18 mg of glucose dissolved to a total volume of 100 ml produces a 1 mM solution. By further decreasing the amount of solute and solution in proportion, even smaller quantities of the same concentration can be made.

To make molar solutions of 100% liquids, you can also use the same weight method by weighing the liquids in grams into a flask (zeroing the weight of the flask) and filling to the appropriate level with distilled water. This method is easier than other methods and compensates for differing densities of liquids. For example, to make a 100 ml of a 0.3 molar solution of 100% solution of ethanol, weigh 1.38 g of ethanol in a flask whose weight has been zeroed (i.e., 0.1 liter solution desired × 0.3 M × MW of 46 for ethanol = 1.38 g/100 ml), and fill the flask to 100 ml with distilled water.

Objectives

The objectives of these introductory experiments are:

1. To become familiar with the preparation of varying molar solutions, the operation of the spectrophotometer and graphing with the computer.
2. To determine the isotonic and hemolytic molar concentrations of electrolytes and nonelectrolytes, and the degree of electrolyte dissociation.
3. To determine the diffusion rate of penetrating molecules of varying size and partition coefficient (lipid solubility) across the cell membrane.
4. To determine the relationship of the molecular size, number hydroxyl groups, and partition coefficient to the diffusion rate of molecules across the cell membrane.

Experiment 1:
Determination of Isotonic and Hemolytic Molar Concentrations of Electrolytes and Nonelectrolytes and Degree of Electrolyte Dissociation

Introduction

Solutions of nonpenetrating nonelectrolytes (e.g., glucose, sucrose, etc.) cause hemolysis (due to influx of water by osmosis) at approximately the same molar concentrations. This is because these solutions have the same number of molecules per liter. Thus nonelectrolyte solutions of the same molar concentrations demonstrate the same osmotic pressure. On the other hand, solutions of nonpenetrating electrolytes (e.g., NaCl) cause hemolysis (due to influx of water by osmosis) at lower molar concentrations than the nonpenetrating nonelectrolyte. This is because an electrolyte can dissociate into two ions (e.g., NaCl into Na^+ and Cl^-), and every ion in the dissociated solution exerts the same osmotic pressure as is produced by the entire NaCl molecule. Therefore, at the same molar concentrations, there would be more molecules per liter in the electrolyte solution than in the nonelectrolyte solution and the solutions would demonstrate different osmotic pressures. Further, different electrolyte solutions can vary in the osmotic pressure they exert, depending on the degree of dissociation of the particular electrolyte in the solvent (Abramoff and Thomson, 1982).

A way to express this relationship of the electrolyte and the nonelectrolyte is by determining the isotonic coefficient. The isotonic coefficient (*i*) is calculated by the following formula:

$$i = \frac{isotonic\ molar\ concentration\ of\ \ glucose}{isotonic\ molar\ concentration\ of\ \ NaCl}$$

For example, if the isotonic molar concentration of glucose (the last molar concentration with full absorbance readings immediately before the hemolytic molar concentration in a concentration series) is 0.1818 M, and the isotonic molar concentration of NaCl is 0.097 M, the isotonic coefficient would be calculated as 1.87 (i.e., 0.1818/0.097 = 1.87). This means that 100 molecules of NaCl exert as much osmotic pressure as 187 molecules of glucose. It further means that 87% of the NaCl molecules were dissociated. In other words, 87 out of every 100 NaCl molecules, appear to have separated into two ions, so that there is a total of 187 particles that are capable of exerting osmotic pressure (i.e., the osmotic pressure exerted is equivalent to the pressure exerted by 187 glucose molecules; Abramoff and Thomson, 1982).

With the same data, it is also possible to calculate the proportion of molecules of NaCl that are dissociated or the degree of electrolyte dissociation, using the following formula:

$$i = 1 + (k - 1)\,a$$

in which *i* is the isotonic coefficient of NaCl, *k* is the number of ions from each molecule of the salt ($k = 2$), and *a* is the degree of electrolyte dissociation (Abramoff and Thomson, 1982).

Operation of a Spectrophotometer

1. Turn on the spectrophotometer, at least 15 minutes prior to use, and set appropriate wavelength.
2. To blank the spectrophotometer: (a) with nothing in the chamber, use the left knob to set the needle to 0% transmittance; (b) next, with the “blank” in the chamber, use the right knob to set the needle to 100% transmittance (or zero absorbance).

3. *Optional:* To test your spectrophotometer skills, do the following: (a) Make at least 3 ml each of a series of dye concentrations (1:2, 1:4, 1:8, 1:16) by measuring dye and distilled water in a graduated cylinder. Also mix an unknown dilution of the dye. (b) With the wavelength set at 500 nm, read the absorbance of 3 ml of each of the dye dilutions. Blank between each reading, using distilled water as the "blank." (c) Graph your results with dye concentration on the *x*-axis and absorbance (Å) on the *y-axis.* (d) Read the absorbance of the unknown dye concentration and determine its concentration from the graph. Also determine the unknown dye concentration by using the formula which compares the absorbance of the unknown to the absorbance of a standard at known concentration (Abramoff and Thomson, 1986).

Experimental Procedure

In this experiment, you will determine the isotonic and hemolytic molar concentrations of nonpenetrating electrolytes (NaCl) and nonelectrolytes (glucose) for sheep red blood cells. From the isotonic molar concentrations of glucose and NaCl, you can calculate the isotonic coefficient and degree of electrolyte dissociation. Do the following to obtain your data (methods adapted from Bakko, 1985):

1. To make the "blank," pipet 0.1 ml of sheep red blood cell suspension (i.e., RBCs suspended in a 0.16 M NaCl solution) into a cuvet containing 3.0 ml distilled water (RBCs settle to the bottom; therefore always *mix* the RBC suspension before pipetting into the cuvet). Allow this cuvet to stand for 15–20 minutes for complete hemolysis to occur. Upon complete hemolysis, this tube will have the minimum absorbance and is therefore the "blank." Set the wavelength to 600 nm. With nothing in the chamber, use the left knob to set the needle to 0% transmittance. Place the "blank" in the chamber and with the right knob, adjust the needle to 100% transmittance (0 absorbance).
2. To determine if the sheep RBC suspension is the correct concentration, fill a second cuvet with 3.0 ml of 0.16 M NaCl. Add 0.1 ml of the sheep RBC suspension to this cuvet (remember to *mix* the RBC suspension before pipetting). This isotonic solution will allow for maximum absorbance of light by full sized RBC at the wavelength of 600 nm. Blank the spectrophotometer with the "blank" as before, and measure the absorbance of the second suspension. The absorbance should read between 0.5 and 0.7, indicating the correct concentration of sheep RBC suspension required for the experiment.
3. Using the instructions for preparation of molar solutions in the Introduction, prepare 100 ml each of the following concentration series of glucose (MW = 180) and NaCl (MW = 58.5): 0.05 M, 0.06 M, 0.07 M, 0.08 M, 0.1 M, 0.125 M, 0.16 M, 0.2 M, 0.25 M, and 0.3 M.
4. To 10 cuvets, add 3.0 ml of the varying NaCl concentrations which you prepared (from 0.05 M to 0.3 M). Add to each cuvet 0.1 ml of *well mixed* RBC suspension and allow to stand for 15–20 minutes for complete hemolysis. In the meantime, construct a data table to record the NaCl concentration and the absorbance (Å). Read and record the absorbance of the varying NaCl concentrations, remembering to "blank" between each reading.
5. Clean the cuvets thoroughly and repeat the above procedure using varying concentrations of glucose (from 0.05 M to 0.3 M). Enter the glucose absorbance (Å) data in the data table provided.

6. Using a computer, graph glucose and NaCl data on one graph with the molar concentration (M) on the *x*-axis and the absorbance (Å) on the *y*-axis. Hemolysis molar concentration can be determined when the absorbance reading decreases to approximately zero as the suspension clears. The isotonic molar concentration is the last molar concentration in a series demonstrating maximal absorption preceding the hemolytic molar concentration. Label the hemolysis and isotonic molar concentrations on your graph.
7. Analyze your data by comparing the NaCl and glucose isotonic molar concentrations. Calculate and explain the meaning of the isotonic coefficient (*i*), and the degree of electrolyte dissociation (*a*) for NaCl in your experiment using the formula in the Introduction for this experiment.

Experiment 2:
Determination of Diffusion Rate of Molecules of Varying Size and Lipid Solubility Across the Cell Membrane

Introduction

Molecules cross membranes by three major routes:

1. Bilipid Layer: This route involves the molecule leaving the aqueous phase on one side of the membrane, dissolving directly in the bilipid layer of the membrane, diffusing across the thickness of the bilipid layer, and finally entering the aqueous phase on the opposite side of the membrane. Molecules which enter the cell via this route are of two main types: (a) small, nonpolar molecules (such as oxygen); and (b) uncharged, polar molecules ranging in size from small molecules (such as water, MW = 18)) to larger molecules such as alcohols (e.g., ethanol, MW = 46, and glycerol, MW = 92), ureas (e.g., urea, MW = 60), and amides (e.g., proprioamide, MW = 73; Alberts et al., 1989; Eckert and Randall, 1988).
2. Aqueous Channels: This route involves maintenance of the molecule in the aqueous phase and its diffusion through aqueous channels or water-filled pores in the membrane. These membrane channels have diameters of less than 1.0 nm (mean size 0.7 nm) and thereby limit the size of the molecules which can diffuse through them. Molecules which enter the cell via this route are charged molecules, including inorganic ions such as Na^+, K^+, Ca^{2+}, and Cl^- (Eckert and Randall, 1988) .
3. Carrier-Mediated Transport: This route involves the combination of the molecule to be carried with a carrier molecule located in the cell membrane. The carrier molecule facilitates the movement of the molecule across the bilipid layer. Some carrier molecules simply facilitate diffusion for specific molecules down the concentration gradient with no expenditure of energy (termed facilitated diffusion). Other carrier molecules utilize energy to move a specific molecule against the concentration gradient (termed “active transport”). Examples of molecules which require a carrier system are larger, noncharged, polar molecules (such as sugars) and large, charged molecules (such as acids; Eckert and Randall, 1988).

Several factors determine the rate of diffusion of a molecule across the membrane depending on the size, polarity and charge of the particular molecule. The rate of diffusion through the bilipid layer for the small, nonpolar molecules is determined by the size and steric configuration or shape of the molecule. The rate of diffusion of uncharged, polar molecules through the bilipid layer is

determined primarily determined by lipid solubility (expressed by partition coefficient), but may be modified by molecular size and steric configuration (Eckert and Randall, 1988).

Lipid solubility, expressed as a partition coefficient, is determined by factors other than simply how easily the molecule dissolves in lipid. For the uncharged, polar molecule to leave the aqueous phase and enter the lipid phase it must first break its hydrogen bonds with water (which requires activation energy in the amount of 5 kcal per broken hydrogen bond) before it can dissolve in the lipid phase. The number of hydrogen bonds a molecule forms with water is determined by the number of polar groups on the molecule, as well as the strength of the hydrogen bonds formed. For example, the polar hydroxyl (-OH) groups form very strong hydrogen bonds with water, the polar amino ($-NH_2$) groups form weaker hydrogen bonds with water, and the polar carbonyl groups of aldehydes (CHO) and ketones (C=O) form even weaker hydrogen bonds with water. Each additional hydrogen bond formed between a polar group and water results in a 40-fold decrease in the partition coefficient, and a resulting decrease in the molecular permeability through the cell membrane. In other words, strongly polar molecules exhibit less lipid solubility due to more polar groups forming hydrogen bonds with the water which hold the polar molecule in the aqueous phase and prevent it from entering the bilipid layer of the cell. This reduces the polar molecule's ability to penetrate the bilipid layer and reduces its diffusion rate across the membrane. Whereas the addition of polar groups decreases penetrating ability and diffusion rate of the molecule, the addition of nonpolar groups increases the penetrating ability and diffusion rate of the molecule by allowing the molecules to enter the bilipid layer more easily (Eckert and Randall, 1988).

The partition coefficient value, which expresses the lipid solubility of a molecule, is derived by shaking the molecule in a test tube with an equal amount of lipid (olive oil) and water and by determining the concentration of the molecule in each of the phases. The partition coefficient (K) is calculated by the following equation:

$$K = \frac{solute\ concentration\ in\ lipid}{solute\ concentration\ in\ water}$$

Thus, the higher the partition coefficient, the greater the solubility in lipid (Eckert and Randall, 1988).

Simple diffusion through the bilipid layer exhibits nonsaturation kinetics, meaning that the rate of influx of penetrating molecules across the membrane increases in direct proportion to the concentration of the solute in the extracellular fluids. Diffusion through aqueous pores does not strictly exhibit nonsaturation kinetics, for as the extracellular concentration of the molecules increases, the aqueous channels can become filled with solute inhibiting free diffusion across the bilipid layer. Therefore, at low extracellular solute concentrations, the rate of influx of solutes through the pores increases in direct proportion to the concentration of the solute in extracellular fluids, but at high extracellular solute concentrations the influx of solutes through the pores decreases slightly. The carrier-mediated route exhibits saturation kinetics, wherein the rate of influx reaches a plateau beyond which a further increase in solute concentration does not increase in the rate of influx. This is because the number of carriers, the rate at which carriers can react with molecules, and the actual transport of the molecule across the membrane is limited. Therefore, the rate of carrier-mediated transport increases in direct proportion at lower extracellular solute concentrations, then reaches a maximal level when the carrier molecules are saturated (Eckert and Randall, 1988).

Urea (MW = 60) is a small, noncharged, polar molecule with an oil:water partition coefficient of 0.00015. Ammonia, which is a toxic breakdown product of amino acid metabolism within the cell, is converted into the less toxic, water soluble urea to be released out of the cell through the cell membrane. Glycerol (MW = 92) composes the three carbon backbones of lipids, and is a slightly

larger, noncharged, polar molecule with an oil:water partition coefficient of 0.00007. Glucose (MW = 180) is a larger, noncharged, polar monosaccharide with an oil:water partition coefficient of approximately 0.00003. Glucose is metabolized in the cell during cellular respiration to produce ATP. Sucrose (MW = 342) is a large, noncharged, polar disaccharide with an oil:water partition coefficient of 0.00003. The above partition coefficients are from Collander (1954).

Experimental Procedure

To determine the diffusion rate of molecules of varying molecular size and lipid solubility (partition coefficient), do the following (methods adapted from Bakko, 1985):

1. Set up cuvets with 3.0 ml of each of the following 0.3 M solutions: glucose (MW = 180), sucrose (MW = 342), urea (MW = 60), and glycerol (MW = 92). The "blank" for this series is 3.0 ml distilled water with 0.1 ml *well mixed* RBC suspension (allow to set for 15–20 minutes for complete hemolysis).
2. Construct a data table with time 0–30 seconds in 5 second intervals, then 60 seconds, and then in 60 second intervals thereafter, for a total of 15 minutes.
3. Blank the spectrophotometer at the wavelength of 600 nm. Place the cuvet containing 3 ml of the urea solution in the spectrophotometer. Add 0.1 ml of *well mixed* RBC suspension into the cuvet as you start the stop watch. Record the absorbance in 5-second intervals for a total of 30 seconds.
4. Next add 0.1 ml of *well mixed* RBC suspension to the tubes with 3 ml of glucose, glycerol, and sucrose solutions. Record the absorbance at time 0 and at 1-minute intervals thereafter, blanking between each reading.
5. Using a computer, graph the time (seconds) on the *x*-axis and the absorbance (Å) on the *y*-axis for all four molecules on one graph.
6. Analyze the data by determining the hemolysis time of the four molecules. Explain the route through the cell membrane by using the information you know about the size and steric configuration, the polarity and number and type of polar groups, and the lipid solubility of the molecules.

Experiment 3:
Determination of the Relationship of Molecular Size, Number of Hydroxyl Groups, and Partition Coefficient to Diffusion Rate of Molecules Across the Cell Membrane

Introduction

As discussed in the Introduction for Experiment 2, the three routes through a cell membrane are: (1) the bilipid layer (used by small, nonpolar molecules or uncharged, polar molecules); (2) the aqueous pores (used by small, charged molecules); and (3) carrier-mediated transport (used by large, uncharged polar molecules and large, charged molecules). Remember that the rate of diffusion of noncharged, polar molecules through the bilipid pathway is primarily determined by its lipid solubility (the higher the partition coefficient, the more lipid soluble the molecule), but can be

modified by factors such as the size of the molecule (the smaller molecule is faster) and the steric configuration or shape of the molecule (the symmetrical or globular molecules are faster than the fibrous molecules). Further, remember that a determining factor of lipid solubility is number of polar groups (hydroxyl, amino, and carbonyl groups of aldehydes and ketones) which can form hydrogen bonds with water molecules, and the strength of the hydrogen bonds formed. The greater the number of polar groups to form hydrogen bonds and the greater the strength of the bonds, the greater hold the water has on the molecules causing the molecule to be less soluble in the bilipid layer. Finally, remember that the addition of nonpolar groups increases molecular permeability (Alberts et al., 1989; Eckert and Randall, 1988; Strand, 1983).

Alcohols are uncharged, polar molecules which vary in size, number of hydroxyl groups, and partition coefficients. Ethanol (C_2H_5OH) has a molecular weight of 46, an oil:water partition coefficient of 0.032, and one hydroxyl group; ethylene glycol ($C_2H_6O_2$) has a molecular weight of 62, an oil:water partition coefficient of 0.00049, and two hydroxyl groups; glycerol ($C_3H_8O_3$) has a molecular weight of 92, an oil:water partition coefficient of 0.00007, and three hydroxyl groups; and erythritol ($C_4H_{10}O_4$) has a molecular weight of 122, an oil:water partition coefficient of 0.00003, and four hydroxyl groups. The above partition coefficients are from Collander (1954).

Experimental Procedure

Do the following to determine the relationship of molecular size, number of hydroxyl groups, and partition coefficient to diffusion rate of the molecule across the cell membrane:

1. Set the wavelength to 600 nm and blank the spectrophotometer (the "blank" for this experiment is 3.0 ml distilled water with 0.1 ml *well mixed* RBC suspension which has completely hemolysed).
2. Add 3.0 ml of 0.3 M ethanol to each of three cuvets. Place the first cuvet of ethanol in the spectrophotometer. Now add 0.1 ml of *well mixed* sheep red blood cell suspension directly into the tube in the spectrophotometer and start the stopwatch. Determine and record the time in seconds required for hemolysis (i.e., the time it takes for the absorbance to reach an absorbance reading of 0). Repeat using the same instructions for the other two cuvets of ethanol. Average the hemolysis time for the three trials, convert to minutes, and enter the mean time into the data table. *Note:* If you want to slow the diffusion rate (so that hemolysis time is easier to record), the concentration of the RBCs can be increased in the solution by using 1.0 ml RBC suspension to 2.0 ml solution with and endpoint of 75% transmittance. If the concentration of RBCs is changed, you would need to make a new "blank" by adding 2.0 ml distilled water to a tube, then adding 1.0 ml of sheep red blood cell suspension and allowing the mixture to completely hemolyse (10–15 minutes).
3. Repeat the instructions above for a 0.3 M ethylene glycol recording three trials and averaging your results in minutes. "Blank" between trials.
4. Set up three cuvets series with 3.0 ml of 0.3 M glycerol, and a series of three cuvets with 3.0 ml of 0.3 M erythritol. Add 0.1 ml of *well mixed* sheep red blood cell suspension to each tube, and time all six tubes at once. Read the absorbance every 5 minutes, and more often as the absorbance nears 0. Determine and record the amount of time for hemolysis in minutes, average the three readings of glycerol and the three readings for erythritol in minutes, and enter the data into your table. Assume that if the RBCs have not hemolysed in 60 minutes, hemolysis is either very slow or there is no hemolysis.

5. Using a computer, graph the mean hemolysis time in minutes on the *x*-axis and the partition coefficient ($\times 10^4$) and molecular weight and number of hydroxyl groups ($\times 10$) on the *y*-axis. (These adjustments will allow you to scale the data so that the data can be entered on the same graph). Do not graph a hemolysis time of greater than 60 minutes.
6. Analyze your data by determining the relationship of hemolysis time to molecular weight, the number of hydroxyl groups, and the partition coefficient of all four of the molecules. Interpret and explain the meaning of your results.

Questions for Investigative Experiments

In the introductory experiments, you learned how to determine the isotonic and hemolytic molar concentrations of electrolytes and nonelectrolytes and the degree of electrolyte dissociation. You also learned how to determine the membrane diffusion rate for molecules of varying sizes and partition coefficients, and how to determine the relationship between the diffusion rate of the molecule and molecular size, number of hydroxyl groups, and partition coefficient. Now your research team will devise a protocol to answer a question about cell membrane diffusion. Some sample questions are:

1. What is the effect of RBC age on diffusion rate or cell membrane fragility?

2. Are there species differences in diffusion rate through RBCs? If there are differences, are the differences proportional between species so that the rules developed from experiments on cells of one species apply to the same cells in other species?

3. What is the effect of the physiological state of the cell, such as temperature, anesthetics, pH, salt imbalance, metabolic poisons (e.g., cyanide, azide, or dinitrophenol), or pump inhibitors (e.g., digitalis or ouabain) on diffusion rate into RBCs? What are the practical implications of these differences?

4. What are the relative diffusion rates for other polar, noncharged molecules not tested (e.g., smaller alcohols, other ureas, amides, or sugars) in sheep RBCs? What is the relationship of molecular weight, number of hydroxyl groups, and partition coefficient to the diffusion rate for these molecules? (Refer to Appendix A for molecular formulae, molecular weights, and partition coefficients for alcohol, urea, amide, and sugar series.)

5. What happens to the diffusion rate when you compare molecules of like partition coefficient and differing molecular weight? What happens to diffusion rate when you compare molecules of like molecular weight and a differing partition coefficient?

6. What are the isotonic and hemolytic molar concentrations, isotonic coefficients and degree of dissociation for electrolytes other than NaCl?

   You may answer any of these questions or your own questions derived from the laboratory manual, textbook, or library literature.

Literature Cited

Abramoff, P. and R. G. Thomson. 1982. Movement of materials through cell membranes. Pages 109–121, *in* Laboratory outlines in biology. W. H. Freeman, New York, 529 pages.

Abramoff, P. and R. G. Thomson. 1986. Appendix C: Spectrophotometry. Pages 493-497, *in* Laboratory outlines in biology. W. H. Freeman, New York, 529 pages.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1989. The plasma membrane. (Chapter 6). Pages 276–337, *in* Molecular biology of the cell (Second edition). Garland Publishing, New York, 1217 pages.

Bakko, E. L. 1985. Cell membrane physiology. *In* Physiology laboratory manual (unpublished). St. Olaf College, Northfield, Minnesota.

Collander, R. 1954. The permeability of *Nitella* Cells to non-electrolytes. Physiologia Plantarum, 7:420–445.

Eckert, R., D. Randall, G. Augustine. 1988. Permeability and transport. (Chapter 4). Pages 65–99, *in* Animal physiology (Third edition). W. H. Freeman, New York, 683 pages.

Giese, A. C. 1963. Movement of solutes through the cell membrane in response to a concentration gradient. (Chapter 12). Pages 223–243, *in* Cell physiology (Second edition). W. B. Saunders, Philadelphia, 592 pages.

Strand, F. L. 1983. The plasma membrane as a regulatory organelle. (Chapter 4). Pages 49–67, *in* Physiology: A regulatory systems approach (Second edition). MacMillan, New York, 670 pages.

Laboratory Report

Include within your laboratory report the following:

1. Title: Be specific and concise to reflect the scope of your experiment(s).

2. Introduction: Introduce the concepts and background information needed to make hypotheses (educated guesses), making sure to reference your sources in the appropriate format. Also indicate, if possible, the importance of the question. At the end of the introduction, you should clearly state the hypothesis or series of hypotheses which you intend to test by experimentation.

3. Methods and Materials: For the introductory experiments, reference the section on laboratory procedures in your laboratory manual. Specifically, explain any modifications of the experimental procedures in the laboratory manual.

   For your investigative experiment, specifically describe the materials, procedures, and equipment you used (including your controls). Describe these in enough detail so that someone else could read this section and repeat your work.

4. Results: The following tables, graphs (with appropriate titles), and calculations should be included in your report:

   (a) Graph and table of NaCl and glucose concentration vs. absorbance. Label the hemolytic and isotonic molar concentrations. Calculate the isotonic coefficient (*i*) and the degree of dissociation (*a*) for NaCl.
   (b) Graph and table of Absorbance vs. time for sucrose, glucose, glycerol, and urea. Label hemolysis time.

(c) Graph and table of partition coefficient, molecular weight, and number of hydroxyl groups vs. hemolysis time for ethanol, ethylene glycol, glycerol and erythritol.
(d) Appropriate graphs, tables, and calculations for your investigative experiments.

This section of your report should also contain a clear and concise narrative description of the data in the tables and graphs, specifically referring to each table and figure (e.g., Table 1 and Figure 1 indicate...). Describe and summarize the trends in the data to the reader, specifically referring to the numerical data.

5. Discussion: Discuss, interpret and explain the data as related to the background information. Indicate whether the data support or refute your hypothesis or series of hypotheses. If you have data which does not support your hypothesis, present an explanation as to why you think they did not support your hypothesis and make an alternative hypothesis to be tested.

6. Literature Cited: List all literature cited (including laboratory manual, textbook, library literature, etc.) following the appropriate format.

Notes for Instructor

Introductory Laboratory

Note that the following results were obtained using sheep red blood cells. Since red blood cell membrane structure differs significantly between species, using red blood cells from other species may give very different results. Also, remind students to fully mix the RBC suspension prior to every pipetting; otherwise the hemolysis times for different molecules cannot be compared with any confidence.

In Experiment 1 sample data, the isotonic molar concentration for glucose and NaCl is 0.25 M and 0.125 M, respectively. This calculates to an isotonic coefficient of 2.0 (i.e., i = 0.25 M/0.125 M), indicating 100% dissociation for NaCl. Other data collected in student labs indicate the isotonic molar concentration for NaCl at 0.16 M, indicating a 56% dissociation for NaCl (i.e., i = 0.25 M/0.16 M). The degree of dissociation for NaCl is actually between these two values, but can only be more specifically determined if smaller increments of molar concentrations are tested.

In Experiment 2, urea enters the cell very fast to cause hemolysis (less than 5 seconds), glycerol enters the cell in approximately 14 minutes to cause hemolysis, and glucose and sucrose never enter the cell to cause hemolysis. This is because urea, even though relatively insoluble in lipid (partition coefficient of 0.00015), is a small (MW = 60), uncharged, polar molecule and can therefore diffuse quickly through the bilipid layer. Glycerol is a larger (MW = 92), uncharged, polar molecule, which is even less soluble in lipid (partition coefficient of 0.00007). This molecule can also diffuse through the bilipid layer; however, it is held in the aqueous phase by hydrogen bonds due to its three hydroxyl groups, thus slowing the diffusion rate. Glucose is an even larger (MW = 180), uncharged, polar molecule (with six hydroxyl groups), which is not very lipid soluble (approximate partition coefficient of 0.00003). Glucose cannot diffuse across the RBC membrane, but enters via a carrier molecule. The glucose carrier molecule exhibits saturation kinetics and limits the amount of glucose entering the cell so that hemolysis does not occur. Sucrose is a very large (MW = 342), uncharged, polar disaccharide, which is not very lipid soluble (partition coefficient of 0.00003). Sucrose cannot penetrate the cell even with the aid of a carrier, and therefore does not hemolyse the red blood cell.

In Experiment 3, ethanol, ethylene glycol, glycerol, and erythritol cause hemolysis in 0.14 minutes, 0.27 minutes, 22 minutes, and more than 24 hours, respectively; these data are supported

by Hober (1945). The hemolysis time increases with an increasing number of hydroxyl groups and decreasing partition coefficient. This increase in hemolysis time is due to the addition of hydroxyl groups (which increases the polarity and is reflected in the decreasing partition coefficients) holding the molecule in the aqueous phase, thereby slowing the diffusion rate. This observation is further supported by plant cell studies performed by Collander in 1954 (Strand, 1983).

Although hemolysis time also increases as molecular weight increases, molecular weight does not account for the differences in the hemolysis time. For example, if molecules of equivalent size but differing number of hydroxyl groups and partition coefficients are compared (such as ethyl alcohol, MW = 46, partition coefficient of 0.032) and ethylene glycol (MW = 62, partition coefficient of 0.00049) with the size difference of one oxygen), the diffusion rates are significantly different (1.5 and 64 seconds, respectively). Therefore, lipid solubility is the primary factor determining the diffusion rate of alcohol molecules with more than one hydroxyl group.

Investigative Experiments

For the following experiments use 0.3 M concentrations of the penetrating molecules tested and 0.1 ml of the RBC suspension, so that the results from the different investigative experiments can be pooled and compared. The following notes aid in answering the questions for the investigative experiments:

1. *What is the effect of RBC age on diffusion rate or cell membrane fragility?*

As a red blood cell membrane ages, the membrane becomes more fragile and ruptures more easily. Therefore, old RBCs hemolyse at higher molar NaCl or glucose concentrations (i.e., lower water concentrations) than young RBCs. Also old RBCs hemolyse faster when they are exposed to the same penetrating molecules as young RBC. Further, there are species differences in fragility due to the shape of the cell (spherical cells are more fragile than flat cell) and the thickness of the membrane (e.g., the order of fragility from most to least fragile is goat, cat, rabbit, dog, and human red blood cells; Davson, 1959).

2. *Are there species differences in diffusion rate through RBCs? If there are differences, are the differences proportional between species so that the rules developed from experiments on the cells of one species apply to the same cells in other species?*

Red blood cell membrane structure differs significantly between species, therefore causing a variance in diffusion rates for the same penetrating molecules. For example, the hemolysis time (in seconds) for various mammalian erythrocytes in 0.3 M glycerol in 0.12 per cent NaCl ranges from 3.5 seconds in the rat to 850 seconds in the sheep red blood cell (refer to Table 7.5 in Appendix B for hemolysis times for other species). Similar ranges in hemolysis times are observed for urea, hexoses, pentoses, and amino acids. For example, erythritol penetrates the mouse cell in less than 5 minutes while it takes more than 24 hours in ox and sheep RBC; human and monkey RBCs are penetrated by glucose, as well as other hexoses and pentoses (with pentoses penetrating faster); mouse RBCs are penetrated by mannitol and sorbitol, but not hexoses; guinea pig RBCs are penetrated by the amino acids glycine and alanine, but not by hexoses; dog RBCs are slowly penetrated by fructose and sorbose and more quickly by pentoses; and ox, pig, rabbit, and rat RBCs are impermeable to sugars and amino acids (Hober, 1945). According to Giese (1968), however, even though there are differences between species, the same cells of the various species often show the same relative differences in permeability to a particular penetrating molecule. These findings

give greater confidence that the rules of permeability developed from experiments on the cells of a fair sample of organisms are likely to apply to other cells in a qualitative manner.

3. *What are the effects of the physiological state of the cell, such as temperature, anesthetics, pH, salt imbalance, metabolic poisons (e.g., cyanide, azide, dinitrophenol), or pump inhibitors (e.g., digitalis or ouabain) on diffusion rate into RBCs? What are the practical implications of these differences?*

As the temperature increases, the diffusion rate into the cell increases due to increased molecular motion, decreasing hemolysis time. Whereas some molecules, such as propyl alcohol, freely diffuse through cell membranes, other molecules require activation energy to enter the bilipid layer. For the molecules requiring activation energy, a 10°C rise in temperature increases the number of molecules with the energy of activation required to cross the barrier by several fold, and thus increases diffusion rate (Giese, 1968). For example, hemolysis time for the sheep red blood cells exposed to 0.3 M glycerol at room temperature was approximately 14 minutes, while at 40°C it was 2 minutes, and at 5°C was 2 hours and 30 minutes.

According to Giese (1968), the effect of anesthetics on the permeability of cells is dependent on the species. For example, butyl alcohol decreases the penetration of glycerol into RBCs of humans, rats, rabbits, guinea pigs, and birds, but it increases the penetration of glycerol into the RBCs of sheep, horses, dogs, bats, pigs, reptiles, and fishes. (Thiourea, on the other hand, has the reverse effects on the same two series of cells). Also, different concentrations of the same anesthetic may have opposite effects on the permeability of the RBC, making a dose response investigation interesting. The mechanism of action is unclear, but these highly lipid-soluble anesthetic molecules may collect on the surface of the cell and alter properties of the membrane, thereby either increasing or decreasing the entry of other molecules. Some anesthetics which can be tested are ethane, acetylene, dimethyl ether, methylchloride, dimethyl acetal, and chloroform (listed in the order of increasing lipid solubility). Further, injury to the cell by heat, anesthetics, pH change, salt unbalance, etc., will increase the permeability of the cell (Giese, 1968).

Metabolic poisons such as dinitrophenol, cyanide or azide inhibit electron transport by binding to the cytochrome oxidase complex, blocking electron transport, and thus ATP production. Cardiac glycosides such as ouabain or digitalis are pump inhibitors. For example, ouabain blocks the K^+-binding site on the surface of the membrane, as well as K^+ activated ATPase activity. This action blocks the Na^+/K^+ exchange pump and release of energy to operate the pump (consequently blocking the transport of glucose). Metabolic poisons and pump inhibitors therefore, inhibit active transport by blocking production or release of energy, but have no effect on diffusion rate of molecules through the bilipid layer. However, if the Na^+/K^+ exchange pump is inhibited due to any factor, the osmotic gradient is disrupted and hemolysis can occur due to osmosis into the cell (Alberts et al., 1989; Eckert and Randall, 1988; Strand, 1983).

4. *What are the relative diffusion rates for other polar, noncharged molecules not already tested (e.g., smaller alcohols, other ureas, amides, or sugars) in sheep RBCs? What is the relationship of the number of hydroxyl groups, the molecular weight, or partition coefficient to diffusion rate for these molecules?*

If molecular weight and partition coefficient are graphed against hemolysis time in sheep RBCs for all series of homologous molecules (i.e., urea, alcohol, and amides), the hemolysis time decreases with increasing partition coefficient, and is not correlated with molecular weight. That is, as the lipid solubility increases the diffusion rate or permeability of the molecule increases independently of its molecular size. This observations is supported by studies performed using

plant cells by Collander in 1954 (Davson, 1959; Eckert and Randall, 1988; Hober, 1945). Therefore, in all molecules the overall property that determines the diffusion rate is the lipid solubility of the molecule, with factors such as molecular weight and shape modifying the diffusion rate across the membrane. Further, the diffusion rate increased in the following order: saccharoses, hexoses, pentoses, erythritol, glycerol, ethylene glycol, and the fastest molecules being ureas, aldehydes, ketones, esters (these data are supported by studies performed by Hedin using ox RBCs [Hober, 1945]), amides with one amino group, and alcohols with one hydroxyl group.

If a homologous series of **alcohols** with one hydroxyl group (methyl, ethyl, propyl and n-butyl) are compared, however, the results are different. In these alcohols, the hemolysis rate is very fast (less than 3 seconds) with the diffusion rates ranging from fastest to slowest: methyl, ethyl, propyl, and butyl (these data are supported by Hober [1945] using rat, pig, ox, and human RBCs). For these molecules, the hemolysis time increases only slightly as the molecular weight and lipid solubility increase significantly (partition coefficient increases, even though all of these alcohols contain one hydroxyl group, due to the lengthening of the nonpolar portion of the molecule, allowing it to enter the lipid phase more easily). In fact, the smaller, less lipid-soluble alcohols diffuse faster than larger, more lipid-soluble alcohols. This indicates that, in the alcohols with one hydroxyl group, the molecular size is an important modifying factor in determining diffusion rate. These results are different than those obtained in Experiment 3 when observing larger alcohols with an increasing number of hydroxyl groups, where lipid solubility was the primary factor determining the diffusion rate independent of molecular size.

In the **urea** series of homologous compounds, the hemolysis time increases in the following order: urea, methyl urea, dimethylurea, diethylurea, and thiourea (these data are supported by Hober [1945] using rat, pig, ox, and human RBCs). The hemolysis time for these ureas are approximately 2.7, 3.6, 3.2, 7.8, and 48.6 seconds, respectively. With the exception of thiourea, the hemolysis time increases linearly as the molecular weight and lipid solubility increases (partition coefficient increases as the number of exposed amino [NH_2] groups which form weaker hydrogen bonds with water are reduced). That is, smaller, less lipid-soluble ureas diffuse faster than larger, more lipid-soluble ureas. Therefore, molecular size is an important modifying factor in determining diffusion rate in the ureas (same results observed in the alcohols with one hydroxyl group). Thiourea is the exception in this series in that sulfur is substituted for an oxygen which leads to weaker hydrogen bonding with water, causing it to be less polar than urea, even though they have the same number of amino groups (partition coefficient of 0.0012 for thiourea and 0.00015 for urea). Thiourea is also the exception, in that its hemolysis time is much slower than the other ureas and does not fit linearly into the series, which is probably due to other factors affecting its diffusion rate. Also note that when comparing alcohol and urea molecules of equivalent size (e.g., ethylene glycol, MW = 62, and urea, MW = 60, that alcohols with the two hydroxyl groups are more polar than ureas with two amino groups (partition coefficients 0.00049 and 0.00015, respectively) indicating that hydroxyl groups form stronger hydrogen bonds with water than amino groups.

In the **amide** series of homologous molecules, the diffusion rate decreases in the following order: acetamide, propionamide, lactamide, and malonamide (these data are supported by Hober [1945] using rat, pig, ox, and human RBCs). In the amides with one amino group (i.e., formamide, acetamide, propionamide, and butyramide), the hemolysis time increases as the molecular weight and lipid solubility increase (the partition coefficient increases as the nonpolar portion of the molecule is lengthened). The smaller, less lipid-soluble amides diffuse faster than the larger, more lipid-soluble amides. Therefore, molecular weight is an important modifying factor for diffusion rate for the amides with one polar groups, as in the alcohols with one hydroxyl group and in the ureas. As the amides add more polar groups (i.e., a hydroxyl group to lactamide, and another amino group to malonamide), the hemolysis time increases as the partition coefficient decreases (0.00075

for lactamide, and 0.00008 for malonamide). Further, note that molecules of equivalent molecular size (propioamide, MW = 73, and lactamide, MW = 89, with the addition of one oxygen to lactamide) have very different partition coefficients (0.0036 and 0.00075, respectively) and very different hemolysis times (approximately 2.9 and 87.2 seconds, respectively). Therefore, in amides with an increasing number of polar groups, lipid solubility becomes the primary factor in determining the diffusion rate (as observed in the alcohols with more than one hydroxyl group).

In the **sugar** series, erythritol acts somewhat like an alcohol and diffuses across the cell membrane very slowly (hemolysis time is more that 24 hours in sheep RBCs). The other large, uncharged, polar hexose and pentose sugars, with an even greater number of polar hydroxyl groups, must enter the cell via the carrier mediated route (if they enter at all). The sugars which enter the cell exhibit saturation kinetics and do not increase to a solute concentration in the cell to cause hemolysis. Sucrose, a very large, uncharged, polar disaccharide does not enter the cell even with the aid of a carrier, and therefore does not cause hemolysis.

In summary, lipid solubility is the primary factor determining diffusion rate when comparing molecules with increasing polarity due to an increasing number of polar groups (larger alcohols and amides with more than one hydroxyl or amino group), and the primary factor determining diffusion rate for all molecules. However, within a series of homologous molecules with one exposed polar group (smaller alcohols and amides) and the ureas, the diffusion rate is modified by molecular size and shape (supported by Eckert and Randall, 1988; Hober, 1945; and Strand, 1983).

5. *What happens to the diffusion rate when you compare molecules of like partition coefficient and differing molecular weight? What happens to the diffusion rate when you compare molecules of like molecular weight and differing partition coefficient?*

If the partition coefficients of the molecules are similar, the molecular size (even if small) has some effect upon permeability. According to Giese (1968), cyanamide (MW = 42), propionamide (MW = 73), succinamide (MW = 116), and diethylmalonamide (MW = 158) have similar partition coefficients. Their molecular weight has a great impact on the permeability of the molecule, with an increase in molecular weight causing a decrease in the diffusion rate (i.e., larger molecules diffuse at a slower rate).

When molecules of similar size and molecular weight, for example, glycerol (MW = 92), lactamide (MW = 89), and dimethylurea (MW = 88), and differing partition coefficients (0.00007, 0.00075, and 0.0023, respectively) are compared, the ability to penetrate and diffusion rate increases with increasing lipid solubility (hemolysis time is 850, 87.2, and 3.2 seconds in sheep RBCs, respectively).

6. *What are the isotonic and hemolytic molar concentrations, isotonic coefficients, and degree of dissociation for electrolytes other that NaCl?*

The degree of electrolyte dissociation, other than NaCl, can be determined by comparing electrolyte solutions to the nonelectrolyte glucose. The preliminary estimate of degree of dissociation can be made using the molar concentrations in Experiment 1. A more specific degree of dissociation can be determined by using small increments of molar solutions around the preliminary isotonic molar concentration.

The higher the activity coefficient of an electrolyte, the more likely the electrolyte is to dissociate. At 0.1 molal concentration, NaCl, KCl, and HCl have high activity coefficients (0.778, 0.764, and 0.796, respectively) indicating that they dissociate to a large extent and are termed strong electrolytes. At 0.1 molal concentration, $CaCl_2$, H_2SO_4, and $MgSO_4$, have lower activity

coefficients (0.528, 0.313, and 0.049, respectively), dissociate to a smaller extent, and are termed weak electrolytes (Eckert and Randall, 1988).

Literature Cited

Abramoff, P. and R. G. Thomson. 1982. Movement of materials through cell membranes. Pages 109–121, *in* Laboratory outlines in biology. W. H. Freeman, New York, 529 pages.

Abramoff, P. and R. G. Thomson. 1986. Appendix C: Spectrophotometry. Pages 493-497, *in* Laboratory outlines in biology. W. H. Freeman, New York, 529 pages.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1989. The plasma membrane. (Chapter 6). Pages 276–337, *in* Molecular biology of the cell (Second edition). Garland Publishing, New York, 1217 pages.

Bakko, E. L. 1985. Cell membrane physiology. *In* Physiology laboratory manual (unpublished). St. Olaf College, Northfield, Minnesota.

Collander, R. 1954. The permeability of *Nitella* Cells to non-electrolytes. Physiologia Plantarum, 7:420–445.

Davson, H. 1959. Permeability and the structure of the plasma membrane. (Chapter 8). Pages 218–256, *in* A Textbook of general physiology (Second edition). Little, Brown and Company. Boston, 846 pages.

Eckert, R., D. Randall, G. Augustine. 1988. Permeability and transport. (Chapter 4). Pages 65–99, *in* Animal physiology (Third edition). W. H. Freeman, New York, 683 pages.

Giese, A. C. 1963. Movement of solutes through the cell membrane in response to a concentration gradient. (Chapter 12). Pages 223–243, *in* Cell physiology (Second edition). W. B. Saunders, Philadelphia, 592 pages.

Hober, R., D. I. Hitchcock, J. B. Bateman, D. R. Goddard, and W. O. Fenn. 1945. The permeability of the cells to organic nonelectrolytes. (Chapter 10). Pages 229-242, *in* Physical chemistry of cells and tissues (First edition). Blakiston Co.,. Philadelphia, 676 pages.

Strand, F. L. 1983. The plasma membrane as a regulatory organelle. (Chapter 4). Pages 49–67, *in* Physiology: A regulatory systems approach (Second edition). MacMillan, New York, 670 pages.

APPENDIX A
Sample Tables and Graphs for Introductory Laboratory

Table 7.1. NaCl and glucose concentration versus absorbance using sheep RBCs.

Solute concentration	Absorbance (Å)	
	NaCl	Glucose
0	0	0
0.05	0.03	0.01
0.06	0.07	0.03
0.08	0.08	0.05
0.1	0.10	0.08
0.125	0.14	0.68
0.16	0.18	0.70
0.2	0.19	0.73
0.25	0.72	0.75
0.3	0.78	0.80

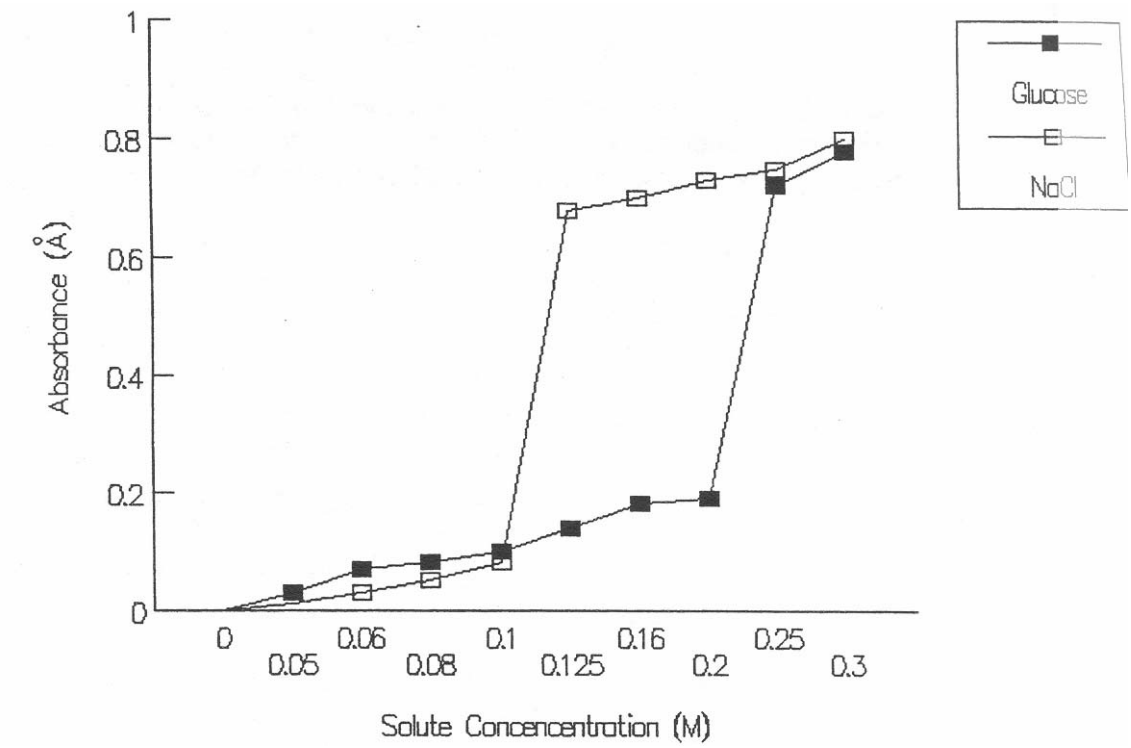

Figure 7.2. Absorbance versus concentration of NaCl and glucose using sheep RBCs.

Table 7.2. Absorbance versus time for urea, glycerol, glucose and glycerol using sheep RBCs.

Time (seconds)	Absorbance (Å)			
	Urea	Glycerol	Glucose	Sucrose
0	0.54	0.60	0.54	0.48
5	0.01	0.60	0.54	0.48
10	0	0.60	0.56	0.48
20	0	0.60	0.56	0.48
30	0	0.60	0.56	0.47
60	0	0.60	0.56	0.48
120	0	0.60	0.56	0.47
180	0	0.60	0.56	0.48
240	0	0.60	0.56	0.48
300	0	0.58	0.56	0.48
360	0	0.56	0.56	0.48
420	0	0.54	0.56	0.48
480	0	0.47	0.56	0.48
540	0	0.37	0.57	0.48
600	0	0.29	0.57	0.48
660	0	0.19	0.57	0.48
720	0	0.10	0.56	0.48
780	0	0.03	0.55	0.48
840	0	0.01	0.54	0.48
900	0	0	0.54	0.48

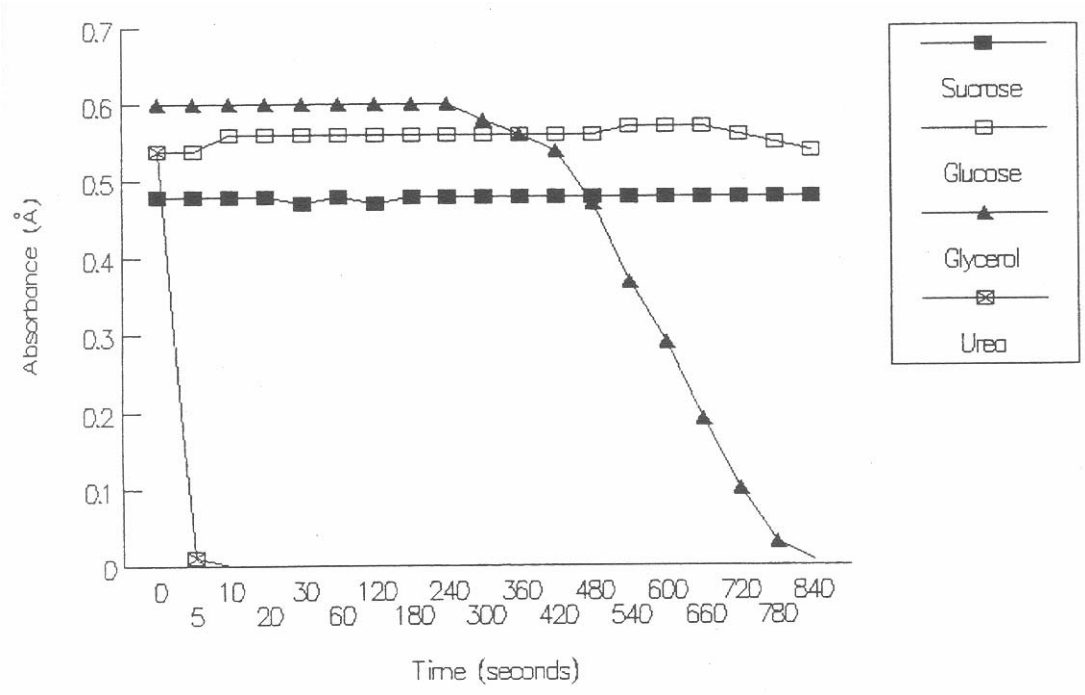

Figure 7.3. Absorbance versus time for urea, glycerol, glucose, and sucrose using sheep RBCs.

Table 7.3. Partition coefficient, molecular weight, and number of hydroxyl groups versus mean hemolysis time using sheep RBCs.

Molecule	Mean hemolysis time (minutes)	Partition coefficient (oil:water) ($\times 10^4$)	Molecular weight	Number of hydroxyl groups ($\times 10$)
Ethanol	0.14	320	42	10
Ethylene glycol	0.27	4.9	62	20
Glycerol	22.0	0.7	92	30

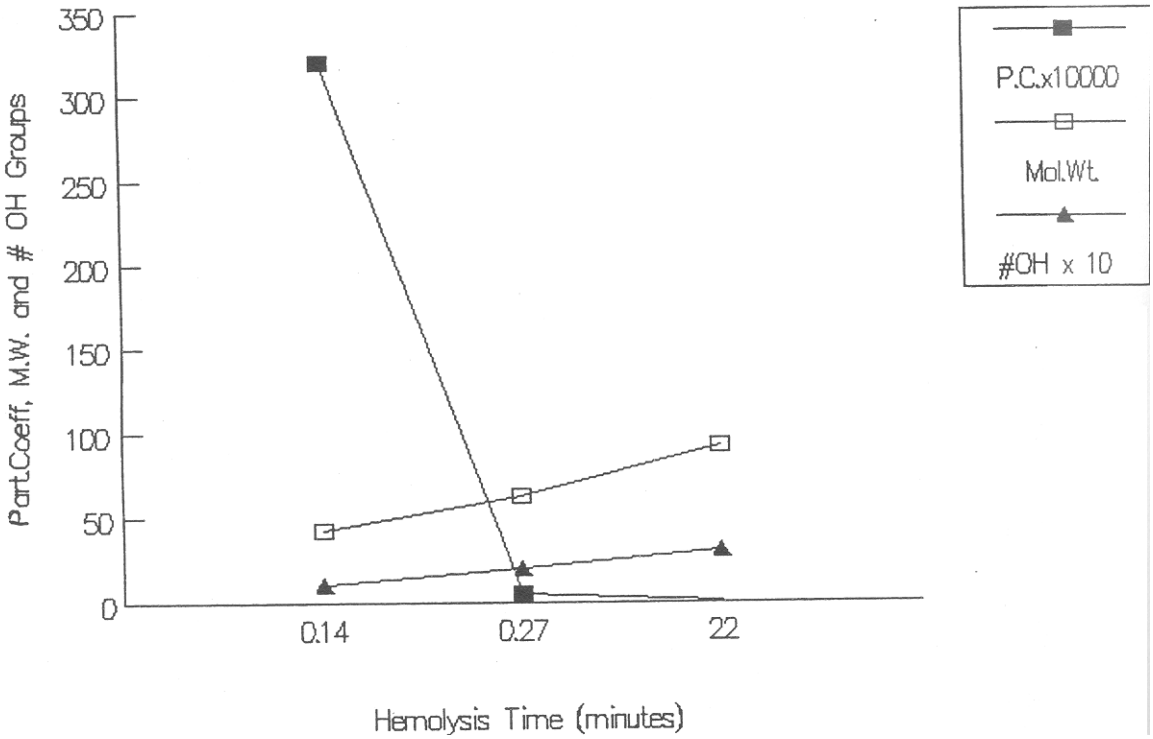

Figure 7.4. Partition coefficient (P.C.), molecular weight (Mol. Wt.), and number of -OH groups (#0H) versus mean hemolysis time using sheep RBCs.

APPENDIX B
Information Tables for Investigative Laboratory

Table 7.4. Molecular formulae and weights and partition coefficients for alcohol, urea, amide, and sugar series.

Molecule and molecular formulae	Molecular weight	Partition coefficient[1] (oil:water)
Alcohol Series		
Methyl (CH_3OH)	32	0.0078
Ethyl (C_2H_5OH)	46	0.032
Propyl ($CH_3CH_2CH_2OH$)	60	0.13
n-Butyl ($CH_3CH_2CH_2CH_2OH$)	74	0.25
Ethylene glycol (CH_2OHCH_2OH)	62	0.00049
Glycerol ($CH_2OHCHOHCH_2OH$)	92	0.00007
Urea Series		
Urea (H_2NCONH_2)	60	0.00015
Methyl Urea ($NH_2CONHCH_3$)	74	0.00044
Thiourea (H_2NCSNH_2)	76	0.0012
Dimethylurea	88	0.0023
($CH_3NHCONHCH_3$)	116	0.0076
Diethylurea ($(C_2H_5)_2NCONH_2$)		
Amide Series		
Formamide ($HCONH_2$)	45	0.00076
Acetamide (CH_3CONH_2	59	0.00083
Propioamide ($CH_3CH_2CONH_2$)	73	0.0036
Butyramide ($CH_3CH_2CH_2CONH_2$)	87	0.0095
Lactamide ($CH_3CH(OH)CONH_2$)	89	0.00075[2]
Malonamide	102	0.00008[2]
($H_2NCOCH_2CONH_2$)		
Sugar Series		
Erythritol	122	0.00003[2]
($CH_2OH(CHOH)_2CH_2OH$)	182	–
Mannitol ($C_6H_{14}O_6$) (with 6-OH)	150	–
Arabinose ($C_5H_{10}O_5$) (with 4-OH)	180	–
Glucose ($C_6H_{12}O_6$) (with 5-OH)	342	0.00003[2]
Sucrose ($C_{12}H_{22}O_{11}$)		

1. Collander (1954)
2. From Collander (1954); data in Davson (1959).
Note: For list of partition coefficients for molecules other than those listed here see Collander (1954).

Table 7.5. Hemolysis time for various mammalian erythrocytes for 0.3 M glycerol in 0.012% NaCl (Giese, 1963).

Mammal	Time (seconds)	Mammal	Time (seconds)
Rat	3.5	Dog	253
Mouse	12.9	Cat	459
Rabbit	21.8	Pig	340
Guinea pig	38.2	Ox	612
Human	5.1	Sheep	850