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Osmosis and Transport

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Rabbit red blood cells (RBCs) are used to study osmosis and membrane transport. In Activity 1, students study red blood cells as osmometers, devices with which they can estimate the relative osmolarity of solutions using a spectrophotometer. Students use their results to estimate the water content of RBCs. Activity 2 explores the relative permeability of RBC membranes to glycerol and the transport of glycerol into RBCs. Activity 3 looks at the facilitated diffusion of electrolytes using the antibiotic valinomycin as a mobile carrier of K+ ions and Activity 4 examines water movement across cell membranes via aquaporin protein complexes.

Keywords: osmosis, transport, rabbit red blood cells, valinomycin, aquaporin

Introduction

The laboratory exercise, **Osmosis and Transport**, is one in a series that are closely coordinated with lectures in Honors Biology I: Biochemistry and Cellular Physiology, the first term of an advanced three term sequence of classes for University of Oregon (UO) Biology and Biochemistry majors. The Biochemistry term is followed by Honors II: Genetics and Molecular Biology and Honors III: Ecology, Evolution and Biodiversity. This set of three courses, each with an associated laboratory component, presents students with the key principles and relationships that underlie much of Biology. The prerequisites for enrollment in the courses are completion of General Chemistry and College Algebra. Many students concurrently enroll in Organic Chemistry. The lab is designed to focus students' intellectual effort on the lab exercise itself, instead of on the lab report. That is, we want students to be intellectually engaged while working on the lab in the lab room, where help is available, not later, while writing the report. In the case of this lab, Osmosis and Transport, we also wanted to address specific aspects of the mechanisms of osmosis and facilitated diffusion and one possible laboratory method of investigation. The exercise is done during a three-hour lab period with 16 students, one faculty member and two teaching assistants (one graduate, one undergraduate). The lab work is structured to create a cooperative environment that encourages both group work and questions from individuals. The text of the lab manual is designed to provide continual intellectual challenge, rather than passive data collection. The lab utilizes common, reliable, simple technology and takes about a day to prepare.

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Student Outline

Osmosis and Transport

Pre-lab Assignment **Name:** ______________________________

Assignment:

- Read the lab exercise, focusing on the Overview section and the introductory material for each activity.
- Answer the questions below.

Questions:

1. What is the osmolarity of each of the following (include units):
   - 1 mM Glucose
   - 1 mM NaCl
   - 1 mM $CaCl_2$

2. Red Blood Cells (RBCs) are isotonic with 150 mM NaCl

   a. What happens when the RBCs are transferred to a 120 mM NaCl solution?

   b. What happens when 120 mM impermeable solute is added to the RBCs in the 120 mM NaCl solution?

3. The following figures show the way in which cell volume changes after the addition of two different permeable solutes (A and B) to cells that were originally in solutions of the same composition.

   a. Which solute had the higher osmolarity?

   b. To which solute are the cells more permeable?

4. What mechanisms do cells use to control their osmotic balance and, hence, water content?

Objectives

- To understand the factors that affect the osmotic balance of cells
- To understand how we can measure the size of RBCs in suspension
- To understand how cells transport materials across their membranes
- To understand how cells regulate their size

Overview

Inside cells the various properties are maintained relatively constant despite a drastically different and often changing environment. The cell membrane lies at the interface of the controlled interior and the fluctuating exterior, and represents not only the structural but also the functional boundary of the cell. One of the important mechanisms by which a cell achieves independence from its environment is by regulating the movement of mass across the surface of the membrane. Despite its delicate thickness (~7.5 nm), a biological membrane can 1) select among different molecular species, slowing down the permeation of some materials while allowing others to pass almost unimpeded, and 2) transport of material either inward or outward.

The first experimental evidence for the existence of biological membranes came from observations in the 1800s that cells swell or shrink when placed in solutions containing lower or higher concentrations of NaCl. The observed changes in cell volume are due to water movement in response to the changed concentration of water (more accurately, change in its thermodynamic activity) caused by the dissolved solute molecules. If the concentration of water is different in two regions, there will be a negative free energy change associated with water movement from the higher concentration (i.e., where there is less solute) to the lower concentration (i.e., where there is more solute). In this respect, water is no different from any other substance; it moves or changes to lower its free energy. The movement of water toward regions where its concentration is lower is called osmosis.

Red blood cells offer several advantages for the study of osmosis and membrane transport. The most important is the absence of internal compartments (nuclei, mitochondria, etc.). In addition, red blood cells are readily available (anyone can grow them) and they are quite stable during storage.

This laboratory is divided into three parts. In Activity 1 we study red blood cells (RBCs) as osmometers, that is, as devices with which we can estimate the relative osmolarity of solutions. In Activity 2 we measure the relative permeability of RBC membranes to glycerol and the transport of glycerol into RBCs. In Activity 3, we examine the facilitated transport of electrolytes. Activity 4 explores protein mediated water movement across the lipid bilayer.

Theoretical Background: Red Blood Cells as Osmometers

The osmolarity of normal blood is approximately 300 milliosmolar (mOsM). Quantitatively, by far the most important solutes are NaCl and $NaHCO_3$. For our purposes we will consider blood as a solution of NaCl (150 mM) at pH 7.4. Red blood cells do not change size while they are in blood or in 150 mM NaCl; this concentration of NaCl is said to be isotonic and iso-osmolar with RBCs. This means that the concentration of impermeant solutes inside RBCs is 300 mOsM and that NaCl is also impermeant (or very nearly so).

When RBCs are placed in a more dilute solution of NaCl (a *hypotonic* solution), water enters the cells by osmosis and the cells swell. If the solution is sufficiently dilute the volume will increase to such an extent that the cell membrane ruptures and the cells lyse (hemolysis). When RBCs are placed in a more concentrated solution of NaCl (**hypertonic**), water leaves the cells and they shrink. When we say red blood cells can be used as osmometers, this is what we are referring to: how much a red blood cell swells or shrinks can tell you something about the osmolarity of the solution it is in.

To be more precise, imagine that RBCs are removed from blood and suspended in a 75 mM solution of NaCl (150 mOsM). Water will flow into the cells until the intracellular solution has been diluted to 150 mOsM; the volume of the intracellular solution obviously will increase two-fold. The cells are again at osmotic equilibrium with the outside. (We assume here, and throughout, that the volume of the outside solution is so large that movements of water or solutes in or out of the cells do not affect the concentrations in the outside solution.) Now imagine that RBCs are suspended in a 300 mM NaCl solution (600 mOsM). In this case, water leaves the cells until the intracellular solution is also 600 mOsM; the volume of the internal solution is reduced two-fold and the cells are again at osmotic equilibrium with the outside. Thus, the volume of the internal solution is inversely proportional to the osmolarity of the external solution. We can express this mathematically in the following equation:

$$V_w = k/C_o$$

where V_W is the volume of the internal solution and C_o is the osmolarity of the external solution. k is a proportionality constant; a plot of V_W vs $1/C_o$ is a straight line with slope k.

If we want to observe this relationship in real cells, there are several complicating factors. First, because real cells are not

just bags full of water, the volume of the internal solution (V_w) is always less than the total volume of the cell (V_c). In RBCs a large fraction of the difference between V_c and V_w represents the volume occupied by hemoglobin, in addition to other cellular components. To account for this volume from which water is excluded, the equation must be modified by making use of the relationship:

$$V_c = V_w + b$$

where b is the volume not occupied by water. Remembering our first equation, we can substitute k/C_o for V_W:

$$Vc = (k/C_o) + b$$

A second complicating factor in observing the effect of changing osmolarity on cell volume is that there isn't an easy way to measure the volume of a cell directly. However, it is possible to measure relative cell volumes of large numbers of cells at different osmolarities. We can express cell volume (V_c) as a fraction of the cell volume in blood or an osmotically equivalent solution (e.g., 150 mM NaCl).
We'll call this:

$$V_c' = \text{the } \underline{\text{relative volume}} = V_c/V_{300}$$

Where V_{300} represents the volume of the cell in an isotonic solution (300 mOsm). If the cell is in a hypotonic solution (< 300 mOsm), the cell will swell and V_c' will be greater than 1; if it is in a hypertonic solution (>300 mOsm), Vc' will be between 0 and 1. When the cell is in an isotonic solution, $V_c' = 1$. As you will see in Activity 1, V_c' can be determined using a spectrophotometer.

Similarly, the concentration of the impermeant solute on the outside (C_o), can be expressed as a fraction of the osmolarity of blood. Thus,

$$C_o' = \text{the } \underline{\text{relative concentration}} = C_o/C_{300}$$

Where C_o is the osmolarity of the solution the blood cells are in and C_{300} is 300 mOsm. Hypotonic solutions will have a C_o' between 0 and 1, hypertonic solutions will have a C_o' greater than 1, and isotonic solutions will have a C_o' equal to 1.

We can use V_o' and C_o' to determine one particular value of biological interest: the fraction of water in a normal blood cell under normal (300 mOsm) conditions in blood. We can call this W. There is a precise mathematical relationship between V_c', C_o', and W, which we can determine using the equations above plus some algebra (see Supplement 1). The resulting relationship is:

$$V_c' = (W/C_o') + (1-W)$$

This equation represents a line, and has a slightly modified y = mx + a format you may be familiar with. $1/C_o'$ is the independent variable, x, which can be plotted on the x-axis of a graph. V_c' is the dependent variable, y, which can be plotted on the y-axis. W is the slope of the line (m), and 1-W is the y-intercept (a). In Activity 1, you will collect data for V_c' at different osmolarities; this will allow you to plot a graph and determine W, the slope of the line.

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Measurement of Cell Volume

The most accurate method to measure the volume of the RBCs is to centrifuge a measured volume of a suspension of cells in a narrow tube of known internal diameter, measure the length of the column of packed cells, and calculate the volume. This is the hematocrit procedure used in clinical blood tests. A more convenient procedure and the one we will use, is based on the fact that the amount of light transmitted by a suspension of RBCs increases as cell volume is increased by water uptake. The swelling reduces the refractive index of the cells and thus increases their ability to transmit, rather than scatter, light. In fact, over a fairly wide range of cell volumes (from ~0.6 to 1.8 times the volume in blood) light transmitted is almost exactly proportional to cell volume. Hence, in the equations above we can replace cell volume (V_c) with per cent transmitted light (% T).

$$V_c' = \%T'$$

$$\%T' = (W/C_o') + (1-W)$$

Thus, we can calculate the relative fraction of the cell volume taken up by water, W, by measuring the slope of %T' vs $1/C_o'$.

Obviously, the transmittance also depends on the cell concentration; the more cells there are the less light the suspension will transmit. However, the cell concentration is the same in all the experiments in this lab so you need not take this factor into account. We use per cent transmitted light rather than optical density because the former is directly related to cell volume whereas the latter is inversely related. This makes it easier to think about the experiments as you are doing them.

General Procedures

We will use rabbit RBCs. Before class, blood was drawn, the cells were centrifuged and the serum discarded. The cells were then washed, equilibrated in 150 mM NaCl and stored on ice until class. Keep the RBC suspensions on ice until just before use. Make all your measurements at room temperature. Before pipetting suspensions of RBCs be sure to mix them well by swirling the flask several times. Before making measurements in the spectrophotometer, mix the solutions well. The cells settle quite rapidly, and if you do not mix the suspension your samples will not be representative.

Activity 1. Red Blood Cells as Osmometers

In this experiment you will determine the relative volume of RBCs in various concentrations of NaCl. Each pair of students should have the following materials:

a. 12 spectrophotometer tubes (1 blank)

b. Suspension of RBCs in 150 mM NaCl (about 4.5 ml)

c. About 20 ml of 450 mM NaCl

d. About 20 ml of Tris HCl buffer (1 mM, pH 7.4)

(Notes: All solutions used in this exercise are made up in Tris HCl buffer, 1 mM, pH 7.4.)

A. Number the tubes and add the volumes of 450 mM NaCl and buffer shown in the table below. DO NOT ADD THE CELLS YET. Mix well. The final concentration of NaCl is shown for the first two tubes; you are to figure out the rest and the osmolarity for all of them. Do this first so that you will be ready to plot the results.

B. Set the spectrophotometer to 680 nm. Zero the spectrophotometer (0% absorption, 100% transmission) with a buffer blank. Mix the cells well to resuspend them. Add 0.3 ml of cell suspension to each tube and mix. After RBCs have been added to all tubes MIX WELL and read the percent transmission (%T) of each tube one after the other. Make your measurement right after inserting the tube into the spectrophotometer. Otherwise the cells will start to settle and change your measurement. Record the results on the following data sheet.

%T vs [NaCl]

Tube	450 mM NaCl	Tris buffer	Cell suspension (in 300 mOsM NaCl)	Final NaCl mM	Final NaCl mOsM (C_o)	%T
1	-	2.7 ml	0.3 ml	15		
2	0.5 ml	2.2 ml	0.3 ml	90		
3	0.7 ml	2.0 ml	0.3 ml			
4	0.9 ml	1.8 ml	0.3 ml			
5	1.1 ml	1.6 ml	0.3 ml			
6	1.3 ml	1.4 ml	0.3 ml			
7	1.5 ml	1.2 ml	0.3 ml			
8	1.7 ml	1.0 ml	0.3 ml			
9	1.9 ml	0.8 ml	0.3 ml			
10	2.1 ml	0.6 ml	0.3 ml			
11	2.3 ml	0.4 ml	0.3 ml			

C. Use the following table to record the results of the following calculations:

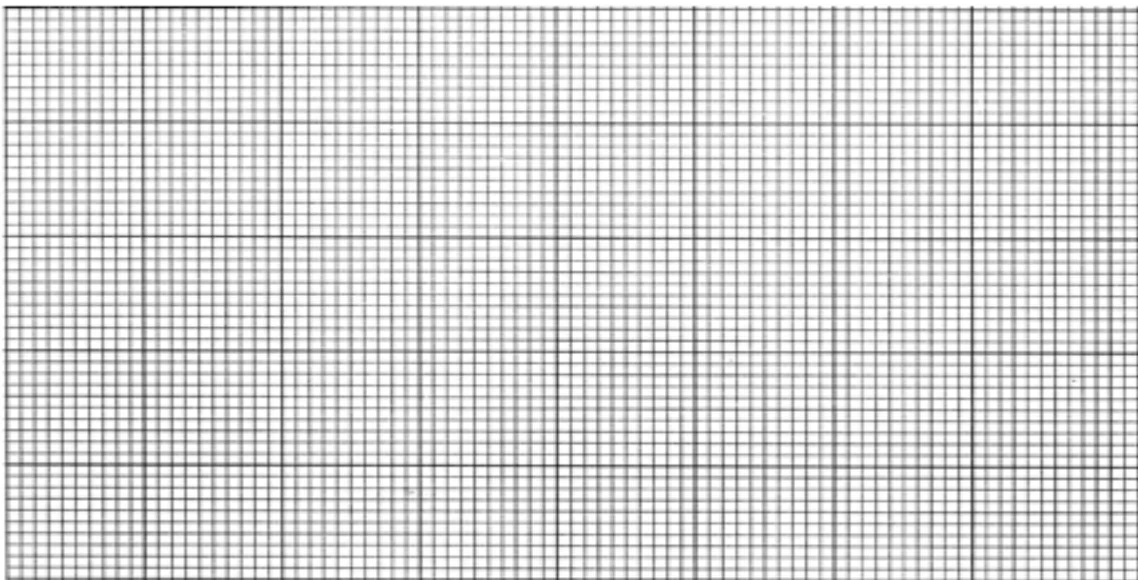
- Calculate the reciprocal of the final osmolarities ($1/C_o$).
- Calculate $1/C_o$' for each tube as a fraction of the reciprocal of the iso-osmolar concentration (300 mOsM); this relative (normalized) value is $1/C_o$'. To do this, divide each $1/C_o$ value by the value obtained for the 300 mOsM tube ($1/C_o$ value from Tube #4).
- Calculate %T as a fraction of %T in iso-osmolar solution; this is %T'. To do this, divide each %T value by the value obtained for the 300 mOsM tube (%T value from Tube #4).

Summary of Calculations

Tube	$1/C_o$	$1/ C_o$’	%T	%T’
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				

D. Plot %T' vs $1/C_o$'. Do not plot the first point (tube 1). Why not?

%T ' vs $1/C_o$'



E. Use your data to estimate W, the fraction of the total cell volume that is water, using two different methods:

$$\text{From: } V_o' = (W/C_o') + (1\text{-}W)$$

1. W = the slope of %T' vs $1/C_o'$

2. W = [1- the %T' intercept (where $1/ C_o'=0$)]

Activity 2. Permeability to Glycerol

We can make use of the osmotic behavior of RBCs to measure the rates of transport of permeant solutes. Imagine a suspension of RBCs in 120 mM NaCl (240 mOsM). The osmolarity of the cell contents will also be 240 mOsM and the cells will be a little larger than they are in blood. This is because the volume of the internal solution will be 300/240 = 1.25 times its volume in blood (see Fig. 1). Now imagine that we add a permeant, non-ionizable solute to the outside at a concentration of 120 mM. The osmolarity of the solution will be 360 mOsM (240 + 120). Because water moves across the cell membrane very much faster than any solute, the volume of the internal solution will very quickly decrease to 83% (300/360) of its initial volume before any of the permeant solute has entered the cells. The concentration of NaCl inside the cells will now be 180 mM (360 mOsM) and the internal and external solutions will be at osmotic equilibrium (see Fig. 2). There is, however, an inwardly directed concentration gradient of the permeant solute (120 mM on the outside, 0 on the inside) and the solute will begin to enter the cells. As it does, the internal osmolarity will tend to increase above 360 mOsM and water will re-enter the cells to maintain osmotic equilibrium. The cell volume will accordingly increase and the rate of this increase will be equal to the rate of entry of the permeant solute. This is the important point for us. Eventually, the cells will return to their initial volume, and the concentration of NaCl will be 120 mM inside and outside and the concentration of the permeant solute will also be 120 mM inside and outside (see Fig. 3). In other words, the concentration of a permeant solute has no effect on the cell volume at equilibrium.

Inside (RBC)		Outside	
KCL	120 mM	NaCl	120 mM
S	0 mM	S	0 mM
240 mOsM		240 mOsM	

Figure 1. RBC in 120 mM NaCl.

Inside (RBC)		Outside	
KCL	180 mM	NaCl	120 mM
S	0 mM	S	120 mM
360 mOsM		360 mOsM	

Figure 2. RBC in 120 mM NaCl and 120 mM permeant solute (S), after osmosis and before solute movement.

KCL 120 mM

S 120 mM

360 mOsM

NaCl 120 mM

S 120 mM

360 mOsM

Figure 3. RBC in 120 mM NaCl and 120 mM permeant solute (S), after reaching equilibrium.

In this activity you will observe the movement of the permeant solute, glycerol, into RBCs. You will add a suspension of cells in 120 mM NaCl to a solution containing 120 mM NaCl and glycerol at 360 mM. Recall that we expect an initial, very rapid decrease in volume (and %T) as water leaves the cells followed by an increase in volume (and %T) as the solute and water enter the cells. The rate of this second phase is a measure of the rate of entry of the solute. Before beginning the experiments consider what deter¬mines whether or not you can observe these predicted effects with our procedure and instrumentation.

A. %T in 120 mM NaCl

Add 0.3 ml of the cell suspension (in 120 mM NaCl) to 2.7 ml of 120 mM NaCl, MIX WELL, and record %T.

%T in 120 mM NaCl: ___________

Retain this tube and recheck %T (after remixing) from time to time to make sure that the instrument hasn't drifted.

B. Glycerol

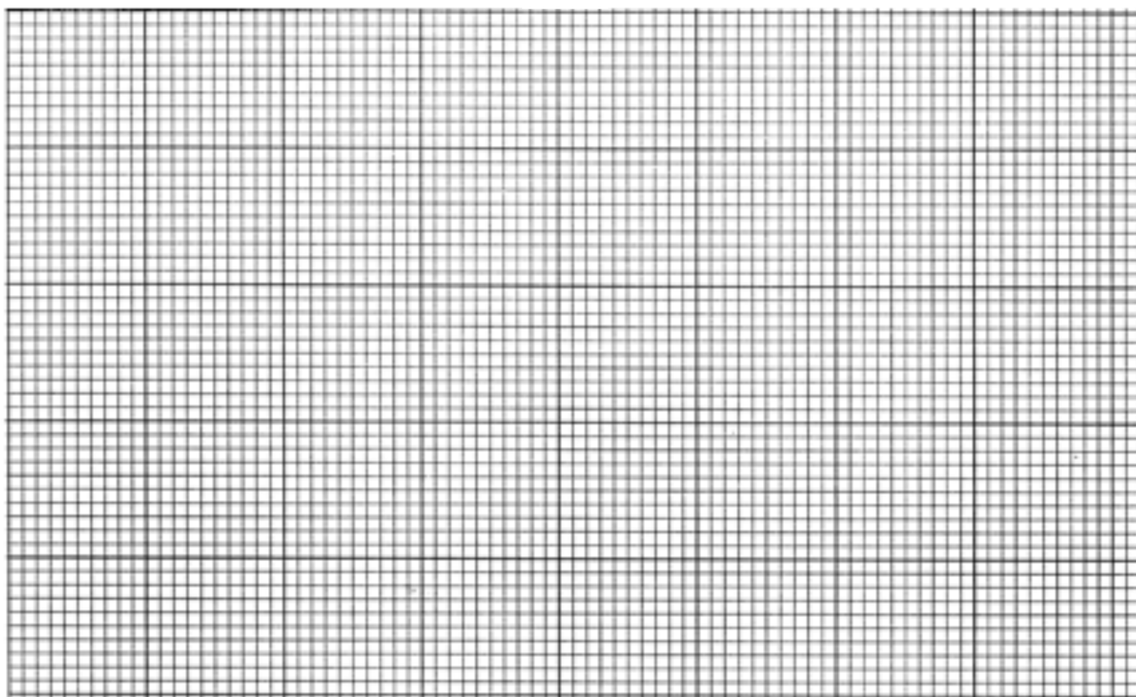
Obtain a tube with 120 mM glycerol in 120 mM NaCl. Add 0.3 ml of the cell suspension, mix rapidly and read %T **as soon as possible** and at 10 second intervals during the first minute. Then (after remixing) measure %T once per min. for about 5 min. Record the results in the following table.

Time	%T	Time	%T

1. How does the value of %T in presence of glycerol compare with the value in 120 mM NaCl?

2. Plot the %T as a function of Time:

%T vs Time



3. Explain what has happened during the first part of the experiment.

4. Why does %T recover towards the value in 120 mM NaCl?

5. What can you say about the rate of entry of glycerol?

Activity 3. Transport of Electrolytes

Background on Ionophores

Our understanding of mediated transport has been enhanced by the study of ionophores, substances that vastly increase the permeability of the membranes to particular ions.

Ionophores May Be Carriers or Channel Formers

Ionophores are organic molecules of diverse types, many of which are antibiotics of bacterial origin. Cells and organelles actively maintain concentration gradients of various ions across their membranes. The antibiotic properties of these ionophores arise from their tendency to discharge these vital concentration gradients.

There are two types of ionophores:

1. Carriers, which increase the permeabilities of membranes to their selected ion by binding it, diffusing through the membrane, and releasing the ion on the other side.
2. Channel formers, which form trans-membrane channels or pores through which their selected ions can diffuse.

Valinomycin is a carrier molecule that specifically binds K+ ions and can transport up to 10^4 K+ ions/second across a membrane. The X-ray structure of valinomycin's K+ complex indicates that the K+ is octahedrally coordinated by the carbonyl groups of its 6 Val residues, which also form its ester linkages. The cyclic, intramolecular hydrogen bonded valinomycin backbone follows a zigzag path that surrounds the K+ ion with a sinuous molecular bracelet. Its methyl and isopropyl side chains project outward from the bracelet to form a spheroidal complex with a hydrophobic exterior that makes it soluble in the hydrophobic core of membranes. Uncomplexed valinomycin has a more open conformation than its K+ complex, which presumably facilitates the rapid binding of K+.

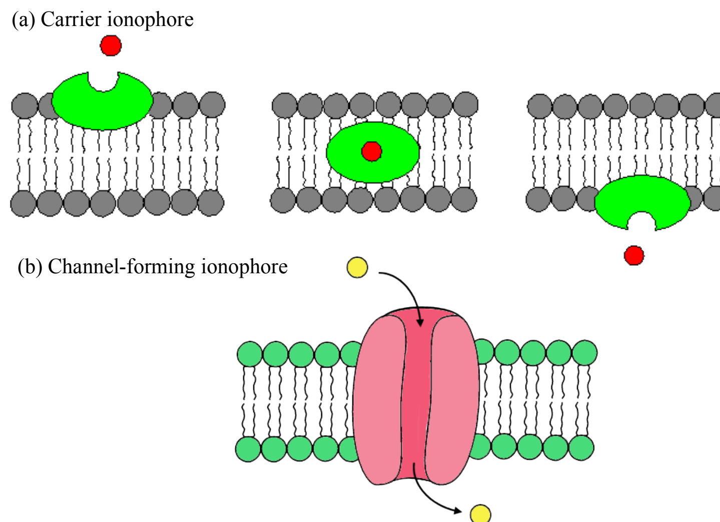


Figure 4. The ion transport modes of ionophores: (a) Carrier ionophores transport ions by diffusing through the lipid bilayer. (b) Channel forming ionophores span the membrane with a channel through which ions can diffuse. (Source for (a): Public Domain image, downloaded from commons.wikimedia.org. Source for (b): image by Danielchemik, used under Creative Commons Attribution-Share Alike 3.0 Unported license, downloaded from commons.wikimedia.org.)

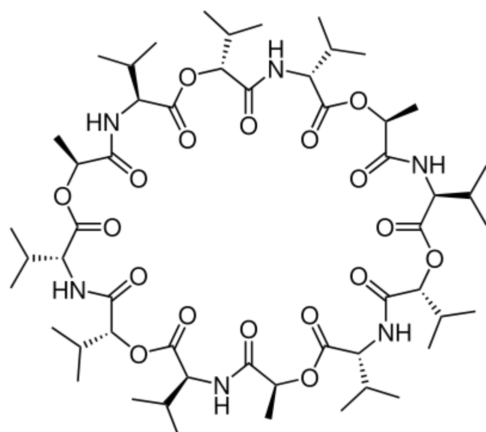


Figure 5. Valinomycin is a cyclic depsipeptide (has both ester and amide bonds) that contains both D- and L- amino acids. (Source: Public Domain image, downloaded from commons.wikimedia.org.)

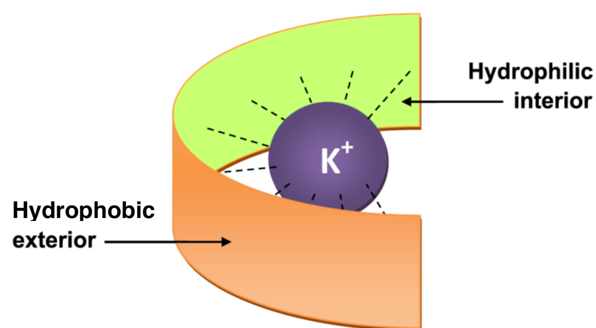


Figure 6. Valinomycin structure, complexed with potassium. (Source: image by Danielchemik, used under Creative Commons Attribution-Share Alike 3.0 Unported license, downloaded from commons. wikimedia.org.)

RBC membranes are virtually impermeable to Na+ and only slightly permeable to K+ ions. The permeability to K+ can be greatly increased by adding valinomycin, a compound that binds K+ ions specifically and transports them across the membrane. In this experiment, you will compare the relative effects of adding valinomycin to RBCs in isotonic Na+ or K+.

Effect of Valinomycin on permeability to Na+ and K+

Obtain four tubes containing the following:

a) 2.7 ml of 120 mM KSCN

b) 2.7 ml of 120 mM KSCN + valinomycin (0.67 µM)

c) 2.7 ml of 120 mM NaSCN

d) 2.7 ml of 120 mM NaSCN + valinomycin (0.67 µM)

We use the thiocyanate salts of K and Na because membranes are quite permeable to the lipid soluble thiocyanate anion; we are concerned only with the permeabilities of cations.

- To tube a and b add 0.3 ml cells in 120 mM KSCN
- To tube c and d add 0.3 ml cells in 120 mM NaSCN

You can make the measurements from all 4 tubes together. For each tube, add the cells, mix, and record the initial %T. Then at 1 min. intervals, remix and record %T for each tube until the %T stops changing.

KSCN				**NaSCN**			
Tube a		**Tube b**		**Tube c**		**Tube d**	
Time	% T	Time	% T	Time	% T	Time	% T

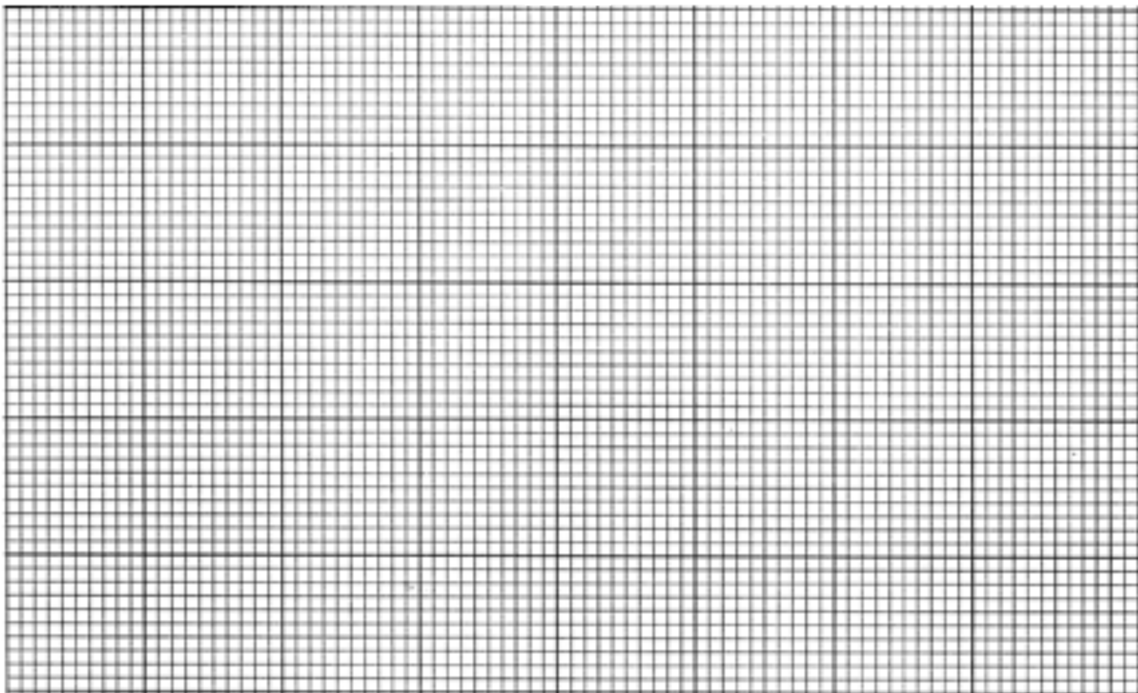
1. In tubes a and b (with KSCN) in what direction is electrolyte moving (if at all)?

2. In tubes c and d (with NaSCN) in what direction is electrolyte moving (if at all)?

3. What is the effect of valinomycin on permeability of the cell to K+? To Na+?

4. Plot the %T as a function of Time for tubes b and d:

%T vs Time



5. Explain what has happened to the cells in tubes b and d.

Activity 4. Aquaporins – The Cell 's Plumbing System

Aquaporins selectively conduct water molecules in and out of the cell, while preventing the passage of ions and other solutes. Also known as water channels, aquaporins are integral membrane pore proteins. An individual aquaporin protein is made up of six transmembrane α-helices arranged in a right-handed bundle (Fig. 7), with the amino and the carboxyl termini located on the cytoplasmic surface of the membrane. Aquaporins form tetramers in the cell membrane to form a water channel (Fig. 8).

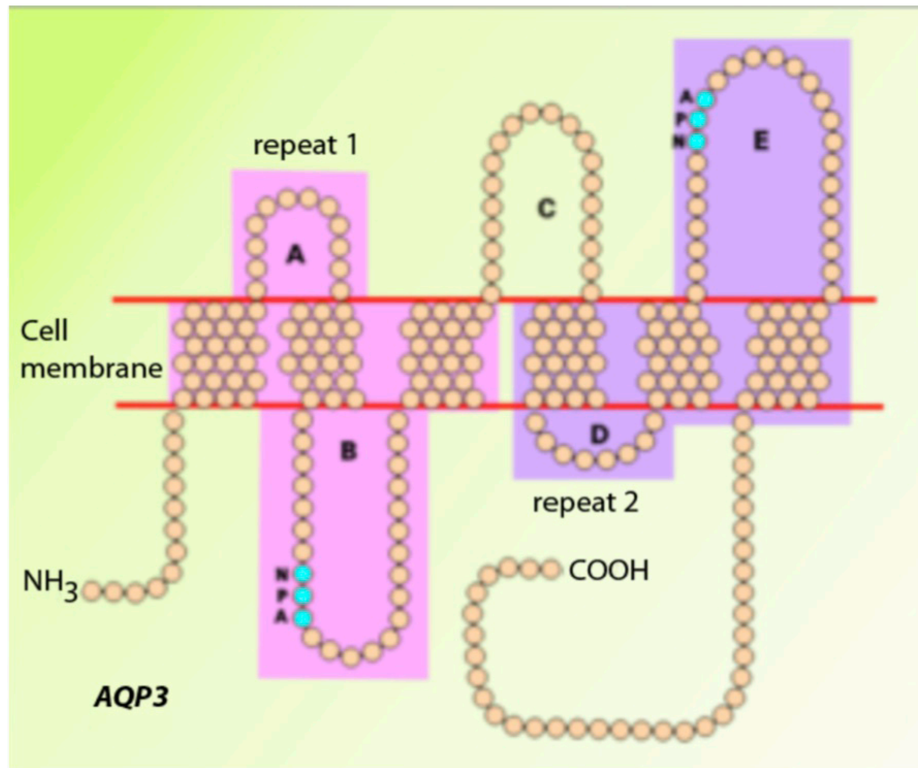


Figure 7. Schematic structure of aquaporin. (Source: image by Opossum58, used under Creative Commons Attribution-Share Alike 3.0 Unported license, downloaded from commons.wikimedia.org.)

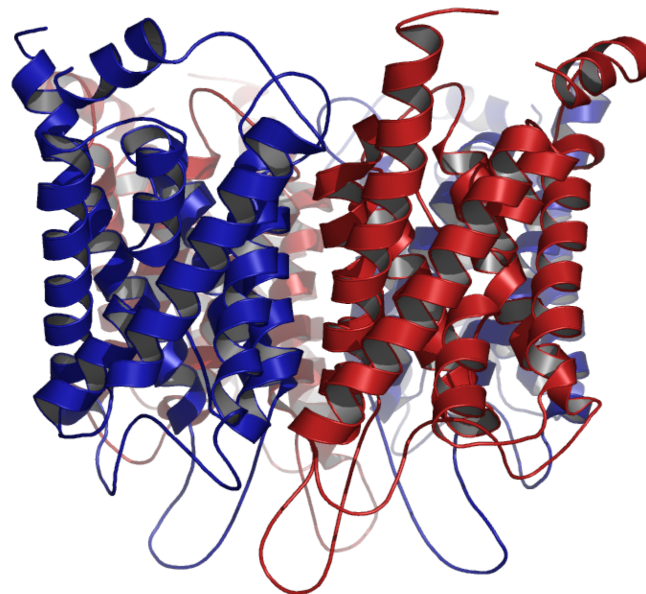


Figure 8. Aquaporin tetramer, side view. (Source: image by Kauczuk, used under Creative Commons Attribution-Share Alike 3.0 Unported license, downloaded from commons.wikimedia.org.)

The presence of water channels increases membrane permeability to water. A single aquaporin channel can facilitate water transport at a rate of roughly 3 billion water molecules per second. Water molecules traverse through the pore of the channel in single file. The narrowest part of the channel is lined by a cluster of amino acids that help bind water molecules (Fig. 9). The narrow pore acts to weaken the hydrogen bonds between the water molecules allowing the water to interact with the positively charged amino acid side chains.

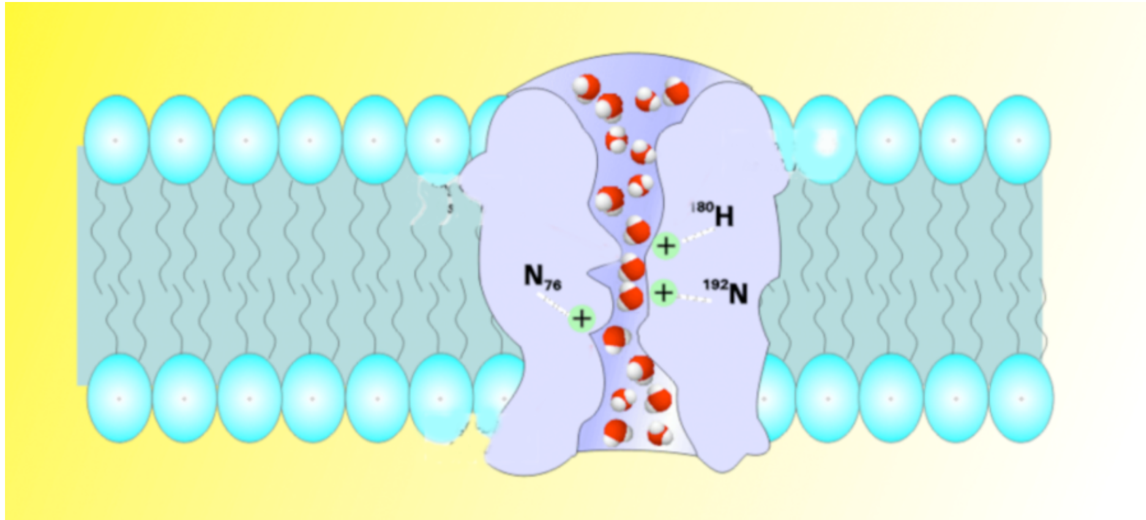


Figure 9. Aquaporin in the cell membrane. (Source: image by Opossum58, used under Creative Commons Attribution-Share Alike 3.0 Unported license, downloaded from commons.wikimedia.org.)

1. Do you expect protons to pass through the aquaporin channel? Why or why not?

2. Is it a problem if they do?

More than 10 different mammalian aquaporins have been identified to date, and additional members are suspected to exist. Two aquaporins, aquaporin-2 and aquaporin-3, are important for function of our kidneys and are expressed by cells that line the kidney ducts, which collect urine for excretion. Duct cells regulate water movement by changing the number of aquaporin-2 molecules in the cell membrane. Aquaporin-2 resides in a pool of membrane vesicles within the cytoplasm (Fig. 10). By inserting aquaporin-2 into the apical membrane, cells are now able to take up water efficiently from the lumen of the kidney duct. Aquaporin-3 is constitutively expressed and inserted into the basolateral membrane of kidney duct cells. When water floods into the cells through aquaporin-2 channels, it can rapidly exit the cells through the aquaporin-3 channels and flow into blood.

3. How does aquaporin-2 get to the apical cell membrane?

4. What do you predict will happen if aquaporin-2 levels are too high?

5. Mutations in the aquaporin-2 gene cause hereditary nephrogenic diabetes insipidus. What do you think the symptoms are and why?

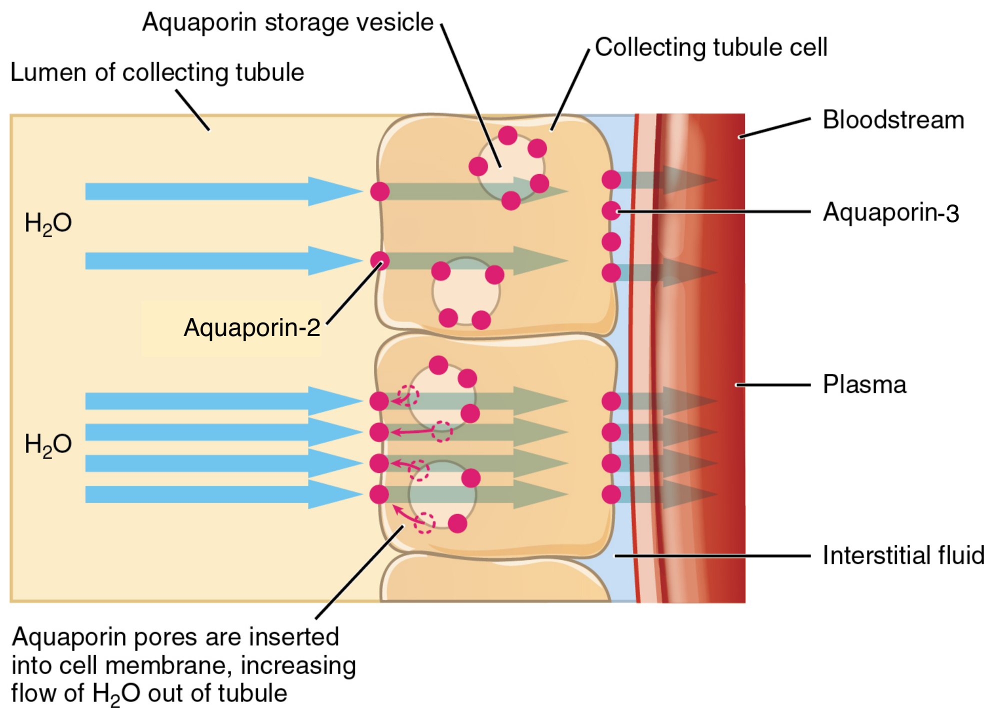


Figure 10. Aquaporins in kidney collecting tubules. (Source: OpenStax College, Anatomy & Physiology, cnx.org/content/col11496/1.6/, used under Creative Commons Attribution-Share Alike 3.0 Unported license, downloaded from commons.wikimedia.org.)

Supplement

Student Handout

This supplement explains the derivation of the equation described in **Theoretical Background: Red Blood Cells as Osmometers**.

We will start by re-writing the main equation relating volume and osmolarity,

$$V_c = (k/C_o) + b$$

in terms of relative volume and osmolarity. Remember that:

$$V_c' = V_c/V_{300}$$

If we solve this equation for V_c, we get:

$$V_c = V_c' \times V_{300}$$

We can then replace V_c in our main equation with the values above:

$$V_c' \times V_{300} = (k/C_o) + b$$

and solve for V_c' :

$$V_c' = (k/(C_o \times V_{300})) + b/V_{300}$$

If we then do a similar set of algebraic manipulations for C_o:

$$C_o' = C_o/C_{300} \qquad C_o = C_o' \times C_{300} \qquad V_c' = (k/(C_o' \times C_{300} \times V_{300})) + b/V_{300}$$

We find that our equation becomes:

$$V_c' = ((k/(C_{300} \times V_{300})) \times 1/C_o') + b/V_{300}$$

Now our equation is written completely in terms of relative volumes and concentrations. Note that this equation still takes the basic form of a line (y = mx + a), with $y = V_c'$, $x = 1/C_o'$, $m = k/(C_{300} \times V_{300})$, and $a = b/V_{300}$. While x and y are variable, m and b are both constants. Although it looks messy, if you step back for a moment and think about what this represents, you can see that if you vary x (the osmolarity), it has an effect on y (the cell volume).

The first term (mx, or $(k/(C_{300} \times V_{300})) \times 1/C_o'$)) represents the contribution of water to the relative cell volume, V_c' , since it varies with relative concentration changes. This means that the second term (a, or b/V_{300}) represents the non-water contribution to relative cell volume.

Now we'll turn our attention to W, which is defined as the fraction of water in a normal blood cell under normal (300 mOsm) conditions in the blood. We can write this in terms of the ratio of water volume to total cell volume at 300 mOsm:

$$W = V_{w300} / V_{c300}$$

Also recall that we have the two equations:

$$V_c = V_w + b \qquad \text{and} \qquad V_c = (k/C_o) + b$$

We can substitute these into our equation for W by first substituting $V_w = V_c - b$ into the top:

$$W = (V_{c300} - b)/ V_{c300}$$

And then $V_{c300} = (k/C_{300}) + b$ into the bottom:

$$W = (V_{c300} – b) / ((k/C_{300}) + b)$$

Finally, we note that $V_c = (k/C_o) + b$ implies that $b = V_c – (k/C_o)$ and we substitute this value for b into our equation for W:

$$W = (V_{c300} – V_{c300} – (k/C_{300})) / ((k/C_{300}) + V_{c300} – (k/C_{300}))$$

This looks messy at first glance but 4 of the terms cancel out leaving us with:

$$W= k/(C_{300} \times V_{c300})$$

Which is exactly the same combination of constants we saw above in the first term of the equation for relative volume. Substituting in W for that collection of constants, our new equation for relative volume is:

$$V_c' = (W\times 1/C_o') + b/V_{300}$$

We 're now close to being able to express W in terms of values we can easily determine in the lab (Vc ' and 1/Co '); we just need to deal with b/V300 somehow. The trick is to realize that at 300 mOsm our equation gets much simpler. In that case both Vc ' and Co ' are 1 and our relative volume equation is just:

$$1 = W + b/V_{300} \text{ (at 300 mOsm)}$$

And with a simple rearrangement:

$$b/V_{300} = 1 - W$$

While we found this relationship in the special case of a 300 mOsm, isotonic, solution it actually applies at all concentrations since all the values are constants (none depends on the concentration). So, we can substitute 1-W in for b/V300 in our expression for relative volume and obtain:

$$\mathbf{V_c' = (W/ C_o') + (1-W)}$$

This equation is the one you will use in Activity 1 today.

Osmosis and Transport

Lab Report **Name** ___________________________________

1. Explain the effects on the size of RBCs of increasing and decreasing the concentration of an impermeable solute.

2. Explain how we can use %T to measure cell volume.

3. What fraction of the total RBC volume was occupied by water under isotonic conditions in your experiment? Show how you calculated this value.

4. Explain the effect of glycerol on RBC volume.

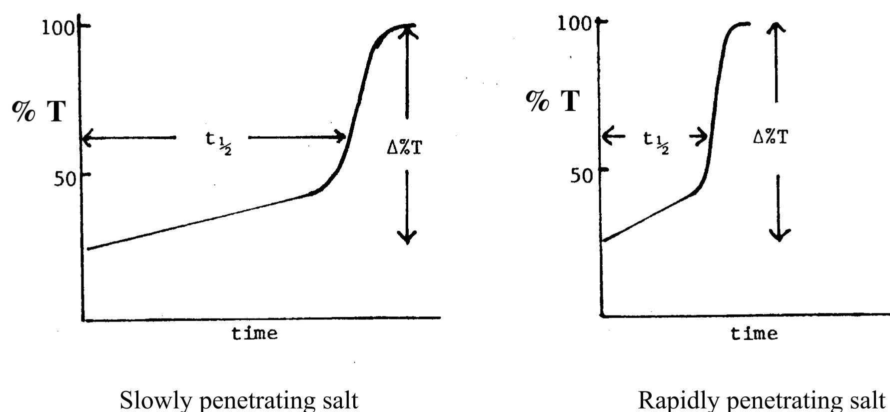
5. Explain how aquaporins can regulate the volume of urine we excrete.

6. Calculate the free energy change that occurs when valinomycin is added to RBCs in 150 mM NaCl + 10 mM KCl. Assume the intracellular concentration of K^+ is 150 mM before addition of valinomycin.

Optional Supplemental Activity

Transport of Ammonium Salts of Organic Acids.

Experimenters studied the rates of penetration of ammonium salts of several organic acids into RBCs. RBCs were added to iso-osmolar solutions of the ammonium salts (at pH 7.2) and the change in %T with time was determined. Typical results are shown for a slowly penetrating salt, left, and for a rapidly penetrating salt, right (Fig. 11). The initial, slower increase in %T is due to swelling of the cells; the abrupt increase is due to hemolysis. The results are recorded as the time for one-half of the total change in %T ($t_{1/2}$).



Slowly penetrating salt   Rapidly penetrating salt

Figure 11. Rates of penetration of ammonium salts.

Other experiments showed that the acids penetrate as the anions not as the undissociated acids.

The following table shows for each salt $t_{1/2}$, the molar volume, the length of the molecule, and the pKs. To refresh your memory, the structures are given (Fig. 12).

Acid	$t_{1/2}$ (min)	Molar Volume (cm^3)	Length (A)	pK_1	pK_2
lactic	6.5	57	7.4	3.08	-
oxalic	1.0	36	5.0	1.23	4.19
malonic	4.0	51	7.6	2.83	5.69
succinic	70.0	64	9.0	4.16	5.61
maleic	9.0	60	6.9	1.83	6.07
fumaric	58.0	60	8.7	3.03	4.44
2-ketoglutaric	∞	82	10.3	4.30	5.4
2-ketovaleric	6.0	81	10.0	4.80	-

List the acids in order of decreasing permeability:

Acid	$t_{1/2}$
1.	
2.	
3.	
4.	
5.	
6.	
7.	
8.	

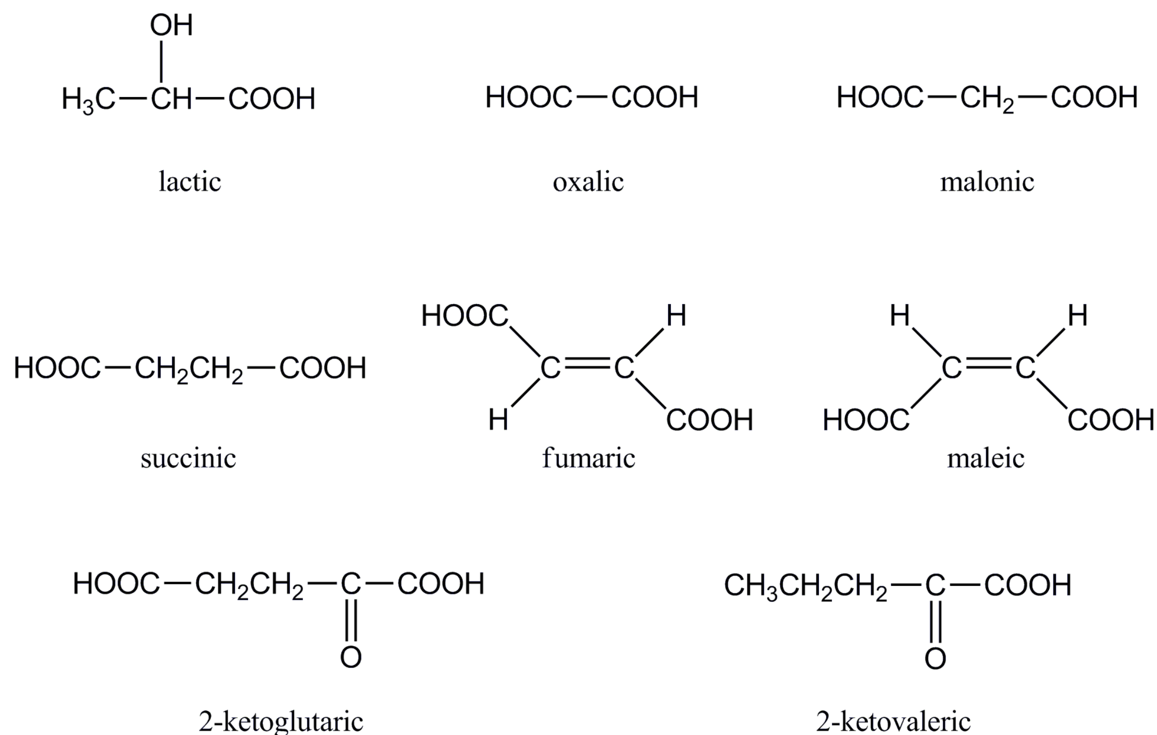


Figure 12. Organic acid structures.

1. In interpreting these results you first need to explain how ammonium salts can penetrate the membrane at all even though it is known that the membrane is highly impermeable to the ammonium ion (NH^{+4}).

2. Provide an explanation (model) for the specificity of the permeability; why are some anions more permeable than others?

   (Hint: Presumably, the transport is mediated by a protein that recognizes (binds) some anions better than others. Your job is to figure out which structural features of the anions the protein recognizes, based on the information given in the table or any other information you think might be useful and wish to look up. What structural features would the binding site on the protein need, to explain the transport data? You should consider the first six acids in the results table first and then refine your model to account for the two keto-acids.)

Notes for the Instructor

Plus Experimental Results

For further background reading, see Brahm (1982) and De Weer (2000).

Activity 1

Students prepare 11 tubes of 2.7 ml of NaCl at varying osmolarities, add 0.3 ml of RBCs equilibrated in 150 mM NaCl and then measure the %T of each of the tubes. Students then calculate $1/C_o$ for each tube and then as a fraction of the reciprocal of the iso-osmolar concentration (300 mOsM); this relative normalized value is $1/C_o$'. Next they calculate %T as a fraction of %T in iso-osmolar solution. To do this they divide each %T by the value obtained for the 300 mOsM tube (#4). This is %T'.

A plot of %T' vs $1/C_o$' is used to estimate the water volume, W, of the RBCs (Fig. 13).

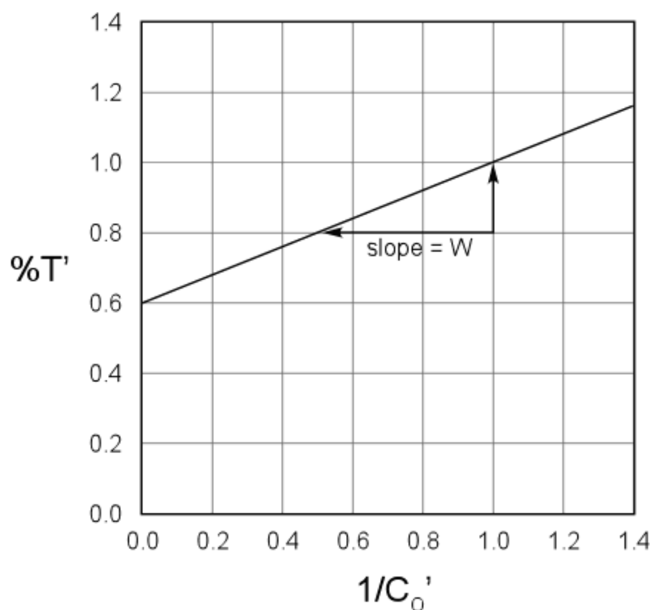


Figure 13. Relationship between $1/C_o$' and %T'.

Precision pipetting is critical to getting good results.

Activity 2

The RBCs shrink in size when transferred to the hypertonic solution (360 mOsM) solution containing glycerol. As glycerol moves down its concentration gradient and enters the RBCs, the RBCs increase in size and the %T increases. Students record %T vs time.

Activity 3

RBCs have a higher [K+] inside than out and a lower [Na+] inside than out.

When RBCs in 120 mM KSCN are added to a solution of 120 mM KSCN plus 0.67 μM valinomycin nothing appreciable happens because there is no gradient of K+ from inside to out.

When RBCs in 120 mM NaSCN are added to a solution of 120 mM NaSCN plus 0.67 μM valinomycin the cells slowly shrink, %T decreases, as K+ moves down its concentration gradient from high inside the RBCs to outside and the %T decreases as water leaves the cell with the K+.

Optional Supplemental Activity

Students examine the $t_{1/2}$ of transport of ammonium salts of some organic acids and predict what structural features would the binding site on a transporter protein need, to explain the transport data? Assuming that the smallest $t_{1/2}$ represents the optimum affinity for the organic anion, a comparison of lactic, oxalic, malonic and succinic acids illustrates that size matters.
A comparison of maleic and fumeric acids demonstrates the effect of shape on affinity. A comparison of 2-ketoglutaric and 2-ketovaleric acids demonstrates the effect of molecular solubility on transport. One possible model of the hypothetical transporter protein is shown in Figs. 14A and 14B.

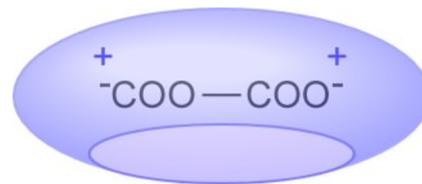


Figure 14A. Hypothetical transporter protein.

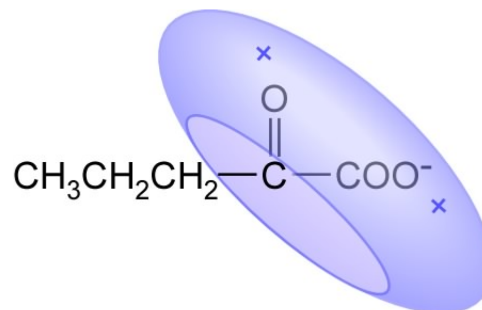


Figure 14B. Hypothetical transporter protein plus 2-ketovaleric acid.

Acknowledgements

The Cellular Biochemistry laboratory series, including this lab, Osmosis and Transport, was created originally by the late Professor William R. Sistrom, a member of the UO Institute of Molecular Biology. Over the years, the lab has been modified and reformatted by myself and others, including UO Professor Monte Westerfield, Institute of Neurosciences, who wrote the activity on Aquaporins. Figures 12, 13 14A and 14 B were created by UO Senior Instructor Alan Kelly and used with his permission.

Literature Cited

Brahm, J. 1982. Diffusional water permeability of human erythrocytes and their ghosts. *Journal of General Physiology*, 79: 791-819.

De Weer, P. 2000. A century of thinking about membranes. *Annual Review of Physiology*, 62: 919-26.

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Appendix A

Required Equipment, Materials and Procedures

1. Spectrophotometers (1/ student pair) at 680 nm in % Transmittance mode.

   Note: At the UO we use Sequoia Turner Model 340 digital spectrophotometers using 13 x 100 mm disposable culture tubes as cuvettes.

2. Test tube racks: 1 pin rack/ pair of students and 3 Wasserman racks or equivalent/ pair of students.
3. Twelve 13 x 100 mm disposable culture tube “cuvettes”/ pair of students for Activity 1.
4. One P-1000 pipettor and tips/ pair of students.
5. One sharpie/ pair of students.
6. One waste container for tips and kimwipes / pair of students.
7. One box of kimwipes/ pair of students.
8. One ruler/ pair of students
9. Rabbit Blood in Citrate from HemoStat Laboratories, PO Box 790, Dixon, CA 95620-0790
10. Solutions required:
    - 1 mM Tris buffer, pH 7.4 (Used for all buffers and (Activity 1) 20 ml/ pair of students)
    - 450 mM NaCl (20 ml/ pair of students)
    - 150 mM NaCl (needed for RBC preparation plus 5 ml/ pair of students)
    - 120 mM NaCl (needed for RBC preparation plus 2.7 ml/ pair of students)
    - 120 mM NaCl plus 120 mM Glycerol (2.7 ml/ pair of students
        - The NaCl solutions were made by diluting 1 M NaCl in 1 mM Tris buffer, pH7.4 with 1 mM Tris buffer, pH 7.4 to the final concentrations.
    - 120 mM NaSCN (needed for RBC preparation plus 2.7 ml/ pair of students)
    - 120 mM NaSCN plus 0.67 µM valinomycin (2.7 ml/ pair of students)
    - 120 mM KSCN (needed for RBC preparation plus 2.7 ml/ pair of students)
    - 120 mM KSCN plus 0.67 µM valinomycin (2.7 ml/ pair of students)
    - The thiocyanate anion solutions are prepared in 0.1 mM Tris buffer, pH 7.4.
    - For the valinomycin solutions, prepare a 1 mM valinomycin stock:
      Add 1.1 mg valinomycin (Sigma V0627) to 1.0 ml methanol.
    - To prepare 150 ml of 0.67 µM valinomycin in 120 mM KSCN add 0.1 ml of valinomcin stock to 150 ml of 120 mM KSCN in 0.1 mM Tris buffer, pH 7.4.
    - To prepare 150 ml of 0.67 µM valinomycin in 120 mM NaSCN add 0.1 ml of valinomcin stock to 150 ml of 120 mM NaSCN in 0.1 mM Tris buffer, pH 7.4.
11. Rabbit RBCs

    To prepare Rabbit RBCs, the following equipment is needed:
    - A spectrophotometer set at 680 nm and cuvettes to measure absorbance of rabbit RBC suspensions.
    - A preparative centrifuge with one rotor for 250 ml centrifuge bottles and another rotor for 30-50 ml centrifuge tubes.
    - Graduated cylinders.

    Example Rabbit RBC Preparation Procedure:
    1. Add 5.0 ml of Rabbit Blood in Citrate (HemoStat Laboratories, PO Box 790, Dixon, CA 95620-0790) to 200 ml of 150 mM NaCl in a 250 ml centrifuge bottle and mix well by inverting several times.
    2. Centrifuge the RBCs in 150 mM NaCl at 3000 x g for 5 minutes at 4°C.

3. Decant the supernatant and resuspend the RBC pellet in 200 ml of 150 mM NaCl in the 250 ml centrifuge bottle and mix well by inverting several times.
4. Centrifuge the RBCs in 150 mM NaCl at 3000 x g for 5 minutes at 4°C.
5. Decant the supernatant and resuspend the RBC pellet in 100 ml of 150 mM NaCl and mix well by inverting several times. Transfer the suspension to a 100 ml graduated cylinder,
6. By diluting samples from the 100 ml RBCs in 150 mM NaCl with 150 mM NaCl, determine what dilution results in an absorbance of 3.0 at 680 nm (a 10x dilution of OD_{680} = 3.0 is 0.3, more easily measured with most spectrophotometers).

   For example, if a 4X dilution of an aliquot of the 100 ml RBC suspension in the graduated cylinder had an OD_{680} = 3.0, then combine 30 ml of the RBC suspension with 90 ml of 150 mM NaCl to a final volume of 120 ml and mix well.
7. Transfer three 20 ml aliquots from the 120 ml suspension to three 30 - 50 ml centrifuge tubes. The remaining 60 ml of RBCs at OD_{680} = 3.0 is used for Activity 1 (5 ml/pair of students; 12 pairs of students)
8. Centrifuge the 20 ml RBC aliquots in the three centrifuge tubes at 3K x g for 5 minutes at 4°C.
9. Decant the supernatant and and resuspend the cells in one labeled centrifuge tube in 30 ml of 120 mM NaCl.
10. Decant the supernatant and resuspend the cells in one labeled centrifuge tube in 30 ml of 120 mM NaSCN.
11. Decant the supernatant and resuspend the cells in one labeled centrifuge tube in 30 ml of 120 mM KSCN.
12. Wash the cells 2x by centrifugation, as before, in their individual buffers and resuspend each pellet of washed and equilibrated cells to a final volume of 20 ml.

These cells (1.0 ml /pair of students) are for activities 2 (120 mM NaCl) and 3 (120 mM NaSCN and 120 mM KSCN).

Labeled Glassware and Reagent Volumes

- 1 each/ pair of students:

Activity 1

- 10 ml Erlenmeyer flask labeled, RBCs in 150 mM NaCl (5.0 ml)
- 50 ml Erlenmeyer flask labeled, 0.1 mM Tris buffer, pH 7.4 (25 ml)
- 50 ml Erlenmeyer flask labeled 450 mM NaCl (25 ml)

Activity 2

- 13 x 100 mm cuvette labeled, RBCs in 120 mM NaCl (1.0 ml)
- 13 x 100 mm cuvette labeled, 2.7 ml 120 mM NaCl
- 13 x 100 mm cuvette labeled, 2.7 ml 120 mM NaCl + 120 mM Glycerol

Activity 3

- 13 x 100 mm cuvette labeled, RBCs in 120 mM NaSCN (1.0 ml)
- 13 x 100 mm cuvette labeled, 2.7 ml 120 mM NaSCN
- 13 x 100 mm cuvette labeled, 2.7 ml 0.67 µM valinomycin in 120 mM NaSCN
- 13 x 100 mm cuvette labeled, RBCs in 120 mM KSCN (1.0 ml)
- 13 x 100 mm cuvette labeled, 2.7 ml 120 mM KSCN
- 13 x 100 mm cuvette labeled, 2.7 ml 0.67 µM valinomcin in 120 mM KSCN

Note: a good label maker makes this much detailed labeling a lot easier and more legible.

Appendix B

Pre-Lab and Report Answer Keys

Osmosis and Transport **___KEY___________________________**

Pre-lab assignment **Name**

Assignment:

- Read the lab exercise, focusing on the Overview section and the introductory material for each activity.
- Answer the questions below.

Questions:

1. What is the osmolarity of each of the following (include units):

   1 mM Glucose **1 mOsM**

   1 mM NaCl **2 mOsM**

   1 mM $CaCl_2$ **3 mOsM**

2. Red Blood Cells (RBCs) are isotonic with 150 mM NaCl

   a. What happens when the RBCs are transferred to a 120 mM NaCl solution?
   120 mM NaCl is a hypotonic solution. The cells increase in size when water enters.

   b. What happens when 120 mM impermeable solute is added to the RBCs in the 120 mM NaCl solution?
   The solution is now hypertonic (360 mOsM) The cells decrease in size as water leaves.

3. The figures below show the way in which cell volume changes after the addition of two different permeable solutes (A and B) to cells that were originally in solutions of the same composition.

   a. Which solute produced the higher osmolarity?
   Solution B

   b. To which solute are the cells more permeable?
   Solution B is more permeable because the time required for the cells to return to 50% of their original volume ($t_{1/2}$) is less than for solute A.

4. What mechanisms do cells use to control their osmotic balance and, hence, water content?

   Semipermeable membranes, carrier molecules and pumps

Osmosis and Transport ___**KEY**___________________________

Lab Report **Name**

1. Explain the effects on the size of RBCs of increasing and decreasing the concentration of an impermeable solute.

   ↑ solute → ↑ osmolarity → H_2O leaves and the cell shrinks until $[S_{total}]In = [S_{total}]out$

   ↓ solute → ↓ osmolarity → H_2O enters and the cell swells until $[S_{total}]In = [S_{total}]out$ or the cells lyse

2. Explain how we can use %T to measure cell volume.

   Small cells reflect more of the light and less is transmitted
   Large cells reflect less of the light and more is transmitted

3. What fraction of the total RBC volume was occupied by water under isotonic conditions in your experiment? Show how you calculated this value.

   Approximately 30% calculated by both slope and 1-intercept methods

4. Explain the effect of glycerol on RBC volume.

   Glycerol ↑ solute concentration outside. Initially H_2O leaves the cells and they shrink. Then, slowly, glycerol is transported into the cells until $[glycerol]_{In} = [glycerol]_{out}$ and the cells return to their original volume.

5. Explain how aquaporins can regulate the volume of urine we excrete.

   By increasing or decreasing the number of aquaporin-2 channels in the apical membrane of kidney duct cells, less or more water, respectively, is excreted in urine.

6. Calculate the free energy change that occurs when valinomycin is added to RBCs in 150 mM NaCl + 10 mM KCl. Assume the intracellular concentration of K+ is 150 mM before addition of valinomycin.

   $$\Delta G = \Delta G_o + RT \ln [10 \text{ mM KCl}] - \Delta G_o + RT \ln [150 \text{ mM KCl}]$$

   $$= RT \ln [10/150]$$

   $$= -1.62 \text{ kcal/mole}$$