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Measuring osmosis and hemolysis of red blood cells

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Goodhead LK, MacMillan FM. Measuring osmosis and hemolysis of red blood cells. *Adv Physiol Educ* 41: 298–305, 2017; doi: 10.1152/advan.00083.2016.—Since the discovery of the composition and structure of the mammalian cell membrane, biologists have had a clearer understanding of how substances enter and exit the cell's interior. The selectively permeable nature of the cell membrane allows the movement of some solutes and prevents the movement of others. This has important consequences for cell volume and the integrity of the cell and, as a result, is of utmost clinical importance, for example in the administration of isotonic intravenous infusions. The concepts of osmolarity and tonicity are often confused by students as impermeant isosmotic solutes such as NaCl are also isotonic; however, isosmotic solutes such as urea are actually hypotonic due to the permeant nature of the membrane. By placing red blood cells in solutions of differing osmolarities and tonicities, this experiment demonstrates the effects of osmosis and the resultant changes in cell volume. Using hemoglobin standard solutions, where known concentrations of hemoglobin are produced, the proportion of hemolysis and the effect of this on resultant hematocrit can be estimated. No change in cell volume occurs in isotonic NaCl, and, by placing blood cells in hypotonic NaCl, incomplete hemolysis occurs. By changing the bathing solution to either distilled water or isosmotic urea, complete hemolysis occurs due to their hypotonic effects. With the use of animal blood in this practical, students gain useful experience in handling tissue fluids and calculating dilutions and can appreciate the science behind clinical scenarios.

hematocrit; handing tissue fluids; osmolarity; tonicity

Objectives and Overview

THE MOVEMENT OF WATER and small molecules across the selectively permeable membranes of mammalian cells is a fundamental concept of physiology. These processes can be difficult for students to visualize and appreciate, and it is often left to images in textbooks or online animations to explain such movements. This practical uses animal blood bathed in solutions with differing osmolarities and tonicities to explore the concept of water movement by osmosis and the resultant hemolysis that can occur when red blood cells are exposed to hypotonic solutions. Students are given the opportunity to handle body fluids, practice preparing dilutions, and make accurate observations.

Background

In 1925, Gorter and Grendel (6) were the first to report the bilayer nature of the cell membrane. The structure of the cell membrane was further advanced by the work of Singer and Nicolson (18), who described the presence and location of proteins in the bilayer and developed the fluid mosaic model. In the mammalian cell membrane, the phospholipid bilayer alone is permeable to some substances such as oxygen, a small nonpolar molecule, and partially permeable to water, but some substances such as charged ions and glucose are impermeant without the additional presence of protein channels and transporters in the membrane. The combined properties of the phospholipid and proteins has resulted in the use of the term the "selectively permeable" membrane (3, 9). The extent to which solutes can cross the cell membrane dictates the tonicity of extracellular fluids and, therefore, the size and shape of cells from the resultant osmotic water movement (19). Knowledge of the structure and function of cell membranes and the movement of substances across the membrane is fundamental to all biomedical science disciplines and is often taught in early parts of undergraduate courses.

Osmosis is the movement of water down its osmotic gradient across a selectively permeable membrane (5). The establishment of an osmotic pressure gradient, i.e., the pressure required to prevent the movement of water down its gradient, is a result of the difference in numbers of impermeant particles in solution on either side of the membrane (14). Water can move directly through the cell membrane; however, due to the lipid bilayer nature of the membrane, this process is relatively slow. It was the discovery of water carrying pore-forming proteins known as aquaporins (16) that helped improve knowledge of how water moves from intracellular to extracellular fluid and vice versa. Water balance is crucial in homeostasis; hormones such as antidiuretic hormone (ADH) and atrial natriuretic peptide are released in response to changes in plasma composition and volume, respectively, and act on the kidney to regulate plasma osmolarity and volume.

The osmolarity of a solution is determined by the total number of particles present, known as osmolyte particles, and is not affected by the identity of these molecules (19). The higher the osmolarity of a solution, the greater the concentration of osmolytes, and the physical properties of a solution such as osmotic pressure and freezing point will be dependent on the concentration of osmolytes in solution. Osmolarity is calculated from the sum of the molar concentration of each solute multiplied by the osmotic coefficient for that solute. The osmotic coefficient is determined by the degree to which a solute (e.g., an ionic compound) dissociates in solution; therefore, an osmotic coefficient of "1" indicates that the solute completely dissociates in solution. For example, to calculate the osmolarity of a 0.9% wt/vol NaCl (mol wt 58.44) solution first the molarity is calculated by:

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$$\text{Molarity of a \%wt/vol solution (M)} = \text{\%solution in g/l} \div \text{molecular mass of the solute}$$

$$0.154 \text{ M} = 9 \text{ g/l} \div 58.44 \text{ g/mol}$$

To calculate the osmolarity, given that NaCl dissociates into two ions (Na^+ and Cl^-) in solution and has an osmotic coefficient of 0.93, the following equation is used:

$$\text{Osmolarity of solution (mosM)} = \text{molarity (M)} \times \text{number of osmoles produced by dissociation} \times \text{osmotic coefficient}$$

$$0.286 \text{ mosM or } 286 \text{ mosM} = 0.154 \text{ M} \times 2 \times 0.93$$

Osmolarity and tonicity are often used interchangeably by students, but they are not the same. Tonicity refers to the effect a solution has on cell volume as a result of the permeability of the membrane to that solute. Tonicity is, therefore, determined by the osmolarity and whether the solute can cross the cell membrane; it is the concentration of the impermeant solutes alone that determines tonicity. When comparing fluid concentrations to that of extracellular body fluid, the terms isotonic, hypertonic, and hypotonic are used rather than osmolarity, as they describe the effect the solution has on cell volume, which is of physiological significance. The tonicity will result in the following: no net movement of water (isotonic), net flow of water out of a cell (hypertonic), or net flow of water into a cell (hypotonic). Two solutions that are isosmotic may not be isotonic. A key example is isosmotic urea and isosmotic NaCl. Both urea and NaCl have the same osmolarity, having the same total number of osmolyte particles; however, the membrane is permeable to urea, which will freely diffuse across the cell membrane, and impermeable to NaCl. An isosmotic urea is, therefore, hypotonic compared with an isosmotic and isotonic solution of the impermeant NaCl. As a result, the volume of a cell is determined by the solution in which it is being bathed and whether the cell's membrane is permeable to the solute. If a membrane is not equally permeable to all solutes, then a difference in water movement will be observed that is not explained by osmolarity alone, and, hence, an additional term, tonicity, is required. Hypotonic solutions lead to cell swelling and eventual rupture or lysis if the resultant osmotic movement of water is great enough. In the case of red blood cells, this is referred to as hemolysis (4).

Knowledge of osmosis and tonicity is crucial in understanding the movement of fluids in the body. These concepts are fundamental in normal physiological processes; one example is that of water reabsorption in the kidney as increases in osmolarity are detected by the hypothalamus and stimulate the secretion of ADH, resulting in greater water retention and excretion of more concentrated urine (7). Osmosis and tonicity are important clinically, as the failure of the body to respond to changes in osmolarity, or the failure to release ADH, results in the condition diabetes insipidus. Another important concept is the diagnosis of the different types of dehydration and the administration of appropriate intravenous fluids (2). In this practical, with the use of easy-to-obtain red blood cells as model cells (1), students can explore the concepts of membrane permeability, osmosis, osmotic pressure, tonicity, and hemolysis while also learning key laboratory skills, such as making dilution series and handling tissue fluids.

Learning Objectives

After completing this activity, the student will be able to:

1. Content Knowledge: Define key terms used in explaining concentration, osmolarity, osmotic pressure, and tonicity
2. Content Knowledge: Calculate the osmolarity of a solution
3. Content Knowledge: Describe and explain the consequences of bathing red blood cells in solutions of differing tonicity
4. Process Skills: Handle mammalian blood samples safely
5. Process Skills: Prepare standard saline solutions
6. Process Skills: Measure hematocrit and estimate hemoglobin concentration
7. Process Skills: Carry out experiments with careful planning, accurate observation and recording of results

Activity Level

This activity is used to teach students in their first year of undergraduate study in physiology. This practical is used on our Physiological Sciences program and Veterinary Science program, but would also be suitable for other biomedical science or healthcare professional programs, such as medicine.

Prerequisite Student Knowledge or Skills

Before undertaking this activity, students should have a basic understanding of:

1. Homeostasis and the proportions of fluid in intracellular and extracellular compartments
2. The definition of a solute, a solvent and a solution
3. The concept of osmosis and the movement of water across a selectively-permeable membrane

Students should know how to:

1. Perform basic calculations to work out volumes required for concentrations
2. Use pipettes to create serial dilutions from stock solutions
3. Collect data carefully and accurately
4. Observe safe laboratory practices

Time Required

This practical is run in a 3-h laboratory time slot. The practical is completed within one session; however, it is expected that students complete their prereading of the laboratory notes, which explain the concepts of osmolarity, tonicity, and how to calculate osmolarity (to aid in achieving content learning *objectives 1* and *2*), and an online prepractical quiz before they come to the practical. This preparation work is expected to take around 1 h.

METHODS

Equipment and Supplies

The following equipment and supplies are needed.

Solutions

1. Distilled water (20 ml per pair of students).
2. 2.7% wt/vol NaCl solution (2.7 g NaCl per 100 ml of distilled water) (20 ml per pair of students plus that required for nonhemolyzed blood preparation). This stock solution is used to prepare all other NaCl solutions in the experiment.
3. Isosmotic urea solution (17.1 g/l) (5 ml per pair of students plus that required for hemolyzed blood preparation).

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4. Fresh mammalian blood. This blood is referred to for the rest of the experiment as nonhemolyzed blood. We find that there are no appreciable differences in the outcome of the experiment depending on which species blood is used, although values of hemolysis can vary. Obtaining mammalian blood supplies can be problematic if obtained locally direct from an abattoir; however, blood can also be purchased online (for example, http://www.rockland-inc.com/blood-products.aspx). For a class of around 200 students working in pairs, ~1.5 liters of blood are required (~11 ml blood per pair of students and allowing extra for repeat experiments if required). The blood must be heparinized before use to prevent clotting by the addition of heparin sodium (5,000 IU/ml per 1.5 liters blood). This blood is then used to produce the hemolyzed and nonhemolyzed blood as follows.
5. Hemolyzed blood. To prepare the hemolyzed blood in manageable volumes, 250 ml of nonhemolyzed blood are measured into a 600-ml beaker, together with 250 ml urea solution (17.1 g/l), and stirred. The tonicity of the urea and resultant osmotic water movement results in hemolysis of the cells, and this will form the blood used for the production of the hemoglobin standards that will be used to assess the degree of hemolysis in the experiment. Decant 10 ml of the hemolyzed blood into 50 centrifuge tubes (one per pair of students), labeled “H” for hemolyzed blood, and centrifuge at 6,000 rpm for 2 min. Repeat depending on quantities of blood required, i.e., if 1 liter is required, repeat once.
6. Nonhemolyzed blood. To prepare the nonhemolyzed blood in manageable volumes, 275 ml of nonhemolyzed blood (from the original heparinized fresh mammalian blood) are prepared by the addition of 275 ml of 0.9% wt/vol saline and stirred gently. This forms the nonhemolyzed blood, which will be used for the main part of the experiment at an equal concentration to the hemolyzed blood. Decant 11 ml of the nonhemolyzed blood into 50 centrifuge tubes (one per pair of students) labeled “N” for nonhemolyzed blood. Repeat depending on quantities of blood required, i.e., if 1 liter is required, repeat once. An assumption is made that the hemoglobin concentration of the original blood sample is 15 g/dl, but, as the hemolyzed blood is diluted 1:1 with isosmotic urea (17.1 g/l) and the equivalent nonhemolyzed blood is diluted 1:1 with isosmotic (0.9% wt/vol NaCl), the hemoglobin concentration of both blood samples is, therefore, assumed to be 7.5 g/dl (75 g/l).

Equipment

1. 600-ml Glass beakers (2 for blood preparation)
2. 500-ml Measuring cylinders (2 for blood preparation)
3. Stirring rods (2 for blood preparation)
4. 25-ml Glass beakers for water, 2.7% wt/vol NaCl and urea distribution (3 per pair of students)
5. 1.5-ml Plastic Eppendorf tubes with hinged cap (11 per pair of students)
6. 10-ml Plastic centrifuge tubes with cap (10 per pair of students)
7. Centrifuge tube racks (1 per pair of students)
8. 75-μl Glass microhematocrit tubes (Hawksley catalog no. 01603) (6 per pair of students)
9. Plasticine
10. Centrifuge with centrifuge tube rotor and microhematocrit tube rotor (Hettich EBA21 centrifuge with 1416 rotor and 1450 hematocrit rotor)
11. Hematocrit readers (Hawksley) or 30-cm rulers (a number of readers/rulers can be shared between pairs of students)
12. 1.5-ml Disposable plastic pipettes or equivalent Gilson pipettes if available (3 disposable pipettes per pair of students)
13. Marker pens (1 per pair of students)
14. White paper (1 sheet per pair of students)

Table 1. *Dilutions calculations for saline solutions*

%Saline Solution, wt/vol	2.7	0.9	0.45
Volume of 2.7% wt/vol NaCl, ml	9	3	1.5
Volume of distilled water, ml	0	6	7.5

Values underlined are calculated by the students in advance of the class.

Table 2. *Dilutions calculations for hemoglobin standards from hemolyzed blood*

%Hemoglobin	100	66	33	7	1
Volume of hemolyzed blood, ml	1.5	1.0	0.5	0.1	1 drop
Volume 0.9% wt/vol NaCl, ml	0	0.5	1.0	1.4	1.5

Values underlined are calculated by the students in advance of the class.

Human or Animal Subjects

The animal blood used in this experiment is obtained as a by-product from a local abattoir, and, therefore, the animals are not slaughtered for the purpose of this experiment.

Instructions

Preparation before the practical. In advance of the class, students must calculate *1*) the volume of distilled water and 2.7% wt/vol NaCl stock solution required to produce 9 ml each of 0.9 and 0.45% wt/vol saline solutions; *2*) the volumes of hemolyzed blood and 0.9% wt/vol NaCl (ml) required to produce 1.5 ml %hemoglobin concentrations; and *3*) the hemoglobin concentration (g/dl) in results Tables 1–3 provided in their laboratory books.

In our programs, this is the first practical that students will have had to calculate and make serial dilutions and handle blood, two key, but challenging, transferable skills.

Making saline solutions and hemoglobin standards. We recommend that students carry out this practical working in groups of two or three. Students begin the practical by making a set of standard solutions of hemolyzed blood of known hemoglobin concentration to use later in the experiment, which they will compare against the unknown hemoglobin-containing solutions they will produce. Hemolyzed blood is used to create these hemoglobin standards, as this contains red blood cells that have already fully lysed in urea, and all of the hemoglobin has been released into the solution. The steps below take the students through the practical:

1) Using the 2.7% wt/vol NaCl and 1.5 ml pipettes provided, prepare 9 ml each of 0.9% wt/vol NaCl and 0.45% wt/vol NaCl solutions in two labeled 10-ml plastic centrifuge tubes from the dilutions calculated in Table 1 (values underlined are calculated by the students in advance of the class).

2) Using the marker pen, label five Eppendorf tubes, 100%, 66%, 33%, 7%, and 1%, which will represent the percentage of hemolyzed blood to be added to these Eppendorf tubes. Using the volumes calculated in Table 2, use 1.5-ml pipettes to add the appropriate volumes of 0.9% wt/vol NaCl solution and hemo-

Table 3. *Hemoglobin concentration in each standard solution*

%Blood Sample	[Hb], g/dl
100	7.5
66	5
33	2.5
7	0.5
1	0.08

Values underlined are calculated by the students in advance of the class. [Hb], hemoglobin concentration.

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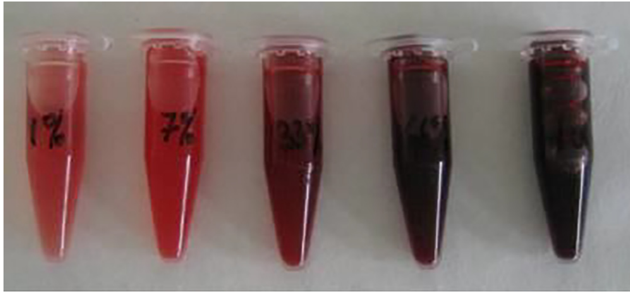

Fig. 1. The hemoglobin standards produced from hemolyzed blood with 1% hemolyzed blood to the *left* of the image and 100% hemolyzed blood on the *right*. [Image courtesy of the University of Bristol.]

lyzed blood to each labeled Eppendorf tube (values underlined are calculated by the students in advance of the class). Gently invert the tube containing the hemolyzed blood before use to ensure the blood is evenly mixed and, once filled, also invert each Eppendorf tube to ensure mixing.

The calculations performed in Table 3 provide reference hemoglobin concentrations for each hemoglobin standard (values underlined are calculated by the students in advance of the class).

3) Lay out the five mixed hemolyzed blood/0.9% wt/vol NaCl solutions in the Eppendorf tubes on a blank sheet of white paper to observe the colors. The colors of the hemoglobin standards should range from translucent pink to translucent red and should look similar to Fig. 1. These hemoglobin standards will be used later in the experiment and should be kept to one side until then.

Investigating the effects of tonicity on red blood cells. The next part of the experiment investigates the effects of membrane-permeable and membrane-impermeable solutions of differing concentrations on whole red blood cells using the nonhemolyzed blood sample. Both hematocrit and %hemolysis will be estimated. The hematocrit will indicate the degree to which red blood cells swell or shrink when exposed to the different solutions but does not take into account if hemolysis has occurred. Percent hemolysis gives a measure of the degree of hemolysis of the samples and can be used to determine whether red blood cells have swollen and burst. Hematocrit alone cannot distinguish between cell shrinkage and a combination of swelling and lysis.

4) Label an additional six 10-ml plastic centrifuge tubes from 1 to 6. Gently invert the tube containing the nonhemolyzed blood several times before use to ensure an even suspension of red blood cells.

5) Prepare the centrifuge tubes as follows:

Tube 1: 1.5 ml nonhemolyzed blood + 1.5 ml 2.7% wt/vol NaCl
Tube 2: 1.5 ml nonhemolyzed blood + 1.5 ml 0.9% wt/vol NaCl
Tube 3: 1.5 ml nonhemolyzed blood + 1.5 ml 0.45% wt/vol NaCl
Tube 4: 1.5 ml nonhemolyzed blood + 1.5 ml distilled water
Tube 5: 1.5 ml nonhemolyzed blood + 1.5 ml isosmotic urea
Tube 6: 3 ml nonhemolyzed blood

When filled, gently invert the centrifuge tubes several times to ensure the blood is mixed and leave for 10 min before proceeding with the next step.

6) The blood solutions are then prepared for centrifuging to allow the measurement of the packed cell volume (hematocrit) of each sample. Label six glass microhematocrit tubes 1–6 to correspond to the samples in the plastic centrifuge tubes. In turn, invert each centrifuge tube several times to ensure even dispersal of red blood cells, and then dip the corresponding microhematocrit tube in the blood until capillary action has filled the glass tube. Seal the bottom of the microhematocrit tube with a small plug of plasticine by twisting the bottom of the tube in a tray of plasticine.

7) Centrifuge the microhematocrit tubes at 6,000 rpm for 2 min using the microhematocrit tube rotor until the cells have packed together at the bottom of the tube, leaving the fluid (supernatant) above.

It is not expected that the centrifuges are operated by the students. In our laboratory, students bring their samples to the shared laboratory centrifuges, and these are run by experienced demonstrators or technicians.

Measuring hematocrit

8) After centrifuging, measure the hematocrit of each sample using a hematocrit reader and read off the %hematocrit. If hematocrit readers are difficult to obtain, a ruler can be used instead. By this method, measure the total length of the column of fluid and the length of the column of packed cells and calculate the proportion of the total column that is made up of packed cells at the bottom. This percentage is the hematocrit. Record the hematocrit readings in the Observed Hematocrit column in Table 4.

9) With the exception of *tube 6*, the hematocrit readings measured are for blood diluted 50:50 with a saline solution. Therefore complete the Dilution Factor column with a dilution factor of 2 for *tubes 1–5* and a dilution factor of 1 for *tube 6*. To calculate the true hematocrit values, complete the final Corrected Hematocrit for Nonhemolyzed Blood column in Table 4 by using the following equation:

$$\text{Corrected hematocrit} = \text{observed hematocrit} \times \text{dilution factor}$$

Estimating hemolysis

10) Following the measurement of hematocrit, estimate the percentage of hemolysis of the red blood cells in the various solutions. To do this, centrifuge the remaining contents of the six plastic centrifuge tubes at 6,000 rpm for 2 min using the centrifuge tube rotor. Take six clean 1.5-ml plastic Eppendorf tubes also labeled 1–6 and pipette 1.5 ml of the supernatant from each correspondingly labeled centrifuge tube into the labeled Eppendorf tube, taking care not to disturb the red blood cell pellet at the bottom of the tube.

11) The colors of the six samples of supernatant can then be compared with that of the known hemoglobin standard solutions prepared at the beginning of the practical. Using the colors of the known hemoglobin standard solutions as a scale, estimate the concentration of observed supernatant hemoglobin, with the darker the color of the sample indicating the greater the amount liberated hemoglobin in the supernatant and hence the greater degree of hemolysis. Using the known hemoglobin concentrations (g/dl) calculated in Table 3, record

Table 4. *Hematocrit measurements for nonhemolyzed blood*

Sample	Observed Hematocrit, %	Dilution Factor	Corrected Hematocrit for Nonhemolyzed Blood, %
Tube 1: blood + 2.7% NaCl			
Tube 2: blood + 0.9% NaCl			
Tube 3: blood + 0.45% NaCl			
Tube 4: blood + distilled water			
Tube 5: blood + isosmotic urea			
Tube 6: nonhemolyzed blood			

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Table 5. *Estimated hemolysis and final corrected hematocrit*

Sample	Observed Supernatant [Hb], g/dl	Estimated %Hemolysis	Corrected Hematocrit, %
Tube 1: blood + 2.7% NaCl			
Tube 2: blood + 0.9% NaCl			
Tube 3: blood + 0.45% NaCl			
Tube 4: blood + distilled water			
Tube 5: blood + isosmotic urea			
Tube 6: nonhemolyzed blood			

these observations for *tubes 1–6* in the Observed Supernatant [Hb] column of Table 5. If the colors are not exact matches, estimate about where the concentration falls between the two standards.

12) To convert the observed hemoglobin concentration into an estimated percentage of hemolysis of the red blood cells, with the exception of the nonhemolyzed blood sample, which contained 7.5 g/dl hemoglobin, the blood in the other mixtures was diluted 50:50 and, therefore, contained one-half the original hemoglobin. To estimate the amount of hemolysis that occurred in each sample, use the following calculations and complete the Estimated %Hemolysis column of Table 5:

$$\text{Diluted samples estimated \%hemolysis} = [\text{observed supernatant Hb concentration}/3.75] \times 100$$

$$\text{Nonhemolyzed blood estimated \%hemolysis} = [\text{observed supernatant Hb concentration}/7.5] \times 100$$

13) The corrected hematocrit recorded in Table 4 was generated by nonhemolyzed red blood cells only as these were the whole cells that would have made up the packed cell volume in the hematocrit tubes. To correct for hemolysis in each sample and allow an estimate of what hematocrit would be had if there had been no cell lysis, use the following calculation and complete the final column (Corrected Hematocrit) of Table 5:

$$\text{Corrected hematocrit } (\%) = [100/(100 - \text{estimated \%hemolysis})] \times \text{corrected hematocrit for nonhemolyzed blood } (\%)$$

14) When all data have been collected, each group should pool their final corrected hematocrit (%) data from Table 5 with the rest of the class using a spreadsheet on a central computer to ensure that group data can be distributed for more comprehensive analysis following the class.

Troubleshooting

A common student mistake in this practical is the incorrect or lack of labeling of tubes and pipettes containing the different solutions during the various steps undertaken. As a result, students lose track of the contents of tubes they are testing and find their results are meaningless. This is an important error to impress upon the students as, if this is kind of mistake occurs in a clinical setting, the outcome could be life threatening. Trained demonstrators should be on hand to spot mistakes early and help students rectify them as soon as possible.

Students also often find it difficult to perform the correct calculations to work out dilutions (15). It is recommended that students be encouraged to attempt these calculations (Tables 1–3) before the practical and come prepared to have these calculations checked by a demonstrator in the practical before proceeding.

Safety Considerations

Despite the risk to humans from animal blood being extremely low, when dealing with blood, standard safety precautions must be taken to minimize the risk of infection. At all times in the laboratory, general laboratory safety rules must be followed, including wearing a laboratory coat and using disposable gloves and hand washing before leaving the laboratory. Any spilled blood or fluids must be wiped up immediately and disposed of in waste bags provided. All sharps should be disposed of in a sharps box.

Unless the students are already trained and experienced in using centrifuges, the centrifuge should only be operated by trained personnel, and students should not be left to spin their samples unsupervised. The centrifuge should be inspected for damage regularly. When using the centrifuge, ensure the tubes are undamaged, firmly sealed, and have not been overfilled. When placing the tubes in the rotor, they must be balanced, and the lid must never be opened while the rotor is moving. The centrifuge should not be left unattended during use.

RESULTS

Expected Results

Nonhemolyzed blood. Tube 6, which contains the nonhemolyzed blood sample prepared in *step 5*, should be used as a control and reference point against which to compare any changes to hematocrit in the other blood samples (*tubes 1–5*) that were exposed to permeant and nonpermeant solutes. Completed sample data tables (Tables 6 and 7) are given here from experiments carried out using pig blood, but caution should be taken when making direct comparisons to the values obtained as, although the relative changes should be the same, the actual values can vary greatly, depending on the blood sample used.

The effects of hypertonic NaCl. In *step 5*, nonhemolyzed blood was exposed to 2.7% wt/vol NaCl solution, which has an osmolarity of 859 mosM and is hypertonic relative to plasma (*tube 1*). When red blood cells are placed in a hypertonic

Table 6. *Completed sample results of hematocrit measurements for nonhemolyzed blood*

Sample	Observed Hematocrit, %	Dilution Factor	Corrected Hematocrit for Nonhemolyzed Blood, %
Tube 1: blood + 2.7% NaCl	3	2	6
Tube 2: blood + 0.9% NaCl	5	2	10
Tube 3: blood + 0.45% NaCl	2	2	4
Tube 4: blood + distilled water	0	2	0
Tube 5: blood + isosmotic urea	0	2	0
Tube 6: nonhemolyzed blood	9	1	9

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Table 7. *Completed sample results of estimated hemolysis and final corrected hematocrit*

Sample	Observed Supernatant [Hb], g/dl	Estimated %Hemolysis	Corrected Hematocrit, %
Tube 1: blood + 2.7% NaCl	0.08	2.1	6.1
Tube 2: blood + 0.9% NaCl	0.08	2.1	10.2
Tube 3: blood + 0.45% NaCl	2.5	66.7	12.0
Tube 4: blood + distilled water	5.0	133.3	0
Tube 5: blood + isosmotic urea	2.5	66.7	0
Tube 6: nonhemolyzed blood	0.08	1.1	9.1

solution, the higher effective osmotic pressure of the bathing solution compared with the intracellular fluid results in water moving down its osmotic gradient and a net movement of water out of the cell via osmosis (10). The red blood cells, therefore, lose their normal biconcave shape and shrink or crenate. This collapse leads to a decrease in the packed cell volume, or hematocrit, of the solution in comparison to that of the nonhemolyzed blood, as the cells take up less space due to the rapid loss of water. Very little hemolysis of the red blood cells in the solution should be observed, as no cells have taken on an additional water load and burst or hemolyzed; however, a few cells may have been damaged during handling and release some hemoglobin.

The effects of isotonic NaCl. In *step 5*, nonhemolyzed blood was exposed to an isotonic solution of 0.9% wt/vol NaCl (osmolarity 286 mosM) (*tube 2*). This environment has an even distribution of osmolyte particles across both sides of the cell membrane as intracellular fluid also has an osmolarity around 286 mosM. There is, therefore, no net water movement between the bathed red blood cells and the NaCl solution. The hematocrit of the solution should be unaffected, and the value similar to that of the nonhemolyzed blood. Similarly, little if any hemolysis of the red blood cells should have occurred.

The effects of hypotonic NaCl. In *step 5*, nonhemolyzed blood was exposed to a low osmolarity (143 mosM) hypotonic solution (0.45% wt/vol NaCl) (*tube 3*). When red blood cells are exposed to these conditions where there is a higher concentration of water and lower effective osmotic pressure outside the cell compared with the intracellular fluid, this results in net movement of water into the cells via osmosis (11). The cells will increase in size and some may hemolyze. In this sample, therefore, a small proportion of hemolysis should have been observed with increased hemoglobin in the supernatant compared with the whole blood, and the remaining cells that had not lysed would increase in size, causing the hematocrit to increase.

The effects of distilled water. In *step 5*, the cells in *tube 4* that were bathed in distilled water underwent complete hemolysis and the estimated %hemolysis should have been 100%. With no ions present in the bathing solution, this solution was very hypotonic, resulting in net movement of water into the red blood cells via osmosis, causing all of the cells to lose the integrity of their membranes and to hemolyze releasing hemoglobin into the supernatant, hence the strong red color of the sample. The resultant corrected hematocrit was 0%, as there were no remaining complete red blood cells to contribute to pack cell volume. Comparing the results of distilled water (*tube 4*) and 0.45% wt/vol (*tube 3*) is a clear example of how the osmotic fragility or susceptibility of red blood cells to hemolysis depends on the degree of hypotonicity of the bathing solution.

The effects of isosmotic urea. In contrast to NaCl, the membrane is permeable to urea. In *step 5*, when red blood cells were bathed in isosmotic urea (286 mosM) (*tube 5*), the effects of the permeability of the membrane to urea on both hematocrit and degree of hemolysis were very different than when red blood cells are exposed to isosmotic NaCl (*tube 2*). In the presence of an isosmotic urea solution, the red blood cells underwent complete hemolysis with a corrected hematocrit of 0%. This is because, although isosmotic, the urea solution is not isotonic, as urea can freely diffuse across the cell membrane into the cell via passive diffusion and through urea transporters (20, 21). This leads to a change in cell volume as a result of osmotic water movement (13). The isosmotic urea solution is, therefore, hypotonic, because the reflection coefficient of the membrane (permeability) for urea is 0.024 compared with a reflection coefficient of the membrane of 0.3 for NaCl. If the membrane is completely impermeable to a solute, the reflection coefficient would be 1. The consequence of this is that the effective osmotic pressure of a urea solution is lower than that of NaCl of the same osmolarity, and, as a result, the osmotic gradient across the cell membrane is increased, and water moves into the red blood cells via osmosis, causing the cell membrane to rupture and the cell to hemolyze. Conversely, NaCl dissociates into Na^+ and Cl^- particles that cannot cross the cell membrane and, therefore, generate an equal effective osmotic pressure between the extracellular fluid and the intracellular fluid. Under these conditions, the osmotic gradient across the cell membrane is maintained, and the solution is both isosmotic and isotonic. The same strong red color of the urea sample in *tube 5* should have been observed as that of the distilled water sample in *tube 4*, as there is 100% hemolysis and 0% corrected hematocrit.

Conclusions. The observations and conclusions that should have been drawn from this practical are fundamental to understanding basic cell physiology. A good grasp of the concepts covered by this practical will help students appreciate the fact that cell membranes are indeed selectively permeable, and that the tonicity and osmolarity of fluids affect cell size and structure. This is essential in understanding the concept of homeostasis and will be referred to in later parts of many physiology courses, including during study of the gastrointestinal tract, regulation of NaCl by the nephron in the renal system, and, in particular, the effect of dehydration on the whole body.

Caution must be taken with the practical to ensure students observe the expected results: common mistakes such as poor labeling of samples and contamination with urea due to pipette confusion can lead to students obtaining results that may not be as anticipated. Careful supervision of students and pooling of data to analyze the class averages should help prevent this.

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Misconceptions

From early on in many physiology-based courses, students struggle with the concepts of osmolarity and tonicity and find it difficult to relate them to the direction of water movement. This practical can help students to visualize different solutions and the effects that these can have on red blood cells. By being able to see the color change of the nonhemolyzed blood samples mixed with various solutions, they can relate the theory of osmosis to what has happened to the cells when water moves out of the red blood cells in a hypertonic solution (*tube 1*), when there is no net movement of water in an isotonic solution (*tube 2*), and when water moves into the cells in a hypotonic solution (*tube 3*). The practical also helps students with misconceptions surrounding hemolysis and hematocrit. The students often mistake the contents of the tubes (4, 5) containing distilled water and urea as having 100% hematocrit due to the dark coloration of the whole sample with little or no visible plasma band rather than the product of 100% hemolysis due to the effects of the tonicity. Trained demonstrators should be on hand to ensure that students are able to relate their findings to the learning outcomes, and a whole-class tutorial on the outcomes of the experiments and their meanings should be scheduled for 1 wk after the practical class.

Evaluation of Student Work

As discussed, there are a number of ways in which students may not get the results expected. To ensure that students have access to some representative data on which to perform any postpractical analysis, students are expected to pool their results with the rest of the group to produce group data for the class before the finish, and this is then shared online with the students to use immediately after the class.

We assess our students on this work through the submission of an online postpractical assessment. This takes the form of multiple choice questions on the background physiology, method, results, and physiological significance of the findings. Questions included in the assessment test whether students have grasped the direction of fluid movement in the presence of different solutions and the physiological reasons for this. With regards to further data analysis, students are expected to plot the class findings in graphical form. They are instructed to produce a column/bar graph of the final corrected hematocrits (%) from all solutions with the error bars as the standard error of the mean, and this is uploaded as part of their online post-practical assessment (see Fig. 2). Students are also given information on how to calculate osmolarity and are expected to perform calculations themselves.

Inquiry Applications

As this practical is run at the start of the students' exploration of physiology at the undergraduate level, there is very limited inquiry in this practical and would be considered "methods" level. The questions being explored and the procedure being followed are clearly set out by the teacher running the practical. Students carry out the practical, with assistance from demonstrators, and analyze the data during the session.

However, there is scope for this practical to become more student centered and to be used at a higher inquiry level by making a number of modifications to the protocol. These could include giving the students less rigid instructions on how and what hemoglobin standard solutions to produce and allowing them to make a wider range of standard solutions to allow more accuracy in estimating the final degree of hemolysis. They could also be asked to predict the impact of the different solutions on the hematocrit and hemoglobin concentration and subsequently test these predictions. More sophisticated techniques could also be used to measure hemolysis in the supernatant, such as spectrophotometry and using light microscopy to visualize the red blood cells after they have undergone crenation or hemolysis, as opposed to the estimates made in this experiment by eye.

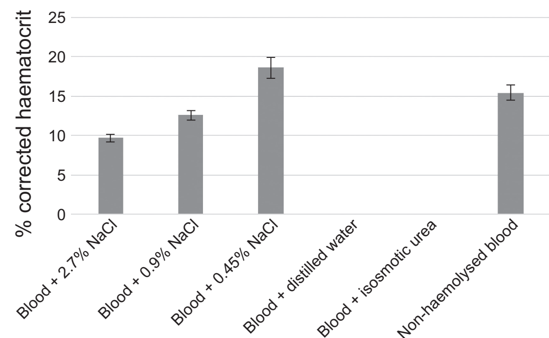

Fig. 2. Mean corrected hematocrit values of blood following exposure to permeable and nonpermeable solutions (n = 64). Values are means ± SE.

This practical could be incorporated into a number of different biomedical programs, from Biology/Physiology/Biochemistry honors programs to aid in the understanding of the fundamental concepts of cell transport and membrane structure, as well as developing vital scientific skills, including handling blood and performing serial dilutions. Professional disciplines, such as medicine and veterinary science, would also benefit from this practical to further explore the concepts of osmotic fragility and the administration of intravenous fluids and the clinical implications that these can have.

The concept of osmotic fragility could be explored further by using a series of hypotonic solutions and recording %hemolysis. From these data, an osmotic fragility curve could be plotted to explore the internal pressures exerted on the cell membrane when water diffuses into a cell. Students could further consider how the shape of red blood cells, e.g., sickle cells may affect the osmotic fragility of red blood cells (8), and how hemolytic diseases, such as thalassemia and hereditary spherocytosis, are a result of changes in osmotic fragility both by extending this practical and the use of further resources (12).

Further examples of the differences between tonicity and osmolarity and the effect of permeable solutes can be used, for instance with the addition of glucose, a particularly clinically relevant solute with regards to intravenous administration of fluids. The clinical application of an understanding of this concept can be emphasized, including those surrounding patient safety (17).

Additional Resources

For additional information on this topic, any undergraduate level physiology textbook should provide relevant background information required to understand the theory on which this practical is based.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

L.G. and F.M. performed experiments; L.G. and F.M. analyzed data; L.G. and F.M. interpreted results of experiments; L.G. and F.M. prepared figures; L.G. drafted manuscript; L.G. and F.M. edited and revised manuscript; L.G. and F.M. approved final version of manuscript.

REFERENCES

1. **Bird GW.** The red cell. *BMJ* 1: 293–297, 1972. doi:10.1136/bmj.1.5795.293.
2. **Choong K, Kho ME, Menon K, Bohn D.** Hypotonic versus isotonic saline in hospitalised children: a systematic review. *Arch Dis Child* 91: 828–835, 2006. doi:10.1136/adc.2005.088690.
3. **Cooper GM.** *The Cell: A Molecular Approach* (6th Ed.). Sunderland, MA: Sinauer, 2013.
4. **Dourmashkin RR, Rosse WF.** Morphologic changes in the membranes of red blood cells undergoing hemolysis. *Am J Med* 41: 699–710, 1966. doi:10.1016/0002-9343(66)90031-3.
5. **Feher JJ, Ford GD.** A simple student laboratory on osmotic flow, osmotic pressure, and the reflection coefficient. *Am J Physiol* 268: S10–S20, 1995.
6. **Gorter E, Grendel F.** On bimolecular layers of lipids on the chromocytes of the blood. *J Exp Med* 41: 439–443, 1925. doi:10.1084/jem.41.4.439.
7. **Hasler U, Leroy V, Martin PY, Féraille E.** Aquaporin-2 abundance in the renal collecting duct: new insights from cultured cell models. *Am J Physiol Renal Physiol* 297: F10–F18, 2009. doi:10.1152/ajprenal.00053.2009.
8. **Hijiya N, Horiuchi K, Asakura T.** Morphology of sickle cells produced in solutions of varying osmolarities. *J Lab Clin Med* 117: 60–66, 1991.
9. **Khan Academy**. *Structure of the Plasma Membrane* (Online). www.khanacademy.org/science/biology/membranes-and-transport/the-plasma-membrane/a/structure-of-the-plasma-membrane [4 Nov 2016].
10. **Kregenow FM.** The response of duck erythrocytes to hypertonic media. Further evidence for a volume-controlling mechanism. *J Gen Physiol* 58: 396–412, 1971. doi:10.1085/jgp.58.4.396.
11. **Kregenow FM.** The response of duck erythrocytes to nonhemolytic hypotonic media. Evidence for a volume-controlling mechanism. *J Gen Physiol* 58: 372–395, 1971. doi:10.1085/jgp.58.4.372.
12. **Kumar S.** An analogy for explaining erythrocyte fragility: concepts made easy. *Adv Physiol Educ* 26: 134–135, 2002. doi:10.1152/advan.00008.2002.
13. **Lezama R, Díaz-Téllez A, Ramos-Mandujano G, Oropeza L, Pasantes-Morales H.** Epidermal growth factor receptor is a common element in the signaling pathways activated by cell volume changes in isosmotic, hyposmotic or hyperosmotic conditions. *Neurochem Res* 30: 1589–1597, 2005. doi:10.1007/s11064-005-8837-5.
14. **Macey RI.** Transport of water and urea in red blood cells. *Am J Physiol Cell Physiol* 246: C195–C203, 1984.
15. **Moni RW, Hryciw DH, Poronnik P, Lluka LJ, Moni KB.** Assessing core manipulative skills in a large, first-year laboratory. *Adv Physiol Educ* 31: 266–269, 2007. doi:10.1152/advan.00020.2007.
16. **Preston GM, Carroll TP, Guggino WB, Agre P.** Appearance of water channels in *Xenopus oocytes* expressing red cell CHIP28 protein. *Science* 256: 385–387, 1992. doi:10.1126/science.256.5055.385.
17. **Silverthorn D.** *Using IV Fluid Therapy to Teach the Principles of Osmolarity and Tonicity* (Online). Washington, DC: AAMC. https://www.mededportal.org/publication/8082 [5 June 2016].
18. **Singer SJ, Nicolson GL.** The fluid mosaic model of the structure of cell membranes. *Science* 175: 720–731, 1972. doi:10.1126/science.175.4023.720.
19. **Strange K.** Cellular volume homeostasis. *Adv Physiol Educ* 28: 155–159, 2004. doi:10.1152/advan.00034.2004.
20. **Wieth JO, Funder J, Gunn RB, Brahm J.** Passive transport pathways for chloride and urea through the red cell membrane. In: *Comparative Biochemistry and Physiology of Transport* (Bolis K, Bloch K, Luria SE, Lynen F, editors). Amsterdam: Elsevier/North-Holland, 1974, p. 317–337.
21. **Yang B.** Transport characteristics of urea transporter-B. *Subcell Biochem* 73: 127–135, 2014. doi:10.1007/978-94-017-9343-8_8.

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