Laboratory Manual
Selected Experiments in General Physiology, Basics of Hematology & Nerve-Muscle Physiology

(For medical students)

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Note – The selection of experiments in this manual represents curricular choices in a module on general physiology, basics of hematology, and nerve-muscle physiology at AIMST University School of Medicine, where the author and the reviewer taught physiology at the time this was written.

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This manual and any updates to it can be downloaded from http://esprakash.wordpress.com

Feedback requested: If you spot an error, please let me know. Comments and suggestions are welcome.

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Blood grouping and Rh typing

Aim: To determine the ABO blood group and Rh blood group of an individual.

Requirements: Sterile cotton; lancet; disinfectant; clean glass slides; 0.9% NaCl (citrated), small tubes for retaining an RBC suspension, anti-A, anti-B and anti-D antisera

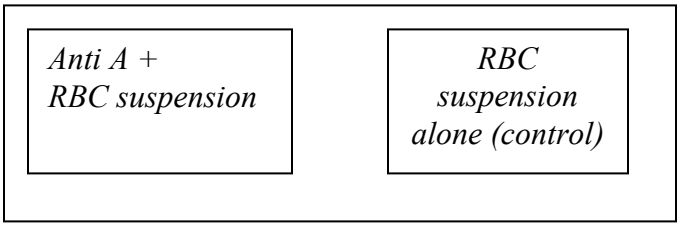
Principle: Red cells which contain A or B or Rh antigens will agglutinate (i.e., red cells are linked with other red cells) when the corresponding agglutinating antibodies are added in sufficient amounts. Blood group is defined on the basis of antigens present on red blood cells.

Method:

1. Take 4 clean glass slides.
2. Obtain a small amount of blood by finger prick using sterile precautions.
3. Add a drop of blood to a small tube prefilled with at least 3 ml of citrated 0.9% NaCl. Shake this tube gently so that red blood cells are adequately diluted in saline. This is called an RBC suspension.
4. In one of the glass slides add a drop of anti-A antiserum *(see the figure below)*
5. In another slide, add a drop of anti-B antiserum.
6. In a third slide, add a drop of anti-D antiserum.
7. Using a pipette, add 1 or 2 drops of RBC suspension to each of these slides.
8. In each of the slides, mix the suspension with antiserum using different edges of a clean glass slide.
9. Also add a small amount of RBC suspension as "control" on both slides.
10. Examine the slides under the microscope (low power magnification) for the presence of agglutination. Also note if there is any agglutination in the control suspension.

Precaution: Don't contaminate antisera. First add a drop of antiserum to the slide and add RBC suspension after that. Use different edges of the slide to mix the suspension with antisera in the three slides.

Note: Sometimes, an RBC suspension is not prepared and instead a small drop of undiluted blood is directly added to antiserum on a slide; although this is simple, it would be sometimes difficult to determine whether RBC have been cross linked by antibodies (true agglutination) or they are simply a clump of RBC. On the other hand, when a RBC suspension is prepared, RBC are sufficiently diluted and the number of RBC is greatly reduced; therefore, any agglutination that occurs is due to cross linking of RBC by antibodies and this can be easily verified by examining the slide under the low power objective of a compound microscope. Furthermore, when an RBC suspension is used, less antiserum is required.



Observations (tick as is appropriate):

Agglutination of RBC occurred with *anti A / anti B / both antisera / neither*

RBC *were / were not* agglutinated by anti D antiserum.

The individual's ABO & Rh blood group is: _____

Student assignment:

Please answer the following questions briefly:

1. Which of these red cell antigens among A, B and D are present on red blood cells of an individual with blood group "O negative"?

2. Red blood cells contain as many as three hundred different antigens. However, only the ABO and the Rh blood group are determined routinely. Why?

3. What is Landsteiner's law?

4. In the determination of blood group, what is the purpose of preparing an RBC suspension?

5. In determining blood group, why should RBC suspension be used as a “control”?

6. Which blood group is called “universal donor” and which is called “universal recipient”? Why?

7. How will you determine if an individual who is O negative can actually donate blood to an individual who is O positive?

8. What fraction of the population is Rh negative?

9. When does Rh incompatibility lead to hemolytic disease of the newborn?

10. What is H substance?

11. What is the Bombay blood group?

Differential Leukocyte Count (DLC)

Aim: To make a smear of peripheral blood and to determine differential leukocyte count

Requirements: Sterile cotton, lancet, disinfectant, clean glass slides, Leishmann's stain, compound microscope, and cedar wood oil.

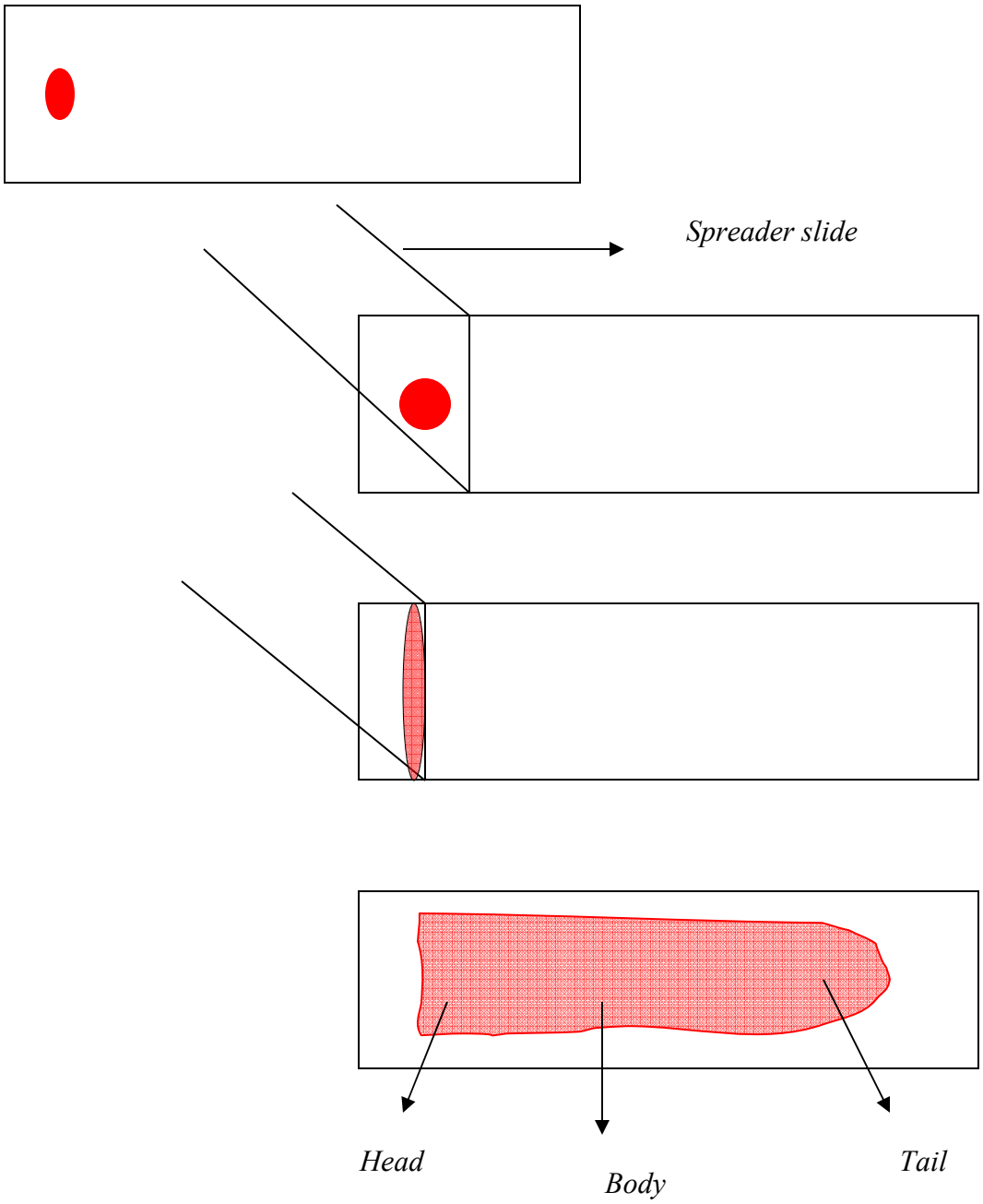
Procedure: This involves the following three steps:
1. Preparation of a blood smear
2. Staining the smear
3. Examining the smear under the microscope

Preparation of a blood smear: Take 4 clean glass slides. Select 1 slide with smooth edges as a spreader. Obtain a small amount of blood by finger prick using sterile precautions. Lay this drop of blood on the slide near one end of the slide. Please see the illustrations on the next page. Lay the edge of the spreader on the slide just in front of the drop of blood at an angle of 45 degrees with the slide. Draw the spreader back until it makes contact with the drop of blood, and blood spreads evenly along the edge of the spreader. Make a smear with a straight smooth forward movement. A well made smear has a head, body and tail, covers nearly 2/3 of the slide, its thickness is optimum, and it does not have obvious striations. For doing a differential leukocyte count, we need a thin smear because the idea is to study the morphology of different types of leukocytes. If we can read print (example the text on this page) through the smear, thickness is optimum. Make at least three smears.

Precautions: If you take more blood, the smear will be unacceptably thick. Also, if the angle between the spreader and the slide is greater than 45 degrees, the smear will be short and thick.

Staining the smear: Air dry the smear. After it has dried, add enough Leishmann's stain to fill the slide. Wait for 2 minutes. Leishmann's stain contains methyl alcohol, a fixative. This will cause cells to attach (fix) to the slide so that they won't be washed away. Now gently add buffer (distilled water) on the slide making sure the stain does not spill over. Add twice the amount of stain you used. After adding distilled water, wait for 8 minutes. Now the cells would take up the stain. After this time, gently wash the slide in tap water for long enough so that excess unused stain is removed. When washing is adequate, the smear looks salmon pink. Don't allow the water to splash straight on the slide. Allow water to run on your thumb and then on to the slide. Wash well enough (it takes about a minute on an average); the idea is to remove excess unused stain. If washing is inadequate, you will observe stain particles in your smear and it will greatly hamper identification of WBC. Also, stain particles will appear in the smear when the stained filled smear dries up before dilution with distilled water. Take care to add stain only after the smear has dried well enough because otherwise the smear will come off when it is washed. After washing the slide well, air dry it.

Leishmann's stain consists of eosin (an acidic dye which stains basic structures in the cell including histones), methylene blue (a basic dye which complexes and stains acidic structures like DNA and RNA), and acetone free methyl alcohol. Methyl alcohol must be acetone free because acetone, an impurity in the production of methyl alcohol, causes lysis of cells.



Examining the smear: Once the stained smear has been dried, examine it under low power objective (10 ×). This is to assess the quality of the smear. As you navigate from head to body to tail, you will appreciate differences in the density of cells in each of these

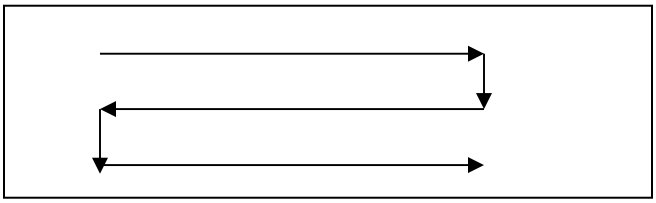
portions of the smear. Add a drop of cedar wood oil on the slide and focus it using the 'oil immersion objective lens (×100)'. This terminology comes from the fact that the lens is just immersed in oil and there is no air interface between the lens and the slide. Cedar wood oil is used because its refractive index is the same as that of glass; so light rays will not bend before being transmitted to the objective. Microscope adjustments for the oil immersion lens include opening the diaphragm, using plane mirror, and raising the condenser. These adjustments are aimed at increasing the illumination of the object.

Morphology of different types of white blood cells as seen in a peripheral smear:
It is common to compare the size of a WBC with that of RBC, the most abundant cell type in blood. WBC have been classified as granulocytes (granules present in cytoplasm) and agranulocytes (granules absent from cytoplasm when seen under 1000 × magnification). When examined using the compound microscope, neutrophils, eosinophils and basophils contain cytoplasmic granules (hence they are called granulocytes) whereas monocytes and lymphocytes do not. A brief description of the morphology of each cell type (as it is seen under the compound microscope) follows:

Granulocytes	
Neutrophil	Roughly twice as large as an RBC, multilobed nucleus (2-6 lobes connected by thin strands of chromatin), fine pink granules in cytoplasm. The fine pink granules may not be readily seen all the time. Use the fine adjustment to determine if granules are present or absent from a cell. The neutrophil is called so because it was thought to be stained by a dye at neutral pH.
Eosinophil	Roughly twice as large as an RBC, typically it has a spectacle shaped nucleus (bilobed) connected by an obvious strand of chromatin; coarse brick red granules in the cytoplasm are unmistakable. Because these granules are stained by eosin, these cells are eosinophils. Because eosin is an acidic dye, the granules themselves must consist of basic substances. One such substance in the eosinophil is the major basic protein. It is bactericidal.
Basophil	Roughly twice as large as an RBC, typically it has a bilobed nucleus. The coarse basophilic granules in the cytoplasm are unmistakable. In fact, the granules may obscure the outline of the nucleus.
Agranulocytes	
Monocyte	It is the largest WBC; the nucleus is prominently indented on one side and is usually kidney shaped, and is placed eccentrically; the cytoplasm is pale and agranular; the nucleus-cytoplasmic ratio is about 50:50. In contrast, in a lymphocyte, the nucleus occupies almost the whole of the cell leaving only a thin rim of agranular cytoplasm.
Lymphocyte	The outstanding feature in lymphocytes is the large nuclear-cytoplasmic ratio; i.e., the nucleus occupies almost the whole of the cell

	leaving a thin rim of cytoplasm in the periphery. The size of lymphocytes varies from 6-14 microns. Accordingly, they are sometimes specifically identified as small lymphocytes (it is just smaller than the RBC) and large lymphocytes (clearly larger than RBC). The feature that differentiates a large lymphocyte from a monocyte is the fact that the chromatin is denser in a lymphocyte compared to a monocyte.

Examine the body of the smear as indicated below. This will ensure that the same cells are not counted again.



Record your observations in this table below:

N	N	L	L	M	L	N	N	N	N

N: neutrophil; L: lymphocyte; M: monocyte; E: eosinophil; B: basophil. For example, your result could be written like this: N70 L26 M2 E2. The total must be equal to 100.

Result: The leukocyte differential is: _____

Interpretation of results: Normally, the differential leukocyte count is: neutrophils 50-70%; lymphocytes 20-40%, eosinophils 1-4%, monocytes 2-8%, and basophils < 1%. It is important not to interpret deviations of leukocyte differential in isolation. For example, a leukocyte differential N80 L16 E2 M2 does not necessarily indicate neutrophilia nor does it indicate lymphocytopenia. To make such an interpretation, we need to know the absolute neutrophil count, absolute lymphocyte count.

For example, if your total leukocyte count (TLC or WBC count) which is determined by hemocytometry is 10000/mm^3, and your leukocyte differential is N50L40E5M5, then:

Absolute neutrophil count (ANC) = 50/100 × 10000 = 5000/mm^3
Absolute lymphocyte count (ALC) = 40/100 × 10000 = 4000/mm^3.

Normal neutrophils constitute 50-70% of WBC and normal TLC is 4000-11000/mm^3.

Thus, the lower limit of normal ANC is 50/100 × 4000 = 2000/mm^3. If the ANC is less than 2000/mm^3, we would call that neutropenia.

The upper limit of normal ANC is 70/100 × 11000 = 7700/mm^3. An ANC above this would be called neutrophilia.

Definitions of lymphocytosis and lymphocytopenia and for the other WBC types are also derived similarly.

Neutrophilia commonly occurs with acute bacterial infections such as pneumonia (an infection of lung parenchyma), tonsillitis, and pharyngitis. This is a physiologic response to infection. In fact, a preponderance of young neutrophils may be seen in the blood stream if the number of lobes in the nuclei of neutrophils is carefully counted. This is sometimes called a “shift to the left”.

Exercise is a physiologic state that is characterized by neutrophil leukocytosis. This is presumably due to demargination of neutrophils when the circulation is hyperdynamic.

Viral infections usually are characterized by lymphocytosis. However, the human immunodeficiency virus (HIV-1) induces immunodeficiency by subverting a specific type of lymphocyte called helper T lymphocyte. Thus, in advanced stages of the resulting condition called AIDS, lymphocyte count is abnormally low.

Neutropenia, lymphocytopenia may occur with anemia and thrombocytopenia (pancytopenia: all blood cell types are deficient) when the bone marrow is affected by malignant disease that interferes with hematopoiesis; cytotoxic drugs that are used in cancer chemotherapy also affect hematopoiesis significantly; similarly, radiation of the bone marrow also inhibits hematopoiesis. Drug allergy is also a common cause of bone marrow depression. The consequences of neutropenia and lymphocytopenia can be predicted from their functions of the respective cells.

Eosinophils mediate immune responses to large antigens (including parasites) that cannot be phagocytosed, and some allergic conditions like hay fever are characterized by eosinophilia. Injections of ACTH reduce eosinophil count presumably by releasing cortisol from the adrenal cortex, and a deficiency of adrenocortical hormones is also associated with eosinophilia.

Assignment:

Please answer the following questions briefly:

1. What is the normal differential leukocyte count?

2. What are the characteristics of a well made smear?

3. What is the composition of Leishmann's stain?

4. Why should methyl alcohol in Leishmann's stain be acetone free?

5. The differential leukocyte count of a patient with fever was N20 L76 M2 E2. Does he have neutropenia or lymphocytosis?

6. The differential leukocyte count of a patient with fever was N20 L76 M2 E2. His total leukocyte count is 2000/mm^3. Does he have neutropenia or lymphocytosis?

7. How is neutrophilia defined?

8. Define eosinopenia.

9. Mention two conditions in which neutrophilia occurs.

10. Mention one cause of eosinophilia and one cause of lymphocytosis.

11. Mention one cause of eosinopenia and one cause of lymphocytopenia.

12. What is a band neutrophil?

13. List the uses of a peripheral smear.

14. Draw a schematic diagram depicting the morphology of various types of WBC.

15. How would you distinguish a monocyte from a large lymphocyte?

Bleeding time and clotting time:

Bleeding time (by Ivy method):

It is the time it takes for bleeding to stop after a standard stab wound is inflicted. There are several methods. The simplest is the Duke's method. In this method, after following sterile precautions, a nick is made in the fingertip deep enough (the tip of the lancet is 3 mm long) to result in bleeding. The test is timed from the time the injury is inflicted. The bleeding site is blotted on a Whatman's filter paper every 30 seconds. The upper limb should be held vertical and pressure applied on the forearm to increase pressure in the finger veins. The time taken for bleeding to cease is noted. Normally, by this method, the bleeding time is about 3 minutes. Though the test is popular, its drawback is the lack of reproducibility because the amount of pressure applied on the forearm is not constant.

In the more standard Ivy method, following sterile precautions, a standard wound 1 mm wide and 3 mm deep is inflicted on the ventral surface of the forearm about 2 inches below the elbow. A blood pressure cuff is applied on the arm on that side and kept inflated at 40 mm Hg to occlude venous outflow from the forearm. The test is timed using a stopwatch. The puncture is blotted with a filter paper every 30 seconds. Caution must be exercised not to cut subcutaneous veins. When bleeding ceases, the BP cuff can be released. Normally, bleeding time by this method ranges from 1-7 minutes. A value exceeding 10 minutes is clearly abnormal.

Clotting time:

After following sterile precautions, a standard wound is inflicted on the tip of a finger to result in bleeding. Freely flowing blood is taken into a capillary tube about 8 cm long. The capillary tube is broken from one end at periodic intervals (every 30 seconds), starting about one and a half minutes from the time of filling it. The time it takes for fibrin threads to appear from the time blood is taken into the tube is the clotting time. Normally, clotting time by this method is 2-6 minutes.

Answer the following questions:

1. What does bleeding time reflect?

2. What does clotting time reflect?

3. Why is bleeding time prolonged in thrombocytopenia?

4. Why is bleeding time prolonged in von Willebrand's disease?

5. What happens to clotting time in von Willebrand's disease? Why?

6. What further tests would you perform when clotting time is increased?

7. Why is clotting time prolonged in patients with chronic liver disease?

8. What is the effect of administration of warfarin on clotting time?

9. Give an example each of *in vitro* and *in vivo* anticoagulants.

10. What is the mechanism of anticoagulant action of heparin?

Determination of motor nerve conduction velocity

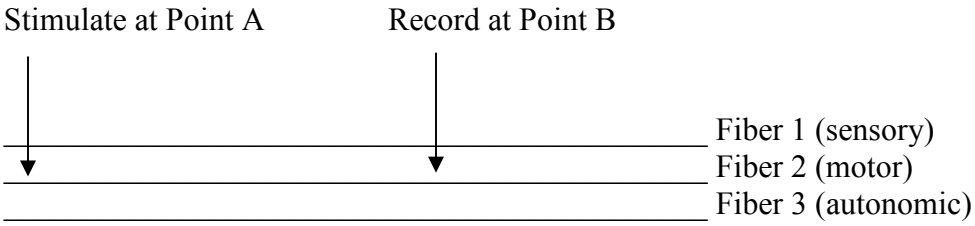
Background: In lectures on nerve and muscle physiology you have been introduced to mechanism of generation of resting membrane potential and action potential in neurons and muscle cells. Furthermore, you would have learnt about different types of nerve fibers and the Erlanger-Gasser classification of nerve fibers. Do you know what a mixed nerve is? Can you give an example? Is the median nerve an example of a mixed nerve? Is the ventral root of T1 spinal nerve a mixed nerve? You would have studied that conduction velocity is different in different types of fibers because these fibers differ from each other in their diameters; some fibers are myelinated whereas type C fibers are not. Can you tell what factors affect conduction velocity in a mixed nerve?

Physiologically, it is interesting, and in certain clinical situations, it is useful to determine conduction velocity in peripheral nerves such as the median nerve, ulnar nerve, tibial nerve. All of these are mixed nerves because they contain sensory, somatic motor as well as autonomic motor neurons. At this stage, you might want to predict the clinical effects of slowing nerve conduction velocity in peripheral nerves. There are several disease states in which this happens. Of course, when a peripheral nerve is completely cut (as may occur with injuries), the muscles supplied by that nerve will be paralyzed, and sensation in the area supplied by that nerve lost.

Objective: *At the end of this demonstration and discussion, you should be able to:*

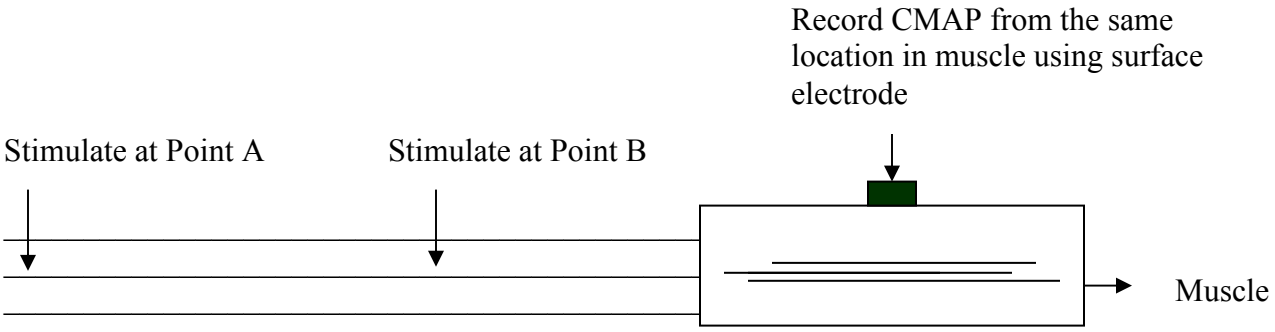
- explain the principle used in determining motor nerve conduction velocity;
- calculate conduction velocity when the required data are provided;
- explain the difference between "action potential" in a muscle cell and compound muscle action potential; and
- enumerate factors that affect nerve conduction velocity and discuss the effects of each of these factors.

How would you estimate conduction velocity in motor fibers of a mixed nerve?
Below is a schematic of a nerve trunk and let us assume it consists of three different types of fibers.



In principle, we could stimulate motor fibers at a point (point A) and record from another point (point B) separated by a distance D. However, there are several technical concerns that make this difficult in routine practice. Peripheral nerves are mixed nerves and it is difficult to precisely stimulate motor fibers alone without stimulating other types of fibers. Second, it is difficult (although not impossible) to record action potentials from nerve fibers.

A simple strategy is used to get over these difficulties. When motor nerve fibers are stimulated (with a stimulating electrode), the muscle supplied by that nerve will be eventually depolarized. It is far less cumbersome to record electrical activity from muscle. It can be done by placing recording electrodes on the muscle, and surface electrodes will suffice for this purpose. This is the principle used for determining nerve conduction velocity.



The same nerve is stimulated at two different points. In both instances, the effects of nerve stimulation are recorded from the same surface electrodes placed on the muscle. Because the electrical activity that is recorded from the surface electrode is a composite of electrical activity in different muscle fibers (in space as well as time), this is called a compound muscle action potential (CMAP; see the schematic below). Latency is the time from the stimulus artifact to the beginning of the deflection. The stimulus artifact is a minor deflection seen in the oscilloscope due to current leak from the stimulating electrode at the time of stimulation. This is followed after a definite latent period by the action potential.

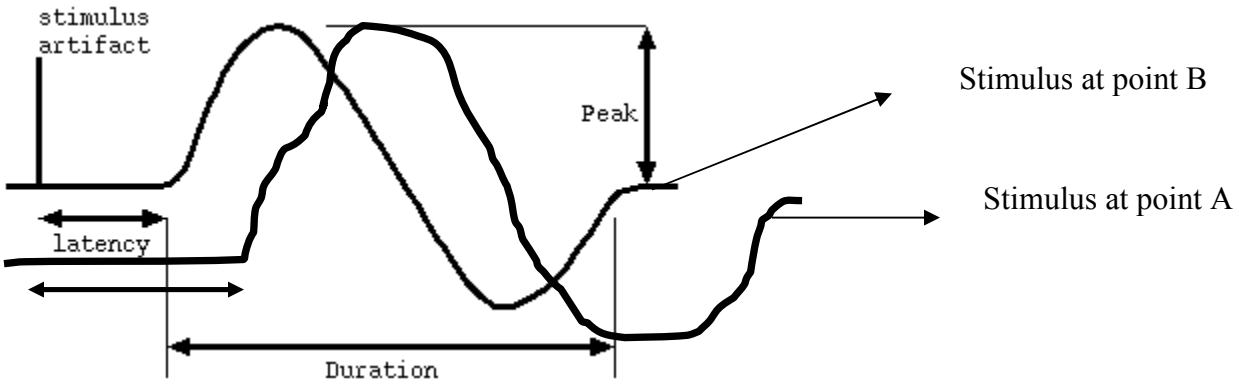


Figure modified from: http://noodle.med.yale.edu/~staib/bme355/neuromusc/cmap.gif; last accessed 9 August 2007.

As one might predict (please note in the above figure that the latency of CMAP will be longer farther the stimulating electrode is from the muscle, here point A is farther from the muscle than point B.

Observations:

This experiment will be demonstrated using the Nicolet VIASYS^{TM} HealthCare data acquisition and analysis system (Madison, USA). For example, in determining motor nerve conduction velocity in the right median nerve, the median nerve is stimulated at the elbow and at the wrist both at known distances from the recording electrode. The effects of nerve stimulation may be tested by placing the recording electrode on thenar muscles.

Conduction velocity in the nerve

$$= \frac{\text{distance between the two points that are stimulated}}{\text{difference in the latency of CMAP when the nerve is stimulated at these two points}}$$

For example,

If distance between the two points (elbow and wrist) at which the stimulating electrodes are placed	25 cm = 2.5×10^{-1} m
If Latency of CMAP when stimulated at elbow	20 ms
If Latency of CMAP when stimulated at wrist	15 ms
Difference in the latency	5 ms = 5×10^{-3} s
Then, motor nerve conduction velocity (right median nerve)	50 m/s

Assignment:
Answer the following questions:
Suggested reading: Chapters 2 and 3, In: Review of Medical Physiology, WF Ganong, Mc Graw Hill, International edition, 2005.

1. What is the Erlanger-Gasser classification of nerve fibers? *Tabulate your response.*

2. In a disease called multiple sclerosis, there is autoimmune destruction of myelin. What effect would this have on conduction velocity in motor neurons?

3. How does temperature affect nerve conduction velocity?

4. What is the difference between action potential from a single skeletal muscle cell and the electromyogram (EMG)?

5. What is compound nerve action potential? What is compound muscle action potential?

6. What is a motor unit?

7. What is the meaning of the term “recruitment of motor units”?

8. What is the “size principle”?

9. Normally, skeletal muscle is excited by acetylcholine released from alpha motor neurons innervating it. Is it possible to directly stimulate skeletal muscle "electrically" and make it contract?

10. What is "fasciculation"? What is "fibrillation"?

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