Determination of hemoglobin concentration in blood
Red blood cell (RBC), white blood cell (WBC) & platelet counts
Determination of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), & mean corpuscular hemoglobin concentration (MCHC)
Measurement of Hematocrit (Hct) / packed corpuscular volume (PCV)
Hemolysis & fragility test of RBC

[Purpose]
1. Learn how to determine the hemoglobin concentration in blood.
2. Learn how to perform RBC, WBC & platelet counts.
3. Learn how to determine MCV, MCH & MCHC.
4. Learn how to measure PCV.
5. Investigate the effects of NaCl solutions of various osmotic pressures on the morphology of RBCs & hemolysis.

[Principle]
Hemoglobin is found in RBCs, each of which contains approximately 280 million hemoglobin molecules. Hemoglobin is an iron-bearing protein that is the red coloring matter of the blood. Anemia occurs when the hemoglobin content of the blood is reduced.

The hemoglobin content of a solution can be estimated by measuring its color, its power of combining with oxygen or carbon monoxide, or by its iron content. The color-matching methods are sufficiently simple & accurate to be practical in clinical routine work, & the advent of photoelectric colorimetry has tremendously increased the accuracy of color-comparison. The most popular methods for hemoglobin estimation in modern clinical laboratories are colorimetric.

Total RBC & WBC counts are a routine part of any physical exam, & most clinical agencies use computers to conduct these counts. Platelets are cell fragments of large multinucleate cells (megakaryocytes) formed in the bone marrow. They appear as darkly staining, irregularly shaped bodies interspersed among the blood cells. Platelets are instrumental in the clotting process that occurs in plasma when blood vessels are ruptured.

Hemocytometer. There are three kinds of hemocytometer: Burker, Thoma & Neubauer. The improved Neubauer counting chamber is the most convenient & is now widely used clinically.
Improved Neubauer Counting Chamber. The improved Neubauer counting chamber (Fig. 2-1) consists of two separate counting chambers which can be used to enumerate both RBCs & WBCs on each side of the chamber without having to set up the apparatus twice. The platform is engraved with a ruled area, 3 mm on each side (9 mm²). This area is divided into 9 large squares. Each of the largest squares is 1 mm² in area. The four large squares (blue in Fig. 2-2) at the four corners of this platform marked with “W” are used for the enumeration of WBC. Each large square is again subdivided into 16 medium squares, each 0.25 mm on a side. The central large square, which is used for enumeration of RBCs, is divided into 25 medium squares & 16 small squares (red in Fig. 2-2).

Count the RBCs in 5 medium squares (4 at the 4 corners & 1 in the center) inside the central large square. Each border of a medium square is marked with three close parallel lines (inner, middle & outer lines). The RBC count is made only within the square bordered by the middle line. Count those cells which touch the upper & left medium lines of the medium squares but not those which touch the lower & right lines.

Fig. 2-2. Ruling of the “improved Neubauer Slide”.

Calculate the number or RBCs in 1 cubic mm of blood from:
- The dilution of the blood 1:200
- The depth of the platform 0.1 mm
- The area of each medium square 0.04 mm²
- Number of medium squares counted 5

EXAMPLE:
- Total RBC count in 5 squares = 600
- Multiply 600 × 5 = 3000 (Total number of cells in central square)
- Multiply 3000 × 10 = 30,000 (Cells per mm³)
- Multiply 30,000 × 200 = 6,000,000 (Dilution factor of 200)
- Total number of RBCs in sample is 6,000,000 cells/ml

Count the number of WBCs in the four large squares at the four corners of the platform. Only
those which lie on the upper & left hand sides of each large square should be include. Ignore those on the bottom & on the right hand line.

Calculate the number of WBCs in 1 cubic mm of blood from:

- The dilution of the blood: 1:20
- The depth of the platform: 0.1 mm
- The area of each large square counted: 1 mm²
- Number of large squares counted: 4

Excessively low values of RBC count, hematocrit, or hemoglobin may be indicative of anemia. There are many different causes of anemia (e.g. loss of blood through hemorrhage, bone marrow disease, iron deficiency, vitamin B12 deficiency, or folic acid deficiency) & some of those are characterized by typically very small or very large RBCs or reduced hemoglobin concentration in each cell. Diagnosis of the type of anemia may be assisted by relating the measurements of RBC count, hematocrit & hemoglobin to derive the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) & mean corpuscular hemoglobin concentration (MCHC).

Blood contains blood cells & plasma. The specific gravity of blood cells is greater than that of plasma. If whole blood, which is mixed with a suitable anticoagulant, is centrifuged sufficiently in a Wintrobe’s hematocrit tube, the blood separates into two layers, an upper layer & a lower layer. The former is called the plasma & the later is referred to as packed blood cells, thus PCV can be obtained. PCV is also called the hematocrit (Hct). Normally PCV is about 45%, that is, the packed cells account for 45 ml in 100 ml blood.

The permeability properties of a membrane can be studied by testing whether various compounds or agents, chemical or physical, can permeate the membrane. One method of doing this is to observe the osmotic effects of compounds on the cell membrane. RBCs require a rather stable osmotic environment, but normal RBCs resist hemolysis due to the fact that the cells are
Experiment II

bi-concave. A slight lowering of the osmotic pressure of the surrounding fluid will not cause them to hemolyze.

The RBCs are suspended in the plasma in the normal condition. They shrink in solutions with an osmotic pressure greater than that of normal plasma, & swell in solutions with a lower osmotic pressure, eventually bursting (lysis). If RBCs do not burst easily in hypotonic solutions, then their fragility is low.

Fig 2-4. Phenomenon of haemolysis.

[Experimental object] Rabbit blood mixed with an anticoagulant (sodium citrate)
[Experimental apparatus]
Tubes, pipettes, centrifuge, test tube rack, 1% sodium chloride (NaCl), distilled water (DW) & droppers.

[Experimental method & procedure]
Part I: Measurement of hemoglobin concentration, RBCs, WBCs, platelets, MCV, MCH, MCHC & PCV with a CA620 Hematology Analyzer.

Part II: Experimental Method & Procedure for Hemolysis & fragility test of RBCs.
1. The test tubes are ranked in the test tube rack & are identified by numbers. Add 1% NaCl solution & distilled water accurately into each tube according to Table 2-1 & then shake evenly & gently.

Table 2-1 Hypotonic solutions

<table>
<thead>
<tr>
<th>Tube number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% NaCl (ml)</td>
<td>1.4</td>
<td>1.3</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>DW (ml)</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.3</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>NaCl (%)</td>
<td>0.70</td>
<td>0.65</td>
<td>0.60</td>
<td>0.55</td>
<td>0.50</td>
<td>0.45</td>
<td>0.40</td>
<td>0.35</td>
<td>0.30</td>
<td>0.25</td>
</tr>
</tbody>
</table>

2. Take the fresh blood, add 1 drop into each tube, shake evenly & gently, & stand for 30 min at room temperature.
3. Judge the results
(1) The liquid of the upper level has no color & the bottom layer is muddy red, indicating no hemolysis.
(2) The upper layer is salmon pink & the bottom layer is muddy red, indicating incomplete hemolysis. In this condition, the sodium chloride concentration reflects the minimum resistance of the RBCs.

(3) The liquid is transparent red & the bottom has no deposited cells, indicating complete hemolysis. In this condition, the sodium chloride concentration reflects the maximum resistance of the RBCs.

**[Discussion]**

1. When does the hemoglobin concentration of blood increase or decrease?
2. What is the function of RBCs? When does RBC count increase or decrease?
3. What is the function of WBCs? When does WBC count increase or decrease?
4. What is the function of platelets? When does platelet count increase or decrease?
5. What is indicated when MCV increases or decreases?
6. What is indicated when MCH increases or decreases?
7. What is indicated when MCHC increases or decreases?
8. What are the differences between blood plasma & serum?
9. When does packed cell volume increase or decrease?
10. Which factors affect the accuracy of the test of RBC osmotic fragility?
11. Which factors affect the osmotic fragility of RBCs?