Tuesday 2nd Nov

Determination of Various Parameters of Human Blood

Aim - To determine the red cell count, haemoglobin concentration and haematocrit value of a sample of blood* and from these obtain information about the volume and haemoglobin content of the average red cell of the sample. These values are useful, especially in conjunction with information obtained from a stained blood film, in differentiating certain blood disorders (e.g. types of anaemia).

* It is important to use the same subject's blood throughout.

Introduction

The oxygen carrying capacity of blood is determined by the amount of normal haemoglobin present in the blood. This in turn is the product of the number of red cells present and the amount of haemoglobin contained within the red cells. It can therefore be appreciated that the number of red cells per unit volume (the red cell count) is a major factor in determining the oxygen carrying capacity of blood.

In health the number of red cells per unit volume (i.e. mm^3) is maintained within well defined limits for a given individual. This clearly shows that in health the rate at which new red cells are produced by the haemopoietic bone marrow must equal the rate at which old cells are destroyed by the phagocytic cells of the reticulo endothelial system.

The two essential points that should be realised about the meaning of a red cell count are:

(a) it reflects the balance between the rate at which new red cells are produced and the rate at which red cells are lost (either by destruction or haemorrhage)

(b) it is related to the plasma volume. Changes in plasma volume will affect the red cell count even in the absence of changes in either the rate of production or loss of red cells.

Normally, however, changes in plasma volume are of lesser importance, and will not be considered further here.

In disease however, the balance between production and loss can be disrupted and will if severe enough lead to changes in the measured red cell count. The most common situation is a fall in the red cell count, which can result from either a fall in the rate of red cell production or an increase in the rate of red cell loss (either by increased destruction or blood loss).

1) Red Cell Count

To carry out a red cell count, a sample of blood (either obtained from venipuncture from a volunteer or individual's finger stab) is diluted by a known amount and the number of red cells in a known volume of the diluted blood is counted under the microscope using a special counting chamber. The true red cell count is then calculated.

Recent development of particle-counting apparatus (the Coulter counter) and electronic means of determination of particle volume (the Channelyzer) has led to great improvements in haematological measurement. Unfortunately, the apparatus used is very expensive so that for all practical purposes use is limited to haematology units such as those in large hospitals.

The Coulter counter can be used for routine red (and white) cell counts. Using the Channelyzer in association with a Coulter counter it is also possible to determine RBC volume and hence M.C.H.C. (see later).

Method

a) Apparatus required

Microscope, haemocytometer (counting chamber and pipette) and diluting fluid.

The red cell pipette has a narrow stem, graduated in tenths with figures indicating 0.5 and 1.0, which widens into a bulb containing a red glass bead. The bulb narrows again, and at this point it is marked 101. Beyond this is a rubber tube with a glass mouthpiece attached.

b) General cleaning of apparatus

It is most important that the pipette should be cleaned and dry. Using a suction pump, suck up distilled water, through the pipette without its rubber tube and mouthpiece. Repeat several times with water and then with alcohol (I MS). Then suck up a little acetone and allow air to flow through the pipette until the interior is thoroughly dry, when the glass bead will roll freely showing no tendency to adhere to the side of the bulb. Replace the tube and mouthpiece on the pipette.

The counting slide consists of a thick glass side with a central platform, on which is ruled a counting grid. On each side of the platform, but separated by a trough there are two parallel supports for the coverglass- which is thus supported exactly one-tenth of a millimetre above the central platform. Clean the haemocytometer and the special thick coverslip carefully with water and then with alcohol. Dry with lens tissue. Both slide and coverslip must be kept free from any trace of grease or fibres from the polishing cloth.

c) Collection of blood

Use either the blood provided, obtained by venipuncture from a volunteer or collect a smaple of your own or your partner's blood by the method outlined below. N.B. It is essential that the red cell count, haemoglobin and haematocrit estimations (and therefore calculation of the red cell indices) be done on the same sample of blood.

Clean the skin at the root of the nail or the pulp of the finger with a swab soaked in alcohol. Allow the skin to dry. Make a quick stab with the disposable sterile lancet (Steriseal) provided. Alternatively blood may be collected from the fleshy edge of the lobe of your partner's ear, after prior cleansing with alcohol and using a fresh sterile lancet. Wipe away the first drop. Do not rub or squeeze if blood does not flow freely; after wiping the puncture site, firmly make a fresh puncture.

When a drop of reasonable size has been obtained, holding the red cell pipette horizontally suck up blood to the 0.5 mark or slightly beyond it. Close the mouthpiece by putting the tongue against it. Take the pipette away from the ear and wipe off any blood adhering to the outside. If the blood is beyond the 0.5 mark, then touch the tip gently against the back of the hand till the blood is exactly at the mark. Draw a small bubble of air into the capillary; this disappears during the next step. Immediately immerse the pipette in the red cell diluting fluid (either Hayem's solution or isotonic saline) and suck up to the 101 mark, rotating the pipette vigorously all the time to mix blood and solution thoroughly.

d) Preparation of film

Disconnect the rubber tube without squeezing it; close the ends of the pipette with the finger and the thumb and shake vigorously for a minute. Replace the rubber tube and blow out a quarter of the contents so as to remove the pure diluting fluid in the stem.

It is most important to avoid getting clotted blood in the pipette as it is exceedingly difficult to clean. If you think that the blood is likely to clot soon, blow it out at once and begin all over again.

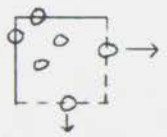
Moisten the glass bars on either side of the counting chamber with the tip of your finger and press the coverslip firmly down on them so that a series of concentrically arranged rings (Newton's rings) is seen. Bring the tip of the pipette quickly but gently onto the bevelled surface of the counting platform where it projects beyond the coverglass; a small amount of the solution will flow under the coverglass. The platform should be covered, but if the fluid flows over the edge of the chamber or if bubbles appear in it, wash up the slide and try again.

As soon as the cells have settled down (i.e. after two minutes), the count can be made as the rulings and the cells are then in the same plane. Examine the counting chamber with the low-power objective and a small iris diaphragm. If the distribution of the cells is not uniform, clean the counting chamber and fill it again. The microscope stage should be horizontal.

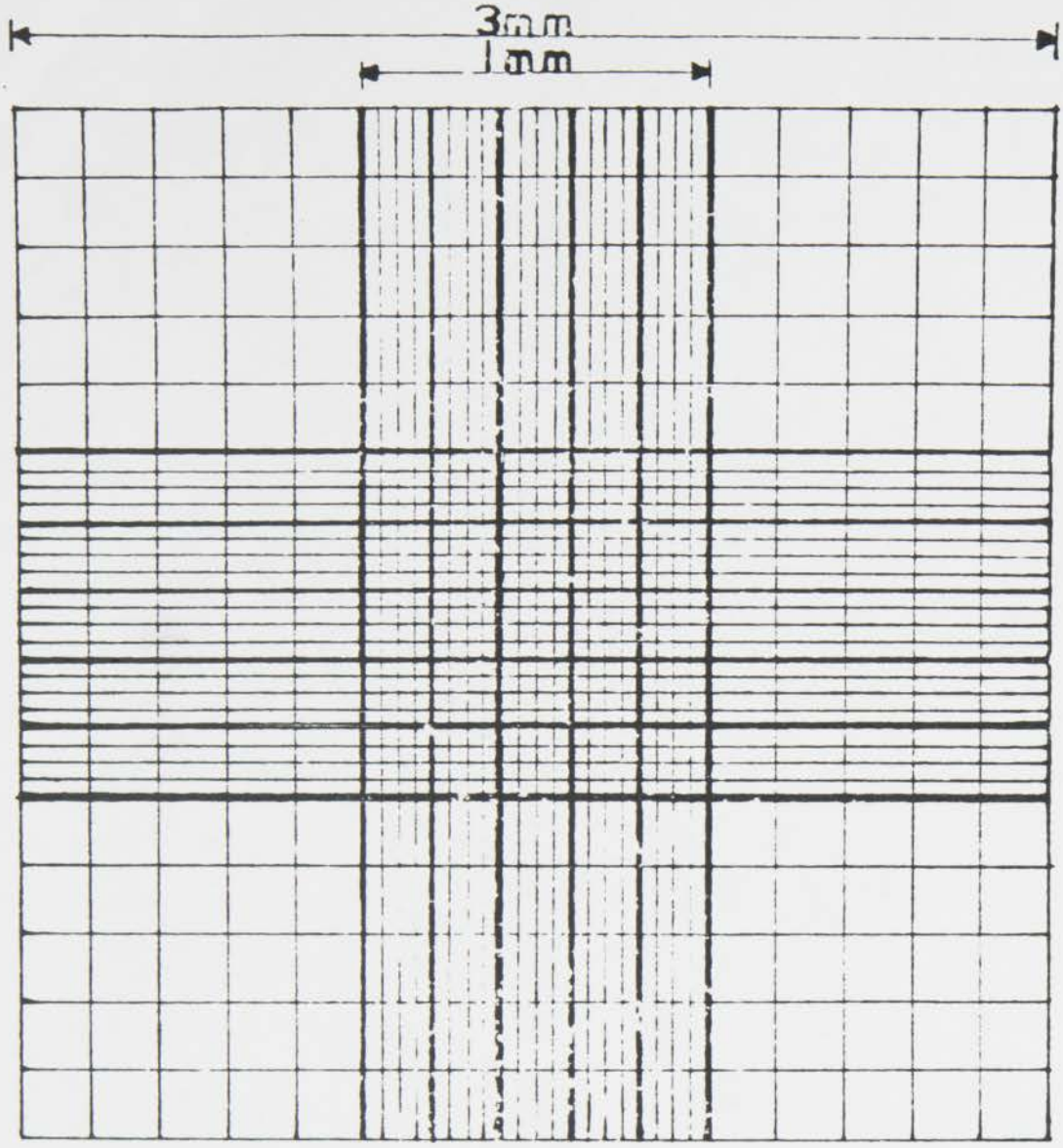
e) Counting

The haemocytometer provided is the improved Neubauer type. The ruling is shown in the diagram.

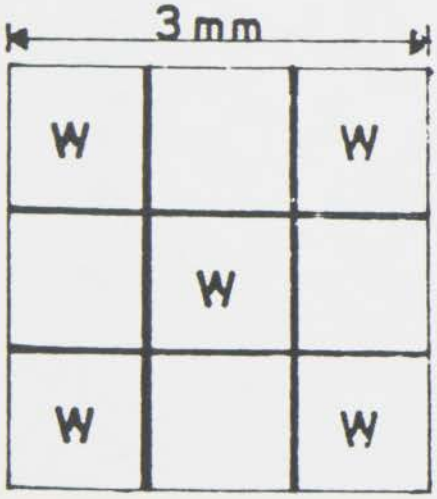
Count under high power the total number of cells in 80 small squares (using hand tally counter). Cells on the upper line and left side of each square are included in the square. See diagram e.g. count 4.



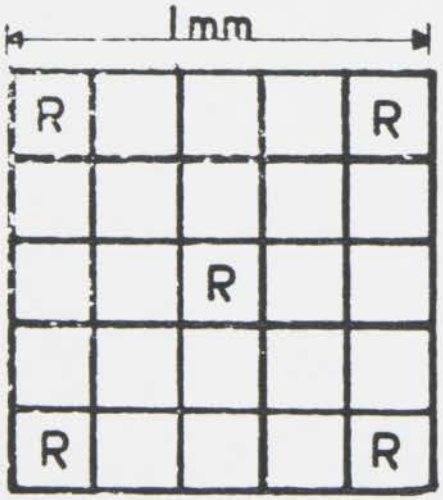
Wash counting chamber and coverslip as soon as count in complete.



Improved Neubauer ruling



white count



red count

NB. thick lines here are triple on slide

. Results

Red cells in 5 groups of 16 small squares

No. of cells	Top left	Top right	Centre	Bottom left	Bottom right	TOTAL
Count 1						
Count 2						

Calculation The depth of each chamber is 1/10 mm

Each square is $1/400 mm^2$

Volume of 1 square is $1/4000\ mm^3$

If n is the number of cells in 80 squares, then the number per mm^3 in diluted blood is $n \times 4000/80$.

Blood is diluted x 200

$\therefore$ total cell per $mm^3 = n \times 4000 \times 200/80$

$= n \times 10{,}000$ per mm^3

($1\ mm^3 = 1\ \mu l \therefore$ total $= n \times 10{,}000$ per μl)

Compare your values with the normal range of red cell count and with the result obtained by other members of the group.

Normal range: $4.6\text{-}6.2 \times 10^6/mm^3$ for males

$3.9\text{-}5.6 \times 10^6/mm^3$ for females

2) Measurement of Red Cell Diameter

Populations of red cells vary appreciably in diameter but characteristic deviations from the normal Price-Jones distribution are found in various anaemias.

Measurement of red cell diameters in dried blood films results in values 8-16% less than when cells are suspended in plasma, and also the distortion may be different in different areas of the film.

Diameters may be measured by projection of the images onto a screen, or photography at known magnification and subsequent measurement. The eye piece micrometer may be employed, but it needs to be calibrated in relation to the microscope first.

A simple, if less accurate, method of determining the mean red cell diameter is by diffraction. Two small lamps are placed 28 in apart at eye level. When they are viewed through a fresh, unstained blood film held near the eye (other eye closed) haloes round each are observed. The size of these diffraction patterns (haloes) is inversely proportional to the size of the red blood cell.

Each has a yellow centre and peripheral red rim, then a rainbow-like halo with red peripherally. This second, larger red circle is used as indicator.

Starting about 8 ft from the lamps, and looking through the film, move backwards and forwards till these indicating red circles just touch. The distance in feet from slide to lamps gives the mean modal daimeter in μm.

e.g. 100 in ≡ 8.33 ft ≡ 8.33 μm

Perform three or four readings and average.

Although not very accurate, this value should reflect any gross departure from normal MCV.

Normal range for dry films: 6.7-7.7 μm (mean 7.2 μm)
for suspensions of cells: 8.1-9.1 μm.

3) Haemoglobin Estimation

Introduction

The haemoglobin concentration of blood gives a true measure of the oxygen capacity of the blood. The amount of haemoglobin contained within a given volume of blood will, of course, be the product of the total number of red cells contained within that volume and the amount of haemoglobin contained within each red cell.

It can therefore be seen that either a fall in the red cell count and/or a fall in the intracellular haemoglobin concentration of the individual red cells will result in a fall in the haemoglobin concentration of whole blood.

There are several different methods of estimating the concentration of haemoglobin in blood. In most methods blood is diluted and the haemoglobin converted to a stable compound whose degree of coloration is compared with a known standard solution.

Method - (Cyanmethaemoglobin Method)

The basis of the method is to dilute blood in a solution containing potassium cyanide and potassium ferricyanide. Haemoglobin, methaemoglobin and carboxyhaemoglobin are all rapidly converted to cyanmethaemoglobin. The optical density of the solution is then measured in a colorimeter, provided with a yellow-green filter (Ilford 625).

0.02 ml of blood is added to 4 ml of modified Drabkin's cyanide-ferricyanide solution (KCN 0.05 g, $K_3Fe(CN)_6$ 0.2 g, distilled water to 1 litre). The tube containing the solution is stoppered with a rubber bung and inverted several times. After being allowed to stand at least 10 minutes at room temperature, the solution of cyanmethaemoglobin is ready to be compared with the standard in a colorimeter. (Set blank to 0 and compare sample to standard).

A commercially available cyanmethaemoglobin solution is generally used as a standard. The concentration of the standard varies slightly from batch to batch; you will be told the concentration of your standard at the start of the practical period.
Standard concentration = g/100 ml.

Results

	Colorimeter readings
Reagent blank	set to zero
Standard solution	

Sample Hb = $\frac{\text{reading unknown}}{\text{reading standard}}$ x conc standard = g/100 ml (= g/dl)

Compare your value with the accepted range of normal (13-18 g/100 ml male, 12-17 g/100 ml female) and with the values obtained by other members of the group.

4) Determination of Haematocrit (PCV)

Introduction

The haematocrit or packed cell volume expresses the percentage of the blood volume that is made up of red cells.

It can be measured by centrifuging blood in a tube, measuring the height of the column of red cells and expressing it as a percentage of the total height of blood in the tube.

The method used in class is a micromethod so that only small samples of blood are needed (i.e. capillary blood from a finger prick can be used instead of requiring larger volumes taken from a vein).

Method

Obtain blood from a finger prick as before. Take blood into the heparinised tube provided, (it will run into the tube by capillarity), and fill to about 1 inch from the end. (If you are using the heparinised venous blood provided, use a non heparinised tube). Seal the unfilled end of the tube in a Bunsen flame or seal with Cristaseal. Place the tube in a numbered slot in the microcentrifuge with the sealed end pointing outwards. All tubes should be touching the outside rim of the head. No balancing of the tubes is required. When all the tubes are in place (maximum 24) replace and screw down the metal cover. N.B. Do not run the centrifuge without this cover. Close the hinged lid and set the automatic time switch to 5'. (The centrifuge develops 12,000 g). When the centrifuge stops, remove the tubes and read the percentage of packed cells on the microhaematocrit reader. (If the tubes are not to be read immediately, place them in a vertical position to preserve strong boundaries between cells and plasma).

Reading of tubes

Position the tube in the slot of the microhaematocrit reader so that the base line of the reader (black line) intersects the base of the red cells. Move the tube holder so that the top line intersects the top of the plasma. Adjust the knob so that the middle line (white) intersects the top of the red cells. Read the percentage packed cell volume on the scale.

Haematocrit =

Compare your results with the normal range (45-55% men, 36-47% women) and with the values obtained by other members of the group.

5) Calculation of MCV, MCH, MCHC

These indices, which may be calculated from your results for the red cell count, haematocrit and haemoglobin content, give information about the volume and haemoglobin content of the average red cell of your blood sample. As mentioned previously, this information is useful in differentiating blood disorders (e.g. types of anaemia).

Mean Corpuscular Volume (MCV) - the volume of the average red cell)

$$MCV = \frac{\text{Volume of packed red cells in ml/litre of blood}}{\text{Red cell count in millions/mm}^3}$$

e.g. $MCV = \frac{450}{5} = 90\ \mu^3$ (90 cubic microns)
(90 femtolitres)

Normal range = 76-96 μ^3. Cells which fall within this range are called normocytes. Cells smaller than this are termed microcytes and larger cells are macrocytes. (N.B. Here the MCV is calculated from the red cell count ∴ not a very accurate index of cell size).

Mean Corpuscular Haemoglobin (MCH) - the average amount of haemoglobin in a red cell measured in picograms

$$MCH = \frac{\text{Haemoglobin in g/litre blood}}{\text{Red cells count in million/mm}^3}$$

e.g. $MCH = \frac{150}{5} = 30$ pg Hb/cell

Normal range = 27-32 pg

This measurement is perhaps not very useful since the amount of the Hb in a red cell is related to the size of the cell as well as the quantity of Hb. In addition MCH is also calculated from red cell count.

Mean Corpuscular Haemoglobin Concentration (MCHC)- the amount of haemoglobin in a red cell expressed as a percentage of the volume of the corpuscle

$$MCHC = \frac{\text{Haemoglobin in g/100 ml} \times 100}{\text{Vol packed rbc in 100 ml}}$$

e.g. $MCHC = \frac{15}{45} \times 100 = 33\%$

Normal range = 32-36% (32-36 g/dl).

Cells with MCHC within the normal range are called normochromic; cells with lower than normal are termed hypochromic.

Of these calculated indices, the MCHC has been considered generally to be the most reliable (and useful) since it is based on haematocrit and haemoglobin estimations. Any calculation involving the red cell count is rather inaccurate since this has a large margin of error unless modern electronic cell counters are used (the standard method of counting is said to have an error of ± 15% i.e. 0.75 million in a count of 5 million).

6) BLOOD - Normal Values

RBC - number per cu mm blood	♀ 3.9-5.6 x 10^6, ♂ 4.6-6.2 x 10^6
WBC - number per cu mm blood	Range 5,000-10,000, average 7,000
Platelets - number per cu mm blood	150,000-400,000, average 250,000
Haemoglobin g/100 ml	♀ 12-17, ♂ 13-18
Haematocrit, PCV -(packed cell volume) ml packed RBC per 100 ml blood	♀ 36-47, ♂ 45-55
Mean corpuscular volume, MCV	$= \frac{\text{vol of RBC per 100 ml blood}}{\text{no of RBC x } 10^6 \text{ per cu mm}} \times 10$
	= 76-96 μ^3 (≡ femtolitres - $10^{-15}\ell$)
MCHC concn of Hb in an average corpuscle as %	$= \frac{\text{g Hb per 100 ml blood}}{\text{vol RBC per 100 ml blood}} \times 100$
	= 32-36% (g/dl)
MCH - amount of Hb in a corpuscle in pg (g x 10^{-12})	$= \frac{\text{g Hb per 100 ml blood}}{\text{RBC x } 10^6 \text{ per cu mm}} \times 10$
	= 27-32 μm
Red cell diameter	Fresh, 8.5 ± 0.4; Fixed and stained, 7.2

These values and their deviations from average may be used to distinguish different types of anaemia.

Changes associated with different types of anaemia

	MCV	RBC	MCHC	Example and Cause
Macrocytic	inc > 94	dec	normal or slight dec > 30	Pernicious anaemia, sprue, due to lack of vitamin B_{12} or folic acid
Normocytic	normal or slight inc 80-94	dec	normal > 30	After haemorrhage
Simple microcytic	normal or slight dec < 80	dec	normal or slight dec > 30	Inflammatory conditions, e.g. after 'flu
Hypochromic microcytic	dec < 30	normal or dec	dec < 30	Nutritional anaemias, e.g. iron deficiency due to dietary lack, poor absorption or continued loss, e.g. heavy menstruation or excessive demand, e.g. close pregnancies or combinations of these.

7) Questions

Answers to these questions may help you with the discussion section of your practical.

(a) Discuss sources of error in the red cell count, haemoglobin and haematocrit estimations. Which is the least reliable method and why?

(b) Can you provide examples of physiological variations (not pathological variations) in the red cell count?

(c) Apart from its oxygen carrying capacity, what other property of blood is greatly affected by the red cell count?

(d) What do you understand by the phrase "a hypochromic anaemia"? How could this arise?

(e) Under what conditions would you expect the haematocrit
i) to rise
ii) to fall?

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