PRACTICAL 6: ELECTROPHORESIS OF HAEMOGLOBIN — USE IN DIAGNOSIS

6.1 Introduction

The **aims** of this practical are:

- to introduce the technique of electrophoresis as a means for separating proteins
- to use electrophoresis to distinguish between normal individuals and those with sickle cell disease and sickle cell trait

6.2 Theory and background

Sickle cell disease

Sickle cell disease is caused by a hereditary defect in the haemoglobin molecule. The two b chains in normal haemoglobin (**Hb-A**) contain a glutamic acid residue at position 6. In people with sickle cell disease, a valine residue occurs in this position due to an **A T transversion mutation** in the glutamate codon GAG to give the valine codon GTG. This residue is on the outer surface of the molecule and this single difference in the sequence of the 146 amino acids of the b chain is enough to produce a "sticky" **hydrophobic** spot on the surface that results in the abnormal quaternary association of the a and b chains of the abnormal haemoglobin (**Hb-S**).

Both deoxygenated Hb-A and Hb-S have a normal, "complementary" sticky patch on the surface of the a chains to which the abnormal hydrophobic spot on deoxygenated Hb-S will bind. This normal patch is masked when the haemoglobin is oxygenated. Thus, when oxygen concentrations fall below a critical level, the Hb-S polymerises into linear, insoluble arrays of fibres within the erythrocyte, which become deformed (sickled) and function abnormally (Figure 1). This only happens in sickle cell homozygotes, since the presence of deoxyHb-A produced by the normal allele in heterozygotes will terminate the polymerisation. Heterozygotes are phenotypically normal but are said to carry the "**sickle cell trait**"

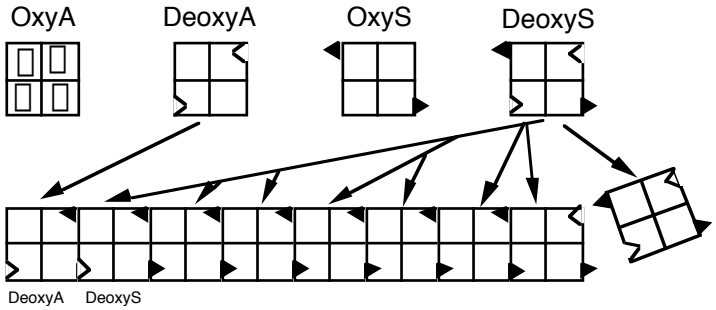

Figure 1: Polymerisation of Hb-S

The Glu Val substitution also causes a charge difference between Hb-A and Hb-S that affects the mobility of the molecule in an electric field. Thus, **electrophoresis** of haemoglobin (or a red cell lysate — **haemolysate** , which is predominantly haemoglobin) can be used as a diagnostic aid and can readily distinguish between normal, sickle cell homozygote and sickle cell heterozygote individuals.

Electrophoresis

Electrophoretic separation encompasses a variety of methods in which solutes (usually charged macromolecules such as proteins, nucleic acids etc.) are resolved on the basis of their mobility in an electric field. In general, an electric field is applied through some supporting medium and the solutes migrate in that field, according to:

- their net charge
- the friction between solute and support (which is dependent upon size)
- the magnitude of the potential difference (the electric field)

The supporting medium may be one of several types:

- liquid (e.g. a dense sucrose solution)
- solid (e.g. paper or cellulose acetate)
- gel (e.g. polyacrylamide, starch or agarose (a seaweed polysaccharide)

Methods of separation and apparatus vary widely, but in general terms a sample is applied to the support in a buffer that allows maximum separation of differently charged solutes — this can be highly dependent upon pH and ionic strength. An electric field is applied and the different components will move in the support, negatively charged molecules towards the anode and positively charged ones towards the cathode (Figure 2).

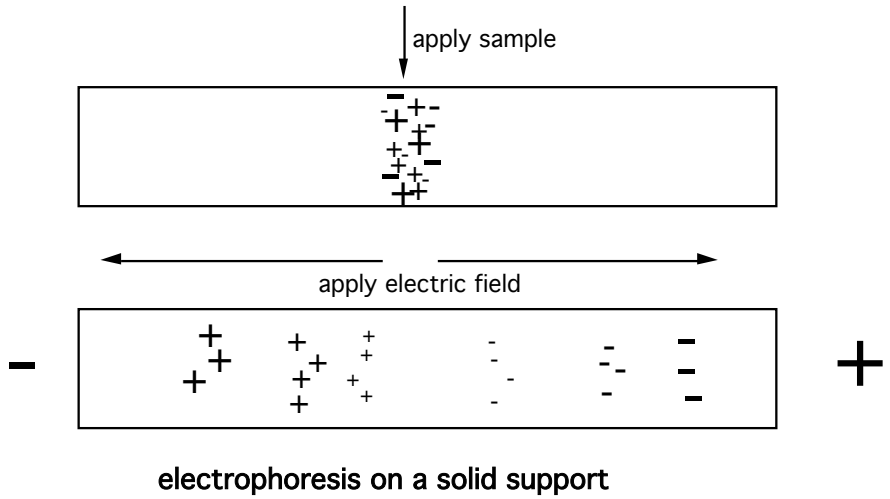

Figure 2: Electrophoresis on a cellulose acetate strip

- *In experiment A, you will separate Hb-A and Hb-S in normal and sickle cell samples by electrophoresis*

6.3 Experiment A: Separation of haemoglobins A and S by electrophoresis on cellulose acetate strips.

Reagents: **Haemolysates A, B and C** (a haemolysate is the contents of the red cells). You will be given a 5 μl sample of each by your demonstrator.
Electrophoresis buffer (TEB — 0.12 M Tris, 5 mM EDTA, 15 mM boric acid, pH 8.9)
Protein stain solution (0.5% Ponceau S in 5% TCA [trichloroacetic acid]). N.B. TCA is corrosive. Take great care with this solution. WEAR GLOVES.
Destain solution (5% acetic acid)

Procedure (work in pairs for this experiment):

N.B. Steps 1 and 2 will probably have been done for you. If so, proceed to step 3.

1. Place TEB electrophoresis buffer (about 500 ml total) into all four compartments of the electrophoresis tank. Ensure that the level is the same in all compartments by carefully lifting the tank so that the buffer laps over the end of the separating walls. Wipe any excess liquid from the walls with a tissue.

2. Thoroughly wet 2 pieces of Whatman No. 3 filter paper (20 x 7.5 cm) with buffer solution and place them over the edges of the shoulder pieces with one edge of the paper running parallel with edge of the shoulder and the other immersed in the buffer of the outer compartment. These pads act as buffer wicks between the buffer solution and the cellulose acetate strips which are placed between them.

3. Take one cellulose acetate strip and, with a blunt pencil (provided), write your initials at one end. Also mark a **faint** starting line (**origin**) lightly across the centre of the strip — see **Figure 3** (*Note: handle strips at the ends only or use the plastic forceps*). Moisten the strip as follows. Add some TEB buffer to a shallow dish and carefully float the strip on top so that it is impregnated with buffer from below by capillary action. When the strip is thoroughly wetted (3-4 min), it can then be submerged in the buffer. It is essential that this procedure is followed exactly since it avoids trapping air bubbles in the pores of the membrane.

4. Remove excess buffer from the strip by blotting lightly on filter paper — do not overdry by pressing too hard.

5. You will be given a 5 μl droplet of each of the three haemolysates (**A**, **B** and **C**) on a small square of Parafilm by your demonstrator. Apply the sample applicator to the surface of droplet **A** then transfer the sample **A** to the left hand one-third of the pencilled origin line on the strip by gently touching the applicator on to the strip (see **Figure 3**). Rinse the applicator with distilled water from a wash bottle, **dry**, then repeat the process for haemolysates **B** and **C** (rinsing between each). Apply **B** to the centre one-third of the origin line and **C** to the right hand one -third (see **Figure 3**).

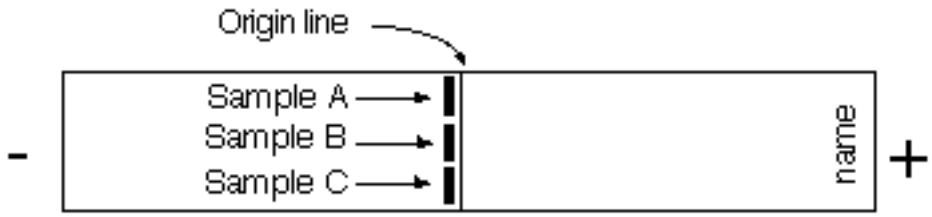

Figure 3: Sample application on cellulose acetate strip

6. Place the strip between the two shoulder pads of the electrophoresis tank, with the origin in the centre and the end with your name towards the anode and carefully pull taut. Press the ends of the strip firmly against the pad to ensure proper contact.

7. Place the Perspex lid on the tank and connect the red and black terminals (male) of the tank to the +ve and -ve terminals (female) of the power supply. Ask a demonstrator to check the correct set-up and the demonstrator will switch on the power supply. Electrophoresis will be carried out at a constant voltage of 150 V (approx. 0.5 mA/cm strip width) for 60 mins.

8. Switch off and unplug the power supply. Remove the strip with forceps and carefully float it on the surface of the Ponceau S staining solution, allowing the stain to impregnate the strip from below. When totally wetted, immerse the strip completely in the stain and leave for 5 mins. Agitate occasionally.

9. To destain the strip, remove it from the stain. Drain off excess stain and rinse in a tray of 5% acetic acid. Change the acetic acid once, agitate for a moment, then finally rinse the strip in tap water.

Diagnosis

Given the nature of the amino acid substitution and the direction in which the protein bands have moved, which of the three samples, A, B and C, represent normal, sickle cell heterozygous and sickle cell homozygous individuals? Why?

A:	B:	C: