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RED, BLUE AND BROWN BLOOD: DEMONSTRATIONS

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This paper reports some simple demonstrations on oxygen exchange between air and blood. Human blood was used to demonstrate the processes of the binding and releasing of oxygen from haemoglobin. However, the procedures should be equally valid for the use of animal blood. The procedures demonstrate the conversion of oxyhaemoglobin (red blood) to methaemoglobin (brown blood) and to deoxyhaemoglobin (blue blood). Moreover, procedures to demonstrate the recharging capacity of haemoglobin (the change of oxyhaemoglobin to deoxyhaemoglobin and back to oxyhaemoglobin) using visual and spectrometric analysis are also reported. The latter demonstration emphasises the transport of oxygen by blood in an organism. The demonstration procedures reported in this paper are simpler, safer and faster than those previously reported.

INTRODUCTION

Demonstration in science has a long history. Uzzell (1978) reported that in 1882 the Education Department (UK) declared that experiments should be used in giving instruction to scholars in science subjects. The experiments aforementioned referred to experiments (demonstrations) performed by teachers rather than by students. Since 1882, practical work in teaching has sometimes been promoted and sometimes dismissed as a waste of time (Lock, 1988; Hodson, 1990). Since the curriculum development projects of the 1960s, which stressed the importance of class practical work, demonstrations have gone somewhat out of fashion.

However, there are many reasons for carrying out demonstrations. They are often spectacular and therefore stimulating and motivating. They enable students to see experiments that they would not be able to perform themselves for a variety of reasons. Some of the reasons are that the experiment may (a) requires skills that are beyond the scope of students; (b) be potentially dangerous in unskilled hands; (c) require expensive apparatus and/or chemicals; (d) require facilities, such as a fume cupboard, which are not available in sufficient number for class practical work. Moreover, they allow students to see a skilled practitioner at work (Lister, 1995).

In most countries of the world including Brunei Darussalam, uses of oxygen, importance of dissolved oxygen in maintaining aquatic life in water, respiration and transport systems are taught in secondary schools (JPK, 1999a & 1999b). The interaction of oxygen with blood and the importance of oxygen for living organisms is often cited by the teachers and they often use diagrams and video-tapes to make the working of the system clear to their students. However, they often are unable to demonstrate the process using blood due to a lack of simple and safe demonstration procedures.

Russo and Sorstokke (1973) developed a procedure to demonstrate interactions between oxygen with blood using red blood cells. The red blood cells were washed with 1.2% NaCl solution. The procedure was repeated till a clean supernatant solution was obtained. The cells were then haemolysed using 1.2 volumes of water and 0.4 volume of toluene (or ether). The mixture was shaken vigorously for several minutes and allowed to stand for an hour or over night at 4.0^{o}C. The toluene layer was then separated and discarded. The resulting solution was then centrifuged to eliminate any precipitates before the stock solution was used for demonstrations. Splittgerber (1974) and Cooke (1976) also used similar procedures that required separation of red blood cells from plasma and washing them with 1% NaCl. Finally the red blood cells were haemolysed with distilled water and ether (or methyl benzene) followed by the use of high speed centrifugation to remove cell wall material. The procedures described above are complex and time consuming. Moreover, the procedures for demonstrating the charging and discharging of haemoglobin with oxygen required preparation of fresh Stokes' solution.

A simpler, quicker and safer procedure to haemolyse the blood as described above has been reported in this study. Ammonium hydroxide solution was used to instantly haemolyse the blood. The advantages of this method over the previous procedures are (a) whole blood in place of red blood cells was used that eliminated the tedious steps requiring removal of plasma, the red blood cells, and their washing, (b) haemolysis occurs instantly, therefore, does not require a wait time of an hour or overnight, (c) the separation of cell wall material was not required because it did not interfere with the reactions, (d) stock solution of blood becomes safe to handle because a highly basic environment is not suitable for any organisms/viruses found in blood, (e) there is no need to prepare fresh stokes solution, and (f) procedures are simple and short.

ROLE OF HAEMOGLOBIN IN OXYGEN TRANSPORT: GENERAL TEACHING TIPS

Diffusion of atmospheric oxygen to the body of the organism is an adequate oxygen transport mechanism for small animals such as insects. However, this process is not suitable to supply sufficient oxygen for large animals. The development of lungs and gills (to provide a large surface area for oxygen exchange with the atmosphere) and the circulation system have provided larger animals with an effective system for transporting oxygen and other nutrients to the parts of the body where they are needed. Although, under the normal atmospheric conditions, the amount of oxygen that can be dissolved in plasma (or water) is insufficient to support life in most of the animals yet some fish that live in Antarctic waters have been reported to have the capacity to dissolve sufficient oxygen in plasma. They therefore, do not use haemoglobin. This is possible because at such low ambient temperatures sufficient oxygen can be dissolved in the plasma or water. However, in most of the animals a mechanism was required to increase the concentration of oxygen that could be achieved. Moreover, this mechanism should meet two contradictory criteria: (i) the oxygen must bind readily with the transporting agent in the lungs and (ii) the transporting agent must release oxygen readily where the oxygen in the body of the organism is required. Haemoglobin (Hb) satisfies both the criteria, therefore is used as an oxygen carrier from the lungs to the tissues in large animals. Haemoglobin is present in red blood cells. It is a protein with four subunits

which give the molecule a complex structure. Each subunit consist of a haeme (iron) unit embedded in the folds of an helical protein called globin. The shape of the haemoglobin molecule is shown in Figure 1.

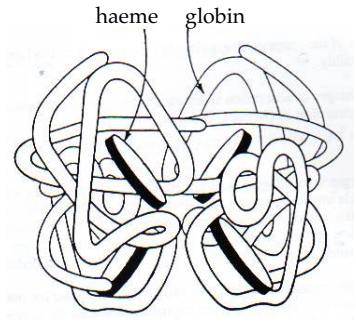


Figure 1: Haemoglobin molecule

Each haeme group consists of ferrous ion (Fe^{2+}) which can form hexa-coordinate complexes. It forms five coordinate bonds with nitrogen ligands in the protein and the sixth place is vacant. The shape of the molecule makes this site small and hydrophobic. Water, therefore, cannot bind at this site because of the hydrophobic nature of the site and of the large size of a water molecule. The size is just right for oxygen or similar small molecules, such as CO and NO. The extension of this work to demonstrate the effects of smoking on blood is in progress.

The dissolved oxygen in plasma is in equilibrium with oxygen attached to haemoglobin. The equilibrium is demonstrated below in equation 1.

$$\underset{\text{Deoxyhaemoglobin}}{Hb\ (aq) + O_2\ (aq)} \quad <==> \quad \underset{\text{Oxyhaemoglobin}}{HbO_2\ (aq)} \qquad (1)$$

In the lungs, the excess of oxygen shifts the equilibrium to the right and more oxyhaemoglobin (HbO_2) is formed. In the tissue, the oxygen diffuses through the cell wall from the plasma to the cells. Therefore, the concentration of oxygen in the plasma is decreased and the equilibrium moves to the left. Hence oxyhaemoglobin changes to haemoglobin commonly known as deoxyhaemoglobin (Hb) and oxygen is released into the plasma.

The release of oxygen from HbO_2 can be achieved either by shifting the above equilibrium to the left by decreasing the dissolved oxygen in plasma or by denaturing the molecule by oxidising the ferrous unit present in the haemoglobin to ferric. Both methods have been reported in this paper, however, the latter one is not reversible and haemoglobin molecules containing ferric unit (methaemoglobin) are not capable of binding to oxygen. Therefore, it is not suitable for the transport of oxygen in an organism. A detailed description of the mechanism is given in the later part of the paper.

AIM AND OBJECTIVES

The aim of this study was to prepare a set of simple demonstrations to illustrate the role of haemoglobin in the oxygen transport mechanism. The objectives of the study were to demonstrate (i) the preparation of oxyhaemoglobin, deoxyhaemoglobin, and methaemoglobin from blood, (ii) the presence of oxygen in oxyhaemoglobin and (iii) the reversible nature of the haemoglobin in charging with and discharging of oxygen.

APPARATUSES, CHEMICALS AND SAFETY

Apparatus

A 500 ml container with lid (volumetric flask or conical flask or glass bottle or plastic bottle)

Nine 50 ml test tubes

A glass rod

A 5 ml syringe

A 10 ml measuring cylinder

Chemicals

Ammonium Hydroxide (NH_4OH), 0.4 M, 250 ml

Tartaric acid, ($C_2H_2(OH)_2(COOH)_2$), 2 g

Ferrous sulphate ($FeSO_4$), 2 g

Potassium hexacyno ferrate ($K_3[Fe(CN)_6]$), 1g

Orthophosphoric acid (H_3PO_4), 4.0 M, 2ml

Sodium dithionite ($Na_2 S_2O_4$), 1g

Blood, 2.0 ml

Special notes

(i) The author had tried the following demonstrations using blood from an expired blood pack obtained from the Blood Bank from a hospital that was declared safe for human-blood-transfusion. The experiment works with animal blood too. The animal blood (excluding pig blood) may be obtained from an abattoir. The blood may also be collected if a live chicken is slaughtered for cooking. Under these conditions, the stock solution should be prepared as soon as possible, especially before the blood coagulates. The stock solution is stable and may be stored at 4°C in a refrigerator. Teachers are recommended to use chicken or any suitable animal blood (not pig blood) to give school students hands-on practice using these procedures as a practical on oxygen-exchange in blood.

(ii) A 50 ml test tube produced better visual effects than a 20 ml test tube. In the case of the latter, colour changes were not clear because of the thin column of the solution.

Safety

Students are to wear goggles to protect their eyes.

Students are to wear gloves (very important when dealing with human blood).

The products from the test tubes may be disposed of down the sink with large quantities of water, since 0.4 M ammonia will disinfect the blood.

PROCEDURE

1. Preparation of oxyhaemoglobin stock solution

Demonstration Procedure

Take a clean dry 500 ml container with a lid.

Add 250 ml of 0.4 M ammonia (NH_4OH) to the container.

Use a 5 ml syringe to add 2.0 ml of blood (see above) to the ammonia solution in the flask.

Close the flask with the lid and swirl it gently to mix.

Ammonia will haemolyse the red blood cells and a red solution of oxyhaemoglobin will be obtained.

Label this solution on as the stock solution.

2. To demonstrate the presence of oxygen in oxyhaemoglobin (red blood)

Demonstration Procedure

Take three 50 ml tubes and label them as A, B and C.

Add 10 ml of stock solution to test tube A and 20 ml to test tube B.

Keep test tube A as a colour reference.

Add 5-6 drops of 4M orthophosphoric acid (H_3PO_4) to the test tube B and mix this solution by swirling the tube.

Add about 10 small crystals of potassium hexacyano ferrate, $K_3[Fe(CN)_6]$, (also known as potassium ferricyanide) to test tube B.

Observe the bubbles of gas (oxygen) coming out of the brown solution.

Compare the colours of the solutions in the test tube B with that of the solution in the reference test tube A. (A colour change from red to brown is observed).

Dissolve the crystals in the solution in test tube B by stirring with a glass rod.

Transfer about 10 ml of brown solution from test tube B to test tube C.

Keep the remaining solution in the test tube B as a second colour reference.

Close the test tube C with a stopper and shake the solution for about one minute to see if the reaction is reversible.

Allow the test tube C to stand for a while so that the foam in the test tube is settles on the top.

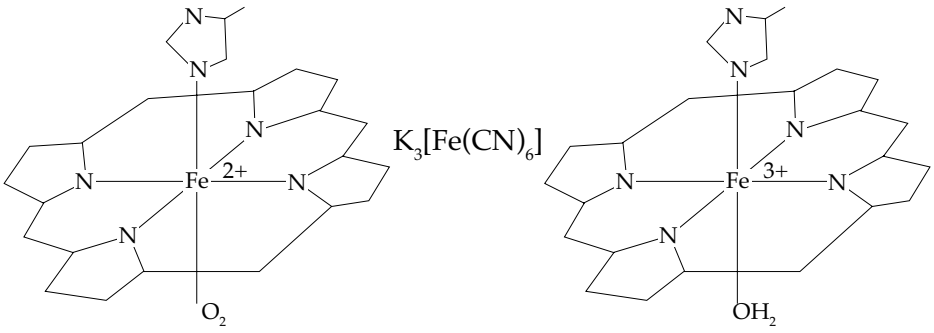
Compare the colour of the solution in C with that in A.

The colour of solution in test tube C will compare with the solution in B but not in test tube A.

A colour change from brown to red will not be observed because the reaction is not reversible.

Specific Teaching Tips

In the following three equations, (2a) shows structural changes to the haemoglobin molecule, (2b) demonstrates equilibrium shift and (2c) represents the oxidation of haeme unit from ferrous ion to ferric ion during the above demonstration. Teachers may omit some or all of these cases depending upon their needs.



Oxyhaemoglobin Methaemoglobin (2a)

HbO_2 (aq) + H_2O (l) <==> $HbOH_2$ (aq) + O_2 (aq) (2b)
Oxyhaemoglobin Methaemoglobin

Oxidation of iron $2Fe^{2+} + O_2 \rightarrow 2Fe^{3+} + 2O^{2-}$ (2c)

Haemoglobin is known as oxyhaemoglobin when oxygen is bonded to it. Oxyhaemoglobin is red in colour. One of the ways to release the bonded oxygen from haemoglobin is to oxidize Fe^{2+} to Fe^{3+}. Potassium hexacyano ferrate, $K_3[Fe(CN)_6]$, is a satisfactory oxidising agent. When potassium hexacyano ferrate is added to the oxyhaemoglobin, it oxidises ferrous ion (Fe^{2+}) present in oxyhaemoglobin to ferric ion (Fe^{3+}). The ferric site is no

more hydrophobic due to the change in the shape of the molecule and a water molecule attaches to Fe^{3+} leaving no place for oxygen to form a bond with the haeme unit in the haemoglobin molecule. Moreover, the shape of the molecule is also changed. Ferric ion is not suitable for binding with oxygen and the shape of the molecule too does not support holding the oxygen molecule in place. Therefore, oxygen is released from the blood. Some of the released oxygen dissolves in the water and some is released in the form of bubbles that can be observed by the students. Haemoglobin containing ferric ion is called methaemoglobin. Methaemoglobuin is brown in colour. Students might have observed this colour while observing an over-exposed and old piece of meat in meat shops or at home. Meat boiled in water also exhibits the same brown colour. The reaction is not reversible because auto reduction of ferric ion to ferrous ion in presence of potassium hexacyno ferrate is not possible.

3. Preparation of deoxyhaemoglobin from the stock solution

Demonstration Procedure

Take three 50 ml test tubes and label them as D, E and F.

Add 10 ml of oxyhaemoglobin stock solution to test tube D and 20 ml to test tube E.

Keep test tube D for a colour reference.

Use a small spatula to add about 0.3g of solid sodium dithionite ($Na_2S_2O_4$) to the test tube E.

Dissolve the solid sodium dithionite by swirling the test tube.

Observe and record the colour change from red to blue.

Pour about half of the solution from test tube E into F.

Keep tube E as a second colour reference.

Close the test tube F with a stopper and shake its content for about one minute.

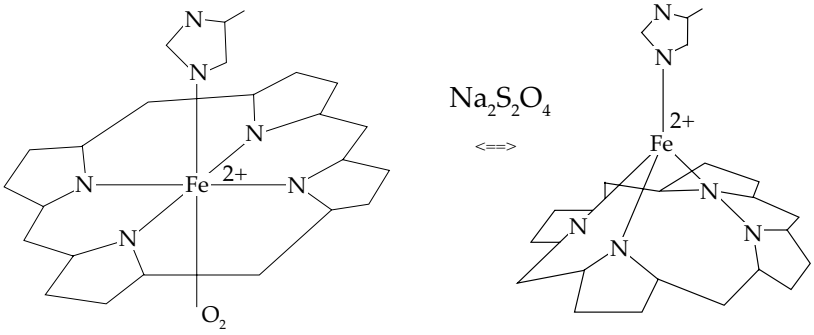
Allow the test tube to stand for a while and compare the colour of the solution in F with that of solution in D and E.

The colour of the solution in F will compare with the colour of solution in E but not in D.

The reaction is not reversible, therefore, colour change after shaking the solution in F was neither expected and nor observed.

Specific teaching tips

In the following equation 3a shows structural changes to the haemoglobin molecule, and equation 3b represents a general equilibrium equation used to demonstrate an equilibrium shift. The readers may omit some or all of them depending upon their needs.



Oxyhaemoglobin Deoxyhaemoglobin (3a)

HbO_2 (aq) <==> O_2 (aq) + Hb (aq) (3b)
Oxyhaemoglobin Deoxyhaemoglobin

Haemoglobin is known as deoxyhaemoglobin, when oxygen is not bonded to it. Deoxyhaemoglobin is violet/blue in colour. Iron present in the dexoyhaemoglobin is still in a ferrous (Fe^{2+}) state. Sodium dithionite ($Na_2S_2O_4$) is also a strong reducing agent. When it is added to the stock solution, the amount of dissolved oxygen in the solution is decreased. As a result, the equilibrium shifts to the right (Le Chatelier's principle), thus forming more deoxyhaemoglobin. Students can observe a colour change from red to blue. Students may observe this colour of blood by observing blue blood vessels on different parts of their body. The reaction is reversible depending upon the quantity of sodium dithionite added. However, in the above experiment the reverse reaction was not possible because of the presence of excessive amount of the reducing agent.

4. Demonstrating the reversible reaction between oxygen and haemoglobin

The rechargeable nature of haemoglobin can be demonstrated by visual and visible spectrophotometer analysis methods.

Demonstration Procedure

Visual Analysis

Take three 50 ml test tubes with stoppers and label them as G, H and I.

Measure 10 ml of stock solution using a measuring cylinder and pour it in test tube G.

Keep this tube as a colour reference as it contains oxyhaemoglobin solution.

Add 20 ml of stock solution to test tube H.

Add about 0.5 gram of tartaric acid to the test tube H and dissolve it with the help of a clean glass rod.

Add about 0.5 gram of ferrous sulphate solid to H and dissolve it with the help of a clean glass rod.

Compare the colours of solutions in test tubes G and H.

The solution in test tube H contains deoxyhaemoglobin which is blue in colour.

Now transfer about half of the solution in test tube H to test tube I.

Keep the test tube H as a second colour reference of deoxyhaemoglobin solution.

Close the test tube I with a stopper and vigorously shake the test tube for about one minute.

Allow the test tube C to stand for a minute to allow the foam to settle on the top of the solution.

Compare the color of solution in test tube I with that of colours in solutions in the test tubes G and H.

The colour of the solution in test tube I will match with that of colour of solutions in both the test tubes G and H.

The test tube I also contains oxyhaemoglobin. (This demonstrate the rechargeable nature of Haemoglobin).

The colours of solutions in the three test tubes are red.

Spectral Analysis

If visible spectrophotometer is available, the visible spectrum of oxy- and deoxy-haemoglobin in the wavelength (λ) range of 500 to 650 nm may be obtained using solutions from the three test tubes (G, H, I). The typical spectrums obtained are reported in Figure 2.

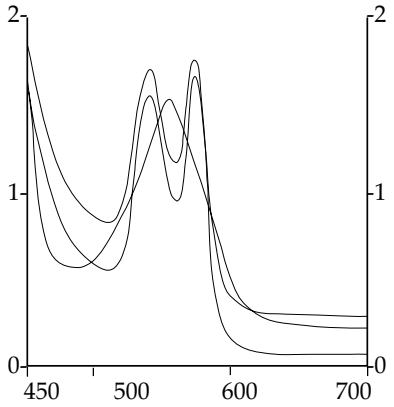
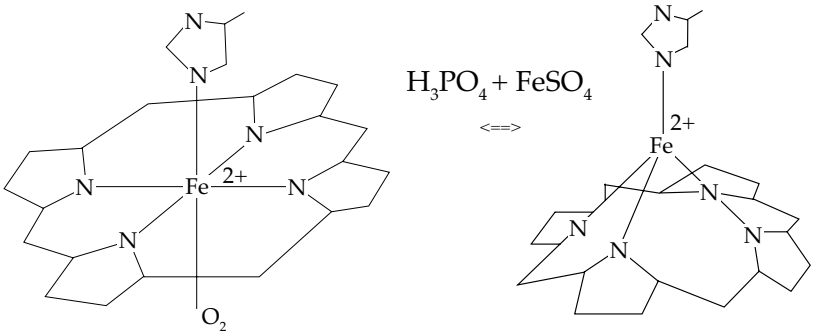


Figure 2: Spectral properties of solutions in test tubes G (— .—; oxyhaemoglobin), H (—; deoxyhaemoglobin) and I (— ; oxyhaemoglobin)

It is known that deoxyhaemoglobin gives peak at 555 nm and oxyhaemoglobin produces two peaks at 541 nm and 577 nm (Russo and Sorstokke, 1973). The graphs from test tubes G demonstrate the binding of oxygen with haemoglobin in stock solution, where as graph from test tube I demonstrates the binding of oxygen to deoxyhaemoglobin after mixing air and solution by shaking. The graphs and peaks from these two graphs are comparable because both are for oxyhaemoglobin. The curve for solution H, peaks at 555 nm showing no binding between haemoglobin and oxygen. These curves support the rechargeable nature of haemoglobin.

Specific teaching tips

In the following equations 4a shows structural changes to the haemoglobin molecule, and 3b represents a general equilibrium equation used to demonstrate equilibrium shift. The readers may omit some or all of them depending upon their needs.



Oxyhaemoglobin Deoxyhaemoglobin (4a)

HbO_2 (aq) <==> Hb (aq) + O_2 (aq) (4b)
Oxyhaemoglobin Deoxyhaemoglobin

Adding tartaric acid and ferrous sulphate to ammonical stock solution helps to prepare fresh Stokes solution in the test tube itself eliminating the need to prepare the stokes solution separately and then use it. Stokes solution is a week reducing agent. When it is added to the stock solution, it reduces the amount of dissolved oxygen in the solution. As a result the equilibrium shifts to the left (Le Chatelier's principle), thus forming more deoxyhaemoglobin. Students can observe the colour change from red to blue. However, when the solution is shaken additional oxygen form the air column in the test tube enters the solution and the equilibrium shifts to the right thus producing more oxyhaemoglobin. Students will thus observe a change in colour from blue to red.