Results of MCD practical MDL1-1

Spectrophotometry and electrophoresis of haemoglobin

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This practical appeared to go very well, especially considering that is was your first appearance in an undergraduate practical laboratory, working with unfamiliar equipment. Well done!

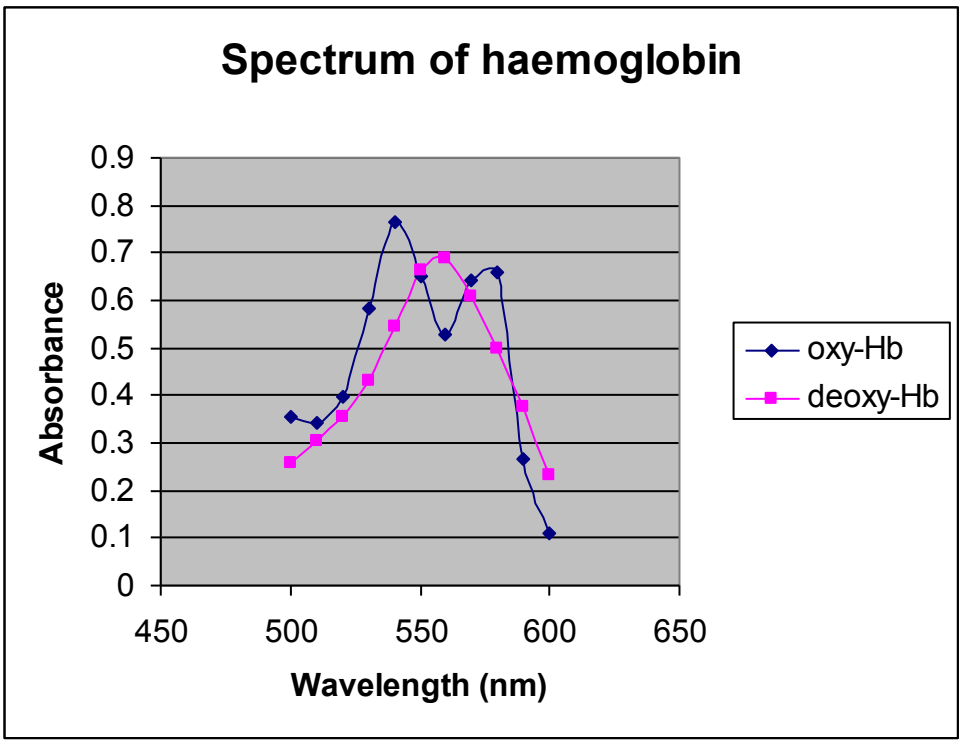
A couple of short housekeeping notes for future practical classes:

1) If, for some reason you cannot attend a particular session then please get in touch with the leader ASAP and we can try to accommodate you in another rotation (preferably during one of your Friday study periods).

2) Please try and read through the script before you attend the session. This greatly increases the productivity of each class.

In the first part of the practical, you gathered into groups of four or five and carried out some electrophoresis in which we compared normal (HbA), sickle (HbS) and a mixture of the two proteins, the comparison being solely on the basis of their charge. This was allowed to run for an hour or so during which students grouped into pairs and set about examining the absorption properties of haemoglobin.

Generation of Absorbance spectra



This seemed to be done rather well, with most students remembering to re-zero the machine against water for each wavelength studied. This needs to be done as the spectrophotometer lamp does not put out the same level of illumination at every wavelength. The graph below shows typical data obtained from a session. It can be seen that oxy-haemoglobin has two main peaks, at 540 and 577 nm (if the investigation had been extended, you may also have found a peak at 412 nm).

In the deoxy form of haemoglobin, the spectrum changes, with a single peak in this range at 560 nm (plus one at 430 nm). A small number of groups failed to see the change in spectrum following the reduction of haemoglobin, presumably because insufficient dithionite was added. This was remedied in a few cases where it was spotted.

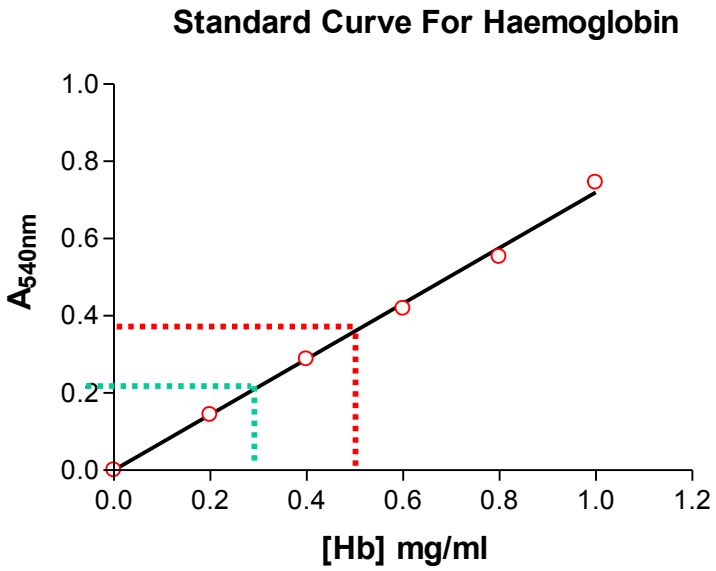
Clearly spectrophotometry can be used to follow changes in oxygen binding by haemoglobin, and this is a method used when checking the respiratory status of new born infants. The spectrum also changes for other forms, which might need to be examined for diagnostically:

- methaemoglobin, where the Fe^{2+} ion is oxidised to Fe^{3+}, occurs in some pathological conditions e.g. haemoglobin M disease, exposure to chlorobenzine
- carboxyhaemoglobin is where the molecule is binding carbon monoxide in place of oxygen

At the main peak the colour of the light is green (I think everyone saw this), just as predicted by the diagram in the course guide.

Determination of the concentration of the "Mystery" haemoglobin solutions, D1 and D2.

This should have been performed at around 540 nm, the wavelength of maximum absorbance that you had hopefully obtained in part 2 of the practical. Within a reasonable range, the Beer-Lambert law holds and absorbance is linearly proportional to concentration. Eventually the spectrophotometer becomes non-linear as absorbance gets too high, but it should always be good up to an absorbance of 1.0, which was the typical range in the practical. Hence the best fit straight line should be drawn to the experimental points. Typical data is illustrated below.

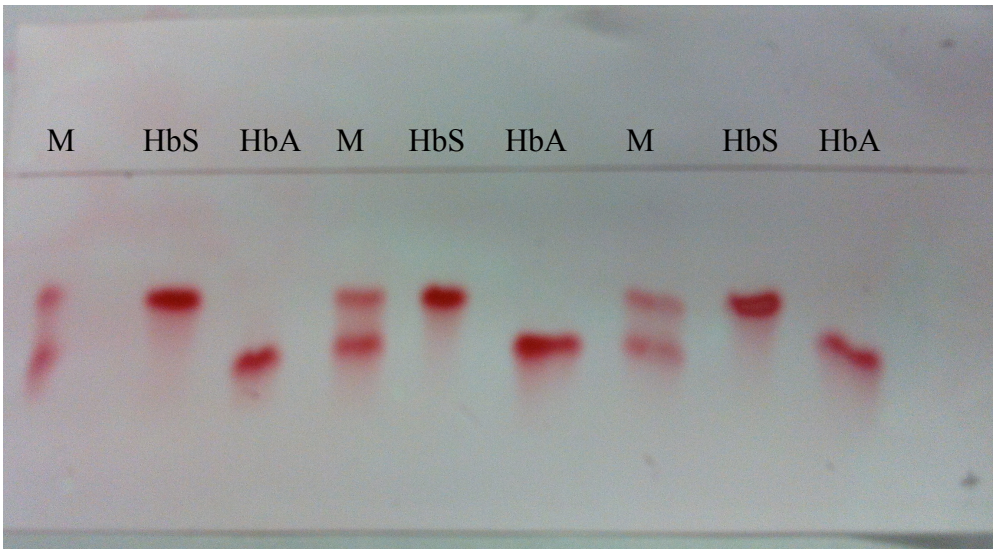


The concentration of the unknowns can now be determined from the standard curve as shown (examples given by dashed lines).

The concentrations of D1 was 0.30 mg/ml and D2 was either 0.50 mg/ml. Although these differences in concentration are almost impossible to determine by eye, the spectrophotometer copes quite well.

Electrophoresis of HbA and HbS

This ran pretty well on the whole. Below is a good specimen obtained by one of the groups in which it can be clearly seen that normal haemoglobin (HbA) ran further towards the positive electrode than sickle haemoglobin (HbS). Since opposite charges attract, it can be concluded that HbA is more negatively charged than HbS.



This difference in charge is due to a point mutation occurring in one amino acid of the β-chain. In this mutation, the amino acid glutamate in the normal protein (negatively charged) is replaced by valine (hydrophobic uncharged). It was kindly brought to my attention the other year that a couple of test books appear to have got these residues the wrong way around, so take care with your additional reading!

The consequences of this mutation can be examined at the molecular level by clicking here.

The mixed sample (M) mimicked a haemoglobin sample obtained from someone who is heterozygous for the condition. The sample contains both HbA and HbS bands. This illustrates nicely the power of this form of electrophoresis, which can separate a mixture of proteins, each consisting of several hundred amino acids, but which differ by *a single* amino acid.

Finally, a word about using the pipettes……..

Some of you struggled initially with the pipettes, which is not surprising as it was most likely your first encounter with them. You will use them again in practical MDL1-3 when we will use them to examine the enzyme malate dehydrogenase, by use of the spectrophotometer. There, they will come in two varieties, one with a yellow button, which maximally dispenses 20µl and one with a blue button, which maximally dispenses 1000µl (1ml). Always remember to use them with a disposable tip (yellow and blue plastic respectively) and to place these tips in the sharps containers when used. Detailed guidance on how to use them is in the course guide. Please also check out the short pipetting video we showed you in the class which can be found here.

See you again in November/December for MDL1-3 !

James Pease