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# PREPARATION OF HAEMOGLOBIN CRYSTALS

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Procedures for the crystallization of human and horse haemoglobins are described.

## 1. Introduction

In the course of our X-ray studies of crystalline haemoglobins my colleagues and I have had to develop procedures for the crystallization of several of its forms. To be suitable for detailed X-ray analysis, protein crystals must have linear dimensions of between 0.3 and 1.5 mm and be free from the kinds of lattice faults which give rise to the splitting of X-ray reflexions. Also, many haemoglobins exhibit polymorphism; only one of the polymorphic forms may be suitable for X-ray analysis and conditions for growing it may have to be found. The procedures outlined here were designed to meet these needs.

The cytoplasm of red blood cells contains 34% haemoglobin in solution. Other proteins are also present in solution, but in much smaller amounts, and they need not be removed before crystallizing the haemoglobin. Essentially, therefore, all one has to do is to separate the red blood cells from the serum by repeated centrifugation and washing with dilute salt solution; to burst the cells by the addition of water; to spin off the cell debris by centrifugation; and finally to remove the added salt by running the haemoglobin solution through an ion exchange column. This procedure generally yields a solution containing about 6% haemoglobin.

The haemoglobin is then precipitated by the addition of ammonium sulphate or sodium–potassium phosphate. At this stage the two decisive factors are *p*H and ionic strength. The *p*H may decide which of several polymorphic forms will grow and may also determine the crystal habit. Ionic strength determines the solubility, and hence the rate of nucleation and crystal growth. Since the solubility is an exponential function of ionic strength, it is difficult to reproduce exactly the conditions under which only a few nuclei will form and develop slowly into crystals of adequate perfection. For this reason a series of tubes with different salt concentrations is used, in the hope that the conditions in one or two of the tubes will be just right for the growth of a few good crystals.

Temperature and presence of impurities seem to have little effect on the growth of the crystals but, since large temperature changes cause them to crack, it is important to grow them at approximately the temperature at which they will be used. In the work described here both crystallization and X-ray analyses were done at room temperature. Any rapid changes in the composition of the suspension medium are also liable to cause the crystals to crack, but gradual changes, such as those produced by diffusion across membrane, are tolerated.

The haemoglobin molecule undergoes a change of structure on combination of its four iron atoms with oxygen, carbon monoxide, or other ligands. Consequently, the ligand-free, or deoxy-form, has a solubility and crystal structure which differs from the one in which the iron atoms are linked to ligands. The haemoglobins of different species also differ in their solubilities and crystal structure (though the mammalian haemoglobins are closely similar in molecular structure). In this note, conditions are described for the crystallization of the deoxy- and oxyhaemoglobins of man and horse.

## 2. Methods

### 2.1. General procedure for purification of haemoglobin solution

Red cells are removed from oxalated blood by centrifugation in a swinging bucket rotor. The supernatant

solution is removed by suction, and the cells are washed four times with 0.9% NaCl and once with 1% NaCl. The cells are then lysed by dilution with an equal volume of water and left 15 minutes or more. 35% NaCl is added to bring the total NaCl concentration to 2%. The solution must immediately be spun at 17000 rpm in a Servall or Spinco centrifuge for one hour. The solution is decanted carefully and the spinning repeated for half an hour.

The solution is run through a G25 Sephadex (fine) column equilibrated against 0.01 M ammonium phosphate of *p*H 7.0. This is made by the addition of 8.5 ml 2 M $(NH_4)_2HPO_4$ plus 1.5 ml 2 M $NH_4H_2PO_4$ to 2 l of water.

The concentration of haemoglobin in the eluate is measured as follows: 0.5 ml of eluate are diluted, first by the addition of about 20 ml of $H_2O$ and then of about 80 ml 0.07 M $K_2HPO_4$ to exactly 100 ml. The solution is reduced by the addition of a little (0.2 g) $Na_2S_2O_4$, then saturated with CO. The absorption coefficients at 540 and 569 m$\mu$ should be equal. The optical density at these wavelengths divided by 8.03 gives the concentration of carbonmonoxyhaemoglobin in g/ml.

Crystallization is carried out at room temperature in tubes of about 8 ml capacity, at a series of buffer concentrations which pass through the critical point where crystallization begins. This point is much the same for oxy, carbonmonoxy, and methaemoglobin of any one species, and different for deoxyhaemoglobin. It differs in different species. The optimum *p*H, buffer and haemoglobin concentrations for human and horse haemoglobin are listed below.

## 2.2. Crystallization of human deoxyhaemoglobin

Use solution C, *p*H = 6.5

0.8 volumes of $(NH_4)_2SO_4$ 4M
0.05 volumes of $(NH_4)H_2PO_4$ 2M
0.15 volumes of $(NH_4)_2HPO_4$ 2M
} Total molarity of buffer: 3.6.

The tubes are filled according to the schedule shown in table 1.

The final mixing of buffer, Fe citrate, and haemoglobin should be done in a glove box under nitrogen. The tubes should be sealed tight with rubber stoppers, and stored in nitrogen-filled specimen jars sealed with well-fitting ground and greased glass stoppers. Under these conditions the crystals can be kept oxygen-free for more than a year.

Table 1

| Tube | Final molarity of buffer | ml solution C | ml deion. $H_2O$ | ml 0.5 M Fe citrate | ml Hb* 6% |
|---|---|---|---|---|---|
| a | 2.8 | 4.65 | 0.25 | 0.1 | 1 |
| b | 2.7 | 4.50 | 0.40 | 0.1 | 1 |
| c | 2.6 | 4.35 | 0.55 | 0.1 | 1 |
| d | 2.5 | 4.15 | 0.75 | 0.1 | 1 |
| e | 2.4 | 4.00 | 0.90 | 0.1 | 1 |
| f | 2.3 | 3.85 | 1.05 | 0.1 | 1 |
| g | 2.2 | 3.75 | 1.15 | 0.1 | 1 |

0.5 M Fe citrate from 2 g $FeSO_4$ + 1.5 g Na citrate + 25 ml water

* The abbreviation Hb in the table stands for haemoglobin.

## 2.3. Human oxy-, carbonmonoxy-, or methaemoglobin

In phosphate buffers. The haemoglobin solution (in 0.01 M ammonium phosphate buffer) is dialyzed against a 1.6 M phosphate buffer. This is made up by dilution from a buffer consisting of 5 volumes 4 M $NaH_2PO_4$ + 7 volumes 4 M $K_2HPO_4$ and has a *p*H of 6.7. Before dialysis, the dialysis sack is boiled twice for 5 minutes in deionized water. After dialysis, the solution is diluted with 1.6 M buffer to obtain a haemoglobin concentration of 4%.

Then tubes are set up as shown in table 2. Two drops of toluene are added to each tube.

Table 2

| Tube | Final molarity of buffer | ml 4 M phosphate buffer | ml $H_2O$ | ml 4% $HbO_2$ in 1.6 M buffer |
|---|---|---|---|---|
| a | 2.75 | 2.35 | 0.65 | 1.0 |
| b | 2.65 | 2.25 | 0.75 | 1.0 |
| c | 2.55 | 2.15 | 0.85 | 1.0 |
| d | 2.45 | 2.05 | 0.95 | 1.0 |
| e | 2.35 | 1.95 | 1.05 | 1.0 |
| f | 2.25 | 1.85 | 1.15 | 1.0 |

## 2.4. Horse deoxyhaemoglobin

3.4 M phosphate buffer of *p*H 6.4 is made up by mixing 0.45 vol $K_2HPO_4$ + 0.55 vol $NaH_2PO_4$ + 0.18 vol $H_2O$ (e.g. 68 ml 4 M $K_2HPO_4$ + 82 ml 4 M $NaH_2PO_4$ + 26 ml $H_2O$); 0.5 M ferrous citrate is used as specified in section 2.2.

To each tube 0.1 ml 0.5 M ferrous citrate is added.

Mixing is done under nitrogen and sealing as specified in section 2.2. Best crystals grow in tubes c–e (table 3).

TABLE 3

| Tube | Final molarity of buffer | ml buffer solution | ml $H_2O$ | ml 6% $HbO_2$ |
|---|---|---|---|---|
| a | 2.73 | 4.90 | 0.10 | 1 |
| b | 2.65 | 4.75 | 0.25 | 1 |
| c | 2.56 | 4.60 | 0.40 | 1 |
| d | 2.48 | 4.45 | 0.55 | 1 |
| e | 2.40 | 4.30 | 0.70 | 1 |
| f | 2.32 | 4.15 | 0.85 | 1 |

## 2.5. HORSE OXY-, CARBONMONOXY-, OR METHAEMOGLOBIN

This system is more complex than the others, due to the existence of two closely related polymorphic forms and the tendency to form multiple crystals. Of the two polymorphic forms, one, grown at *p*H ~ 7.5, has a doubled *b* axis coupled with a lattice disorder which makes it useless for X-ray analysis. At *p*H ~ 7.0, on the other hand, multiple crystals prevail. Sometimes it is possible, by keeping to just the right *p*H between those two limits, to grow crystals which are free from either fault. Often it is easier to grow good single crystals at a *p*H nearer 7.5, which may have a doubled *b* axis, and to cure them of the doubled *b* axis afterwards by acidifying the suspension medium with $NH_4H_2PO_4$. (Addition of 0.3 ml of a 1.8 M solution of $NH_4H_2PO_4$ to 4–5 ml of the suspension medium will normally cure them.)

For a medium of *p*H ~ 7.5 make up *solution A*, consisting of 2 vol 4 M $(NH_4)_2SO_4$ + 1 vol 2 M $(NH_4)_2HPO_4$.

For a medium of *p*H 7.0–7.3 make up *solution B*, consisting of 2 vol 4 M $(NH_4)_2SO_4$ + 1 vol of phosphate buffer consisting of 0.95 vol 2 M $(NH_4)_2HPO_4$ + 0.05 vol 2 M $(NH_4)H_2PO_4$.

The solutions are mixed as shown in table 4.

TABLE 4

| Tube | Final molarity of buffer | ml solution B or C | ml 2% |
|---|---|---|---|
| a | 2.0 | 3.0 | 2.0 |
| b | 1.94 | 2.8 | 2.0 |
| c | 1.89 | 2.6 | 2.0 |
| d | 1.82 | 2.4 | 2.0 |
| e | 1.75 | 2.2 | 2.0 |
| f | 1.67 | 2.0 | 2.0 |

## 3. General notes

If one has to preserve the reactivity of the SH groups of cysteine, distilled and deionized water should be used throughout. Good crystals take about a month to grow.

### UNIT CELLS AND SPACE GROUPS OF WET CRYSTALS GROWN BY THESE METHODS

*Human deoxyhaemoglobin*: Space group $P2_1$; 2 molecules/cell.

$a$ = 63.4 Å, $b$ = 83.6 Å, $c$ = 53.9 Å, $\beta$ = 99°15′.

Muirhead and Perutz[1]).

*Human oxyhaemoglobin*: Space group $P4_12_12$; 4 molecules/cell.

$a$ = 53.7 Å, $c$ = 193.5 Å.

Perutz et al.[2]).

*Horse deoxyhaemoglobin*: Space group $C222_1$; 4 molecules/cell.

$a$ = 77.0 Å, $b$ = 81.7 Å, $c$ = 92.6 Å.

Perutz et al.[3]).

*Horse oxyhaemoglobin*: Space group C3; 2 molecules/cell.

$a$ = 108.1 Å, $b$ = 63.2 Å, $c$ = 54.5 Å, $\beta$ = 110°50′.

The crystals were originally described by Bernal et al.[4]), but the unit cell dimensions given here which I have re-measured recently on a 3-circle diffractometer are more accurate.

## References

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