

*Full Length Research Paper*

# Effect of temperature on the solubility of haemoglobin modified with acetyl-3,5-dibromosalicylic acid

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**Haemoglobin A was acetylated using acetyl-3,5-dibromosalicylic (dibromoaspirin). The acetylated haemoglobin showed enhanced solubility compared to the unmodified haemoglobin and its solubility was found to increase with temperature in the range 13°-37°C. From the plot of log solubility against the reciprocal of temperature, the  $\Delta H^\circ$  was calculated to be 23.74 and 30.06 kJmol<sup>-1</sup>, for the modified and unmodified haemoglobin respectively. This indicates a reduction in the  $\Delta H^\circ$  for the modified species. Since the solubility of haemoglobin is enhanced by acetylation with dibromoaspirin, dibromoaspirin might be an effective antisickling agent when administered at optimum temperature.**

**Key words:** Haemoglobin, acetyl-3,5-dibromosalicylic acid, protein, sickle cell anaemia, antisickling agent.

## INTRODUCTION

Sickle cell anaemia is a genetic blood disorder arising from a point mutation in the  $\beta$ -globin gene that leads to the replacement of glutamic acid residue by valine at the sixth position of the  $\beta$ -chain of haemoglobin. At low oxygen tension, the mutant haemoglobin, sickle haemoglobin, polymerises inside the red blood cells into a gel or further into fibres leading to transformation of the shape (sickling) of the red blood cells (Zaugg et al., 1980). These defective cells are fragile and have a shorter than average lifetime, making the patient anaemic. Since the molecular basis of the disease is known, research efforts are being made to develop medical therapy to prevent the sickling phenomenon. Antisickling agents are classified, based on the target to be modified, into three major classes: the gene, the haemoglobin molecule, and the red cell membrane modifiers (Mehanna, 2001). The haemoglobin modifiers or anti-gelling agents interfere with the mechanism or the kinetics of the haemoglobin polymerisation. It is recognized from experimental data that substantial stochastic fluctuations in conformation occur constantly in a protein molecule, even at equilibrium (Gurd and Rothgeb, 1979; Beece et al., 1980). Deviations in energy may be in tens of kcal/mol in magnitude. Chemotherapeutic approaches to control sickle-cell anaemia using anti-gelling agents aim at diminishing protein-protein interactions that favour aggregation of haemoglobin, thereby enhancing the solubility of haemoglobin. This

may be achieved with minor perturbations in the energetics of such interactions. It has been pointed out by Klotz et al. (1970) that a change as little as 1 kcal/mol in the  $\Delta G^\circ$  of association reactions can shift the solid-solution equilibria markedly. In solid-solution equilibrium, the solubility could be altered, by a two-fold factor with a change of only 0.4 kcal in the  $\Delta G^\circ$ . Such a small alteration in free energy of interaction could be achievable with minor changes in structure. Such disruption would increase the solubility of deoxyhaemoglobin and even small changes in solubility will greatly retard the kinetics of polymer formation.

The substitution of electron-withdrawing Br atoms into aspirin increases its reactivity towards small amines (e.g. propylamine) about eight-fold over that for unmodified aspirin. The observed augmentation in the rate of acetylation of haemoglobin is much greater however (Walder et al., 1979). It is possible that some selectivity is generated from dipolar interactions of the aromatic C-Br bond with loci of cationic lysyl residues in the haemoglobin macromolecule. X-ray diffraction studies have been employed as tool to reveal the haemoglobin binding sites of antisickling agents (Wireko and Abraham, 1991). Transfer of the acetyl group of dibromoaspirin to amino groups of haemoglobins A and S seems to occur predominantly at just two or three sites on these proteins and acetylation appears to occur at relatively specific sites on the protein

(Walder et al., 1977). A substantial fraction of the attached acetyl group is lodged at Lys 144 of the  $\beta$ -chain (Nabaldian et al., 1972; Zaugg et al., 1980), which is in the vicinity of the cavity of the portal between the two  $\beta$ -subunits. Also Lys 90 of the  $\alpha$ -chains are substantially acetylated (Shamsuddin et al., 1974).

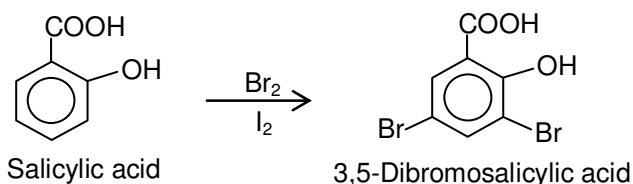
The gelation and sickling phenomenon can be regarded as one of relative solubilities under physiological conditions. Deoxyhaemoglobin S has a solubility of 160 g/L (2.48 mM tetramer) (Ross et al., 1977), whereas oxyhaemoglobin S and both states of normal haemoglobin have solubilities in excess of 500 g/L (7.76 mM tetramer) (Noguchi and Schechter, 1981). The solubility of deoxyhaemoglobin S also is greater at 0°C than at room or body temperature. Eaton et al. (1976) found that when a solution of deoxyhaemoglobin S is warmed rapidly from 0° to 25°C, a considerable delay time ensued before it sets into a gel of ordered fibres. Any pharmacologically active compound that could increase the gelation or delay time would help ameliorate the severity of the sickle cell disease. The solubility of a given substance depends on the relative affinity of solute molecules for each other (solute-solute interaction) and for the solvent molecules. Factors that decrease the solute-solute interactions will tend to increase solubility.

Schwert and Neurath (1950) found that factors such as high protein concentration, decreasing temperature and increasing pH promote precipitation and therefore decrease solubility. They found that the solubility of proteins also depends on ionic strength. In the present study, the effect of temperature on the solubility of both the modified and the unmodified haemoglobin species has been examined.

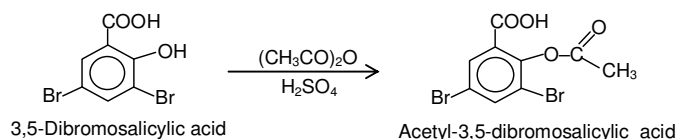
## MATERIALS AND METHODS

### Preparation of acetyl-3,5-dibromosalicylic acid

Acetyl-3,5-dibromosalicylic acid (dibromoaspirin) was prepared by brominating salicylic acid using liquid bromine. Iodine was used as the bromine carrier according to the method described by Robertson (1902). Salicylic acid was obtained from British Drug House and used without further purification. The bromination resulted in a mixture of 3,5-dibromosalicylic acid (melting point 226°-228°C) and 5-bromosalicylic acid (melting point 163°C). The mixture was separated by fractional crystallization from glacial acetic acid.



Acetyl-3,5-dibromosalicylic acid (melting point 156°C) was synthesised by acetylation of the 3,5-dibromosalicylic acid using acetic anhydride and concentrated sulphuric acid as catalyst according to the method of Walder et al. (1977).

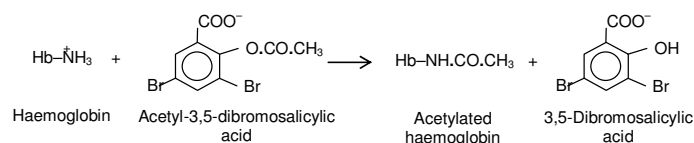


The solid product was recrystallised from benzene. The dibromoaspirin crystals were confirmed pure by checking the melting point using a standard melting point apparatus and recording the infrared spectrum on a Unicam SP 3-300 infrared spectrophotometer, using pressed KBr disc. The melting point and infrared spectrum obtained corresponded with those reported in literature (Robertson, 1902).

### Preparation and acetylation of Hb A with dibromoaspirin

Human blood samples homozygous for haemoglobin A were obtained from the Blood Bank at the University College Hospital, Ibadan and were used to prepare haemoglobin solutions adopting the methods of Walder et al. (1979). The determination of the concentration of the stock haemoglobin solution was done by standard methods (Cameron, 1965).

To acetylate haemoglobin, the procedures of Walder et al. (1979) were adopted. 0.0338 g of dibromoaspirin was suspended in Tris buffer (pH 7.4) of ionic strength 0.05, followed by addition of 2.8 cm<sup>3</sup> of the 1.786 mM (tetramer) stock haemoglobin solution. The solution was then made up to 5 ml with the buffer. This gave rise to 1 mM (tetramer) haemoglobin and 20 mM dibromoaspirin in the final solution. These were incubated at 37°C in a Wilkens-Anderson thermostated water bath for 2 h. After this, the solubility of the haemoglobin solution was determined.



### Effect of temperature on the solubility of haemoglobin

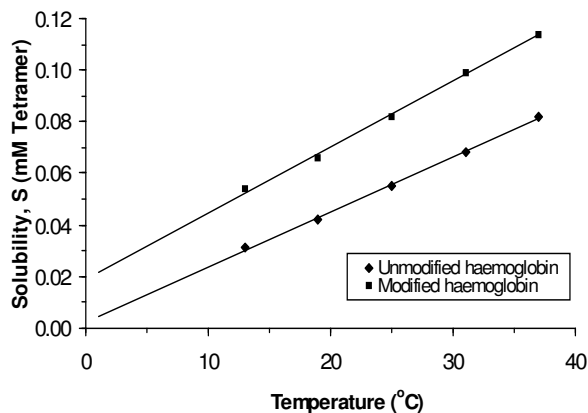
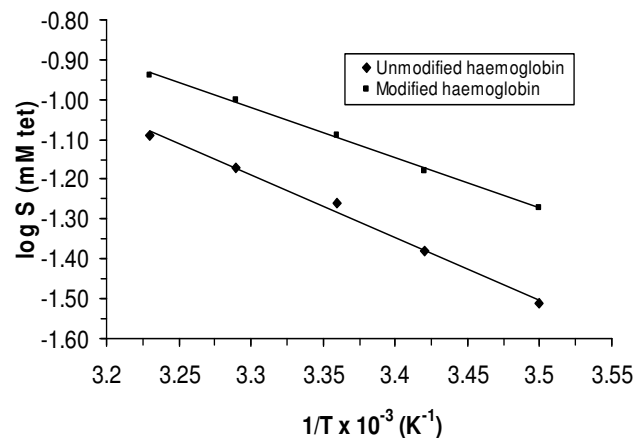
To determine the effect of temperature on the solubility of both modified and unmodified haemoglobins, the incubations were done with temperatures maintained at 13°, 19°, 25°, 31°, and 37°C. At all temperatures, the pH was maintained at 7.4 by the use of Tris buffers of ionic strength 0.05. The pH of 7.4 used for this study is the isoelectric point (I.E.P.) of haemoglobin where the solubility of haemoglobin is a minimum. This is because at the I.E.P., there is minimum electrostatic repulsion between protein molecules and the crystal lattice forces in the solid state are at a maximum and the molecules tend to aggregate and precipitate out of solution and usually giving minimum solubility.

### Determination of solubility of haemoglobin

The solubilities of both modified and unmodified haemoglobin solutions were determined using polyethylene glycol (PEG) according to the methods of Middaugh et al. (1979) with minor modifications. After equilibrations with PEG, the supernatant haemoglobin concentrations were measured spectrophotometrically as cyanmethaemoglobin. This was done using a Perkin Elmer Lambda 3B UV/Visible spectrophotometer, at 540 nm where the molar extinction coefficient of cyanmethaemoglobin is 4.36 × 10<sup>4</sup> (Cameron, 1965). 0.1 cm<sup>3</sup> of the samples were pipetted into 3 cm<sup>3</sup> of Drabkin's solution and the absorbance of the resulting solutions were taken at 540 nm. The concentrations in terms of mM (tetramer) were calculated from the equation:

**Table 1.** Effect of temperature on solubility of modified and unmodified haemoglobin at pH 7.4

| Temperature, T<br>(°C) | 1/T x 10 <sup>-3</sup><br>(K <sup>-1</sup> ) | Unmodified haemoglobin      |       | Modified haemoglobin        |       |
|------------------------|--|-----------------------------|-------|-----------------------------|-------|
|                        |  | Solubility, S (mM tetramer) | Log S | Solubility, S (mM tetramer) | Log S |
| 13                     | 3.50   | 0.031                       | -1.51 | 0.054                       | -1.27 |
| 19                     | 3.42   | 0.042                       | -1.38 | 0.066                       | -1.18 |
| 25                     | 3.36   | 0.055                       | -1.26 | 0.082                       | -1.09 |
| 31                     | 3.29   | 0.068                       | -1.17 | 0.099                       | -1.00 |
| 37                     | 3.23   | 0.082                       | -1.09 | 0.114                       | -0.94 |

**Figure 1.** Effect of temperature on solubility of modified and unmodified haemoglobin.**Figure 2.** Plot of log solubility against 1/T for modified and unmodified haemoglobin.

$$C = \frac{A_{540}}{4.36} \times \frac{(3 + 0.1)}{0.1} \times 10^{-1}$$

Where C = concentration of haemoglobin in mM (tetramer);  $A_{540}$  = absorbance at 540 nm; 3 = volume of Drabkin's solution in cm<sup>3</sup>; 0.1 = volume of haemoglobin sample in cm<sup>3</sup>.

## RESULTS AND DISCUSSION

The enhanced solubility of the acetylated haemoglobin shows that the haemoglobin molecules were appreciably acetylated at the pH value used for the experiment. The acetylation gave rise to an increase in the intrinsic solubility of the haemoglobin (Table 1). The graph of the solubility in mM (tetramer) of both modified and unmodified haemoglobin against temperature at pH of 7.4 is shown in Figure 1. The solubilities of both modified and unmodified haemoglobin at pH 7.4 were observed to increase with temperature. This finding is in line with previous reports which indicated much acetylation at higher temperatures. (Schwert and Neurath, 1950; Klotz and Tam, 1973). From the Van't Hoff's isochore:

$$\frac{d \ln K}{dT} = \frac{\Delta H^\circ}{RT^2} \quad (1)$$

and by integration we obtain:

$$\ln K = \frac{-\Delta H^\circ}{RT}; \quad \log K = \frac{-\Delta H^\circ}{2.303RT} \quad (2)$$

Likening K to the solubility of the haemoglobin species (S), a plot of log S against 1/T, at pH 7.4, is approximately linear within the region of the temperatures studied. This is shown in Figure 2 for both the modified and unmodified haemoglobin species. The points were fitted into straight lines by the method of least squares. From the gradients of the lines in Figure 2, the  $\Delta H^\circ$  was calculated, for the modified and unmodified haemoglobin, to be 23.74 and 30.06 kJmol<sup>-1</sup> respectively. This indicates that the  $\Delta H^\circ$  for the modified haemoglobin is lower than that of the unmodified haemoglobin. Because the plot of log solubility against the reciprocal of temperature is linear (Figure 2), the assumption that the enthalpy of solution,  $\Delta H^\circ$  is independent of temperature is valid.

Acetylation of haemoglobin using acetyl-3, 5-dibromosalicylic acid is an example of non-covalent modification of haemoglobin aimed at improving its solubility. The results clearly established that acetyl-3, 5-dibromosalicylic acid serves as an effective reagent for the chemical modification of haemoglobin. Modification of haemoglobin with dibromoaspirin increases the solubility of haemoglobin.

globin to some reasonable extent. Since the sickling phenomenon is one of relative solubility, administration of dibromoaspirin could have effect on sickling. If the potency of dibromoaspirin is as a result of its polarisability or its electron-withdrawing ability, then alternative derivatives of this compound that promise to be more potent could be tried in the future. This study also shows that the solubility of both the modified and unmodified haemoglobin increased at higher temperatures within the range of temperature studied. This is because at higher temperatures, more acetylation is achieved thereby increasing the solubility. This study provides *in vitro* background for appropriate *in vivo* experiments.

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