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SOLUBILITY STUDIES OF HAEMOGLOBIN A MODIFIED WITH ACETYL-3,5-DIBROMOSALICYLIC ACID

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ABSTRACT

Acetyl-3,5-dibromosalicylic acid (dibromoaspirin) was prepared by standard methods. Incubation of Haemoglobin A samples with dibromoaspirin leads to acetylation of haemoglobin *in vitro*. Solubility was observed to increase with increase in dibromoaspirin concentration and maximum modification was obtained at about 12 mM dibromoaspirin to 1 mM (tetramer) of haemoglobin. The acetylated haemoglobin showed a marked increase in solubility compared to the unmodified protein at all pH values studied. From the plot of logarithm of solubility (log S) versus polyethylene glycol (PEG) concentration, true solubility of the modified and unmodified haemoglobin were obtained by valid extrapolation to be 794.33 ± 2.04 g/l and 676.08 ± 2.09 g/l respectively. This represents an increase of about 17.5 % in the solubility of haemoglobin when modified with acetyl-3,5-dibromosalicylic acid at pH 7.4 and 37° temperature. The pH study also showed that the solubility was lowest at about the isoelectric point of haemoglobin for both the modified and unmodified species.

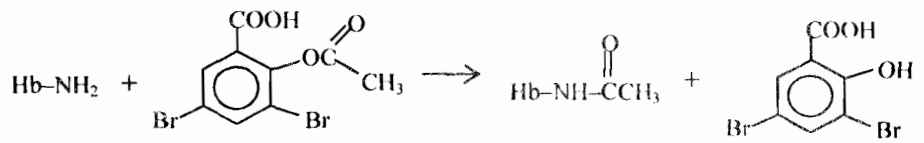
KEYWORDS: Haemoglobin, Solubility, Acetyl-3,5-dibromosalicylic acid, Acetylation, Sickle cell disease.

INTRODUCTION

Sickle-cell anaemia is a classical point mutation disease. Glutamate at the sixth position in the β chain of normal haemoglobin (Hb A) is interchanged with valine in sickled haemoglobin (Hb S). This position is located on the exterior of the haemoglobin molecule. The hydrophobic group of valine is the key to the sickling phenomenon. Hb S has a hydrophobic R group in place of the normal hydrophilic R group of glutamate (Ingram, 1956). Because deoxyhaemoglobin has a complementary hydrophobic site for the R group of this valine between the E and F helices but oxyhaemoglobin does not, high concentration of the deoxygenated form of haemoglobin S induce aggregation (Zaugg, et al., 1980). Within the erythrocyte, these fibres distort the cell into various abnormal shapes among which is the characteristic sickled form. These defective cells are fragile and have a shorter than average lifetime, making the patient anaemic and placing a heavy load on the erythrocyte-generating machinery of the bone marrow.

Now that the molecular basis of the anaemia is known, intense research efforts are being made to develop medical therapy to prevent the sickling phenomenon or to alleviate sickle-cell crisis. Previous investigations have shown that chemical modification of the haemoglobin molecule might inhibit aggregation of deoxy Hb S. Analysis of the effects of additives on the solubility of deoxy Hb S have become one of the standard methods for studying potential therapies for sickle-cell anaemia. Any such compound that would make the hemoglobin molecules less "sticky" might be expected to decrease the viscosity of the solution, increase the solubility of deoxyhaemoglobin S molecules; and even small changes in solubility will greatly retard the kinetics of polymer formation (Hofrichter, et al., 1974; Ross and Subramanian, 1977).

It has been found that acetylation of amine groups of haemoglobin by acetylsalicylic acid (aspirin) leads to increased concentrations of oxyhaemoglobin by increasing the oxygen affinity of the hemoglobin and should therefore defer the onset of sickling (Klotz and Tam, 1973). de Furia et al. (1973) reported later that though acetylsalicylic acid acetylates hemoglobin, it does not increase its oxygen affinity. Further work done by Walder et al. (1977), indicated that an alternative aspirin, acetyl-3,5-dibromosalicylic acid (dibromoaspirin) acetylates haemoglobin at a rate even greater than that of acetylsalicylic acid and increase the oxygen affinity of the haemoglobin. In this work, it was found that modification of haemoglobin with dibromoaspirin increases the solubility of haemoglobin to some reasonable extent, which would have effect on sickling. The aim of chemical approaches to antisickling is to diminish protein-protein interactions that favour aggregation of haemoglobin.



Haemoglobin | Dibromoaspirin | Acetylated Haemoglobin | Dibromosalicylic acid

It has been shown that sickle haemoglobin show susceptibilities to modification with salicylates that are fully comparable to those of normal haemoglobin (Wood et al., 1981).

MATERIALS AND METHODS

Preparation of 3,5-Dibromosalicylic Acid and Acetyl-3,5-dibromosalicylic Acid

Salicylic acid obtained from British Drug Houses was used as sourced without further purification. Salicylic acid was brominated by addition of bromine, using iodine as bromine carrier according to the method of Robertson (1902). The resulting mixture consisted of 3,5-dibromosalicylic acid (melting point, 226°–228°C) and 5-bromosalicylic acid (melting point, 163°C) (Robertson, 1902). Separation was by fractional crystallization from glacial acetic acid. The equation of the reaction is as follows:

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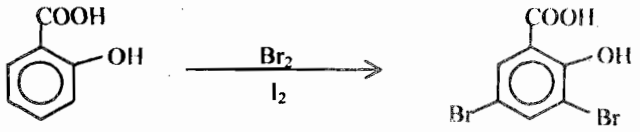
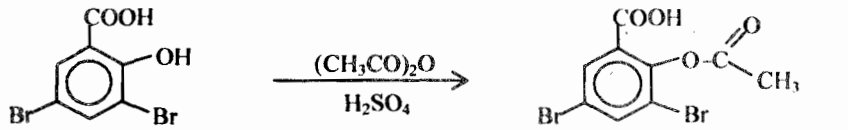


Salicylic acid 3,5-Dibromosalicylic acid

3,5-Dibromosalicylic acid was then acetylated using acetic anhydride and concentrated sulphuric acid as catalyst by the method of Walder et al. (1977). The crystals of the acetyl-3,5-dibromosalicylic acid formed were recrystallised from benzene: melting ting point 155.5°–156.5°C; literature 156°C (Robertson, 1902). The equation of the reaction is as follows:



3,5-Dibromosalicylic acid Acetyl-3,5-Dibromosalicylic acid

The melting points of both 3,5-dibromosalicylic acid and acetyl-3,5-dibromosalicylic acid were confirmed to ascertain their purity using a standard melting point apparatus. The infrared spectra of the two samples on pressed KBr disc were recorded on a Unicam SP 3-300 infrared spectrophotometer, which further confirmed the purity.

Preparation and Acetylation of Hb A with Dibromoaspirin

Normal human blood homozygous for haemoglobin A was obtained from Blood Bank at the University College Hospital, Ibadan. Haemoglobin solutions were prepared according to standard procedures (Walder et al., 1979) with little modifications. The concentration of the stock haemoglobin solution was determined by the method of Cameron (1965).

Acetylation of haemoglobin samples with dibromoaspirin were carried out by incubating Hb with 20 mM dibromoaspirin according to procedures of Walder et al. (1979). The effect of concentration of dibromoaspirin on solubility of haemoglobin was determined by incubating 1 mM (tetramer) of haemoglobin with 0–20 mM of dibromoaspirin for 2 hours. These were done at pH of 7.4 and temperature maintained at 37°C using a Wilkens-Anderson thermostated water bath. The solubilities of the haemoglobin solutions were determined according to the method of Middaugh et al. (1979) with minor modifications.

Effect of pH on the Solubility of Haemoglobin A

The effect of pH on the solubility of both modified and unmodified haemoglobin A was determined by incubating 1 mM (tetramer) of haemoglobin with 20 mM of dibromoaspirin. Reactions were run at temperatures of 37°; and varying pH values of 6.0 – 9.0 using bis-Tris buffers (6.0 – 7.2) and Tris buffers (7.4 – 9.0). The solubility of both modified and unmodified haemoglobin was determined using polyethylene glycol (PEG) according to the methods of Middaugh et al. (1979).

Determination of Solubility of Haemoglobin A

Solubility of both modified and unmodified haemoglobin A was determined using the methods of Middaugh et al. (1979). PEG was used as a protein precipitant and its precipitatory action is by excluded volume principle (Ingham, 1977; Miekka and Ingham, 1978). The haemoglobin solutions were equilibrated with PEG in appropriate buffers at 37°C for 4 hours when precipitation had occurred. This was done with gentle stirring. The solid phases were cleanly separated from the aqueous phases by filtration with Sartorius-membranefilter GMB having pore size of 3.0 μ. 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 cyanmet-haemoglobin is 4.36×10^4 (Cameron, 1965). 0.1 cm^3 of the samples were pipetted into 3 cm^3 of Drabkin's solution and the absorbances of the resulting solutions were taken at 540 nm against distilled water. The concentrations in terms of mM (tetramer) were calcultated from the equation

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

where C = concentration of haemoglobin in mM (tetramer); A_{540} = absorbance at 540 nm; 3 = volume of Drabkins solution in cm^3; 0.1 = volume of haemoglobin sample in cm^3.

RESULTS

Figure 1 shows the effect of dibromoaspirin concentration on solubility of haemoglobin A. This shows that solubility of haemoglobin increases as the concentration of dibromoaspirin increases till a certain point when further increases in dibromoaspirin concentration did not increase the solubility. Between 12 mM and 20 mM concentrations of dibromoaspirin, there was no significant increase in solubility of haemoglobin with increase in dibromoaspirin concentration and the curve attains its maxima at this range. Maximum modification was obtained at about 12 mM dibromoaspirin to 1 mM (tetramer) of haemoglobin.

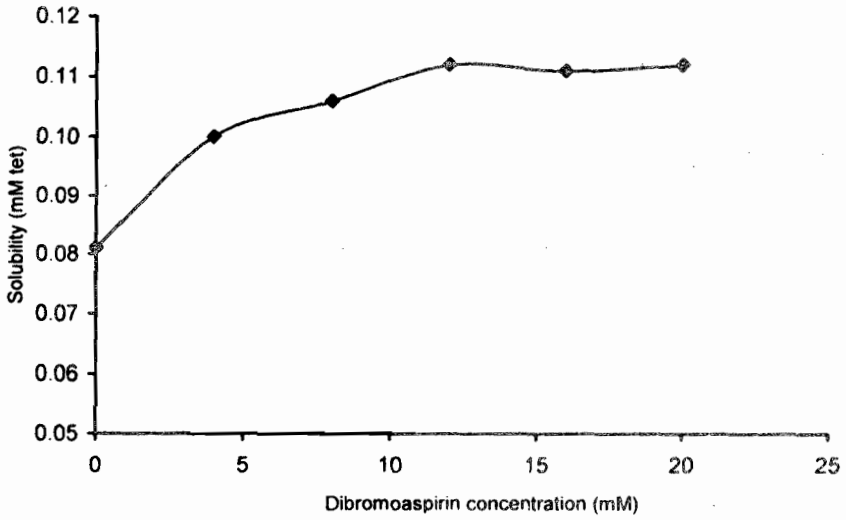


FIGURE 1: Effect of dibromoaspirin concentration on solubility of haemoglobin A

The solubility of modified and unmodified haemoglobin A was studied as a function of pH. The graph of the solubility in mM (tetramer) of both modified and unmodified haemoglobin against pH at 37°C is shown in Figure 2. The solubility-pH profile of the modified and unmodified haemoglobin at 37°C gave rise to "u" shaped curves which show minima around the isoelectric point (I.E.P) of the haemoglobin species.

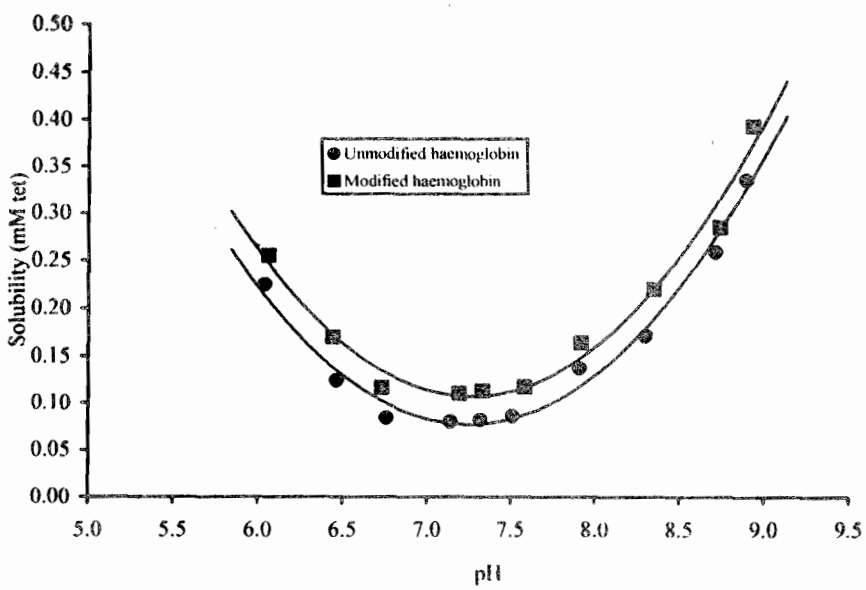


FIGURE 2: Effect of pH on solubility of modified and unmodified haemoglobin A at 37°C

The results of the solubility measurements for unmodified and modified haemoglobin determined at different PEG concentrations, temperature of 37°C and pH of 7.4 is shown in Table 1. The plots of log S against PEG concentration for both modified and unmodified haemoglobin are shown in Figure 3 to be linear. Data in the linear region of log S versus [PEG] plots were fitted to straight lines by the method of least squares. Errors in the extrapolated solubility at 0 % PEG were estimated to be less than 1 %. For the unmodified haemoglobin, the extrapolated solubility at 0 % PEG is 676.08 ± 2.09 g/l while for the modified haemoglobin, the extrapolated solubility at 0 % PEG is 794.33 ± 2.04 g/l being higher than the unmodified haemoglobin.

Table 1: Effect of polyethylene glycol (PEG) concentration on the solubility of modified and unmodified haemoglobin A at pH 7.4 and 37°C temperature

% PEG	Unmodified Hb A				Modified Hb A			
	A_{540}*	S** (mM tet.)	S (g/l)	log S (g/l)	A_{540}	S (mM tet.)	S (g/l)	log S (g/l)
12	0.574	0.408	26.32	1.42	0.722	0.513	33.11	1.52
13	0.445	0.316	20.41	1.31	0.574	0.408	26.32	1.42
14	0.362	0.257	16.60	1.22	0.477	0.339	21.88	1.34
15	0.262	0.186	12.02	1.08	0.370	0.263	16.97	1.23
16	0.190	0.135	8.71	0.94	0.281	0.200	12.89	1.11
17	0.158	0.112	7.25	0.86	0.190	0.135	8.71	0.94
18	0.114	0.081	5.23	0.72	0.165	0.117	7.57	0.88
19	0.089	0.063	4.08	0.61	0.114	0.081	5.23	0.72
20	0.069	0.049	3.16	0.50	0.097	0.069	4.45	0.65

* Absorbance at 540 nm

** Solubilty

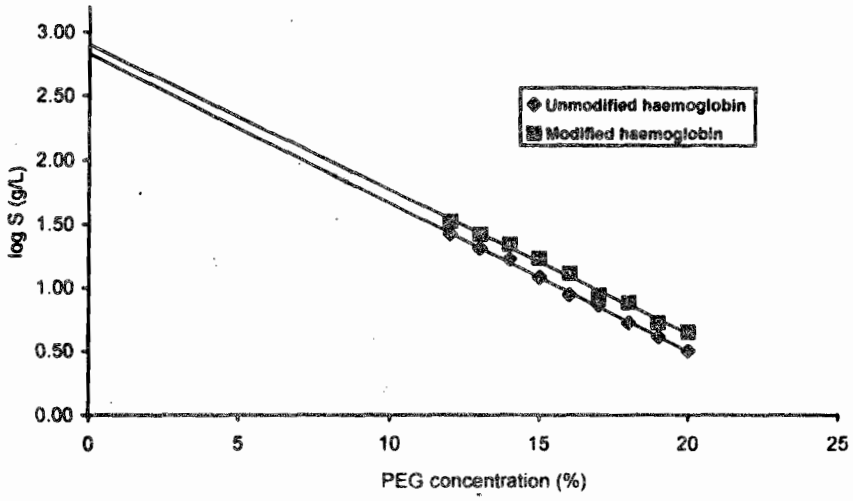


FIGURE 3: Effect of polyethylene glycol (PEG) concentration on solubility of modified and unmodified haemoglobin A

DISCUSSION

Modification of haemoglobin A with varying concentrations of dibromoaspirin showed that no increase in solubility was obtained above 12mM of dibromoaspirin (Figure 1). This indicates that the maximum achievable modification was obtained at about this concentration under the condition of experiment used for modification.

The solubility-pH profile of the modified and unmodified haemoglobin at 37°C gave rise to "u" shaped curves which show minima around the isoelectric point (I.E.P.) of the haemoglobin species (Green et al., 1935) (Figure 2). These results show that the presence of PEG has no effect on the water structure, which affects the stability of hydrophobic bonds in proteins. Minimum solubility are obtained around the I.E.P. of haemoglobin 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 the minimum solubility. On either side of the I.E.P., there will be repulsion between either predominantly negative charges or predominantly positive charges on the molecules leading to less aggregation and therefore increase in solubility.

PEG reduces the solubility of proteins in a regular and quantitatively definable manner. This in turn will permit some

type of valid extrapolation to true solubility. To this end, we have used PEG (molecular weight, 8,000) over a range of concentration (12 % to 20 %). Over this range, the logarithm of solubility (log S) versus PEG concentration was linear, using a constant protein concentration at pH 7.4. This allowed the extrapolation of true solubility of the modified and unmodified haemoglobin to be 794.33 ± 2.04 g/l and 676.08 ± 2.09 g/l respectively. This represents an increase of about 17.5 % the in solubility of haemoglobin when modified with acetyl-3,5-dibromosalicylic acid at pH 7.4 and 37°C temperature. The fact that the slopes of the two plots (Figure 3) are different derives from the different extent of the *excluded volume* effect of PEG on the different haemoglobins which in turn depends on the solubility of each species of haemoglobin. Comparatively, at a particular pH, temperature and PEG concentration, the solubility of the modified species is higher than that of the *unmodified one*. The solubility extrapolations were made with the assumption that the thermodynamic activity of the protein is equivalent, to a first approximation, to the solubility over the range of PEG concentrations used. This derives from the equation below (Middaugh et al., 1979), which was used to analyse the data obtained.

$$\log S_p = \log A_0 - A_{12}[PEG]$$

S_p is the solubility of the protein at any PEG concentration where the protein solid phase is in equilibrium with the PEG/protein saturated aqueous phase. A_0 is the activity of the protein in PEG-free solution and parallel the solubility of the protein when the *activity coefficient* approaches 1. [PEG] represents the concentration of polyethylene glycol while A_{12} is a constant representing the slope of the linear plot of log S_p against [PEG] within the region of the PEG concentration studied. At higher and lower PEG concentrations than those used for the experiment in this work, a tendency towards non-linearity was observed. This might be due to solute-PEG interaction in these concentration ranges (Tisel et al., 1980). The extrapolated solubility of the unmodified haemoglobin is in excess of the solubility of Hb A estimated to be about 500 g/l (Noguchi and Schechter, 1981). This gives an activity coefficient of 1.35 under the conditions of the *experiment*. Such factors as the presence of secondary solutes, that is buffer used, the temperature of experiment and water activity must have played a role in this increase. Since the determination of solubility of both the modified and unmodified haemoglobins was performed under similar conditions, the percentage increase in solubility of the modified haemoglobin over the unmodified one will not be affected by any of such factors. That is to say, in a comparative technique, the activity coefficients introduced by the use of PEG in determination of solubility are not manifested.

SUMMARY AND CONCLUSION

It is evident that modification of haemoglobin A could cause a dramatic change in the state of its aggregation. Dibromoaspirin clearly serves as an effective reagent for the chemical modification of *haemoglobin* A. Modification of haemoglobin A with dibromoaspirin increases the solubility of haemoglobin to some reasonable extent.

It has been shown that dibromoaspirin at 5 mM concentration, modified intracellular and extracellular haemoglobin to equivalent extents (Zaugg et al., 1980). The cell membrane presents little or no barrier to free diffusion of this compound. Thus, the administration of dibromoaspirin will surely enhance the solubility of haemoglobin. Because of the obvious structural similarity of dibromoaspirin to aspirin, we do not expect the toxicity of dibromoaspirin to be proscriptive.

The immediate future holds within its possibilities different alternatives that could be used as acetylating agents. Further work could also be done using Hb S samples.

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