A Rapid Method for Preparing Crystalline Human Hemoglobin and the Separation of Crystalline Hemoglobin A in Quantity*

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Summary: A rapid method is described for the large scale preparation of crystalline human hemoglobin. The use of toluene and rapid stirring are essential for rapid crystallization. The minor hemoglobin component A_2, present in crystalline hemoglobin, is removed by a batch procedure of adsorption on DEAE cellulose and fractionation by elution under conditions determined by column chromatography.

A number of methods have been described for the preparation of human hemoglobin in quantity.[1,2,3,4,5,6] The method of Drabkin[3,4] has been widely used to prepare crystalline human hemoglobin, and that of Rossi-Fanelli, Antonini and Caputo[5], for preparing hemoglobin solutions for physiological and chemical studies. No information concerning homogeneity of prepared hemoglobin is given by the authors of either of these methods. Using chromatographic methods, Clegg and Schroeder[7] reported that crystalline and non-crystalline hemoglobin were equally heterogeneous.

In order to carry out comparative solubility studies on normal human hemoglobin and sickle cell hemoglobin, it was necessary to prepare sizeable quantities of proteins, free from minor hemoglobin components. This paper describes a rapid method for preparing crystalline normal hemoglobin, and the removal of hemoglobin A_2, as shown by gel electrophoresis, by a batch chromatographic method, using DEAE cellulose.

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METHODS AND RESULTS

Preparation of Hemoglobin: The plasma from human blood was removed by centrifugation and the red cells were washed three times with 1.2% sodium chloride solution, and the supernatant liquid aspirated. Packed red cells were hemolyzed by adding 1.2 volumes of water and 0.4 volumes of toluene. The mixture was shaken vigorously for several minutes in a closed bottle and allowed to stand for an hour at room temperature or overnight at 4°. The cell debris and stroma were separated from the hemoglobin solution by centrifugation, and removed with most of the toluene by aspirating the top layer. The hemoglobin solution was then filtered through absorbent cotton. The stroma-free hemoglobin solution, pH 6.8-7.0, was saturated with oxygen if oxyhemoglobin was to be prepared and with carbon monoxide for the preparation of carboxyhemoglobin. For crystallization, the solution was placed in a beaker at room temperature and stirred with a magnetic bar. Two volumes of 2.9 molar potassium phosphate, pH 6.9, or two volumes of an approximately 3 molar neutralized ammonium sulfate solution (prepared by diluting 3 volumes of saturated ammonium sulfate with 1 volume of water) were added, followed by the addition of toluene to make the solution about 3% toluene. Crystals usually formed within 15 minutes using vigorous stirring, having the characteristic bi-pyramidal form as described by Drabkin.[3] After standing for 30 minutes, the crystals were removed by filtration on a Buchner funnel. Excess toluene in the crystalline filter cake was removed by pressing on filter paper. The moist hemoglobin crystals, containing ammonium sulfate, were wrapped in a compact form with Saran wrap, placed in a closed container, and stored at 4°. For recrystallization, hemoglobin was dissolved in water to give a 6-10% solution. Any insoluble material present was removed by centrifugation. The supernatant was then adjusted to pH 6.8-7.0 and the hemoglobin recrystallized by the addition of two volumes of 3 molar ammonium sulfate or 2.9 molar potassium phosphate and toluene.

This method was used to prepare crystalline hemoglobin with as little as 10 ml of blood but usually 500 ml were used. The yield of crystalline hemoglobin was about 90%. It contained a mixture of the A and A_2 components in essentially the same ratio as in the red cell hemolysate as shown by gel electrophoresis using the method of Raymond[8] (Fig. 3). No separation of these components was obtained by repeated fractional crystallization.

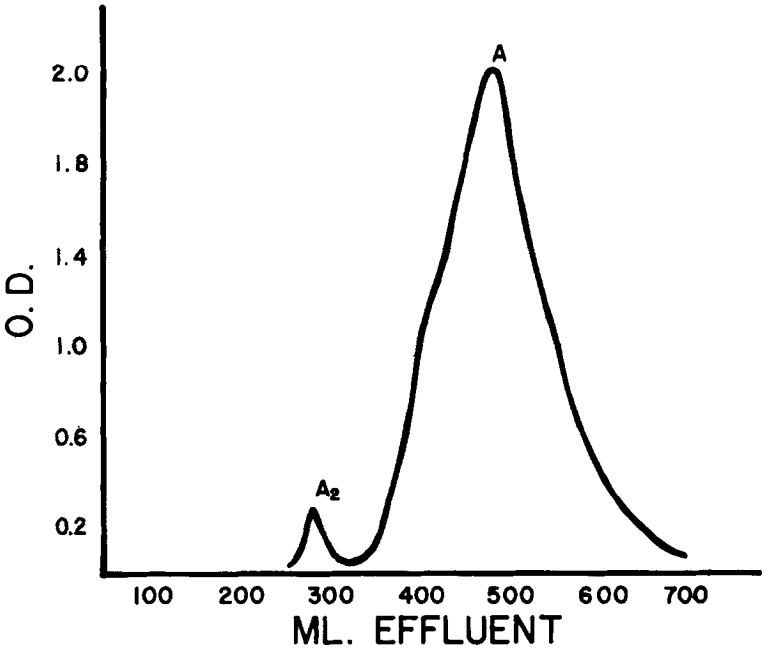

Fig. 1. Column chromatographic separation of hemoglobin A and A_2 on DEAE cellulose using a pH gradient in 0.05M Tris buffer, pH 8.5.

Separation of Hemoglobin A: A number of methods have been devised for separating the components of hemoglobin by column chromatography.[7,9,10] The method described by Huisman and Dozy[10] using DEAE cellulose in 0.05 molar Tris, was found to be effective in separating hemoglobin A and A_2. Separation was verified by gel electrophoresis on the hemoglobin peaks obtained by pH gradient elution (Figs. 1, 2). Hemoglobin A_2 was removed from the DEAE cellulose between pH 8.4 and 8.1 and hemoglobin A was

removed at pH values below 8.0. However, only small amounts of hemoglobin could be separated by this method, and the difficulty of recovering the hemoglobin in the native state made it necessary to adapt this method to give larger amounts of purified hemoglobin. This was done by using DEAE cellulose in 250 ml centrifuge bottles rather than in a column. In other respects the essential conditions for the removal of hemoglobin A_2 from A were the same as was used in the separation by column chromatography.

The following batch procedure for the purification of hemoglobin A was devised: commercial type 40 DEAE cellulose, was washed consecutively through a Buchner funnel, with 1N NaOH, water, and 1N HCl. The DEAE cellulose was then washed with water until the pH was about 4. It was then suspended in water and solid Tris was added with stirring, until a pH of 8.5 was obtained. The supernatant was removed by centrifugation in 250 ml bottles giving a packed volume of DEAE cellulose of about one-third the volume of the centrifuge bottle. The DEAE cellulose was then washed by centrifugation with 0.05M Tris-HCl, pH 8.5 using about 150 ml for each centrifuge bottle.

A concentrated hemoglobin solution was prepared for purification on DEAE cellulose, by removing the salt from crystalline hemoglobin by dialysis against water, adjusting the pH to 8.5 by adding solid Tris, and subsequent dialysis agains 0.05M Tris-HCl, pH 8.5 at 4° for 18 hours. The concentration of the hemoglobin solution was determined spectroscopically, and it was then diluted with 0.05M Tris-HCl buffer, pH 8.5 to make a 4% solution of hemoglobin. One hundred millileters of the 4% hemoglobin solution were added to each of four 250 ml centrifuge bottles containing equilibrated DEAE cellulose. The hemoglobin solution was thoroughly mixed with the DEAE cellulose and allowed to stand for 5 minutes, then centrifuged. Under these conditions about 95% of the hemoglobin was removed from the solution by the DEAE cellulose. Hemoglobin A_2 was then removed from the DEAE cellulose by washing 5 times by centrifugation with 0.05M Tris buffer,

pH 8.1, until the pH of the supernatant became 8.1. Filtration on a Buchner funnel was found to be a quicker method than centrifugation for removing hemoglobin A_2. This was done by transferring the DEAE cellulose after absorption of the hemoglobin and the removal of the excess pH 8.5 buffer by centrifugation, to a beaker with 400 ml of 0.05M Tris buffer, pH 8.1. After mixing, the DEAE cellulose was removed by filtration on a Buchner funnel. Care was taken to remove as much supernatant liquid as possible by packing the filter cake. This operation was repeated twice, using 400 ml of the pH 8.1 buffer each time. Finally, hemoglobin A was extracted by mixing the filter cake with 200 ml of 0.2M monobasic potassium phosphate. After filtering on a Buchner funnel and pressing, the filter cake was removed and mixed with 100 ml of 0.2M monobasic potassium phosphate and the filtration procedure repeated. The volume of the combined filtrate obtained by phosphate extraction was about 350 ml and had a pH of 6.5. The solution was saturated with oxygen if oxyhemoglobin was being prepared or with carbon dioxide when carboxyhemoglobin was being prepared and then the pH was adjusted to 7.0 by adding solid dibasic potassium phosphate. For each 100 ml of hemoglobin solution, 25 grams of solid ammonium sulfate was slowly added while stirring. The pH was again adjusted to 6.8-7.0 by adding solid dibasic potassium phosphate and toluene was then added to make the solution 3% toluene. Usually the hemoglobin crystallized within 15 minutes during rapid stirring, however, occasionally the insoluble hemoglobin was amorphous. If the hemoglobin failed to crystallize, 3 molar ammonium sulfate solution was added gradually, until crystallization took place. The crystalline hemoglobin A was collected on a Buchner funnel and the excess toluene removed by blotting with a filter paper. The moist hemoglobin A was then wrapped in a compact form with Saran wrap, and stored in a closed container at 4°. From 16 grams of crystalline hemoglobin, a yield of 8-10 grams of hemoglobin A with a negligible amount of methemoglobin was obtained with only one electrophoretic component as shown in Fig. 3.

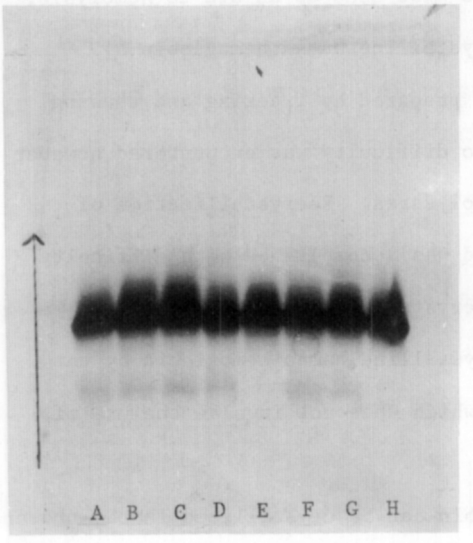

Fig. 2. Slots A & B - 1X cryst.Hb.
Slots C,D.F,G. - no separation of Hb.A & A_2 by recrystallization
Slots E & H - Hb A separated by column chromatography on DEAE cellulose

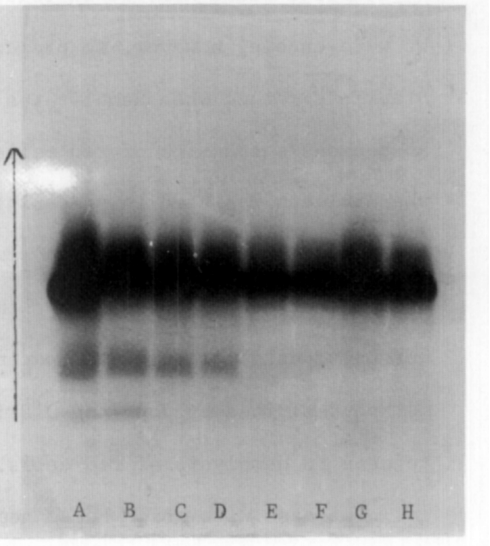

Fig. 3. Slots A & B-red cell hemolysate
Slots C & C-1X cryst. Hb.
Slots E & F-Hb.A (batch method)
Slots G & H-partially separated Hb A by salt precipitation

The DEAE cellulose used in adsorbing 16 grams of hemoglobin amounted to 36 grams. The DEAE cellulose was regenerated by washing extensively with water followed by washing with 1N HCl, and then with water to a pH reached of 3-4. DEAE was then suspended in water, and solid Tris was added to make the pH of the suspension 8.5. The supernatant was removed by centrifugation and the DEAE cellulose washed with 0.05M Tris buffer, pH 8.5.

DISCUSSION

In 1922 Heidelberg[11] introduced the use of toluene in the preparation

of crystalline hemoglobin. He considered its beneficial effect on the preparation of hemoglobin to be essentially that of an improved hemolyzing reagent. In the extensively used method of Drabkin[3,4] for preparing crystalline human hemoglobin toluene is also used as an aid to hemolysis. We were unable, however, to prepare crystalline human hemoglobin by Drabkin's method when hemolysates were prepared by freezing and thawing an aqueous suspension of red cells. No difficulty was encountered however when toluene was used in preparing hemolysates. Recrystallization of hemoglobin prepared by Drabkin's method was accomplished with difficulty, and in fact he did not describe the recrystallization of hemoglobin. We were also unsuccessful in preparing crystalline human hemoglobin by the method described by Jope and O'Brien, which does not involve the use of toluene in hemolysis of red cells.

The ease with which human hemoglobin can be crystallized in the presence of toluene with stirring is remarkable; even solutions as low as 1% hemoglobin can be crystallized in a few minutes. No deleterious effects of toluene on the properties of hemoglobin have been observed. The use of toluene in the crystallization of bovine hemoglobin and β lactoglobulin by salts was explored, however, no beneficial effect of toluene in the crystallization of these proteins was observed. Also, the effect of organic solvents other than toluene, such as benzene, xylene and heptane, was found to be ineffective in hastening crystallization of human hemoglobin. These results indicate that toluene is unique in accelerating the crystallization of human hemoglobin by salts.

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