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STUDIES ON THE HETEROGENEITY OF HEMOGLOBIN

XV. SEPARATION OF FETAL HEMOGLOBIN AND THE NORMALLY OCCURRING MINOR ADULT HEMOGLOBINS BY CHROMATOGRAPHY ON DEAE-CELLULOSE

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SUMMARY

A modification of the DEAE-cellulose chromatographic procedure for the quantitation and isolation of human hemoglobins is described. The method uses a new form of the anion exchanger DEAE-cellulose, namely, microgranular (preswollen) DE-52 (Whatman Biochemicals, W. & R. Balston, Maidstone, Great Britain and marketed in the U.S.A. by H. Reeve Angel, Clifton, N.J.) and 0.05 *M* Tris–HCl developers with different pH values. Separation of the hemoglobins A_0, F_0, A_1, and F_1 is greatly improved, which makes the method valuable for the isolation of Hb-F from large volumes of blood containing low percentages of this fetal protein.

INTRODUCTION

The major human fetal (Hb-F_0) and normal adult (Hb-A_0) hemoglobins can readily be separated by various forms of ion-exchange chromatography, such as CM-cellulose[1,2], Amberlite IRC-50[3–6], CM-Sephadex[7], DEAE-cellulose[8,9], and DEAE-Sephadex chromatography[9,10]. Unfortunately, minor hemoglobins (Hb-A_1) related to Hb-A_0 chromatograph similarly to Hb-F_0, and the chromatographic determination of low amounts of Hb-F in red cell hemolysates is, therefore, subject to considerable error. Moreover, the presence of these Hb-A_1 components in the zone of Hb-F often prevents the isolation of a Hb-F that is sufficiently pure to allow analyses which are part of our study of the chemical characterization of Hb-F in various hematological conditions[11,12]. In this publication we will show that Hb-F_0 can be separated from the Hb-A_0 and Hb-A_1 components on columns of DEAE-cellulose using a newer form of this anion exchanger. The modification is particularly useful for the isolation of Hb-F from red cell hemolysates containing low quantities of this fetal protein.

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MATERIALS AND METHODS

Blood samples

Blood was obtained from subjects with various hematological disorders which included the hereditary persistence of Hb-F (HPFH), β-thalassemia, and Fanconi's anemia (for references see refs 11 and 12), and from newborns. Five millilitres of blood were collected with EDTA as anticoagulant for analytical chromatography and the larger amount (100–450 ml) required for preparative chromatography was collected in an appropriate volume of ACD solution. The red cells were isolated by centrifugation, washed three times with 0.9 g% NaCl solution, and lysed by addition of an equal volume of distilled water and 0.2 volume of carbontetrachloride. The resulting erythrocyte hemolysate was cleared from cellular debris by centrifugation and dialyzed at 4° overnight against a large volume of 0.05 *M* Tris–HCl buffer, pH 8.5, containing 100 mg KCN/1000 ml.

Analytical chromatography

DEAE-cellulose (Whatman DE-52, microgranular, pre-swollen) was used. The anion exchanger was equilibrated with the 0.05 *M* Tris–HCl, pH 8.5, developer* and stored at room temperature as a suspension so that the settled volume of the resin is one-half of the total volume of the slurry. A 0.9 × 60 cm glass column was filled till a resin height of about 55 cm had been attained; the column was equilibrated overnight with an 0.05 *M* Tris–HCl, pH 8.3, developer. An appropriate quantity of the dialyzed hemolysate containing 40 to 50 mg hemoglobin was applied to the column and the chromatogram was developed at room temperature with a pH gradient obtained by supplying an 0.05 *M* Tris–HCl, pH 7.9, developer from a separatory funnel to a 250-ml mixer which contained the pH 8.3 developer. After 400 to 450 ml developer had passed through the column the pH 7.9 developer in the funnel was replaced by a similar developer but with a pH value of 7.5. The flow-rate was maintained at 20 ml/h. The effluent was collected in 4-ml fractions and analyzed as described before[1,8–10]. Measurement of the pH of the effluent was made with a Radiometer PH-4 pH meter (Radiometer, Copenhagen, Denmark).

Preparative chromatography

Larger columns (2.5 × 60 cm) were used. The chromatogram was developed with an 0.05 *M* Tris–HCl, pH 8.0, developer at a flow-rate of approximately 50 ml/h. In many experiments the hemoglobin was not eluted but sections of the DE-52 with the various hemoglobin components were removed from the tube, and the hemoglobin was eluted from each section individually with the 0.05 *M* Tris–HCl, pH 7.0, developer. When a gradient was applied to a preparative DE-52 column the buffers were the same as those used for the development of an analytical chromatogram; the volume of the mixer, however, was increased to 500 ml.

Regeneration of the DE-52 cellulose requires repeated washings of the resin in a büchner funnel with a twenty-fold volume of a 5 g% NaCl solution followed by similar treatment with distilled water until the filtrate is free of chloride ion. The anion exchanger is finally equilibrated with the 0.05 *M* Tris–HCl, pH 8.5, developer.

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* All developers contain 100 mg KCN/1000 ml.

Other methods

DEAE-Sephadex chromatography and starch gel electrophoresis followed previously published methods[9,10,13]. The method of Betke *et al.*[14] was used for the estimation of Hb-F by alkali denaturation (% $Hb\text{-}F_{AD}$). The method developed by Schroeder *et al.*[15], which determines the proportion of Hb-F in mixtures by amino acid analysis for isoleucine, was used for the accurate determination of Hb-F in isolated hemoglobin components (% $Hb\text{-}F_{IIc}$). Pooled fractions of zones from chromatograms were concentrated on columns of CM-Sephadex by the method described before[15].

RESULTS AND DISCUSSION

Fig. 1 depicts the chromatographic separation of the Hb components in three freshly prepared hemolysates containing different amounts of Hb-F. The new features of the chromatograms are the position of Hb-F, which is eluted between the major $Hb\text{-}A_0$ and the minor $Hb\text{-}A_1$ components, and that of the minor $Hb\text{-}F_1$, which is eluted last. The elution pH values for the Hb components differ slightly; $Hb\text{-}A_0$, for instance,

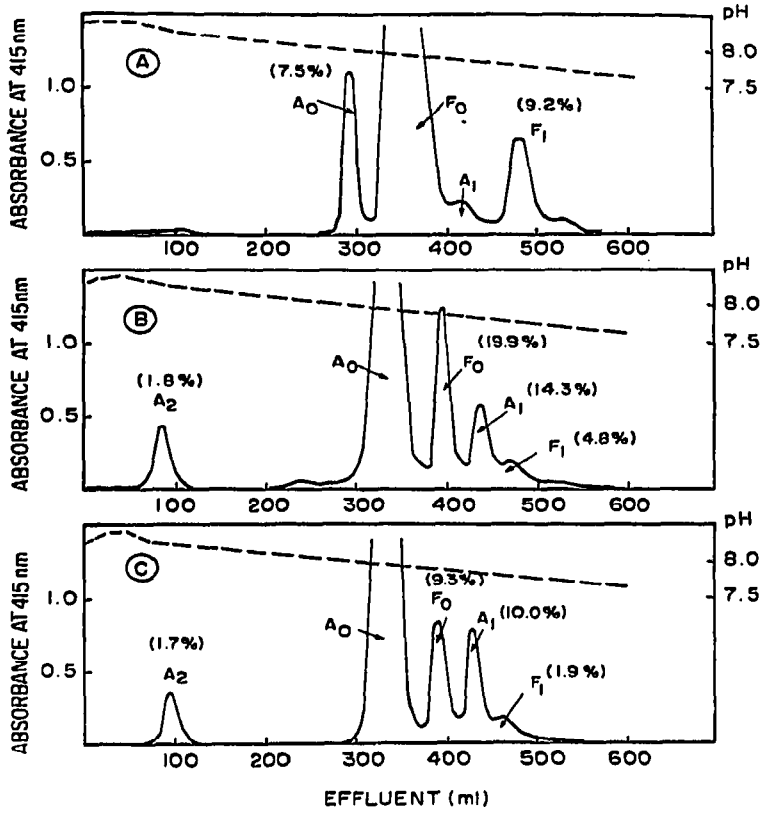

Fig. 1. DE-52 cellulose chromatograms of human red cell hemolysates. (A) Cord blood sample. (B) Subject with a HPFH heterozygosity. (C) Patient with Fanconi's anemia. Broken lines represent pH values of effluents. For further details see text.

is eluted at a pH of 8.05, Hb-F_0 at pH 7.98, Hb-A_1 at pH 7.92, and Hb-F_1 at pH 7.85. Despite this improved separation, a considerable overlap can be expected, and starch gel electrophoresis and amino acid analyses of the isolated Hbs F_0, A_1 and F_1 from the chromatograms B and C of Fig. 1 have shown that the Hb-F_0 zones contain some Hb-A_0 and Hb-A_1 and that a small amount of Hb-F_0 is still present in the Hb-A_1 zones. These observations indicate that the determination of Hb-F in hemolysates by this chromatographic method is still subject to considerable error. Despite the improved separation of the Hb-F and Hb-A components, a more accurate estimation of Hb-F still requires the determination of Hb-F in all minor zones by amino acid analysis for isoleucine (see ref. 15).

Figs. 2 and 3 illustrate the usefulness of the DE-52 chromatography for the isolation of Hb-F from large amounts of blood.

Subject W.D.P. Subject W.D.P. (Fig. 2) has a special type of hereditary per-

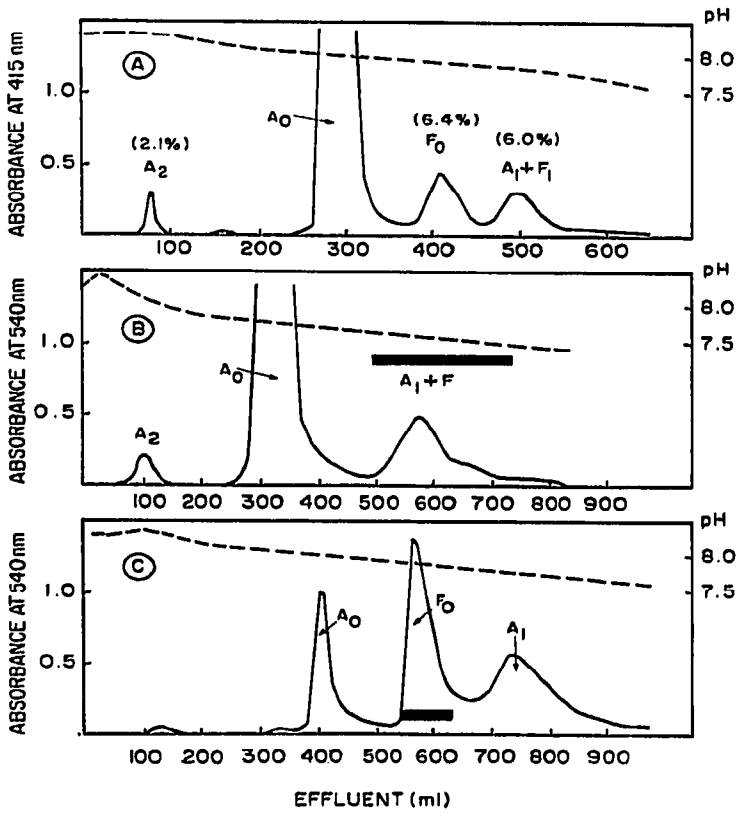

Fig. 2. Isolation of Hb-F from red cell hemolysate of patient W.D.P. containing 4.6% Hb-F_{AD} by DE-52 cellulose chromatography. (A) Analytical DE-52 cellulose chromatogram. (B) Preparative DEAE-Sephadex chromatogram of whole red cell hemolysate. (C) Preparative DE-52 cellulose chromatogram of the A_1 and F zone which was isolated by DEAE-Sephadex chromatography. The patient is described in the text. The two preparative chromatograms were developed with a pH gradient to allow the graphic demonstration of the hemoglobin zones; normally, a single developer was used and the hemoglobins were eluted from sections of the ion exchanger that were removed from the glass tube.

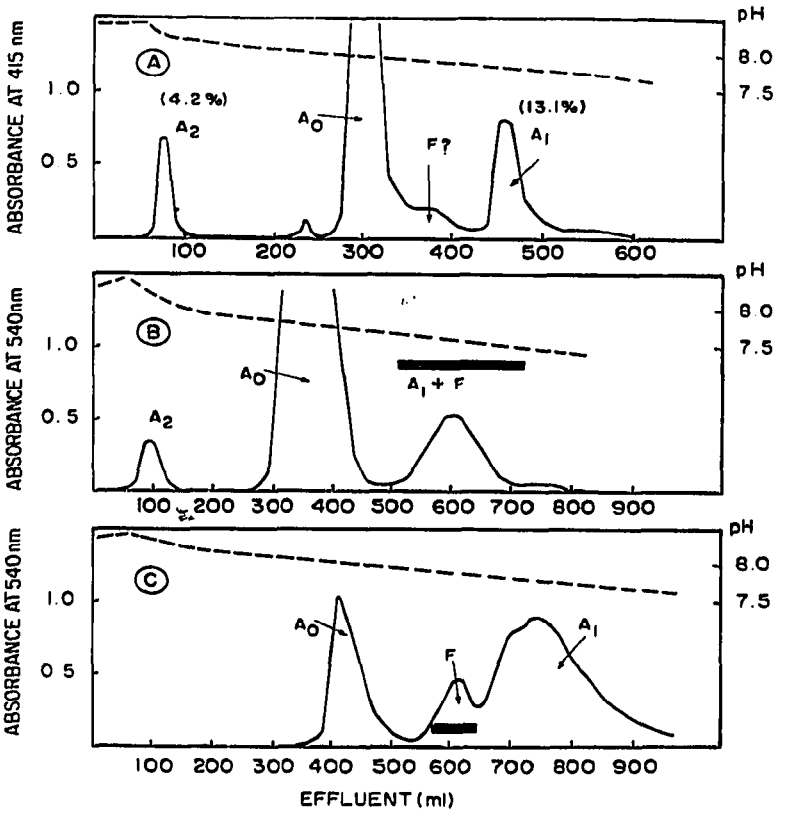

Fig. 3. Isolation of Hb-F from red cell hemolysate of patient K.B. containing 1.6% Hb-F_{AD} by DE-52 cellulose chromatography. (A) Analytical DE-52 cellulose chromatogram. (B) Preparative DEAE-Sephadex chromatogram of whole red cell hemolysate. (C) Preparative DE-52 cellulose chromatogram of the A_1 and F zone which was isolated by DEAE-Sephadex chromatography. The patient is discussed in the text. See also legend of Fig. 2.

sistence of Hb-F (HPFH) with 5–6% Hb-F and with an accompanying mild erythrocytosis[16]. He donated one pint of blood, which was collected in ACD. The total amount of Hb available was 76 g, which contained 4.6% Hb-F_{AD} corresponding to 3.5 g. Fig. 2A depicts the analytical chromatogram showing an almost complete separation of Hb-F_0 and the Hb-A_1 components. However, because of the high cost of the DE-52 anion exchanger (US$ 6–7 per column) the Hb-A_1 and F zones were first isolated by preparative DEAE-Sephadex chromatography using an 0.05 *M* Tris–HCl, pH 7.85, developer[9,10]. Totally 47 columns were required. Fig. 2B depicts a representative chromatogram. The A_1 and F zones were combined, concentrated on columns of CM-Sephadex[15], and dialyzed against the 0.05 *M* Tris–HCl, pH 8.5, developer. Re-chromatography of this material required 16 DE-52 columns; separation of Hb-F from accompanying Hb-A_0 and Hb-A_1 was possible with the 0.05 *M* Tris–HCl, pH 8.0, developer (Fig. 2C). Starch gel electrophoresis showed that the isolated Hb-F_0 zone contained less than 10% Hb-A_0 and Hb-A_1, an observation which was confirmed by amino acid analysis. The total recovery of Hb-F was 1760 mg corresponding to about 50% of the amount present in the blood sample.

Subject K.B. Subject K.B. (Fig. 3), with a β-thalassemia trait, donated 125 ml blood which was collected with EDTA as anticoagulant. The total volume of 125 ml contained 14.5 g Hb. The percentage of Hb-F_{AD} was 1.4, indicating that about 200 mg Hb-F was present in the sample. Fig. 3A presents the analytical chromatogram showing an incomplete separation of Hb-A_0 and Hb-F, and an elevated level of Hb-A_2. Isolation of the A_1 and F zones by DEAE-Sephadex chromatography required ten columns (Fig. 3B depicts a representative chromatogram) and re-chromatography of this zone was made on four preparative DE-52 cellulose columns. The Hb-F zone was incompletely separated from the Hb-A_1 zone (Fig. 3C) and further analyses showed that it contained approximately 60% Hb-F and 40% Hb-A_1. The total recovery of the zone was 120 mg, or 72 mg Hb-F, corresponding to 35% of the amount present in the blood sample.

CONCLUSIONS

The DE-52 cellulose chromatographic procedure is a modification of a previously published procedure[8,9] and has the advantage of an improved separation between the fetal and adult hemoglobins. Although this property does not significantly improve the quantitative determination of Hb-F by analytical chromatography it greatly facilitates the isolation of Hb-F from mixtures. Application of this technique to blood samples from subjects with various hematological disorders involving slight elevations in the level of Hb-F will make it possible for sufficient amounts of the fetal protein to be isolated for further chemical characterization.

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