CHROM. 3900

STUDIES ON THE HETEROGENEITY OF HEMOGLOBIN

XIV. CHROMATOGRAPHY OF NORMAL AND ABNORMAL HUMAN HEMOGLOBIN TYPES ON CM-SEPHADEX

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SUMMARY

A new chromatographic procedure for the separation and quantitation of human hemoglobin components is described. The method utilizes columns of carboxymethyl-Sephadex G-50 and 0.05 *M* Tris–maleic acid buffers as developers; application of a simple pH gradient is preferred over stepwise elution. The procedure allows the separation of Hb-A_2 from the hemoglobins S and C, and of Hb-D_2 from the slow moving hybrid component SDα. Fetal hemoglobin is eluted in front of the normal Hb-A. The minor hemoglobins of normal red cell hemolysates were fractionated into at least four components, well separated from the major hemoglobin component. The procedure has the disadvantage of requiring several days to complete the chromatographic separations.

INTRODUCTION

The procedures most commonly used for the chromatographic separation of normal human hemoglobin types and of their variants make use of the anion exchangers DEAE-cellulose[1,2] and DEAE-Sephadex[2,3], and of the cation exchangers Amberlite IRC-50[4–7] and CM-cellulose[8–10]. Each of these techniques has their advantages and disadvantages. Chromatography on Amberlite IRC-50, for instance, is particularly useful for the isolation of the various minor hemoglobin components from normal adult and cord blood red cell hemolysates, while DEAE-Sephadex chromatography offers a rapid and reliable technique for the isolation and quantitation of hemoglobin variants from blood samples of patients with different hemoglobinopathies. In this paper we introduce a CM-Sephadex chromatographic procedure, which is of value for the isolation and quantitation of certain major and minor hemoglobin components with closely identical electrophoretic mobilities. The method does not lend itself easily to routine application because of the time required to complete a chromatogram. This disadvantage makes a careful selection of the material to be analyzed a necessity.

MATERIALS AND METHODS

Hemoglobin samples

Blood samples were obtained from the following sources:

(1) Normal adults and cord blood samples from Negro babies.

(2) One cord blood sample from a Negro baby with a heterozygosity for the β chain variants S and C (patient N.C.).

(3) One homozygous sickle cell anemia patient (J.G.) and one patient with SC disease (patient S.C.).

(4) One heterozygous β-thalassemia carrier with elevated levels of Hb-A_2 and Hb-F (patient W.O.).

(5) One hemoglobin C-β-thalassemic individual (patient G.S.); starch gel electrophoretic examination showed the presence of small amounts of Hb-A and Hb-F in addition to Hb-C.

(6) One adult patient with a heterozygosity for an α chain variant (Hb-Dα) and for the β chain variant Hb-C (patient L.R.). Four major hemoglobin fractions were demonstrated by starch gel electrophoresis, namely Hb-A or $\alpha_2\beta_2$, Hb-Dα or $\alpha_2 D\beta_2$, Hb-C or $\alpha_2\beta_2 C$, and Hb-CDα or $\alpha_2 D\beta_2 C$. Two additional minor fractions, Hb-A_2 or $\alpha_2\delta_2$ and Hb-D_2 or $\alpha_2 D\delta_2$, were not observed with this technique because of the identical electrophoretic mobilities of Hb-C and Hb-A_2, and of Hb-CDα and Hb-D_2. The father of this individual (patient R.R.) exhibited a homozygosity for Hb-C and a heterozygosity for Hb-Dα; only two components were observed by starch gel electrophoresis. Blood samples from both patients were kindly supplied by Dr. R. B. SQUIRES, Baptist Hospital, Pensacola, Fla.

(7) One individual with a heterozygosity for Hb-C-Harlem (patient G.S.). This β chain variant has been identified as an abnormality with two amino acid residue substitutions, namely a Glu to Val substitution in position 6 and an Asp to Asn substitution in position 73 (ref. 11). A blood sample from an individual with an apparently similar Hb-C-Harlem heterozygosity, but in combination with a Hb-C ($\alpha_2\beta_2{}^{6\mathrm{Lys}}$) heterozygosity, was also studied. Starch gel electrophoresis of the hemoglobin of this last patient (patient P) showed only one component in the position of Hb-C. The blood samples of patients S and P were made available to us by Dr. W. A. SCHROEDER, California Institute of Technology, Pasadena, Calif.

(8) The hemoglobins F_0, A_0, A_2, S_0 and C_0, isolated by DEAE-Sephadex chromatography[2,3]. The minor hemoglobins A_{Ia+b} and A_{Ic}, isolated by Amberlite IRC-50 chromatography[7], were kindly supplied by Dr. B. F. HORTON, University of Tennessee, Knoxville, Tenn.

CM-Sephadex chromatography

CM-Sephadex (C-50, capacity: 4.5 $\pm$ 0.5 mequiv/g, Pharmacia Fine Chemicals, lot No. 9494) was used. The cation exchanger was equilibrated with the first developer and stored at room temperature. The buffer was changed once weekly. The developers were 0.05 *M* Tris-(hydroxymethyl)aminomethane–maleic acid buffers of pH varying between 6.5 and 8.0. These developers were prepared from 1 *M* stock solutions of both chemicals, by titrating the 0.05 *M* Tris solution with the concentrated maleic acid solution to the desired pH value. Each developer also contained 100 mg KCN/1000 ml. A column of 60 $\times$ 0.9 cm was used in most experiments; the resin column was usually

equilibrated with a pH 6.5 developer, and in certain instances with a pH 6.7 developer. Seventy to 150 mg of hemoglobin (in 2 to 3 ml), dialyzed overnight at 4° against the first developer, was chromatographed. A pH gradient was applied by supplying a buffer of higher pH value (usually 7.0) from a separatory funnel to a 250 ml mixer which contained the first developer. The selection of additional developers and the time of introduction depended on the elution pattern observed. The flow rate was maintained at approx. 20 ml/h; an increase was not possible due to an undesirable packing of the column under increased pressure. The time required to complete the chromatogram (5 to 7 days!) made it necessary to run the analyses at 4°. The effluent was collected in 8 ml fractions and analyzed as described before[1–3,8–10]. Measurements of the pH of the effluent were made at room temperature with a Radiometer PH-4 pH meter within 24 h after collection.

Additional techniques

Several isolated hemoglobin components were further characterized by starch gel electrophoresis and by analyses of their total amino acid composition. The components were concentrated by ultrafiltration under reduced pressure at 4°, dialyzed overnight against distilled water, whereafter the appropriate amounts were hydrolyzed for 24 h with 6 *N* HCl at 110° under reduced pressure. Amino acid analyses were made with a Spinco model 120B automatic amino acid analyzer. Procedures for the preparation of hemolysates, starch gel electrophoresis, analytical and preparative chromatography using DEAE-Sephadex have been described before[2,3,12].

RESULTS AND DISCUSSION

Separation of an artifical mixture of the hemoglobins A, F, S, C and A_2

The chromatogram was initially developed with a 6.7 to 7.0 pH gradient; the pH 7.0 developer was replaced in the separatory funnel by similar buffer solutions but with pH values of 7.3, 7.5, 7.7 and 8.4 after approximately 350, 1100, 1450 and 1600 ml of developer passed through the column, respectively. Fig. 1 illustrates the chromatogram; the five major hemoglobin fractions were eluted at pH values as indicated. The identity of each component was determined by starch gel electrophoresis; the relative electrophoretic mobilities, presented in Fig. 2, indicated a sequence of F, A, A_2, S and C in the elution of these components. The identities of components III (or Hb-A_2) and V (or Hb-C) were confirmed by amino acid analyses of their 24 h acid hydrolysates. The electrophoretic pattern also indicated that each component was pure, except the Hb-A_2 and Hb-S fractions, which were contaminated to a minor extent with each other. The elution pattern is rather different from that observed in CM-cellulose chromatography, namely A, F, S, C + A_2 (ref. 9). Application of CM-Sephadex chromatography, therefore, offers an unique opportunity to separate the hemoglobins A_2 and C from red cell hemolysates. The separation of Hb-F and Hb-A is comparable to that observed in Amberlite IRC-50 chromatography.

Separation of minor hemoglobin components from normal red cell hemolysates

Approximately 130 to 150 mg of hemoglobin was chromatographed. The majority of the hemolysates were freshly prepared, while others were either aged for 40 days at 4° or treated with oxidized glutathione, as described in previous papers of

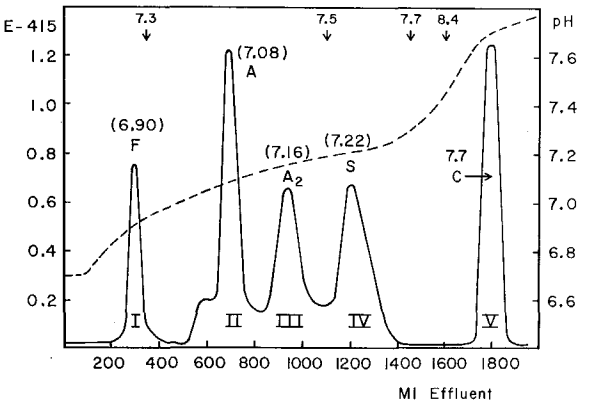

Fig. 1. Chromatography of an artificial mixture of the hemoglobins F, A, A_2, S and C on a column of CM-Sephadex at 4°. An initial pH gradient with 0.05 *M* Tris–maleic acid developers of pH 6.7 to 7.0 was applied; the second buffer of this gradient system was replaced by additional developers with increasing pH values (7.3, 7.5, 7.7, 8.4) as indicated. The values between parentheses represent the pH elution values of the individual zones. The broken line represents the pH of the effluent. The flow rate was 20 ml/h.

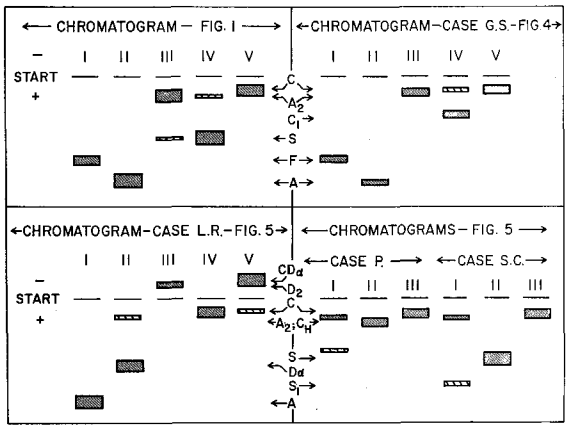

Fig. 2. Schematic presentation of the mobilities of isolated hemoglobin components in starch gel electrophoresis.

this series[13,14]; such treatment will result in the formation of a mixed disulfide, which has been identified as $\alpha_2(SH)_2\beta_2$ $(SH)_2(SSG)_2$ (ref. 13). The chromatograms were developed with a 6.5 to 7.0 pH gradient, while a pH 7.4 buffer was introduced after approximately 1500 ml of developer passed through the columns. Four, and probably five, minor components were observed in a normal freshly prepared hemolysate; these fractions were eluted at pH values of 6.80, 6.85, 6.92 and 7.01, respectively (Fig. 3). The figure also lists the ranges of the relative amounts for each component, calculated from 10 chromatograms. Aging of the hemolysate and incubation with oxidized glutathione resulted in a marked increase in component IV (chromatograms 2

and 3 of Fig. 3), which identified this fraction as the A-GSSG complex. Chromatography of the Hb-A_{Ia+b} and of the Hb-A_{Ic} components, isolated by Amberlite IRC-50 chromatography, identified the components II and III (chromatograms 4 and 4a of Fig. 3). The nature of the minute component I and of component IIIa is not known; amino acid analyses of 24 h hydrolysates of component IIIa indicated the probable presence of a fetal hemoglobin component. A comparison of these data with those observed in other types of cation exchange chromatography indicates a notable improvement of the resolution of the minor hemoglobin fractions over that seen in CM-cellulose chromatography[9,10]. The elution pattern is more or less comparable to that observed in Amberlite-IRC-50 chromatography.

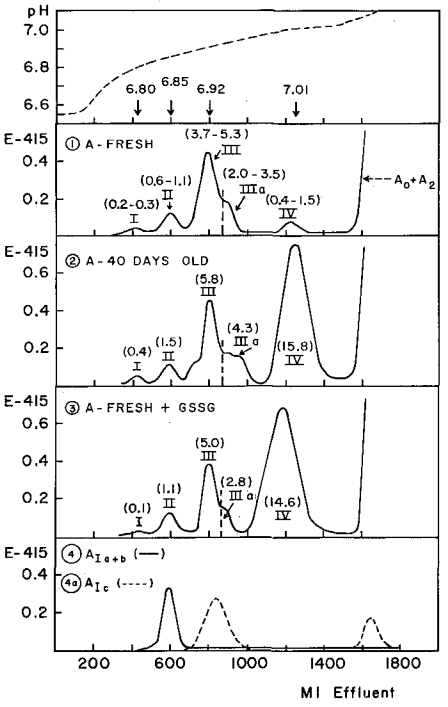

Fig. 3. Chromatography of the minor hemoglobin components on columns of CM-Sephadex. (1) Freshly prepared hemolysate. (2) Same hemolysate after storage at 4° for 40 days. (3) Same hemolysate after treatment with oxidized glutathion. (4) Hb-A_{Ia+b} and (4a) Hb-A_{Ic}, isolated by Amberlite IRC-50 chromatography. The values between parentheses represent the relative amounts of the individual zones; the elution pH value of each individual zone is given at the top of the first chromatogram. For further details see text.

The separation of fetal hemoglobin components from cord blood hemolysates, and from red cell hemolysates of patients with SS anemia, β-thalassemia trait, and β-thalassemia-Hb-C disease

These chromatograms were initially developed with a pH 6.5 to 7.0 gradient, while the pH 7.0 buffer was replaced by similar buffer solutions but with pH values of 7.3, 7.5 and 8.0 after approximately 1500, 2300 and 2600 ml passed through the column, respectively. Each sample was simultaneously analyzed by DEAE-Sephadex

chromatography[3]. The chromatograms obtained with these two techniques are compared in Fig. 4; case N.C. represents a newborn baby with a heterozygosity for both Hb-S and Hb-C, case J.G. a patient with sickle cell anemia, case W.O. a patient with β-thalassemia trait, and case G.S. a patient with Hb-C-β-thalassemia disease. Careful examination of these chromatograms allows the following conclusions: (a) Fetal hemoglobin was observed as two, incompletely separated, components, which are eluted at pH values of 6.91 and 6.94 respectively. The total amounts of fetal hemoglobin calculated from the CM-Sephadex chromatograms, corresponded rather well with those calculated from the DEAE-Sephadex chromatograms. (b) The separation of the Hb-A_2 from Hb-S and Hb-C was (almost) complete, thus allowing its quantitation in patients with a Hb-C heterozygosity or homozygosity. (c) The resolution observed by CM-Sephadex chromatography was in most instances greater than that seen in the DEAE-Sephadex chromatograms. This is particularly apparent in the chromatograms of the β-thalassemic individual (case W.O.) and of the Hb-C-β-thalassemic patient (case G.S.). The electrophoretic mobilities of the components I through V of the chromatogram from case G.S. are presented in Fig. 2; the observations made are in agreement with those described for the isolated fractions of the

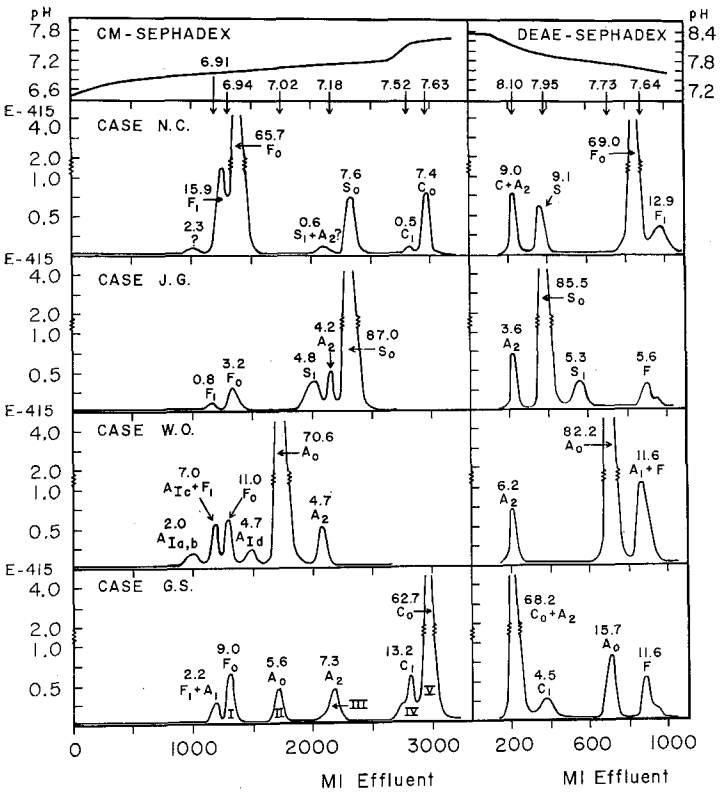

Fig. 4. Chromatography of red cell hemolysates containing fetal hemoglobin on columns of CM-Sephadex and of DEAE-Sephadex. Case N.C.: cord blood from a Negro baby with an heterozygosity for the hemoglobins S and C. Case J.G.: sickle cell anemia. Case W.O.: β-thalassemia trait. Case G.S.: Hb-C-β-thalassemia trait. For further details see legend to Fig. 3 and text.

chromatogram given in Fig. 1. (d) The correlation between the relative amounts of the various hemoglobin components, calculated from the CM-Sephadex and the DEAE-Sephadex chromatograms was rather good, except for that of Hb-A_0 in case G.S., which remains unexplained.

Chromatographic separation of some rare hemoglobin components

The cases, of which chromatograms are presented in Fig. 5, concerned case L.R. with a heterozygosity for the β chain variant Hb-C and for the α chain variant Hb-D; case R.R. with a homozygosity for Hb-C and a heterozygosity for Hb-Dα; case G.S. with a heterozygosity for Hb-C-Harlem; case P with a heterozygosity for Hb-C-Harlem and for Hb-C, and case S.C. with Hb-S-Hb-C disease. The chromatograms were developed in a similar way as described for those presented in Fig. 4. The following remarks seem appropriate:

Case L.R. The separation of the hemoglobins A,Dα, C_0 and the hybrid compo-

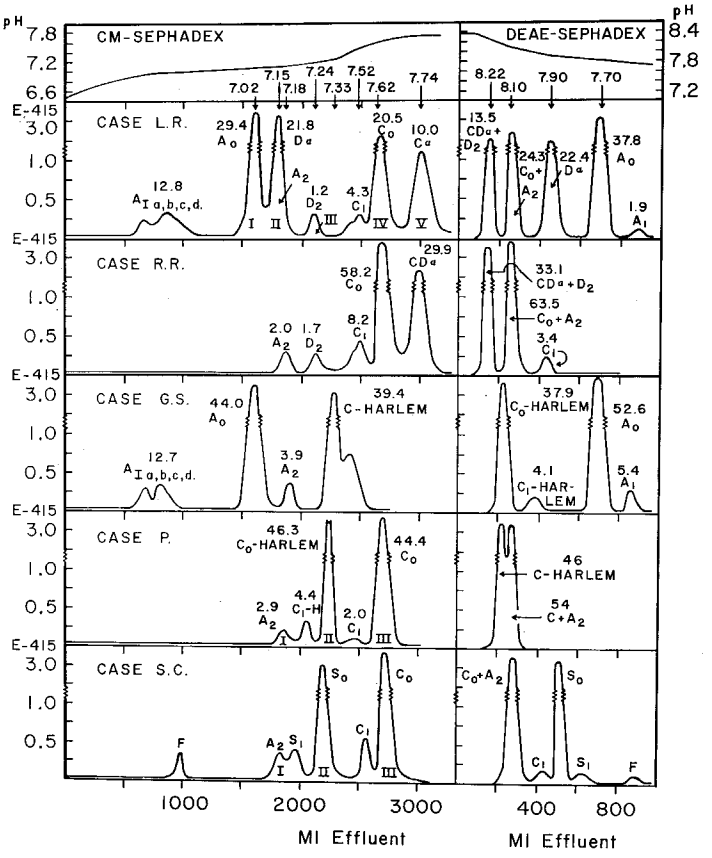

Fig. 5. Chromatography of red cell hemolysates containing some rare hemoglobin types on columns of CM-Sephadex and of DEAE-Sephadex. Case L.R.: heterozygosity for the α chain variant Dα and the β variant Hb-C. Case R.R.: heterozygosity for the α chain variant Dα and homozygosity for the β chain variant Hb-C. Case G.S.: heterozygous C-Harlem carrier. Case P.: Hb-C-Hb-C-Harlem heterozygosity. Case S.C.: Hb-S-Hb-C disease. For further details see legend to Fig. 3 and text.

nent CDα was complete in both types of chromatography. CM-Sephadex chromatography allowed also the isolation of Hb-D_2 (or $\alpha_2D\delta_2$) but not of Hb-A_2, which had a mobility similar to that of Hb-Dα (or $\alpha_2D\beta_2$). The identity of several of these isolated fractions is based on the differences in their electrophoretic mobilities (Fig. 2); the presence of Hb-A_2 in the isolated component II is clearly demonstrated by this procedure.

Case R.R. The absence of normal Hb-A and of Hb-Dα was confirmed in both chromatograms. CM-Sephadex chromatography allowed the complete separation of the minor components Hb-A_2 and Hb-D_2; the relative amounts of these hemoglobins were 2.0 and 1.7 % respectively. Hb-D_2 was eluted at approximately the same pH value as observed for Hb-S (Fig. 1).

Case G.S. The elution pH value for Hb-C-Harlem was found to be approximately 7.33, thus allowing a complete separation of this variant and of the minor Hb-A_2 component.

Case P. A rather incomplete separation of Hb-C-Harlem and Hb-C was observed by DEAE-Sephadex chromatography; the separation of these two components and of Hb-A_2 was complete in the CM-Sephadex chromatogram. The level of Hb-A_2 in this blood sample was 2.9 %; no trace of Hb-F was observed. The electrophoretic mobilities of the isolated components I, II and III are shown in Fig. 2.

Case S.C. This chromatogram closely resembled that of patient P; it may be that Hb-S was eluted slightly faster than Hb-C-Harlem. The separation of Hb-A_2 and the minor Hb-S component was incomplete, preventing an acceptable calculation of the relative amount of Hb-A_2. The relative mobilities of the fractions I, II and III in starch gel electrophoresis are also presented in Fig. 2. It is noteworthy that a small amount of Hb-F (1.6 %) was present in this blood sample.

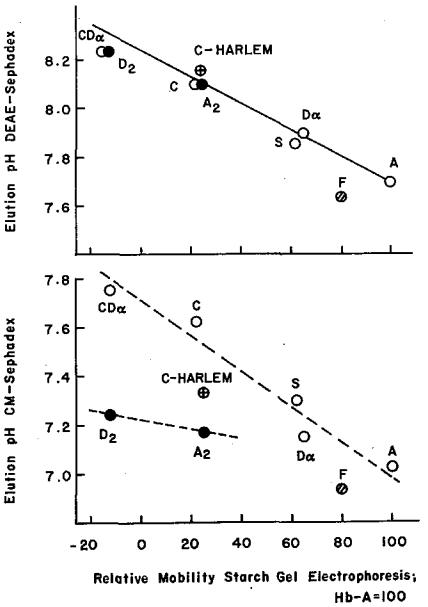

Fig. 6. The relationship between the relative mobilities of various hemoglobin variants and their elution pH values in CM-Sephadex and in DEAE-Sephadex chromatography.

In reviewing the several chromatograms it becomes evident that CM-Sephadex chromatography shows a selectivity, which is considerably greater than that observed by the DEAE-Sephadex chromatographic procedure. This is even more apparent when the relative mobilities of the various components in starch gel electrophoresis are plotted against their respective elution pH values in both types of chromatography (Fig. 6). The separation of the hemoglobin components (with the exception of Hb-F) by DEAE-Sephadex chromatography seems primarily to be based on charge differences between the molecules; additional properties of the structurally different hemoglobin components apparently influence the adsorption of the proteins to the CM-Sephadex cation exchanger to a much greater extent than is the case for the DEAE-Sephadex anion exchanger. This phenomenon can successfully be used for a complete separation of components showing (almost) identical mobilities in starch gel electrophoretic separations, such as Hb-CDα and Hb-D_2, Hb-C and Hb-C-Harlem and Hb-A_2, and perhaps Hb-S and Hb-D.

ACKNOWLEDGEMENT

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