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CHROMATOGRAPHY OF HEMOGLOBINS ON CM-CELLULOSE WITH BIS-TRIS AND SODIUM CHLORIDE DEVELOPERS

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SUMMARY

CM-Cellulose as an ion-exchange medium with Bis-tris as buffer and a gradient of sodium chloride provides a versatile system for the chromatography of hemoglobins. Changes in pH, Bis-tris concentration, and slope of the sodium chloride gradient provide means for markedly altering chromatographic behavior for special separations. Examples are given of the application of the method to normal samples and to those with hemoglobinopathies.

INTRODUCTION

Shortly after the first preparation of an ion-exchange cellulose by Sober and co-workers[1,2], CM-cellulose was applied to the chromatography of hemoglobin by Huisman *et al.*[3,4]. Later, as improved forms of CM-cellulose became available commercially, modifications have been described[5]. These procedures have separated the hemoglobins by means of a gradient of pH that was produced by phosphate buffers. In the development of microchromatographic methods for hemoglobins[6], buffers with Tris or Bis-tris and sodium chloride have been successfully applied. Indeed, with these buffers, human fetal hemoglobin (Hb-F) and adult hemoglobin (Hb-A) were well separated in contrast to their minimal separation in phosphate buffers with a pH gradient. Consequently, we have examined the behavior of human hemoglobins in this new system on more conventionally sized columns in order to ascertain its applicability to the analysis of complex mixtures.

MATERIALS AND METHODS

Blood samples

Blood from normal individuals and from those with various types of hemoglobinopathy were collected with EDTA as anticoagulant. Solutions for chromatography were prepared from blood by washing the cells three times with 0.9% NaCl, by hemolyzing with water equal to 1.5 times the packed cell volume plus 0.4 volume of carbon tetrachloride for 20 min at room temperature, by centrifuging twice to remove cellular debris, and finally by dialyzing the sample against a large volume of the appropriate developer overnight at 4°.

Solutions

Developer I is 0.03 M Bis-tris–HCl–0.03 M NaCl–0.01% KCN at pH 6.1 and contains 6.28 g Bis-tris [N,N-bis-(2-hydroxymethyl)-iminotris-(hydroxymethyl)-methane], 1.75 g NaCl, 0.1 g KCN, and HCl to pH 6.1 in 1 l.

Developer II is 0.03 M Bis-tris–HCl–0.12 M NaCl–0.01% KCN at pH 6.1 and is prepared by adding 5.25 g NaCl per liter to Developer I and adjusting the pH.

Developer III is 0.03 M Bis-tris–HCl–0.085 M NaCl–0.01% KCN at pH 6.1 and is prepared by adding 3.22 g NaCl per liter to Developer I and adjusting the pH.

Preparation of ion exchanger

A 50-g portion of CM-cellulose (CM-52, microgranular and pre-swollen; Whatman, Clifton, N.J., U.S.A.) was suspended in 300 ml of Developer I. After the ion exchanger had been settled twice in this buffer to remove fines, the pH of the stirred suspension was adjusted to pH 6.1, another settling was done, pH was checked, and finally the volumes of settled resin and supernatant fluid were adjusted to a ratio of 1:2. All chromatographic operations were done at room temperature.

Chromatographic procedure

A 20 × 1 cm column was poured from the slurry of equilibrated CM-52 and 50–100 ml of Developer I was passed through. After most of the liquid had been removed, the sample was carefully layered on the column and allowed to flow in. The tube above the column and the line through the pump to the gradient device were filled with Developer I. Development was accomplished with a linear gradient from a two-vessel system of which the mixer contained 650 ml of Developer I and the second vessel 650 ml of Developer II; this is the full gradient. Developer was passed through the column at 50 ml/h with a peristaltic pump. Fraction size was 5 ml. Absorbance was read at 415 nm for all fractions and at 280 nm in some instances. Conductance was determined on every tenth fraction. Because the conductance is a linear function of NaCl concentration, the NaCl concentration at any fraction of the chromatogram can be calculated easily from the measured conductance of the two solutions of a gradient.

If Hb-C is not present in the sample, the gradient may be decreased; 375 ml each of Developers I and III provide a shortened gradient of the same slope as the full gradient.

Other procedures

DEAE-Sephadex chromatography[7,8], starch gel electrophoresis[9], and amino acid analysis by Beckman amino acid analyzer followed published procedures.

RESULTS AND DISCUSSION

Figs. 1–5 depict the separations that may be obtained from a variety of normal and abnormal hematological conditions. The identity of the components was established in one or more of four ways: starch gel electrophoresis, comparison with DEAE-Sephadex chromatography or with microchromatography, and/or amino acid analysis.

Cord bloods with common abnormal hemoglobins

The data in Fig. 1A derive from the cord blood of a newborn infant with sickle cell trait. The separation of Hb-F, Hb-A, and Hb-S is excellent in this system and Hb-F_1 separates from Hb-F_0 as it does in most chromatographic systems. The newborn child with Hb-C trait has the pattern in Fig. 1B. The conditions of chromatography were chosen to provide a relatively rapid movement of hemoglobins on the column. If an electrophoretically fast moving hemoglobin at alkaline pH were present, it would be virtually unadsorbed under these conditions. However, as will be discussed below, a pattern such as that in Fig. 1A can be translated along the volume axis by changing the NaCl gradient at constant pH and Bis-tris and KCN molarity and thus the rate of movement of more rapidly moving components can be retarded.
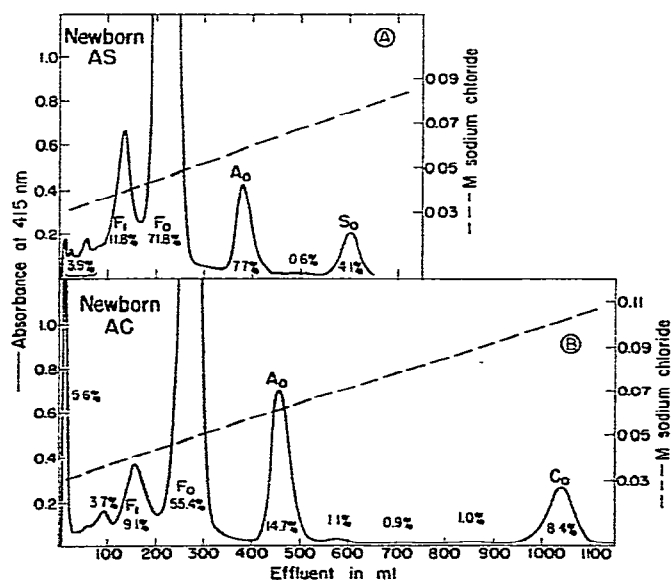

Fig. 1. Separation of hemoglobins in cord blood from an infant with sickle cell trait (A) and an infant with Hb-C trait (B). The shortened and full gradients respectively were used.

Adult samples with common abnormal hemoglobins

The hemoglobins of normal adults and of adults with combinations of hemoglobins A, S, and C yield chromatograms that are depicted in Figs. 2 and 3. In addition to Hb-A_2, a number of other minor components separate from the major Hb-A_0 of the normal individual (Fig. 2A). The very rapidly moving zones may contain some enzymes of the red cell[4] as well as the pyridoxal complex[10]. Traces of Hb-F_0 and minor components of Hb-A are not separable. Because no detailed study has been made of minor components and because they (with the exception of A_2 and F_1) have not been correlated with minor components as detailed by other chromatographic methods or by electrophoresis, they are labelled as A_x, A_y, etc. in the figures.
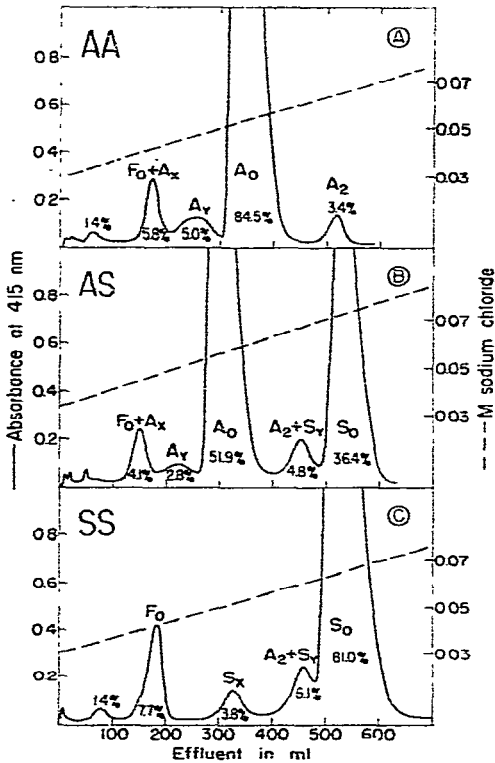

Fig. 2. Separation of hemoglobins of a normal adult (A) and of adults with sickle cell trait (B) and sickle cell anemia (C) by the shortened gradient.

When Hb-S is present, the separations in Figs. 2B (sickle cell trait) and 2C (sickle cell anemia) are obtained. A minor component(s) related to Hb-S (S_y) overlaps Hb-A_2 although in some chromatograms there may be partial separation (Fig. 4B). Hb-A_2, therefore, cannot be quantitatively determined in the presence of Hb-S by this method.

When Hb-C is present and the full gradient is used, Hb-C emerges, as shown in Figs. 1B, 3A, and 3B, before the end of the gradient. The separation of Hb-A_2 from
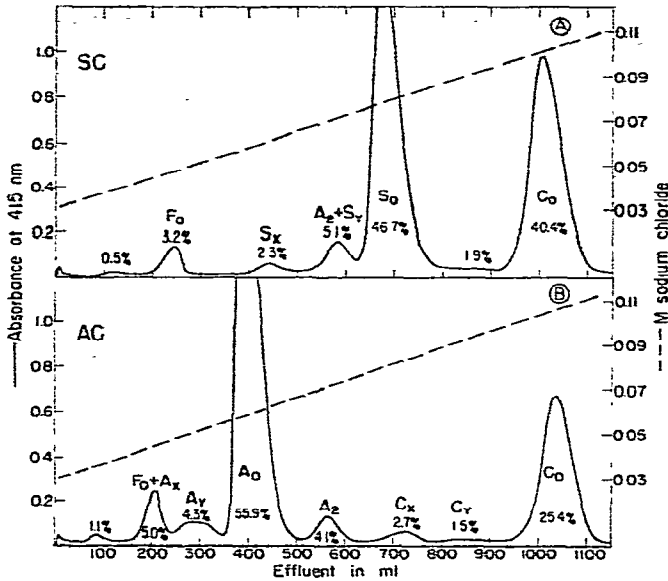
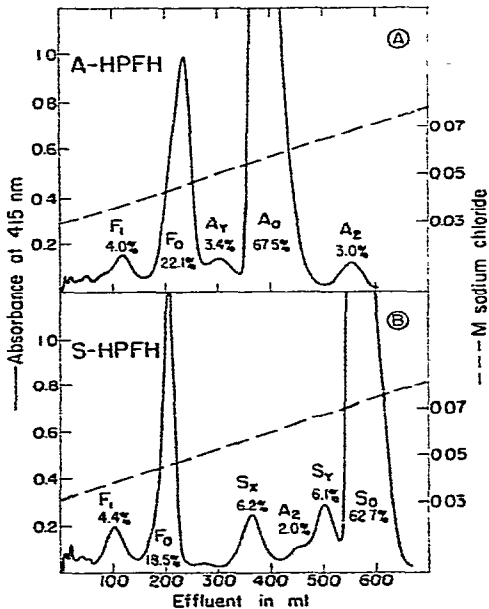Fig. 3. Separation of hemoglobins of adults with SC disease (A) and Hb-C trait (B) by the full gradient

Fig. 4. Separation of hemoglobins in a heterozygote for the hereditary persistence of fetal hemoglobin (HPFH) (A) and in an HPFH individual with Hb-S (B) by the shortened gradient.

Hb-C is excellent and permits the quantitative determination of Hb-A_2 in the presence of Hb-C. Previous chromatographic procedures for this determination required about four days[5] in contrast to about a day by the present method.

Although the reproducibility of the procedure is good, slight changes in pH
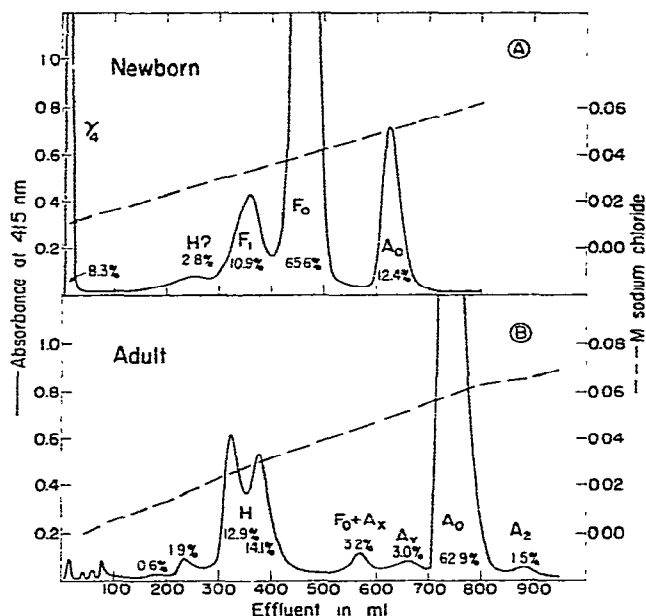
Fig. 5. Separation of hemoglobins in a newborn infant with Hb-Bart's (A) and in the father who has Hb-H disease (B) with a modified gradient (see text).

or NaCl concentration are responsible for the slightly different effluent volumes at which a given peak emerges. The NaCl molarity at the volume of emergence of the peak of any hemoglobin has been as follows: Hb-F_0, 0.039–0.050; Hb-A_0, 0.050–0.063; Hb-A_2, 0.060–0.074; Hb-S_0, 0.064–0.079; and Hb-C_0, 0.093–0.106. From these data as well as from a comparison of various chromatograms in Figs. 2 and 3, it is apparent that Hb-A_0 in an AS sample will be contaminated with a minor component of Hb-S (note the position of S_x in Fig. 2C and Fig. 3A as compared to Hb-A_0 in Fig. 2B). On electrophoresis on starch gel at pH 9, S_x does not behave like A_0 but is heterogeneous and moves like S_0 and F.

Other hemoglobinopathies and abnormal hemoglobins

The adult heterozygote for the hereditary persistence of fetal hemoglobin (HPFH) will have 15–30% Hb-F. The increased Hb-F of such a heterozygote is apparent in the chromatogram of Fig. 4A. When HPFH coexists with Hb-S, the chromatogram of Fig. 4B is obtained.

If Hb-S coexists with β-thalassemia, Hb-A is absent in the type termed S-β°-thal and present in S-β^+-thal. The chromatogram of the former would have much the appearance of Fig. 4B except for a lower percentage of Hb-F. However, in S-β^+-thal, the S_x peak would contain Hb-A_0 and be present to the extent of 15–25%, and Hb-F would vary from case to case.

When other hemoglobins were chromatographed (not depicted), it was found that an Hb-D (possibly $D_{Los\ Angeles}$) moved more rapidly than Hb-S in a distinct peak although incompletely separated from Hb-S. Hb-Lepore (probably Lepore$_{Washington}$) has the mobility of Hb-A_2 as does Hb-E.

Figs. 5A and 5B present data from a Thailander in whom Hb-H(β_4) was detected by starch gel electrophoresis, and from his newborn child in whose hemoglobin Hb-Bart's (γ_4) was present. The mother had no electrophoretically abnormal hemoglobin. Because of the presence of these electrophoretically fast moving hemoglobins, the gradients were modified. For the chromatogram in Fig. 5A, the NaCl concentration in the two buffers was 0.01 M and 0.08 M in 0.03 M Bis-tris and 0.01 % KCN at pH 6.1, the total gradient was 1000 ml, and the column had been equilibrated with 0.01 M NaCl. For that in Fig. 5B, the column was equilibrated with 0.01 M Bis-tris–no NaCl–0.01 % KCN; 25 ml of this solvent was used for initial development with a subsequent 1000-ml gradient between no NaCl and 0.08 M NaCl in 0.03 M Bis-tris and 0.01 % KCN at pH 6.1. The movement of hemoglobins is retarded under these conditions but the NaCl molarity at which a given peak emerges is within the range that is observed with the other gradient. The virtually unabsorbed peak in the chromatogram of the newborn (Fig. 5A) is Hb-Bart's and there is little or no Hb-H. Hb-H formed two peaks (Fig. 5B) which were readily identified by amino acid analysis. Because of the lability of Hb-H, different methods of preparing hemolysates gave varying ratios of the two peaks of Hb-H in other experiments.

Technical considerations

Bis-tris has been chosen as a buffer for these procedures because its pK_a is 6.5 and therefore the buffer capacity at the pH of choice is good. A few chromatograms in this study used only 0.01 M Bis-tris with a linear gradient of 1300 ml total volume and NaCl molarities of 0.03 and 0.12 at pH 6.1 in the gradient vessels. In this system, reproducibility of point of emergence was somewhat variable probably because of poorer pH control at this concentration of Bis-tris. Consequently, 0.03 M Bis-tris has been used and the reproducibility has improved.

A slower flow-rate or a longer column does not improve separations. Most of the chromatograms in the figures used a flow-rate of 25 ml/h, but in more recent work, the flow-rate has been 50 ml/h. Consequently, the chromatogram is complete in about a day. In fact, the flow-rate may be increased to 75 ml/h without significant deterioration in the separations. Visual observations of the movement of the hemoglobins on the column suggest that most of the separation occurs in the upper 10 cm, and that each hemoglobin washes virtually unretarded through the lower half of the column. The columns have been re-equilibrated as many as five times and used again successfully.

The developing conditions with the linear gradient of Developers I and II provide a good and rapid separation of the normal hemoglobins and common variants. However, modification of the NaCl gradient or Bis-tris concentration provides a versatility that can be used with less common mixtures of hemoglobins. As an example, Fig. 5A may be compared with Fig. 2A. The slope of the gradient was identical but the start at 0.01 M NaCl instead of 0.03 M NaCl retarded Hb-A$_0$ about 275 ml of effluent. Similar retardation occurs if the gradient is started at 0.03 M NaCl but the Bis-tris is 0.01 M instead of 0.03 M. In summary, an increase in pH, NaCl or Bis-tris concentration, or in slope of the NaCl gradient speeds the movement of any hemoglobin and vice versa. With some experience, these variables may be adjusted to provide correct conditions for special separations.

If the Bis-tris–NaCl system is replaced by a gradient of 0.02–0.08 M phosphate

at pH 6.1 or a gradient of 0.0 M-0.1 M NaCl in 0.02 M phosphate at pH 6.1, the separations are similar but less satisfactory.

ACKNOWLEDGEMENTS

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