STUDIES ON THE HETEROGENEITY OF HEMOGLOBIN

XIII. CHROMATOGRAPHY OF VARIOUS HUMAN AND ANIMAL HEMOGLOBIN TYPES ON DEAE-SEPHADEX

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In previous papers of this series[1,2] we described the use of the anion exchangers DEAE-Cellulose and DEAE-Sephadex for the chromatographic isolation of different hemoglobin components from red blood cell hemolysates of various origins. Considerable additional experience has been gained with these techniques, while a marked improvement in the resolution of various hemoglobin fractions was noted when a beaded form of DEAE-Sephadex was used as chromatographic medium. A summary of our most recent chromatographic data is the subject of this communication.

MATERIALS AND METHODS

Table I lists the various blood samples that were available. The human samples included those from carriers of β-chain abnormal hemoglobins (S, C), of α-chain abnormal hemoglobins (Dα, Gα, Bibba) and of δ-chain abnormal hemoglobins (B_2, Flatbush); from carriers heterozygous for two hemoglobin abnormalities (S and B_2; S and Gα); and cord blood samples with various abnormal fetal hemoglobin types. The animal blood samples were obtained from different sources and included several variants observed in cattle (A, B, C, D), in sheep (A, B, C, F) and in goats (A, Bα, D and F). Red cell hemolysates were prepared by lysis of the washed red blood cells (three times with 0.85 % NaCl solution) with an equal volume of water and 0.4 volume of carbon tetrachloride. The hemoglobin solutions were cleared from debris by centrifugation for 20 min at 10.000 r.p.m. at 4°. All hemolysates were studied by starch gel electrophoresis[3] prior to chromatography.

Chromatographic analyses were carried out exclusively with a beaded form of anion exchanger: DEAE-Sephadex A-50, capacity 3.5 $\pm$ 0.5 mequiv./g, particle size 40–120 μ (Pharmacia Fine Chemicals, Inc.). Several lot numbers were tested with identical results. The dry material was suspended in a large volume of 0.05 *M* tris-(hydroxymethyl)aminomethane (TRIS)–HCl buffer, pH 8.1 to 8.5, and stirred continuously for several hours. The pH of the suspension was adjusted with 0.05 *M* TRIS solution to 8.5, whereafter the suspension was stored at room temperature. Columns of 65 $\times$ 1.0 cm were used for analytical purposes; the resin height varied from 50 to 53 cm. In many analyses the columns were reequilibrated with an 0.05 *M* TRIS–HCl buffer of selected pH value (7.9 to 8.4) for several hours, although such

TABLE I

SYLLABUS OF THE VARIOUS BLOOD SAMPLES USED IN THIS STUDY

Human blood samples	*n*[a]	*Hemoglobin types*[b]	*Ref.*	*Animal blood samples*	*n*[a]	*Hemoglobin types*[b]	*Ref.*
AA	10	A_2; A_0; A_1		Bovine AA	5	A	
AAA_2B_2	15	$B_2\delta$; A_2; A_0; A_1	4	Bovine AB	5	A; Bβ	10
AS	38	A_2; Sβ; A_0; A_1		Bovine AC	2	A; Cβ	10
ASA_2B_2	6	$B_2\delta$; A_2; Sβ; A_0; A_1	4	Bovine CC	2	Cβ	10
SS	4	A_2; $S_0\beta$; $S_1\beta$; (F)		Bovine DB	2	Dβ; Bβ	10
AAA_2Flatbush	3	A_2; Flatbush-δ; A_0; A_1	5	Sheep AA	4	A	11
AC	6	A_2 + Cβ; A_0; A_1		Sheep BB	4	Bβ	11
CC	2	A_2 + $C_0\beta$; $C_1\beta$		Sheep AB	10	Bβ; A	11
ADα	2	$D_2\alpha$; A_2; Dα; A_0; A_1	6	Anemic sheep AA	30	Cβ; A	11
AGα	1	$G_2\alpha$; A_2; Gα; A_0; A_1	7	Anemic sheep AB	10	Cβ; Bβ; A	11
GαS	3	$G_2\alpha$; A_2 + GαSβ; Gα + Sβ; A_0; A_1	7	Lamb AB	30	Cβ; Bβ; F; A	11
A Bibba	5	$Bibba_2$; A_2; A-Bibba; A_0; A_1	8	Goat AA	10	A	12
Cord blood	26	(A_2); F; A		Goat AB	20	Bα; A	12
Cord blood + Bart's	2	(A_2); F; A; Bart's; (H)	9	Goat AD	4	Dβ; A	7
Cord blood + Xγ	3	(A_2); Xγ; F; A	7	Kid AB	20	Bα; A; F-b; F-a	12
Cord blood + Yγ	3	Yγ; (A_2); F; A	7				
Cord blood + FGα	2	($G_2\alpha$); (A_2); FGα; Gα; F; A	7				

[a] Number of chromatographic analyses; in many instances one hemoglobin sample was repeatedly analyzed.

[b] As observed by starch gel electrophoresis[3]; the various hemoglobin components are listed in order of increasing anodic mobility. The Greek letters β, γ, δ and α are used to indicate if the structural variation of a specific type is located in the β chain (abnormal Hb-A or $\alpha_2\beta_2$), in the γ chain (abnormal Hb-F or $\alpha_2\gamma_2$), in the δ chain (abnormal Hb-A_2 or $\alpha_2\delta_2$) or in the α chain (abnormal Hb-A_2, Hb-A or Hb-F).

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reequilibration was not a necessity. Variable amounts (30–80 mg, 40 mg being the most favorable quantity) of oxyhemoglobin were chromatographed. When the pH of the column effluent was 8.35 or higher it was found unnecessary to dialyze the hemolysate against a selected TRIS–HCl buffer, although in most instances the use of dialyzed hemolysates was preferred. The chromatograms were developed at room temperature by applying a pH gradient to the column in the same manner as described before[2]; the flow rates were kept at 14 to 16 ml per h. The effluent fractions were analyzed for hemoglobin concentration by measuring the optical densities at 415 mμ, while the development of the chromatogram was also followed by measuring the pH of several effluent fractions using Radiometer pH meters model 4. All buffers contained 100 mg KCN per 1000 ml.

RESULTS AND DISCUSSION

Since the purpose of this communication is to demonstrate the usefulness of the chromatographic technique for the separation of the several major and minor hemoglobin components found in red cell hemolysates of human and animal origin, several graphs (Figs. 1 to 4) have been constructed, in which the chromatographic results are compared with those obtained by the starch gel electrophoretic procedure. As was stated before[2], the elution of a certain hemoglobin component in this type of chromatography was found to be determined exclusively by the pH of the developing gradient. The accuracy of the measurement of the effluent pH values depends on many factors, such as temperature, the pH meter, operator, and others. It is, therefore, not surprising that relatively large ranges of effluent pH values were observed when several chromatograms were compared. Such a comparison is presented in Fig. 1, in which the solid curves summarize data of chromatograms selected at random (the numbers of analyses were: 21 (B_2), 82 (A_2), 42 (S_0), 4 (S_1), 79 (A_0), 63 (A_1)), while the hatched curves were composed from data of chromatograms developed under more consistent conditions, such as constant temperature, one operator, and one type of pH meter. In most instances the separation of the various hemoglobin fractions was complete, while moreover the differences in the elution pH values were constant.

In Fig. 2 a comparison is made between the effluent pH values of several α, β, or δ chain abnormal human hemoglobin components and their relative mobilities in starch gel electrophoresis, while in Fig. 3 a similar comparison is given for normal and abnormal human fetal hemoglobin types. The direct relationship between the elution pH values and the electrophoretic mobilities of these components, already discussed previously (Fig. 6 of a previous communication[2]), was again confirmed as was the observation that the elution of the γ chain containing hemoglobin types (F_V or $\alpha_2\gamma_2^V$, FGα or $\alpha_2^G\gamma_2$, F_X or $\alpha_2\gamma_2^X$, F_0 or $\alpha_2\gamma_2$, and F_1 or $\alpha_2\gamma\gamma^{acetyl}$) required a lower pH of the developing buffer system than expected from their relative electrophoretic mobilities. With only a few exceptions, such as Hb-A_2 and Hb-GS (or $\alpha_2^G\beta_2^S$), Hb-A_2 and Hb-C, Hb-A_0 and Hb-S_1, Hb-F_0 and Hb-A_1, a complete separation of the various hemoglobin types in the various red cell hemolysates could be obtained.

Fig. 4 summarizes similar data for the different hemoglobin types in cattle, sheep, and goats. Separation of the adult bovine hemoglobin types A, B, C, D was

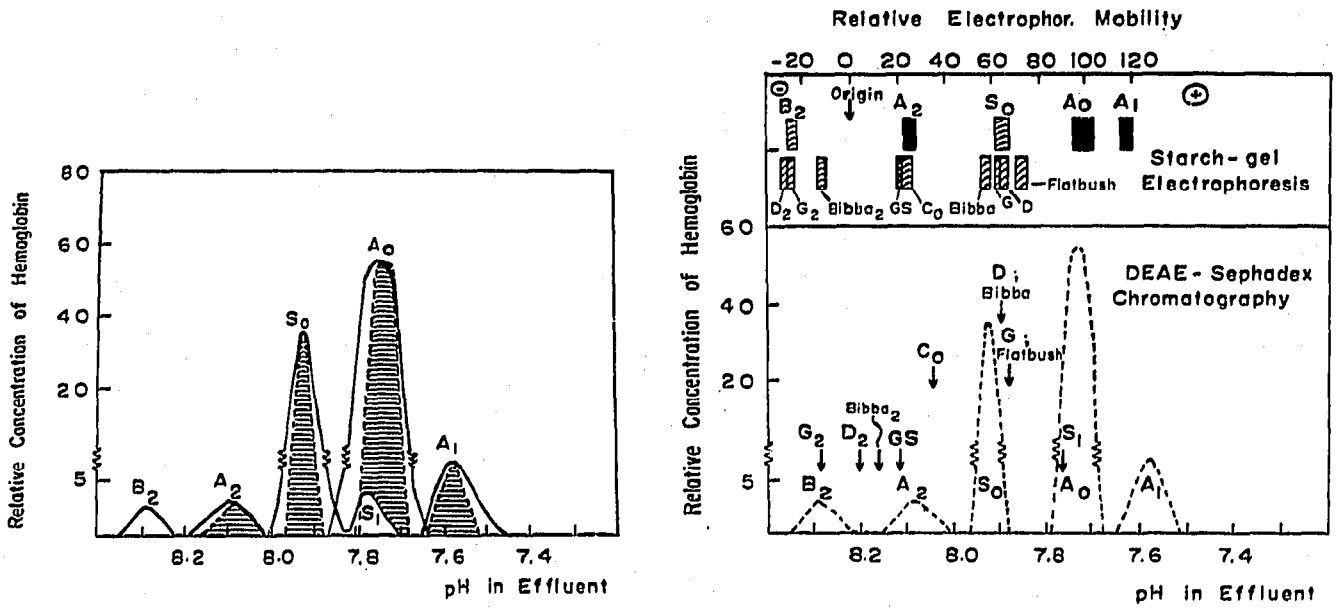

Fig. 1. Comparison of the elution pH values of six human hemoglobin components observed in a large number of chromatograms, selected at random (solid curves), and observed in a series of chromatograms, developed under optimal conditions (hatched curves).

Fig. 2. Comparison of the elution pH values of several human adult hemoglobin types, observed in DEAE-Sephadex chromatography, and of their relative mobilities in starch gel electrophoresis.

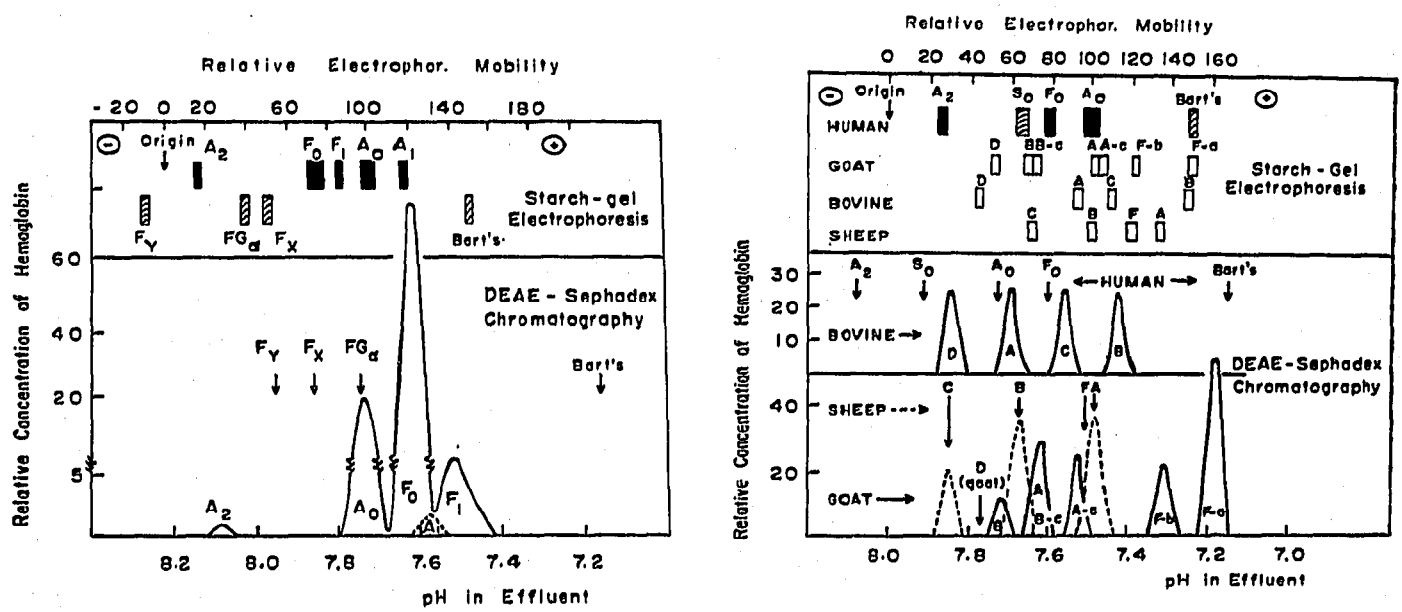

Fig. 3. Comparison of the elution pH values of six human fetal hemoglobin types, observed in DEAE-Sephadex chromatography, and of their relative mobilities in starch gel electrophoresis.

Fig. 4. Comparison of the elution pH values of several bovine, sheep and goat hemoglobin types, observed in DEAE-Sephadex chromatography, and of their relative mobilities in starch gel electrophoresis.

complete as were those of sheep hemoglobins C, B and A, and goat hemoglobins D (or $\alpha_2\beta_2^D$), B (or $\alpha_2^B\beta_2^A$), and A. The separation of sheep Hb-A from sheep Hb-F was not possible. The DEAE-Sephadex chromatographic technique proved to be particularly useful in separating the β-C containing hemoglobin types, which are produced in the goat during severe blood loss anemia[12]. These hemoglobin types, B-c or $\alpha_2^B\beta_2^C$, A-c or $\alpha_2^A\beta_2^C$, showed electrophoretic mobilities only slightly different from those of Hb-B or $\alpha_2^B\beta_2^A$ and Hb-A or $\alpha_2^A\beta_2^A$; despite these minor electrophoretic differences complete separations of $\alpha_2^B\beta_2^A$ (Hb-B) and $\alpha_2^B\beta_2^C$ (Hb-B-c), and of $\alpha_2^A\beta_2^A$ (Hb-A) and $\alpha_2^A\beta_2^C$ (Hb-A-c) were observed. The two forms of fetal hemoglobin, F-b or $\alpha_2^B\gamma_2$ and F-a or $\alpha_2^A\gamma_2$ observed in a newborn AB goat, were eluted as separate bands at the low pH values of 7.30 and 7.19 respectively.

The comparisons presented in Figs. 2, 3 and 4 may serve as a guide for the selection of the proper pH gradient to be applied to DEAE-Sephadex chromatography of these and other hemoglobin components. It should be noted that the technique can easily be modified to a preparative scale; as much as 2 to 3 g of hemoglobin can be successfully chromatographed on 50 × 3 cm columns using a stepwise pH elution.

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SUMMARY

A modification of the anion exchange chromatography of various hemoglobin types using DEAE-Sephadex is presented. It has been observed that the replacement of the DEAE-Sephadex A-50, 100 to 270 mesh, by a similar preparation, but particle size 40 to 120 μ, greatly improved the resolution of several human and animal hemoglobin types.

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