STUDIES ON THE HETEROGENEITY OF HEMOGLOBIN

IV. CHROMATOGRAPHIC BEHAVIOR OF DIFFERENT HUMAN HEMOGLOBINS ON ANION-EXCHANGE CELLULOSE (DEAE-CELLULOSE)

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Various authors have demonstrated that human normal adult hemoglobin, fetal hemoglobin and abnormal hemoglobin types are heterogeneous. The evidence for the existence of these inhomogeneities is based on results obtained in electrophoretic studies, in solubility and alkali denaturation experiments and in column chromatographic investigations. The results of older investigations are diverse and sometimes irreconcilable (a summary on the heterogeneity of Hb-A is presented in ref.[1]). In recent studies[1-4], however, a close relationship between the heterogeneities demonstrable by starch electrophoresis and by column chromatographic procedures (Amberlite IRC-50 and carboxymethylcellulose (CMC)) was found. For human normal adult hemoglobin the following fractions appear to be present in addition to the main hemoglobin fraction (A_0) and the distinct minor component A_2: a fast moving fraction (A_1) present for 10 to 15%, small amounts of fetal hemoglobin (F) and a non-hemoglobin fraction designated as V_1 (CMC-chromatography). Similar heterogeneities were demonstrated for cord-blood hemoglobin, sickle cell hemoglobin and other hemoglobin abnormalities.

Column chromatographic fractionation of human hemoglobin fractions and also of animal hemoglobins[5] has been performed with the use of weak cation exchangers as for instance, Amberlite IRC-50 and carboxymethylcellulose. The cellulose anion exchangers, such as diethylaminoethyl (DEAE) cellulose, triethylaminoethyl (TEAE) cellulose and ECTEOLA-cellulose[6-11] have proved to be useful chromatographic media for many proteins, particularly serum proteins. In the fractionation of serum proteins on DEAE-cellulose, application of buffer gradients of increasing concentration and decreasing pH results in a separation of many components appearing in the effluent in order of increasing electrophoretic mobility. The present communication presents the results of a detailed study of the behavior of human normal adult hemoglobin, cord blood hemoglobin and electrophoretically abnormal hemoglobins on columns of DEAE-cellulose. A successful separation of different hemoglobin fractions was achieved by application of a gradient principle similar to that used in serum

protein fractionation. In addition, the heterogeneity observed with the use of this new chromatographic procedure was compared with that found in CMC-chromatography and in electrophoresis (starch- and starch-gel-electrophoresis).

MATERIALS AND METHODS

A large quantity of DEAE-cellulose (Selectacel-DEAE, Type 40*) was carefully equilibrated by repeated washings with 0.005 *M* sodium phosphate buffer pH 8.6 containing 100 mg KCN/l. The slurry of the freshly equilibrated anion exchanger was diluted with an equal volume of deionized water and then poured into columns (0.9 × 60 cm), and packed under atmospheric pressure until a final height of 45 cm was obtained. Prior to chromatography, hemoglobin solutions were dialyzed for 24 h against 1 l of the buffer used for the equilibration diluted one half. Twenty to 90 mg of oxyhemoglobin dissolved in 1 to 5 ml of buffer were chromatographed.

Chromatographic fractionation was carried out at 10°. Flow rates of 8 to 12 ml/h were used with 2 to 3 ml fractions. The following elution systems were applied:

(a) A gradient of NaCl with little change in pH was obtained with the use of 0.005–0.009 *M* sodium phosphate buffers of a pH ranging from 8.6 to 6.0 and a variable gradient device ("varigrad"), which was also used in earlier studies[3]. This apparatus consists of ten cylindrical chambers of 150 ml, each filled with 120 ml of the phosphate buffer of the pH and the sodium chloride concentration indicated in Fig. 1. Since ferrihemoglobin cyanide and oxyhemoglobin showed no difference in chromatographic behavior, small amounts of potassium cyanide (100 mg/1000 ml) were added to all buffers to prevent the formation of extraneous zones of methemoglobin on the columns.

(b) Similar gradients were obtained by introducing a 0.009 *M* sodium phosphate buffer pH 8.1 (to which 0.04 *M* NaCl was added) from a supply bottle into a constant volume (50 ml) mixing chamber containing the 0.005 *M* sodium phosphate buffer pH 8.6. After 60–80 ml of effluent was collected, the buffer in the supply bottle was replaced by a 0.009 *M* sodium phosphate buffer pH 7.1 (0.15 *M* NaCl added). This buffer was finally replaced after a total collection of about 150 ml by sodium phosphate buffer pH 6.0 to which 0.30 *M* NaCl was added. In some instances the gradient elution was finished after the elution of a desired hemoglobin fraction, the remaining hemoglobin being recovered by a direct introduction of the 0.009 *M* sodium phosphate buffer pH 6.0 (0.30 *M* NaCl added) to the column.

(c) Small columns (15 × 0.9 cm) can be used for special purposes, particularly the determination of the minor hemoglobin component A_2. Smaller amounts of hemoglobin (10 to 25 mg) are analyzed in this type of chromatography, which is described in detail in a separate paper[12].

Fractions were diluted with deionized water to 4 ml and routinely examined at 415 mμ in the Beckman spectrophotometer. Occasionally, U.V. absorption at 280 mμ

* Brown Company, 550 Main Street, Berlin, New Hampshire.

was measured. Volume, pH values, and sodium concentration were determined on the contents of individual tubes. Calculation of the percentage amounts of the hemoglobin components, carried out in a similar way to that used in CMC-chromatography[3], was possible since more than 98% of the total hemoglobin added to the column was recovered.

The presence of fetal hemoglobin was determined on aliquots from pooled fractions by using U.V. spectral absorption measurements as well as the alkali denaturation procedure at 415 mμ specially developed for dilute hemoglobin solutions, and described in detail earlier[3]. Concentration of the dilute fractions was performed with the use of small CMC columns following the procedure mentioned in an earlier paper[3]. Occasionally short DEAE columns (3 × 0.9 cm) instead of CMC were used, particularly when rechromatography of isolated fractions on DEAE-cellulose was desired. In these experiments the pH of the diluted Hb-solution was adjusted to 8.6–9.0 and the sodium concentration was decreased below 0.005 *M* by addition of deionized water. The hemoglobin adsorbed on top of these small columns was either eluted with a 0.009 *M* sodium phosphate buffer pH 6.0 (0.30 *M* NaCl added) or was transferred, while still adsorbed onto the anion exchanger, to a 45 × 0.9 cm column of DEAE for rechromatography. In some instances larger quantities of a certain hemoglobin were prepared by using larger columns (35 × 2.0 cm) and specific elution gradients. The system used depends upon the component desired; an example will be presented later in this paper.

The original hemoglobin samples as well as the isolated hemoglobin fractions were also studied by CMC-chromatography according to the procedures mentioned earlier[3,4]. Paper electrophoresis at pH 8.6, starch electrophoresis according to GERALD AND DIAMOND[13] and a starch gel electrophoretic technique[14] adapted to hemoglobin studies[15] also were used. The amount of alkali-resistant hemoglobin present in the original hemoglobin samples was determined by the spectrophotometric procedure of JONXIS AND VISSER[16].

A procedure described earlier[17] was used for hybridization experiments. The dissociated units were recombined at pH 8.0 and the mixture was finally dialyzed against the first buffer solution of the gradient system used in the DEAE-chromatography. The separation of the hemoglobin components present in this mixture was carried out with the automatic gradient system.

Blood samples obtained from the following sources were used:

(a) Normal laboratory workers. (b) Heterozygous Hb-S and heterozygous Hb-C carriers and homozygous Hb-S carriers admitted to the Eugene Talmadge Memorial Hospital of the Medical College of Georgia. (c) Two heterozygous Hb-J (Georgia) carriers belonging to a family described in detail elsewhere[18]. (d) Cord blood samples from Negro and white babies delivered in the Department of Obstetrics and Gynecology, Medical College of Georgia. (e) Two patients homozygous for thalassemia and four patients with the Hb-E-thalassemia disease, the samples being kindly supplied by Dr. C. C. DE SILVA, Ceylon, and by Dr. K. PUNT, The Netherlands. (f) Carriers heterozygous for the abnormal persistence of fetal hemoglobin and one individual heterozy-

gous for this persistent Hb-F abnormality as well as for Hb-S. The hematologic and genetic details obtained in studying these cases will be reported separately[19]. (g) Heterozygous thalassemia carriers. These samples were obtained from patients in local hospitals or were sent to us by Dr. K. PUNT, The Netherlands. (h) Hb-B_2 heterozygotes. Samples from family members described in a previous paper[20]. (i) Patients heterozygous for the Hb-B_2 abnormality as well as for thalassemia. A detailed report on the family study will appear separately. (j) Members of a family with the abnormal Hb-lepore[22]; these samples were made available through the kind cooperation of Dr. V. P. SYDENSTRICKER (Veterans Administration Hospital, Augusta, Ga.) (k) Two cases with an unknown minor Hb-abnormality; these samples were provided by Dr. K. BETKE (Germany).

RESULTS

A. The behavior of normal adult hemoglobin in DEAE-chromatography

In Fig. 1 is shown the elution diagram obtained when the hemoglobin of an Hb-B_2 heterozygous carrier was chromatographed with the use of the automatic gradient

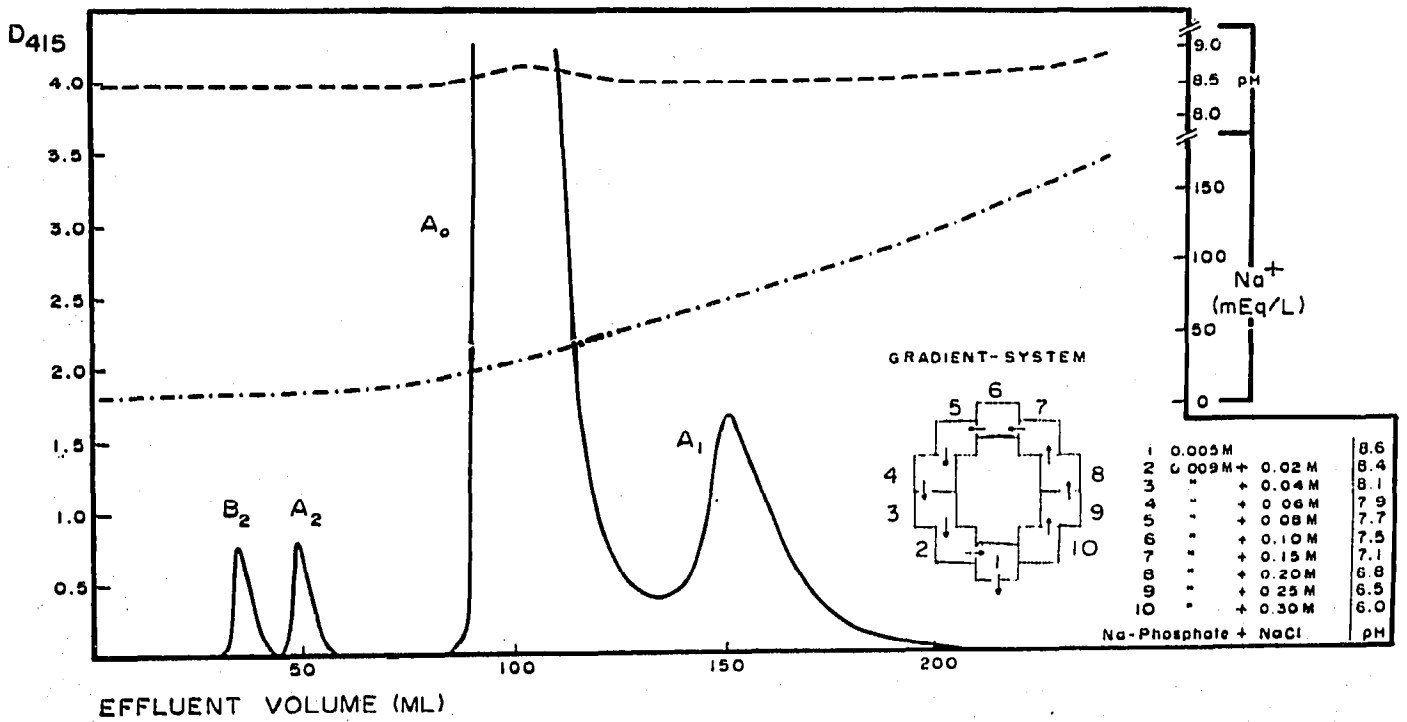

Fig. 1. The behavior of the hemoglobin of a Hb-B_2 trait carrier in DEAE-cellulose chromatography. – – – pH values of the effluent; –·–·– Na^+ concentrations of the effluent.

device. The main component (A_0) was eluted at about 100 ml, while a slower moving fraction (A_1) was recovered at about 150 ml. In front of the main fraction two minor hemoglobin fractions were eluted; the first component had an electrophoretic mobility of the abnormal minor component B_2 and the second was identical with the normal minor Hb-A_2. Proof for identity of fractions obtained by DEAE-chromatography with fractions separated by CMC-chromatography or by electrophoretic techniques

will be presented in a later section of this communication. It seems, therefore, that the order of elution in this type of anion-exchange chromatography (B_2–A_2–A_0–A_1) is opposite to that found with the use of the cation exchanger CM-cellulose (A_1–A_0–A_2–B_2), which is as expected. From the curves representing the pH values and the sodium concentrations in the effluent, it is seen that under our experimental conditions—*i.e.* the use of phosphate buffers of low ionic strength and decreasing pH and increasing sodium chloride concentrations—the pH of the effluent has remained fairly constant, while a large rise in sodium concentration is present. It seems, therefore, that the gradual increase in ionic strength is the best way to decrease the affinity of the anion exchange adsorbent for the hemoglobin fractions. Early experiments had indicated that a decrease in the pH of the buffer system without addition of salt or a rise in molarity of the phosphate buffers when lower concentrations of salt were added was less effective although each of these procedures resulted in some degree of resolution.

The usual amount of hemoglobin added to the column varied from 30 to 60 mg but was decreased to 15 mg and increased to 95 mg without loss in good resolving power. As shown in Fig. 2 (II) the effluent volumes for Hb-A_2 (mean value 48 ml), for

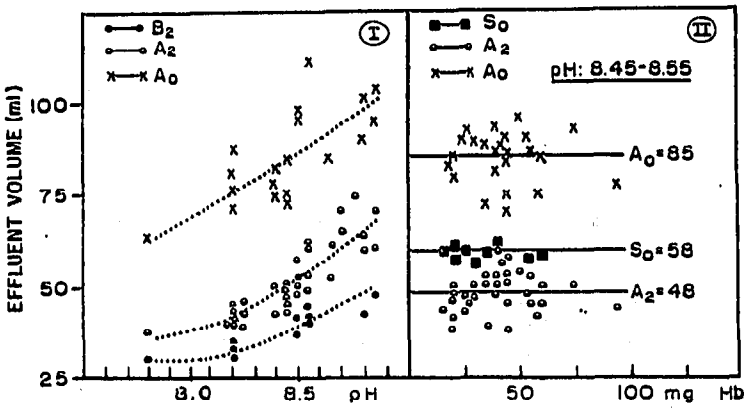

Fig. 2. The relation between the effluent volumes of some hemoglobin types and the initial pH of the DEAE-cellulose (I) and the total amount of Hb chromatographed (II). Column 45 × 0.9 cm; automatic gradient system.

Hb-S (mean value 58 ml) and for Hb-A_0 (mean value 85 ml) were not greatly influenced by differences in column charges. The values presented in this figure were obtained using the automatic gradient device, while the pH of the anion-exchange adsorbent varied between 8.45 and 8.55. Differences in the initial pH values of the DEAE-cellulose had a greater influence on the effluent volumes of the Hb-fractions. Some experiments in which the adsorbent was equilibrated with phosphate buffers of pH values ranging from 7.8 to 8.85 have shown a more or less linear relation between these pH values and the effluent volume Fig. 2 (I). At the low pH values of 7.8 to 8.2, the Hb-B_2 fraction, which is eluted first, is not adsorbed at all; its effluent volume was found almost identical with the column volume of about 26 ml. With a fixed equilibration pH value of 8.5 to 8.6 and the elution schedule presented in Fig. 1, the reproducibility of the positions and the magnitudes of the different

hemoglobin peaks were excellent even when hemoglobin samples from different donors were chromatographed.

Since the recovery of hemoglobin from the columns averaged 98 %, quantitation of the different fractions is possible. The mean percentages found for 6 samples of normal human Hb-A are listed in Table I. The amount of Hb-A_2, for instance,

TABLE I

THE PERCENTAGES OF DIFFERENT Hb-FRACTIONS IN NORMAL INDIVIDUALS AND IN PATIENTS WITH SOME HEMOGLOBINOPATHIES AS ESTIMATED BY DEAE-CHROMATOGRAPHY

Case	*n*	*A_2*	*A_0*	*A_1**	*Abnormal Hb*	*A_2 (CMC)*	*A_2 (Starch)*	*F (Alk. den.)*
Normal	6	2.3 (1.9–2.4)	84.6 (79.4–88.2)	13.1 (9.2–18.6)		2.1 (1.8–2.6)	2.0 (1.5–2.3)	< 2
Hb-S-trait	2	2.2–2.4	56.9–55.3	8.9–7.8	Hb-S: 32.0–34.5	—	—	< 2
Hb-C-trait	2	?	52.1–53.6	10.9–8.9	Hb-C: ** 37.0–37.5	—	—	< 2
Hb-J-trait	2	2.0–2.2	47.4–45.0		Hb-J: 50.6–52.8	2.2–2.4	—	< 2
Hom. Hb-S	20	2.45 (1.7–3.0)	0	0	Hb-S_0: 81.25 (65.5–93.2)	Hb-S_1: 10.1 (4.0–16.8)	Hb-F_s***: 6.2 (0–15.5)	9.8 (2–18
Case H.C.		4.6	0	0	Hb-S_0: 69.0	Hb-S_1: 21.0	Hb-F_s***: 5.4	8.0

* Including small amounts of Hb-F (see Table III).

** Including Hb-A_2.

*** Amount of Hb eluted multiplied with $\frac{\%\text{ alkali resistance of the fraction}}{100}$.

was 2.3% with a range of 1.9–2.4%, this value being in close agreement with those found by CMC-chromatography and starch electrophoresis. The percentage of Hb-A_1 was also comparable with that found by CMC-chromatography in earlier studies[4].

B. Chromatographic studies of abnormal adult human hemoglobins

The behavior of three abnormal hemoglobin types in DEAE-chromatography is presented in Fig. 3. Hb-S was found to be eluted between Hb-A_2 and Hb-A_0, the mean elution volume being 58 ml (Fig. 2). The separations between Hb-A_2 and Hb-S and between Hb-A_0 and Hb-S were almost complete although some overlap may occur. In particular it is possible that small amounts of a minor sickle cell Hb component (Hb-S_1) are present in the Hb-A_0 fraction. This component, which was eluted at a volume almost identical to that of Hb-A_0 and which showed an electrophoretic mobility between Hb-F and Hb-A, was always demonstrable in cases homozygous for Hb-S. The separation between Hb-C and normal Hb-A_0 was almost complete, the abnormal hemoglobin being eluted at about 50 ml. No separation between Hb-C and Hb-A_2 was possible under these conditions. Only partial resolution was obtained

when mixtures of Hb-S and Hb-C, as found in cases suffering from the Hb-S–Hb-C disease, were studied. The abnormal, electrophoretically fast moving Hb-J (Georgia) was easily separated from the normal Hb-A since its elution volume was as high as 170–180 ml. From the elution diagram presented in Fig. 3, it is evident that the Hb-J

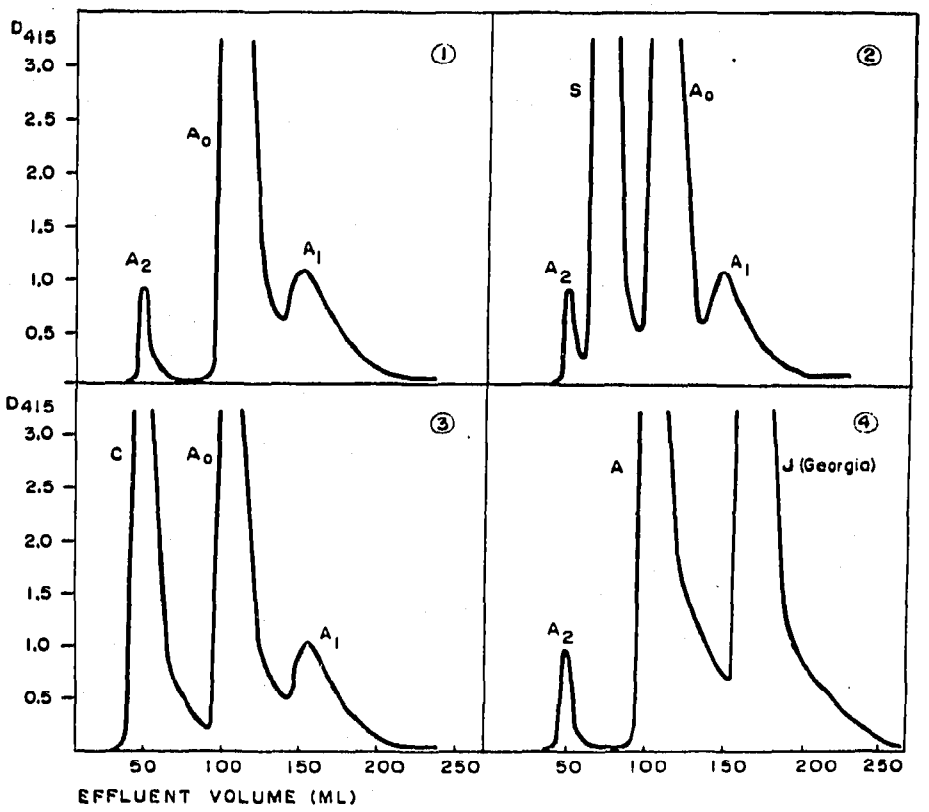

Fig. 3. The chromatographic separation of normal Hb-A and some major abnormal hemoglobin types on DEAE-cellulose. (1) Normal adult; (2) Hb-S trait carrier; (3) Hb-C trait carrier; (4) Hb-J (Georgia) trait carrier.

(Georgia) fraction may partly overlap the normally occurring minor Hb-fraction A_1. Examples of quantitative data are given in Table I. The values of Hb-A_2 in cases with the sickle cell trait and the Hb-J (Georgia) trait are in the normal range of 2 to 2.5 %. The amounts of Hb-S and of Hb-C in heterozygous carriers were found to be about one third of the total amount of hemoglobin, which is in accordance with the findings obtained by electrophoresis as well as by CMC-chromatography[4]. The data obtained for the two cases of Hb-J (Georgia) trait are in close agreement with those found by CMC-chromatography[18]; with both chromatographic methods the amount of the abnormal component was higher than that of normal Hb-A.

Fig. 4 presents the elution diagrams of the hemoglobins of two patients with sickle cell anemia. In the first sample four different components were detectable; Hb-A_2 (elution volume 48 ml), the main Hb-fraction Hb-S_0 (58 ml), the minor Hb-S_1 (120 ml) and a minor fraction designated Hb-F_S (150 ml). The same fractions were detectable in the second sample with the exception of the Hb-F_S component, which was almost absent. The separation of Hb-A_2 and Hb-S was not complete; in many

eases a greater overlap than presented in the figure was encountered. Application of a slower gradient (using the non-automatic system described earlier) resulted in complete separation of the two Hb-components but had the disadvantage of a poor resolution in the S_1–F_S region of the elution diagram. Combination of the two methods

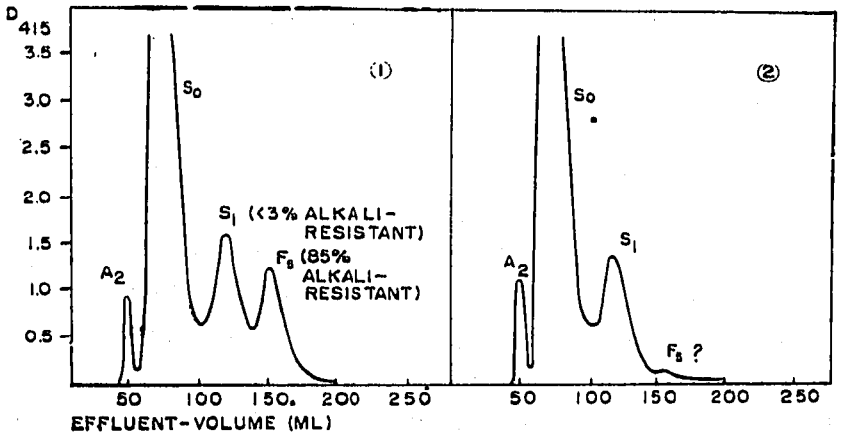

Fig. 4. Chromatographic behavior of the hemoglobins of sickle cell anemia patients on DEAE-cellulose. (1) Hb of a patient with high percentage (11 %) of Hb-F; (2) Hb of a patient with no Hb-F.

(the automatic gradient system for the separation of the S_1 and F_S components and the non-automatic system for the Hb-A_2 resolution) was necessary particularly when quantitative data were required. The minor fractions S_1 and F_S eluted after the main fraction showed different properties. Hb-F_S was resistant to denaturation with alkali, its rate of denaturation being identical to that of fetal hemoglobin of cord blood. The presence of 80–90 % alkali resistant hemoglobin present in this fraction indicated only a slight overlap between the alkali non-resistant Hb-S_1 and Hb-F_S. The possible identity of Hb-F_S and fetal hemoglobin was supported by identical electrophoretic mobilities and U.V. absorption spectra.

Hemoglobin samples from 20 patients who had sickle cell anemia and had not received blood transfusions in six months prior to this study were analyzed with the use of the two gradient systems. In all cases Hb-A_2 and Hb-S_1 were present in addition to the main fraction Hb-S_0. In two samples the Hb-F_S fraction was completely absent, in nine samples its percentage was less than 5 %, while only five samples showed amounts of Hb-F_S higher than 10 %. No normal adult hemoglobin was detectable. A summary of the quantitative results is presented in Table I. The mean value of Hb-F_S was 6.2 % with a range of 0 to 15.5 %; the values obtained by the alkali denaturation procedure were distinctly higher. The Hb-S_1 component averaged about 10 % ranging from 4 to 16.8 %. Of particular interest are the values of the Hb-A_2 fraction: a mean value of 2.45 % with a wide range of 1.7 to 3.0 % was found. The study of one special case (case H.C.), who was considered despite incomplete family studies to be homozygous for sickle cell hemoglobin, revealed values that are listed separately in Table I. The Hb-A_2 level repeatedly was high, above 4.5 %, while the amount of the alkali-resistant fraction Hb-F_S was always below 10 %.

The percentages of fetal hemoglobin found in each sample of the 20 patients having sickle cell anemia were compared with those obtained by the spectrophotometric alkali denaturation procedure. The results of the two procedures correlated reasonably well only in cases with relatively high percentages of Hb-F_s, *i.e.* greater than 10%. However, when fetal hemoglobin was present in amounts below 6-7% the spectrophotometric alkali denaturation procedure gave results too high. The insensitivity of this technique for the determination of low percentages of Hb-F[23,24] may offer the explanation for this discrepancy.

C. Chromatographic studies of hemoglobin samples containing fetal hemoglobin

Fig. 5 presents the elution diagrams of the hemoglobins present in four different cord blood samples. In all four samples fetal hemoglobin was eluted behind normal Hb-A_0 at an elution volume of about 150 ml. In addition to this main Hb-F_0 fraction a minor component (Hb-F_1) was present being eluted at a volume of about 190 ml. Both fractions, which showed the same high rate of resistance towards alkali, were eluted without contamination of non-resistant hemoglobin. Minute amounts of Hb-A_2 were sometimes detectable. The elution diagram of the hemoglobin of a cord blood sample containing the electrophoretically fast moving Bart's hemoglobin, which is known to be composed solely of γ chains[25], showed the presence of four distinct

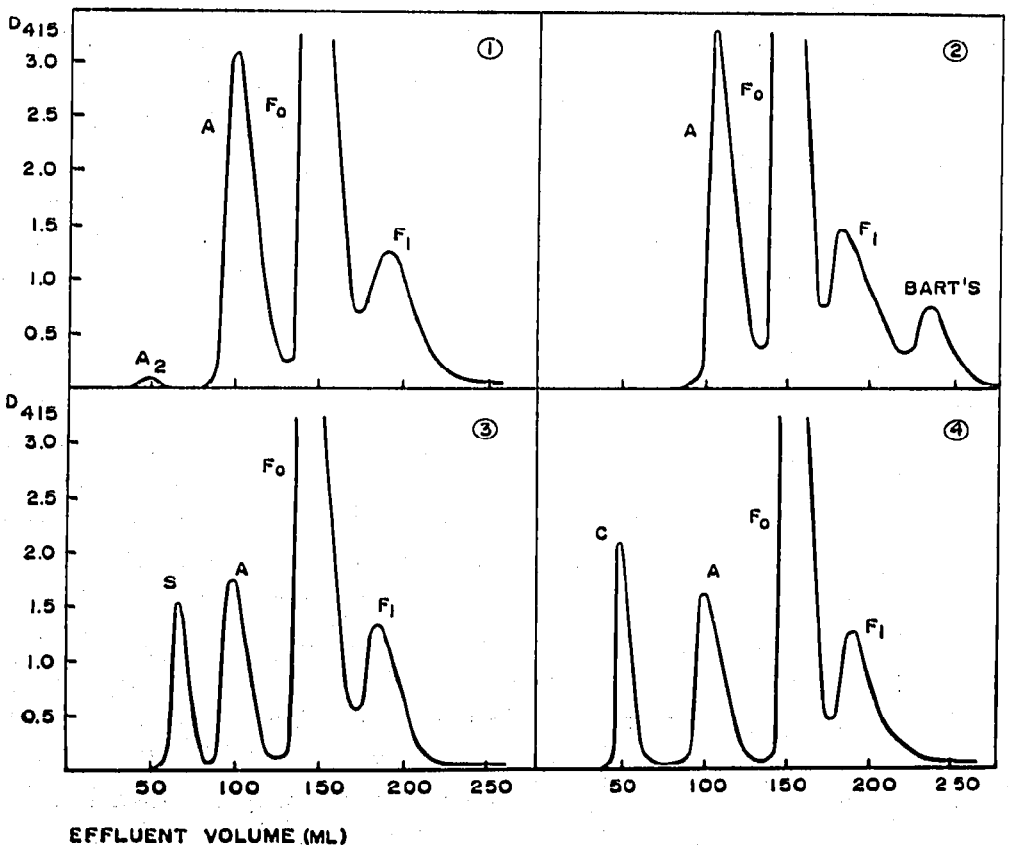

Fig. 5. Chromatography of the hemoglobins of cord bloods by DEAE-cellulose. (1) Normal cord blood; (2) cord blood hemoglobin with 6% of the abnormal Bart's Hb; (3) cord blood from baby heterozygous for Hb-S; (4) cord blood from baby heterozygous for Hb-C.

fractions. The abnormal component was eluted behind the normal fetal fractions at an elution volume of about 240 ml. Its identity with Hb-Bart's was supported by the electrophoretic mobility of the isolated component and by its rate of denaturation by alkali, being intermediate between those of Hb-A and Hb-F. The chromatographic separation of the different hemoglobin fractions present in cord blood samples from newborn babies heterozygous for either Hb-S or Hb-C was almost complete, the abnormal components being eluted in front of Hb-A.

Quantitative analysis of eight cord blood samples are presented in Table II. In most cases a close relationship was present between the sum of the two fetal

TABLE II

THE PERCENTAGES OF DIFFERENT Hb-FRACTIONS IN CORD BLOOD SAMPLES AND IN VARIOUS ABNORMALITIES WITH HIGH FETAL HEMOGLOBIN LEVELS IN ADULT LIFE (DEAE-CHROMATOGRAPHY)

Case	A_2	A_0	F_0*	F_1*	*Abn. Hb*	A_2 *(CMC-chrom)*	*F (Alk. den.)*	*F (CMC-chrom)*
Cord 1	0	3.1	79.0	17.9			92	
Cord 2	0	16.6	66.6	16.8			81.5	
Cord 3	0.2	9.5	80.2	10.1			88	
Cord 4	0	7.8	80.2	12.0			98	
Cord 5	0	18.8	66.6	8.4	Barts: 6.2	0	87	Barts: 6.6
Cord 6	0	9.2	75.5	9.3	S: 6.0		83	(CMC)
Cord 7	?	9.4	72.2	11.1	C: 7.3		80.5	
Cord 8	?	25.0	42.7	11.7	C: 20.6		57	
Thal. major 1	3.3	42.5	48.6	5.6			48.5	
Thal. major 2	3.0	45.8	45.0	6.2			45	
E-Thal. 1	?	45.6	10.9		E: 43.5		14	
E-Thal. 2	?	60.9	10.1		E: 29.0		11	
E-Thal. 3	?	48.9	9.9		E: 41.2		10	
E-Thal. 4	?	10.4	42.8	6.4	E: 40.4		45.5	
"High-F" gene 1	1.7	62.9	35.5			1.6	37	34.5
"High-F" gene 2	1.6	68.8	29.6			1.7	33.5	27.1
"High-F" gene 3	1.3	70.8	27.9			1.5	30.5	25.2
SF comb.	1.7	0	31.6		S: 66.7	1.3	34	31.0

* Amount of Hb eluted multiplied with $\frac{\%\text{ alkali resistance of the fraction}}{100}$.

components (F_0 and F_1) and the percentages of Hb-F determined by the spectrophotometric alkali denaturation procedure. The amount of the Hb-Bart's was equal to the percentage determined by CMC-chromatography. In most samples no Hb-A_2 was detectable. In cases heterozygous for either Hb-S or Hb-C the percentage of Hb-A was higher than that of the abnormal hemoglobin component.

Similar elution diagrams as presented for cord blood hemoglobins were obtained in studying samples derived from patients with hemoglobinopathies characterized by an increased production of fetal hemoglobin in adult life. Examples of chromatographic separations of the hemoglobin fractions found in cases with thalassemia major, thalassemia Hb-E disease, the persistent high Hb-F abnormality and the combination of this anomaly with sickle cell hemoglobin ("SF") are given in Fig. 6. The fetal hemoglobin components were eluted as separate fractions. In thalassemia Hb-E

disease and persistent high Hb-F abnormality small amounts of the minor Hb-A_1 fraction were also present, which became evident from the determination of the percentages of alkali resistant hemoglobin present in the isolated fetal fractions. The separation of Hb-A_2 in cases with thalassemia major and with the persistent high

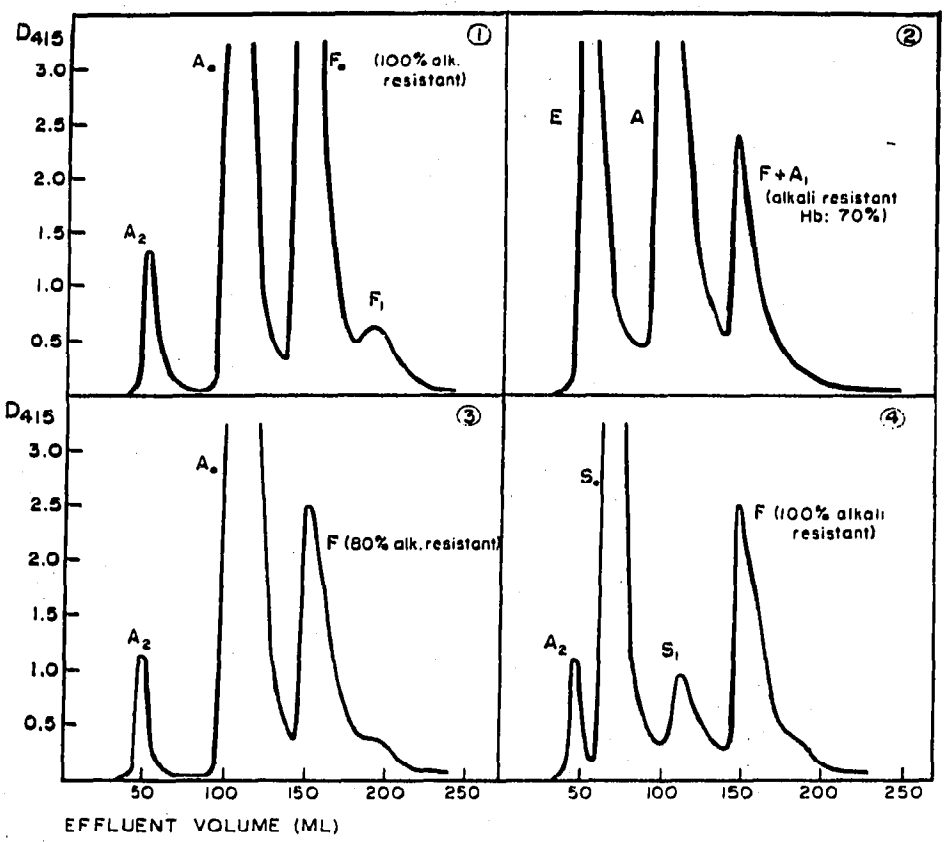

Fig. 6. Chromatography of hemoglobins of adult subjects showing increased amounts of Hb-F. (1) Patient with thalassemia major; (2) patient with Hb-E-thalassemia disease; (3) patient with the persistent high Hb-F anomaly; (4) patient with Hb-S combined with the persistent high Hb-F anomaly.

Hb-F abnormality was complete. No separation was possible between Hb-E and Hb-A_2 The elution diagram of the hemoglobin from a case with the "SF" combination was almost identical with that found in sickle cell anemia patients, with the exception of the presence of higher amounts of a pure Hb-F fraction. Normal Hb-A, which is eluted at a similar elution volume as the Hb-S_1 fraction, was apparently absent in this sample, since the quantitative relationship between the Hb-S_1 and Hb-S_0 components was comparable with that found in hemoglobin samples of cases with the homozygous sickle cell disease. Electrophoretic studies of the isolated Hb-S_1 fraction also failed to show any normal Hb-A.

Quantitative data obtained in the study of these cases are also presented in Table II. Again it was found that the relatively high percentages of Hb-F were in reasonable agreement with the values obtained by the spectrophotometric alkali denaturation procedure and also by CMC-chromatography. The percentages of Hb-A_2

in the two cases with thalassemia major (3.3 and 3.0% respectively) were higher than those found for normal adults. The Hb-A_2 levels in the blood of patients with the persistent fetal hemoglobin abnormality, on the contrary, were in the low range of normal; the values agreed closely with those determined by CMC-chromatography.

D. Chromatographic studies of hemoglobin samples with abnormal minor hemoglobin components

Hemoglobin samples from different abnormal cases were studied; examples of electrophoretic separations by starch gel electrophoresis are presented in Fig. 7. This

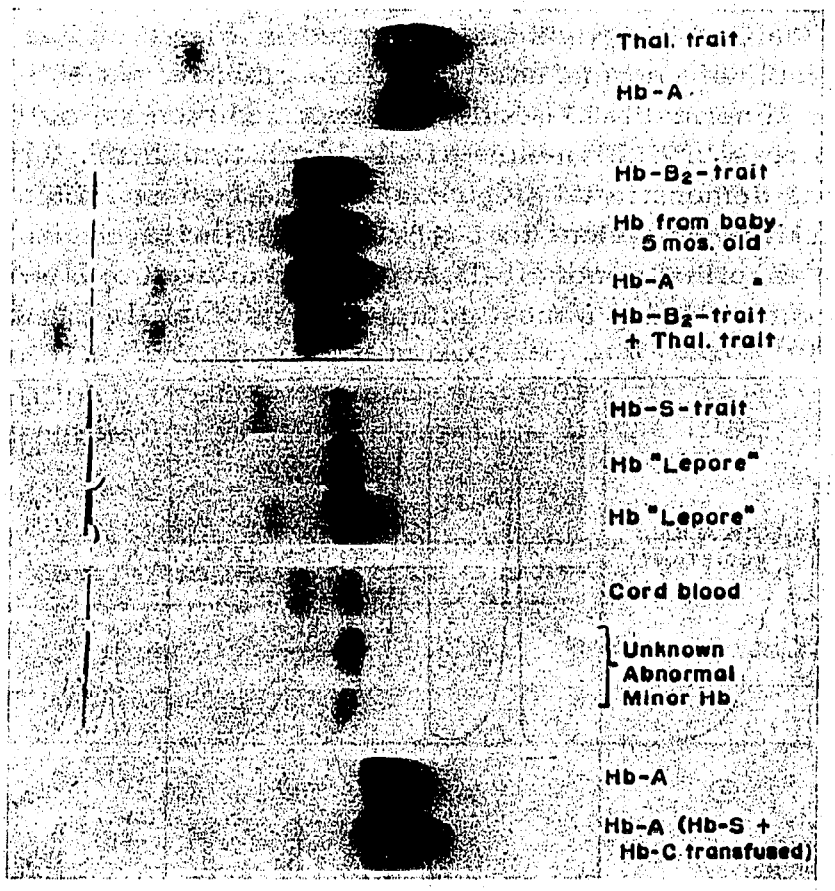

Fig. 7. Starch gel electrophoretic separations of hemoglobins with different abnormal minor Hb-fractions.

study included (a) samples from seven patients with thalassemia minor showing an increased quantity of Hb-A_2 and (b) samples from four individuals heterozygous for the Hb-B_2 abnormality. This slow moving hemoglobin component, which was proven to be an abnormal form of Hb-A_2[20], was present for about 1%. The percentages of Hb-A_2 were equally reduced and comparable with that present in the blood of a 5 months old baby. (c) Samples from seven cases heterozygous for thalassemia trait as well as the Hb-B_2 abnormality[21]. The percentages of Hb-A_2 and of Hb-B_2 were about 2% and therefore comparable with those of Hb-A_2 found in normal adults.

(d) Samples from four members of an Italian family, in which the "Lepore" abnormality[22] was present. This hemoglobin abnormality showed an electrophoretic mobility almost identical to that of Hb-S. (e) Hemoglobin samples from two cases, which showed the presence of an unknown minor hemoglobin component with an electrophoretic mobility between those of Hb-S and Hb-A_2. Characterization of this fraction will be the subject of a separate study. (f) A blood sample from a patient, who was accidentally transfused with blood from sickle cell trait and Hb-C trait carriers. The presence of small amounts of these two abnormal components was proven by a study of blood samples from his donors as well as by repeated analysis of the blood of the recipient.

Examples of elution diagrams are presented in Fig. 8 and are compared with that of the hemoglobin of a normal adult. The hemoglobin of patients with thalassemia minor showed two abnormalities. Besides the expected increase of the Hb-A_2 component a definite increase in the amount of alkali resistant hemoglobin (Hb-F) in the Hb-A_1 fraction was demonstrated (Fig. 8, 2). In most cases the percentages ranged from 10–20 % which is equivalent to 1–3 % of Hb-F in the original sample. As discussed before, the abnormal minor Hb-B_2 was eluted in front of Hb-A_2, the separation of these two components being almost complete (Fig. 8, 3). In cases with the double

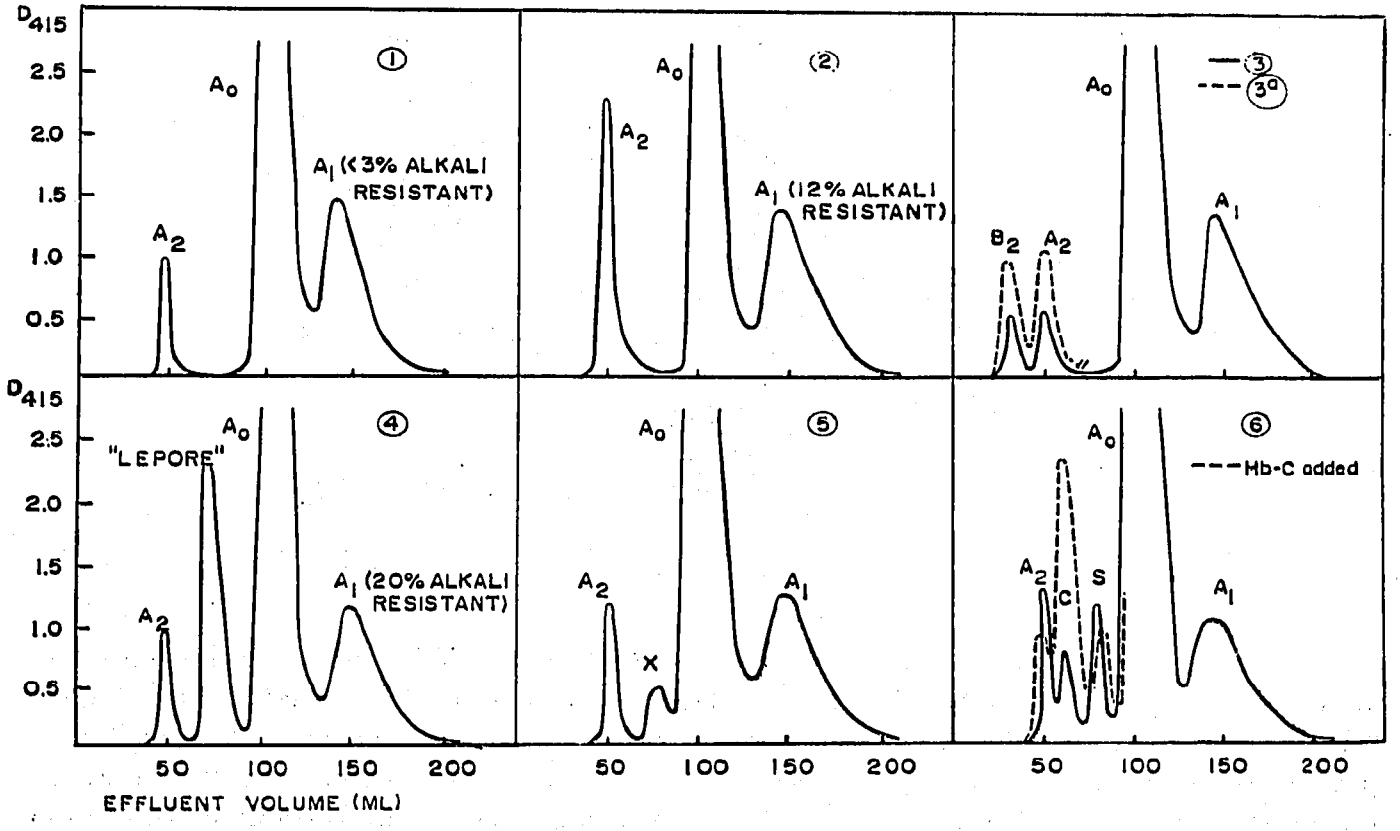

Fig. 8. Chromatographic separations of hemoglobins of several cases with abnormal minor Hb-components. (1) Normal adult; (2) thalassemia minor; (3) Hb-B_2 heterozygosity; (3a) Hb-B_2 heterozygosity combined with thalassemia trait; (4) Hb-Lepore carrier; (5) case with unknown minor Hb-fraction; (6) Hb from patient transfused with blood from Hb-S and Hb-C carriers. In several cases the percentages of alkali-resistant hemoglobin present in the Hb-A_1 fraction is given.

heterozygosity (thalassemia minor and Hb-B_2 abnormality) the amounts of Hb-B_2 and of Hb-A_2 were doubled, while their mobilities were identical to those observed in the hemoglobin samples of the heterozygous Hb-B_2 carrier (Fig. 8, 3 *a*). The elution diagrams of the hemoglobins of the "Lepore" trait carriers were characterized by a complete separation of the abnormal component from Hb-A_2 and Hb-A_0 and by increased percentages of alkali-resistant hemoglobin (Hb-F) in the Hb-A_1 fraction (Fig. 8, 4). The unknown abnormal minor fraction present in the blood of the adults of German origin was also eluted between the Hb-A_2 and Hb-A_0 fractions, its separation being incomplete. The presence of small amounts of the hemoglobins S and C in the hemoglobin sample of the patient who received multiple blood transfusions was easily demonstrated (Fig. 8, 6). An incomplete separation of the small amounts of Hb-A_2, Hb-C and Hb-S was obtained. The finding that small amounts of Hb-C showed a slightly slower elution rate than Hb-A_2 was proven by addition of small amounts of pure Hb-C to the sample. A larger increase of the percentage of Hb-C resulted in a complete overlap of the two components.

Table III summarizes the quantitative data. The Hb-A_2 percentages in the cases with thalassemia minor ranged from 4.7 to 5.8 %. In two cases Hb-A_2 determinations were also performed by CMC-chromatography; comparative results were obtained. The percentages of Hb-F were increased in five cases and ranged from 1 to 2.9 %. No satisfactory correlation between these values and those determined by the spectrophotometric alkali denaturation procedure was seen. In the Hb-B_2 heterozygotes the

TABLE III

THE PERCENTAGES OF DIFFERENT Hb-FRACTIONS IN CASES WITH THALASSEMIA MINOR, Hb-B_2 HETEROZYGOSITY, AND Hb-LEPORE TRAIT AS ESTIMATED BY DEAE-CHROMATOGRAPHY

Case	*n*	A_2	A_0	A_1*	*F***	*Abn. Hb.*	A_2 *(CMC-chrom.)*	*Abn. Hb. (CMC-chrom.)*	*F (Alk den.*
ɔrmal	6	2.3	84.6	13.1	< 0.5		2.1		< 2
ıal. 1									
ınor.		5.5	76.7	14.9	2.9				8
2		5.5	79.3	13.7	1.5		5.1		
3		5.8	84.0	9.2	1.0				2
4		5.5	78.0	16.5	< 0.5				< 2
5		5.2	78.7	14.6	1.5		4.8		4
6		5.5	77.7	16.8					
7		4.7	75.9	17.9	1.5				3
ɔ-B_2	4	1.0	87.1	11.0	< 0.5	Hb-B_2: 0.9	1.0	B_2: 0.9	< 2
terozygotes		(0.8–1.1)	(85.4–89.3)	(9.2–12.9)		(0.7–1.0)	(0.9–1.3)	(0.8–1.1)	
ɔ-B_2	7	2.0	85.7	10.5		Hb-B_2: 1.8	2.0	B_2: 1.9	
terozygotes		(1.7–2.2)	(83.8–87.5)	(8.5–11.7)		(1.6–2.1)	(1.7–2.8)	(1.7–2.2)	
Thal. trait.									
ɔ-Lepore 1		2.3	76.5	9.0	2.0	Lepore: 10.2	1.8	Lepore: 12.5	4
2		1.9	69.8	14.3	2.7	11.3			5
3		1.8	71.0	7.8	9.5	9.9			6
4		2.3	62.7	23.8	1.3	9.9			< 2

* Total amount eluted minus Hb-F.

** Amount of Hb-A_1 eluted multiplied with $\frac{\%\text{ alkali resistance of the fraction}}{100}$.

mean values of Hb-A_2 (1.0 %) and of Hb-B_2 (0.9 %) were in close agreement with those determined by CMC-chromatography. The same was true for the double heterozygous cases. The mean values of Hb-A_2 and Hb-B_2, as determined by both chromatographic procedures, were about 2 %. The percentages of Hb "Lepore" were determined in four cases; a range between 9.9 and 11.3 % was found. In close agreement was the percentage of 12.5 % found when one sample was analyzed by CMC-chromatography. Also of interest are the relatively high percentages of Hb-F (1.3 to 9.5 %) being eluted together with the Hb-A_1 fraction. The Hb-A_2 values found in these cases fell in the normal range.

E. Comparative studies of DEAE-chromatography, CMC-chromatography and starch gel electrophoresis

In previous studies[2-4] using the CMC-chromatographic procedure, the hemoglobins of a normal individual, of cord blood and of patients with various hemoglobin anomalies were separated into different fractions each possessing distinctly different electrophoretic properties. In the present studies attempts were made to correlate these fractions with those found by DEAE-chromatography. Fig. 9 presents examples of elution diagrams obtained when the hemoglobins of a normal adult, of cord blood and of a sickle cell anemia patient were chromatographed on CM-cellulose. Normal adult

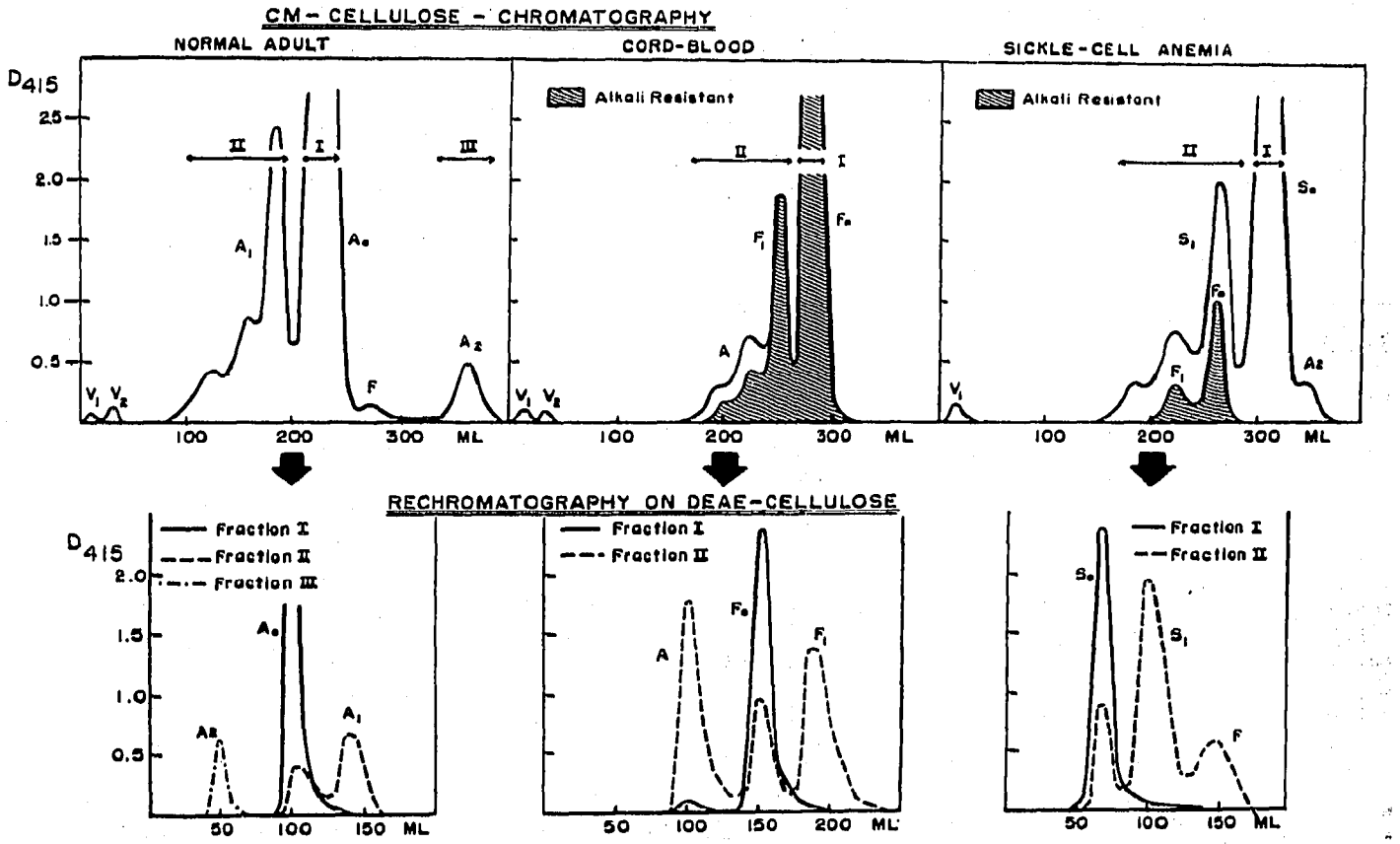

Fig. 9. Chromatographic separations of the hemoglobins of a normal adult, cord blood and a sickle cell anemia patient on CM-cellulose and rechromatography of several isolated fractions on DEAE-cellulose.

hemoglobin was fractionated into the electrophoretically fast moving Hb-A_1, the mean fraction Hb-A_0, small amounts of Hb-F and the electrophoretically slow moving Hb-A_2 component. Similarly, cord blood hemoglobin was separated into the mean component Hb-F_0 and an electrophoretically fast fraction which was composed of the alkali-resistant Hb-F_1 and the alkali non-resistant Hb-A_0 components. The elution diagram of the hemoglobin of the sickle cell anemia patients revealed, in addition to the mean Hb-S_0 and the Hb-A_2 components, a fast moving fraction consisting of alkali-resistant hemoglobin and alkali non-resistant hemoglobin. The different fractions, as indicated in Fig. 9, were isolated, concentrated with the use of small DEAE-cellulose columns and rechromatographed on DEAE-cellulose using the automatic buffer gradient device. Representative results of this study are also presented in Fig. 9. They can be summarized as follows: (a) Normal adult hemoglobin: Fraction I (Hb-A_0 in CMC-chromatography) was eluted as one single component at an elution volume similar to that of Hb-A_0 (DEAE-cell.). Fraction II (Hb-A_1 in CMC-chromatography) was found to consist of two components; the main fraction was identical to the Hb-A_1 fraction (DEAE-cell.), while the minor component possessed an elution rate identical to that of Hb-A_0 (DEAE-cell.). Fraction III (Hb-A_2 in CMC-chromatography) was eluted as one single component at an elution volume characteristic for Hb-A_2. (b) Cord blood hemoglobin: Rechromatography of fraction I

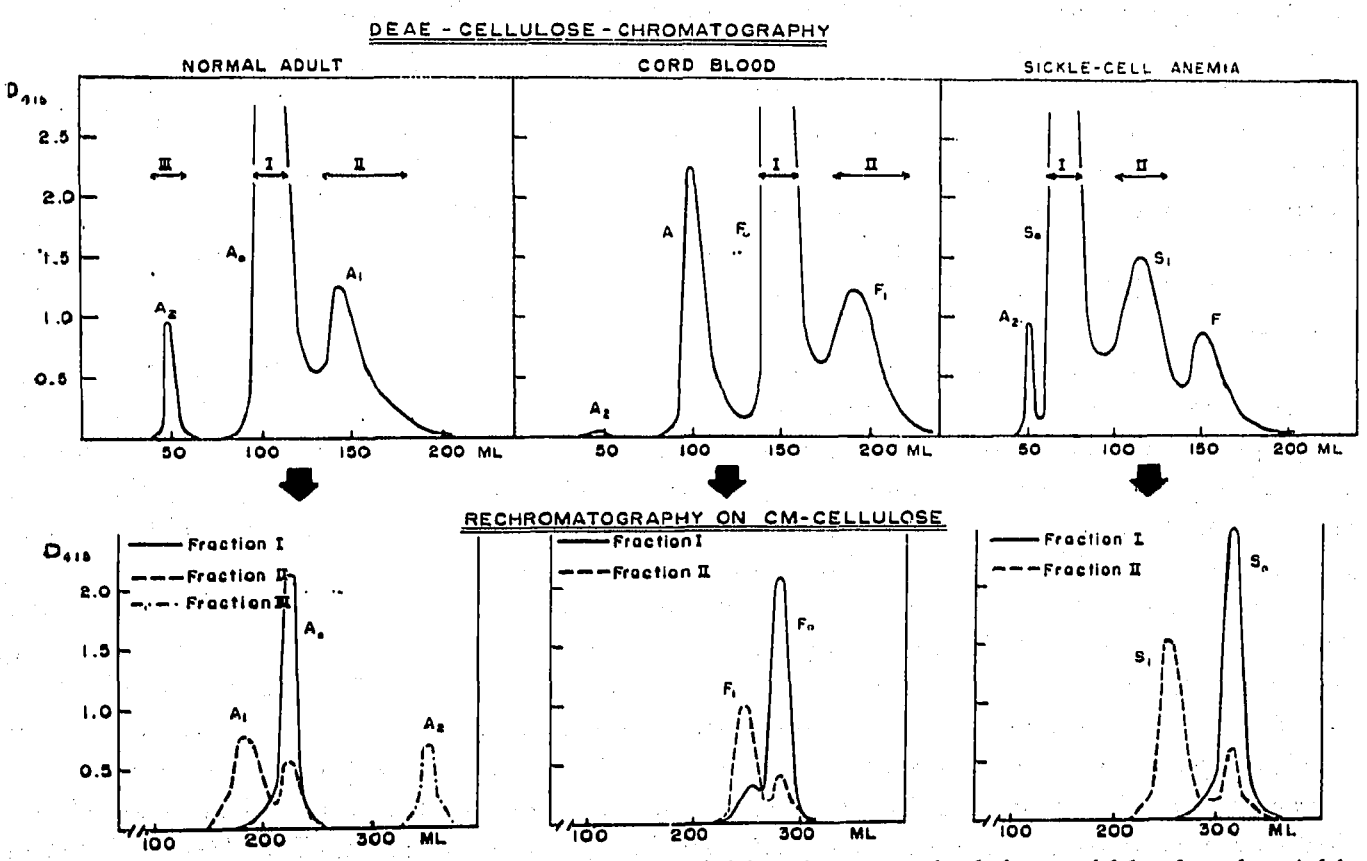

Fig. 10. Chromatographic separation of the hemoglobin of a normal adult, cord blood and a sickle cell anemia patient on DEAE-cellulose and rechromatography of several isolated fractions on CM-cellulose.

($Hb\text{-}F_0$ in CMC-chromatography) resulted in the elution of one major fraction ($Hb\text{-}F_0$, DEAE-cell.), while the presence of a small amount of a Hb-A-like component was also demonstrated. Fraction II (mixture of $Hb\text{-}F_1$ and Hb-A, CMC-chromatography) was found to consist of three components. The two major fractions showed elution rates similar to those of $Hb\text{-}A_0$ and $Hb\text{-}F_1$ (DEAE-cell.), while a third, minor component was eluted at an elution volume characteristic for $Hb\text{-}F_0$ (DEAE-cell.). (c) Sickle cell hemoglobin: The main fraction I (S_0 in CMC-chromatography) was found to be one single component, its elution volume being identical to that of $Hb\text{-}S_0$ (DEAE-cell.). The complex fraction II ($Hb\text{-}S_1$ and Hb-F in CMC-chromatography) was resolved into three components. In addition to the major $Hb\text{-}S_1$ component small amounts of $Hb\text{-}S_0$ and of Hb-F were demonstrated (DEAE-cell.).

Examples of reversed experiments are presented in Fig. 10. The different fractions isolated by DEAE-cellulose chromatography were concentrated with the use of short CMC-columns[3] and transferred, while still adsorbed onto the cation exchanger, to 45 × 0.9 cm columns of CMC for rechromatography. The results are as follows: (a) Normal adult hemoglobin: Fraction I ($Hb\text{-}A_0$ in DEAE-cell.) was identical to $Hb\text{-}A_0$ in CMC-chromatography. Fraction II ($Hb\text{-}A_1$ in DEAE-cell.) consisted of two fractions, the major with the mobility of $Hb\text{-}A_1$ and the minor with the mobility of $Hb\text{-}A_0$ (CMC-chromatography). Fraction III ($Hb\text{-}A_2$ in DEAE-cell.) was eluted as one single fraction with a mobility similar to that of $Hb\text{-}A_2$ in CMC-chromatography. (b) Cord blood hemoglobin: Both fractions (I = $Hb\text{-}F_0$ and II = $Hb\text{-}F_1$ in DEAE-cell.) were heterogeneous in CMC-chromatography. A small amount of $Hb\text{-}F_1$ was present in the $Hb\text{-}F_0$ fraction, while a small quantity of $Hb\text{-}F_0$ was detectable in the $Hb\text{-}F_1$ component. (c) Sickle cell hemoglobin: Fraction I ($Hb\text{-}S_0$ in DEAE-cell.) was rechromatographed as one single component with a similar mobility to $Hb\text{-}S_0$ (CMC-chromatography). Fraction II ($Hb\text{-}S_1$ in DEAE-cell.) consisted of two fractions, in addition to the major $Hb\text{-}S_1$ component a small amount of $Hb\text{-}S_0$ was demonstrated (CMC-chromatography).

Electrophoretic examination of different fractions isolated by DEAE-chromatography revealed results, of which examples are presented in Fig. 11. The fractions A_0, A_1 and A_2, isolated from the blood of normal individuals, the fetal components F_0 and F_1 found in cord blood samples, and the fractions S_0, S_1 and F_S present in the hemoglobin of sickle cell anemia patients were studied with the use of the starch gel electrophoretic technique. Their electrophoretic mobilities were compared with those of hemoglobin components present in hemoglobin samples prepared from cord blood, blood of normal adults and patients with various hemoglobinopathies including the Hb-E-thalassemia disease, sickle cell anemia and the sickle cell-persistent high Hb-F combination. The main hemoglobin fraction $Hb\text{-}A_0$ was found to be electrophoretically pure; neither $Hb\text{-}A_2$ nor $Hb\text{-}A_1$ were detectable. The $Hb\text{-}A_1$ fraction, on the contrary, consisted of a major fraction with a distinctly higher mobility than $Hb\text{-}A_0$ and of a minor fraction with a mobility similar to that of $Hb\text{-}A_0$. The $Hb\text{-}A_2$ fraction consisted of one single component. It should be noted that the mobility of the isolated $Hb\text{-}A_2$ fraction was always slightly less than that of Hb-E. Electrophoretic examina-

tion of the Hb-F_0 and Hb-F_1 fractions, prepared from cord blood hemoglobin, revealed two distinctly different components. Hb-F_0 showed a mobility similar to that of the main Hb-fraction of cord blood, while Hb-F_1 was faster than Hb-F_0 and slightly slower than Hb-A_0. Electrophoretic studies of the Hb-S_0, Hb-S_1 and Hb-F_s fractions

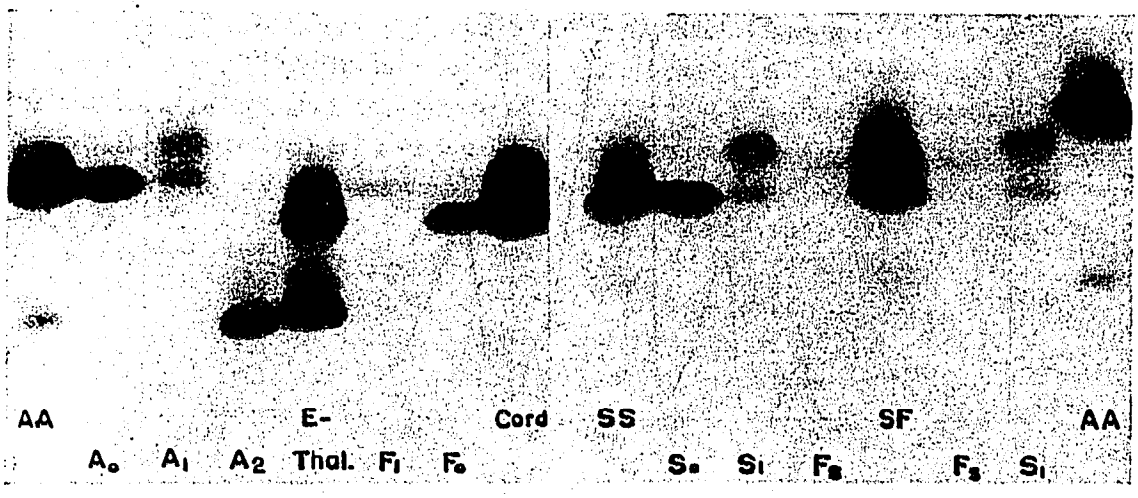

Fig. 11. Electrophoretic behavior (starch gel electrophoresis) of several hemoglobin fractions isolated by DEAE-cellulose chromatography. The mobilities were compared with those of components present in the blood of normal adult (AA), a case with Hb-E-thalassemia disease (E-thal), a homozygous Hb-S patient (SS), a case with the Hb-S persistent high Hb-F combination (SF) and cord blood.

isolated from the blood of patients with sickle cell anemia revealed the following results: Hb-S_0 consisted of one single fraction with an electrophoretic mobility similar to that of the major Hb-fraction present in the original hemoglobin sample. The Hb-S_1 fraction was composed of two components. The minor fraction was identical to the Hb-S_0, while the mobility of the major fraction was intermediate between those of Hb-F_0 and Hb-A_0 and problably slightly less than that of Hb-F_1. The alkali-resistant Hb-fraction found in the blood of patients with sickle cell anemia showed an electrophoretic mobility identical to that of Hb-F_0 of cord blood and distinctly less than that of the Hb-S_1 fraction. The relative mobilities of the different isolated components in starch gel electrophoresis can be summarized as follows: $A_2 < S_0 < F_0 = F_s < S_1 < F_1 < A_0 < A_1$, while their rates of elution as measured by volume of eluent in DEAE-chromatography were found to be: $A_2 > S_0 > A_0 = S_1 > A_1 = F_0 = F_s > F_1$.

F. Isolation of hemoglobin components by DEAE-cellulose chromatography

From the results discussed in the previous section, it will be evident that the DEAE-cellulose chromatographic procedure offers new possibilities for the preparation of electrophoretically pure Hb-fractions. Application of larger quantities of hemoglobin (200 mg) to a column of 45 × 0.9 cm resulted in the isolation of as much as 100 mg of Hb-A_0, Hb-S_0, Hb-F_0 and other major hemoglobin types.

The preparation of larger amounts of purified minor hemoglobin fractions such as Hb-A_2, Hb-B_2 and Hb "Lepore", however, required the use of larger columns in

order to permit an increase in the load of hemoglobin. An example of such a purification is presented in Fig. 12. A large amount of 4.5 g of the hemoglobin from a patient with the hemoglobin "Lepore" dissolved in 40 ml was dialyzed in the usual way and applied to a column of 34 × 2 cm. The elution of the hemoglobin was per-

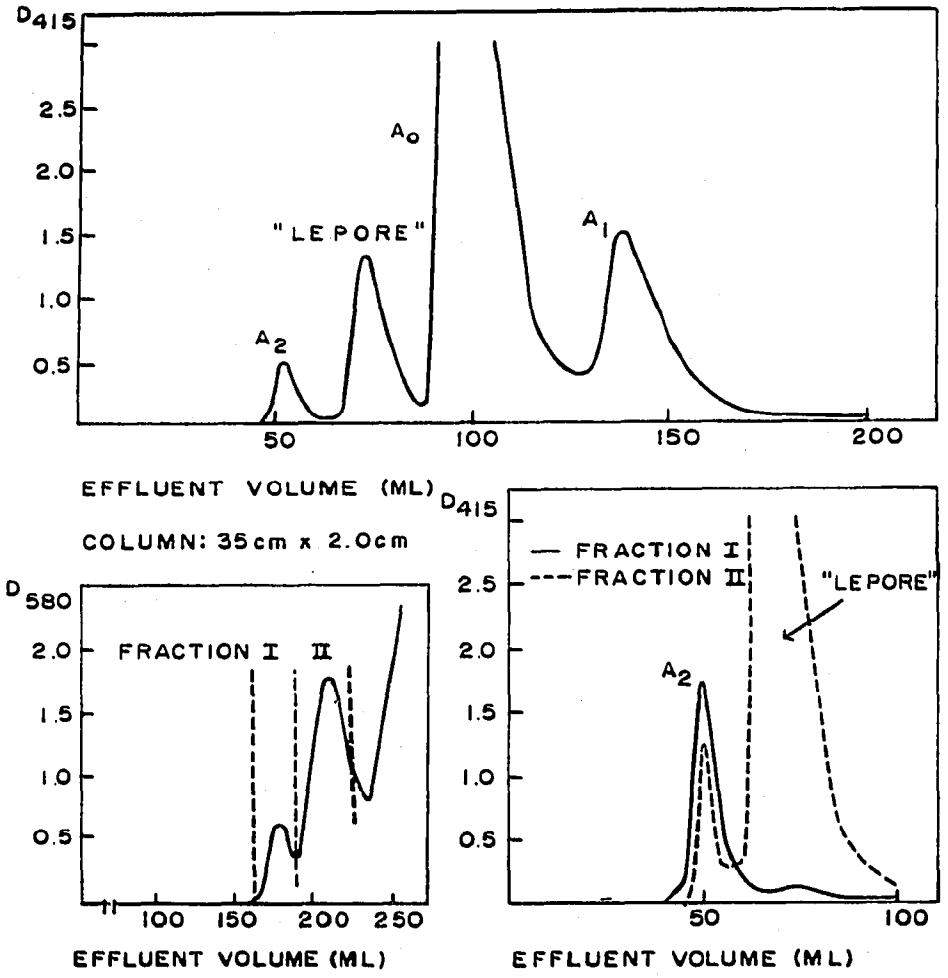

Fig. 12. Preparation of larger quantities of Hb-Lepore by DEAE-cellulose chromatography. Top: Separation of Hb-Lepore on a 45 × 0.9 cm column. Bottom left: Separation of Hb-A and Hb-Lepore on a column of 35 × 2.0 cm (4500 mg of Hb chromatographed). Bottom right: Rechromatography of the Hb-A_2 and Hb-Lepore fractions on 45 × 0.9 cm columns.

formed by application of a 0.01 *M* sodium phosphate buffer pH 8.6 at a flow rate of 35 ml/h. The resulting elution diagram, which is also presented in Fig. 12, showed an incomplete separation of the minor Hb-A_2 component (fraction I), the abnormal Hb "Lepore" (fraction II) and the major Hb-A_0. The isolated fractions I and II were concentrated on short DEAE-cellulose columns and rechromatographed on 45 × 0.9 cm columns of DEAE-cellulose with the use of the automatic gradient system. As shown in the figure, fraction I consisted almost entirely of Hb-A_2, while the second fraction was composed of Hb-A_2 as well as Hb "Lepore". Normal Hb-A_0 was not detectable. The isolated Hb-A_2 and Hb-"Lepore" components were also tested for purity by starch gel electrophoresis. The results presented in Fig. 13 support the evidence that

after rechromatography the Hb-A_2 as well as the Hb "Lepore" consisted of single components each with a specific electrophoretic mobility. It will be noted that the preparation of large quantities of pure Hb-A_2 from blood of normal adults and Hb-B_2 from blood of a homozygous Hb-B_2 carrier could be achieved by one single chromatographic separation using the 35 × 2 cm columns.

As shown by different investigators[7,26–31] hybrid hemoglobins may be formed when two different hemoglobins are together dissociated at acid pH and subsequently recombined by neutralization of the acid solution. The DEAE-cellulose chromatographic procedure was found of use for the isolation and purification of these hybrid hemoglobins permitting a detailed study of the isolated components. Purified "Lepore'

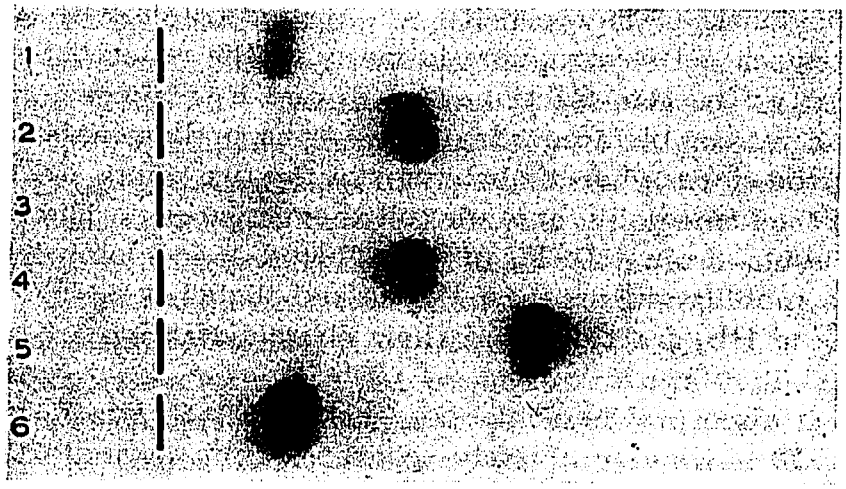

Fig. 13. Electrophoretic behavior (starch gel electrophoresis) of the isolated Hb-A_2 and Hb-Lepore fractions. 1 = Hb-A_2 (rechromatographed); 2 = Hb-Lepore (impure); 3 = Hb-A_2 (impure); 4 = Hb-Lepore (rechromatographed); 5 = Hb-A; 6 = Purified Hb-E.

Hb, for instance, was mixed with Bart's hemoglobin, which is known to be composed of four γ polypeptide chains[25], and hybridized. Chromatographic analyses on DEAE-cellulose revealed the appearance of a new Hb-fraction, which was eluted at an elution volume characteristic for fetal hemoglobin. Its identity with Hb-F was supported by U.V. spectral adsorption measurements and by its slow denaturation rate in alkaline solution. It seems, therefore, that Hb-"Lepore" contains similar α polypeptide chains as are present in fetal hemoglobin.

DISCUSSION

The differences in behavior of various hemoglobin types in anion-exchange chromatography with DEAE-cellulose as adsorbent are generally in accordance with the differences in their isoelectric points. The relative elution rates of some hemoglobins in DEAE-cellulose chromatography as measured by their elution volumes were $B_2 > A_2 = E > S = \text{Lepore} > A_0 > A_1 = F > J$, while the relative electrophoretic mobilities of the same components at pH 8.6 were found to be $B_2 < A_2 = E < S = \text{Lepore} < F < A_0 < A_1 < J$. Hb-F behaves exceptionally, since this Hb-type was eluted

at a rate slower than Hb-A and slightly faster than Hb-J. It may be that the greater structural differences of Hb-F when compared with Hb-A and other abnormal Hb-types are responsible for this phenomenon. It is noteworthy that this effect is not seen in chromatography on the cation-exchanger CM-cellulose; the relative elution rates of the same Hb-types in CMC-chromatography were found to be: $B_2 < A_2 = E < S = \text{Lepore} < F < A_0 < A_1 = J$[3].

The DEAE-cellulose chromatographic procedure offers new possibilities for the study of the inhomogeneity of Hb-types. In addition to the Hb-A_2 fraction and the major hemoglobin component (designated as Hb_0-fraction), the presence of minor Hb-component (designated as Hb_1-fraction) was demonstrated. Comparative studies of DEAE-cellulose and CMC-chromatography and starch gel electrophoresis have indicated that an identical heterogeneity was demonstrable with these procedures. When tested by starch gel electrophoresis many of the isolated fractions showed a high degree of purity; the Hb_1 fractions (A_1, S_1 and F_1), however, were found to be contaminated with reasonable amounts of the corresponding major Hb-fractions. Of special interest is the electrophoretic behavior of the two minor fractions (Hb-S_1 and Hb-F_s) present in the hemoglobin of sickle cell anemia patients. The Hb-F_s component was indistinguishable from Hb-F_0 of cord blood hemoglobin, while the Hb-S_1 fraction showed a mobility faster than Hb-F_0 and slightly less than Hb-A_0. Further purification of these Hb_1 fractions and their characterization are at present under study.

Since different abnormal hemoglobins, such as Hb-S, Hb-E, Hb-C, Hb-J, Hb-Lepore and also Hb-F were completely separated from normal Hb-A, the DEAE-cellulose chromatography was found useful for the quantitation of the percentages of abnormal Hb. Examples for many abnormal hemoglobins such as those of AS, AC, AJ, SS carriers, cord bloods, patients with Hb "Lepore", thalassemia major, Hb-E-thalassemia, the persistent high Hb-F abnormality and the sickle cell high Hb-F combination have been presented. The results are in close agreement with data obtained by moving boundary electrophoresis and CMC-chromatography[4]. It is worth noting, that the complete absence of Hb-A in cases with the combined Hb-S high Hb-F abnormalities[32,19] was confirmed by application of DEAE-cellulose chromatography and subsequent study of the isolated fractions with starch gel electrophoresis. However, incomplete separation of Hb-C and Hb-S makes the application of the DEAE-cellulose chromatography less useful in Hb-S–Hb-C disease.

An important application of the DEAE-cellulose chromatography is the quantitative determination of the minor Hb-A_2 fraction since separation of this Hb component and many other hemoglobin types are complete. The analytical results obtained for normal adults, patients with thalassemia minor and the persistent high fetal hemoglobin abnormality are in essential agreement with the observations obtained from starch gel electrophoresis and CMC-chromatography[1,3]. Since the separation of Hb-A_2 from Hb-A, Hb-F and the electrophoretically fast moving Hb-components was complete, a simplified method for the Hb-A_2 determination using 15 × 0.9 cm columns and two elution buffers has been devised[12]. This method preserves the resolving

capacity of the DEAE-cellulose column while making the procedure more suitable for clinical purposes. Hb-A_2 and some electrophoretically slow moving Hb-types appear to be separated by anion-exchange chromatography only with the use of 45 × 0.9 cm columns and the automatic gradient system described. Examples of separations of Hb-A_2 from Hb-S and Hb-Lepore have been presented; fractionation of Hb-A_2 and the Hb's C and E was impossible. The quantitative findings in these different cases, although preliminary, suggest that the Hb-A_2 percentages of sickle cell trait carriers, of Hb-J (Georgia) trait carriers and of the patients with the Hb-Lepore abnormality are in the normal range. In the two cases of thalassemia major the Hb-A_2 level was slightly increased, while the percentages of Hb-A_2 in three patients with the persistent high Hb-F abnormality fell in the low range of normal. The seven carriers of the thalassemia trait showed the expected high values ranging from 4.7 to 5.8 %.

The almost complete fractionation of abnormal minor hemoglobin fractions such as Hb-B_2, Hb-Lepore and the unknown minor abnormality is another interesting characteristic of DEAE-cellulose chromatography. The quantitative data presented for the Hb-B_2 heterozygotes and the Hb-B_2-thalassemia trait carriers are in excellent agreement with those obtained by CMC-chromatography[20,21]. The percentages of 9.9 to 11.3 found for the abnormal component in the individuals with the Hb-Lepore abnormality are of the same magnitude found by PEARSON, GERALD AND DIAMOND[22] using starch block electrophoresis.

In some instances, quantitative determination of fetal hemoglobin was possible because of an almost complete fractionation of Hb-F and many other Hb-types but excluding Hb-J. The rather large amounts, 10% and higher, found in cord blood samples and in blood samples of patients with thalassemia major, Hb-E-thalassemia disease and the persistent high Hb-F abnormality were in close agreement with the results obtained by the spectrophotometric alkali denaturation procedure. When low percentages of Hb-F were present along with large quantities of normally occurring Hb-A_0 and Hb-A_1 fractions, the Hb-F and the Hb-A_1 were eluted as one fraction. Application of a spectrophotometric alkali denaturation method to this isolated fraction presented possibilities for a more nearly exact quantitative determination of Hb-F in such hemoglobin samples. Small increases in the percentages of Hb-F in cases of thalassemia trait and Hb-Lepore abnormality have been demonstrated, the values being distinctly lower than those determined by the direct application of the alkali denaturation procedure to the untreated Hb-samples. Similar results were obtained for the determination of Hb-F in the hemoglobin samples of sickle cell anemia patients. Since the fetal hemoglobin is almost completely separated from the sickle cell hemoglobin components, the DEAE-cellulose chromatography has greatly facilitated an accurate determination of the Hb-F fraction in these samples.

The potentially of DEAE-cellulose chromatography in hemoglobin fractionation is perhaps best illustrated by the examples given for the preparation of large quantities of pure minor Hb-components. A relatively simple procedure allows the isolation of large amounts of components such as Hb-B_2, Hb-A_2, Hb-Lepore, and others.

It seems that both chromatographic procedures, one with the cation exchanger

CM-cellulose and the other with the anion exchanger DEAE-cellulose as adsorbent, have considerable value as analytical and preparative methods in hemoglobin studies. The data presented in this paper suggest that the DEAE-cellulose chromatography is particularly valuable for the fractionation of electrophoretically slow moving components and of fetal hemoglobin; the CMC-chromatography is preferable for the fractionation of electrophoretically fast moving hemoglobin fractions.

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SUMMARY

1. A new column chromatographic procedure for the separation of different hemoglobin fractions using DEAE-cellulose as adsorbent is described. Except for fetal hemoglobin the elution rates of the hemoglobin components were found to be in accordance with the differences in electrophoretic points. The elution rates, as measured by their elution volumes, are within certain limits independent of the total amount of Hb chromatographed; the elution rates were greatly influenced by the initial pH values of the adsorbent.

2. Examples of hemoglobin separations are presented for cases with the following Hb-abnormalities: AS, AC, AJ (Georgia), SS, SC, normal cord blood, cord blood samples from babies heterozygous for Hb-S and for Hb-C, cord blood sample with the abnormal Bart's Hb, Thalassemia minor and major, Hb-E-thalassemia disease, persistent high Hb-F abnormality, persistent high Hb-F abnormality combined with Hb-S, Hb-B_2 trait, Hb-B_2 trait associated with thalassemia, the Hb "Lepore" abnormality, and a case with an unknown abnormal minor Hb-component.

3. Since small amounts of Hb-F are generally eluted with the minor Hb-A_1 fraction, application of an alkali denaturation method to this isolated fraction offered a new and fairly accurate procedure for the determination of small amounts of Hb-F. Applications to cases with thalassemia minor and the Hb "Lepore" abnormality have been presented.

4. The quantitative data obtained with the procedure were in agreement with the results obtained by starch block electrophoresis and carboxymethylcellulose chromatography. It was emphasized that the method was of particular use for the minor Hb fractions: A_2, B_2, Lepore and of small amounts of Hb-F.

5. The heterogeneity of the hemoglobins of normal adults, sickle cell anemia patients, and cord blood samples as observed by DEAE-cellulose chromatography

was found to be closely related to those found by electrophoretic methods and by CM-cellulose chromatography.

6. The DEAE-cellulose chromatography was found of great use for the preparation of large quantities of pure Hb-fractions: examples for Hb-A_2 and Hb-Lepore have been presented.

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