CHROMATOGRAPHY OF HUMAN HEMOGLOBIN

FACTORS INFLUENCING CHROMATOGRAPHY AND DIFFERENTIATION OF SIMILAR HEMOGLOBINS

RICHARD T. JONES* AND WALTER A. SCHROEDER

*Division of Chemistry and Chemical Engineering**, California Institute of Technology, Pasadena, Calif. (U.S.A.)*

(Received July 30th, 1962)

Several chromatographic procedures that utilize Amberlite IRC-50 or carboxymethylcellulose have been described for the detection of heterogeneity and the separation of different hemoglobins[1–12]. The procedure described by ALLEN, SCHROEDER AND BALOG[6] has been used successfully in these laboratories for the past five years during which time a number of modifications and applications have resulted[9,11,13]. Several important factors which influence the chromatographic behaviour under these conditions have become apparent. The purpose of this paper is to review briefly the procedure of ALLEN, SCHROEDER AND BALOG and its modifications, to emphasize factors that influence the chromatography of hemoglobins, and to describe a method for differentiating very similar hemoglobin components by radioactive tracers.

ALLEN, SCHROEDER AND BALOG[6] employed sodium phosphate buffers of different ionic strength and pH as chromatographic developers. All chromatograms used one developer throughout but each developer was designed for a specific task. Thus, developer No. 1 was used for the separation of normal adult hemoglobin into two zones, designated A_I and A_{II} in order of elution whereas developer No. 4 was designed to resolve the A_I zone into its components on rechromatographing.

CLEGG AND SCHROEDER[9] in extending the method devised a developer which separated normal adult hemoglobin into 6 hemoglobin fractions in one chromatogram. This developer (No. 5) was similar to the weak developers of ALLEN, SCHROEDER AND BALOG and effected a resolution of the rapidly moving minor components (A_I components) at a temperature of 6°. When the front of the main component, A_{II}, approached the lower end of the chromatographic column, the remaining components were eluted rapidly by warming the column to room temperature. With this modified procedure, CLEGG AND SCHROEDER demonstrated the presence of a small zone A_{III} that moved more slowly than the main component, A_{II}. SCHNEK AND SCHROEDER[11] separated this A_{III} zone into two components and correlated each component from the entire chromatographic separation of normal adult hemoglobin with the components obtained by starch block electrophoresis.

The procedure of ALLEN *et al.*[6] and its modification[9,11] have also been applied to studies of the chromatographic behaviour of a number of abnormal hemoglobins

* Present Address: Division of Experimental Medicine, University of Oregon Medical School, Portland, Oregon.

** Contribution No. 2877.

including Hbs, C, D, H, and S[13,14]. The problem of identifying hemoglobins on the basis of their chromatographic behavior and the factors which influence this behavior will be considered in the present paper.

MATERIALS AND METHODS

Preparation of hemoglobin solutions

Oxyhemoglobin solutions were prepared by DRABKIN's method[15] as modified by ALLEN *et al.*[6] and CLEGG AND SCHROEDER[9]. Immediately after preparation, all hemoglobin solutions were dialyzed against a chromatographic developer at 4° and stored thus until used.

Ferrihemoglobin cyanide (cyanomethemoglobin) was obtained by dialyzing solutions of oxyhemoglobin against 0.01 *M* $K_3Fe(CN)_6$ in developer No. 4 for 8 h at 4°. Excess $K_3Fe(CN)_6$ was then removed by dialysis against one of the chromatographic developers.

Radioactive hemoglobins were prepared by the procedure of BORSOOK *et al.*[16] as modified by VINOGRAD AND HUTCHINSON[17]. L-Leucine uniformly labeled with ^{14}C was the radioactive amino acid in all instances.

The concentration of hemoglobin solutions was determined with a Beckman spectrophotometer, Model DU, at a wave length which depended upon the nature of the hemoglobin derivative. The concentration of hemoglobin in mg per ml was calculated by multiplying the optical density for a 1 cm length of solution by an appropriate factor shown in Table I[18,19].

TABLE I

FACTORS FOR ESTIMATION OF HEMOGLOBIN CONCENTRATION[18,19]

Wave length (mμ)	*Hemoglobin*	*Factor*
522	Carbonmonoxyhemoglobin	1.77
522	Ferrihemoglobin cyanide	1.77
540	Ferrihemoglobin cyanide	1.44
542	Oxyhemoglobin	1.14
542	Carbonmonoxyhemoglobin	1.14
563	Oxyhemoglobin	1.77
563	Ferrihemoglobin cyanide	1.77

Preparation of chromatographic columns

Amberlite IRC-50 resin was prepared, and columns were poured according to the procedure of ALLEN *et al.*[6].

Particles of mesh size 200–250 and 250–325 have been used in different columns without apparent influence on the chromatographic properties of hemoglobin. Columns 1 × 35 cm in dimension were used for analytical work and 1.5, 2.5, and 3.5 cm in diameter by 35 cm in length for preparative work. These were maintained at 6° either by circulating water through the jackets or by using in a cold room. Before each newly poured column was used or when a column was converted from one chromatographic developer to another, the appropriate buffer was passed through at 6° at a

rate of 3–12 ml per cm^2 of cross sectional area of resin per hour until 0.8–1.5 l per cm^2 area had passed.

Bio-Rex 70 (equivalent to Amberlite IRC-50) of appropriate mesh size may be obtained from Bio-Rad Laboratories (32nd and Griffin Ave., Richmond, Calif. U.S.A.). One lot with a particle range of 200–230 mesh (sized wet and in the hydrogen form but shipped in the sodium form) was used as supplied without further treatment. It was simply suspended in developer, the pH was adjusted to the original pH of developer (with phosphoric acid in this instance), and the column was poured and equilibrated.

The least mixing of effluent as it emerges from the column may be obtained with the chromatographic columns of SPACKMAN, STEIN AND MOORE[20]. These may be purchased from Scientific Glass Apparatus Co., Inc. (catalog No. SJ-1665-1-B, item No. JC-2800, 45 cm in length above disc). The effluent may be led from the column to a fraction collector through small-bore Teflon or polyethylene tubing.

Chromatographic developers

The chromatographic developers were prepared exactly as described by ALLEN *et al.*[6] from anhydrous Na_2HPO_4 and $NaH_2PO_4 \cdot H_2O$. Substitution of these reagents by other hydrates has given unreliable results. The composition and pH of the developers which were employed are listed in refs. 6, 9 and 11. In general, the flow of developer through the column has been controlled by the hydrostatic head that was produced by raising or lowering the reservoir. It is perhaps more convenient to use a constant volume pump such as that manufactured by the Milton Roy Company, Philadelphia, Pa. (CHMMl-B-29 HC Simplex Chromatographic Minipump).

Operation of column

Prior to each chromatogram, the column was carefully adjusted to a vertical position with a level. The top 1 to 3 cm of resin bed was then stirred, the suspended resin was allowed to settle, and the supernatant developer was removed. Chromatograms were started only if the surface of the freshly settled resin was even and horizontal. Small irregularities in the surface often resulted in the formation of distorted zones and therefore poor resolution of components.

When developer still remained above the surface of the column to a depth of 1 to 3 mm, the hemoglobin solution was pipetted slowly down the side of the glass tube onto the resin bed with a bent-tip pipet. This method of application resulted in the least disturbance of the top of the resin bed, but, in the event that the surface of the resin was disturbed, the effect could be minimized by restirring the top of the column into the overlying hemoglobin solution before the sample had run in. Instead of permitting the sample to drain into the column and then rinsing with developer as described by ALLEN *et al.*[6], the hemoglobin sample was carefully layered over with one or two ml of developer, and the whole was permitted to drain into the column before the main development was begun. A sharp interface between hemoglobin solution and developer could be obtained if the latter was added very slowly onto the wall of the tube several mm above the existing fluid surface. The overlayering has resulted in more even zones and more rapid application of samples. The sample did not exceed a concentration of 100 mg/ml and was applied in a volume of 0.5–2.0 ml/cm^2 of cross sectional area of resin.

The chromatograms were developed essentially as previously described[6,9,11]. The flow rate of developer was generally 5 or 6 ml/h at 6.0° ± 0.2° for the 1 × 35 cm columns and was often doubled for the elution of slowly moving hemoglobins. When the columns were warmed to 28°, the flow rate was maintained at 8–10 ml/h. One to 10 ml fractions were automatically collected.

When zones were to be rechromatographed, the effluent was collected in chilled vessels and then refrigerated. Prior to rechromatographing, pooled fractions were concentrated by ultracentrifugation according to a procedure of VINOGRAD AND HUTCHINSON[17] and SCHNEK AND SCHROEDER[11], and then dialyzed against fresh developer. Recently, fractions have been concentrated by ultrafiltration according to a method described by SMITH[21]. The latter procedure is more convenient than concentrating by ultracentrifugation.

Analogous procedures were used for the larger preparative columns. In preparative work, especially if only one hemoglobin is to be isolated, the desired hemoglobin frequently may be isolated in fairly concentrated solution by the following procedure. The desired hemoglobin is chromatographed normally at 6° until its leading edge approaches the lower end of the column and all less strongly adsorbed material has been eluted. If more strongly adsorbed hemoglobins are present, the resin that contains them is removed from the column. The portion of the column that remains is then warmed to 40° for 10 min after which the desired hemoglobin is washed from the column in a few minutes at the maximum flow rate of developer.

Spectrophotometry and measurement of radioactivity

The optical density of each fraction of the chromatogram was measured in a 1-cm cuvette either in a Beckman model DU spectrophotometer at 280 and 415 mμ or in a Beckman model B spectrophotometer at 415 mμ. In certain cases, optical density at 522, 542, or 563 mμ was also measured. The transfer of fractions to and from the spectrophotometer cuvette has been facilitated by the use of an "automatic transferator" (Gilson Medical Electronics, Middleton, Wis.).

Radioactivity was measured with one of two counting systems. Some radioactive countings were made with Nuclear-Chicago model C1OOB automatic sample changer equipped with a model D-47 gas flow geiger counter. Other countings were made with a Nuclear-Chicago model C115 low background automatic sample changer. "Micromil" windows were used in both detector systems. Volumes of 0.5–1.0 ml of hemoglobin solution were pipetted onto aluminum, stainless steel, or copper planchets. These samples were then evaporated to dryness with an infra red lamp. In general, less than 0.5 mg of protein was placed on these planchets so that correction for self absorption was unnecessary[17]. Corrections were not made for the absorption by the buffer salts or for the absolute counting efficiency of the system. However, the same kind and volume of developer and the same counting equipment were used for any one experiment. Therefore, the relative activity of all samples within any given experiment was comparable.

RESULTS AND DISCUSSION

Factors influencing the chromatography of hemoglobins

The chromatographic behavior of hemoglobins when studied by the procedure of

ALLEN, SCHROEDER AND BALOG is dependent upon the pH and ionic concentration of the chromatographic developers, the state of equilibrium of the IRC-50 resin with the developer, and the temperature during equilibration and chromatography[6,9,11]. Observations which will not be presented confirm the importance of these factors. In addition, it has now been noted that the amount of hemoglobin on the chromatogram influences the rate of movement during chromatography. This effect of load was apparent when differences were observed in two chromatograms that were identical except for the amount of hemoglobin applied. When 50 mg of sickle

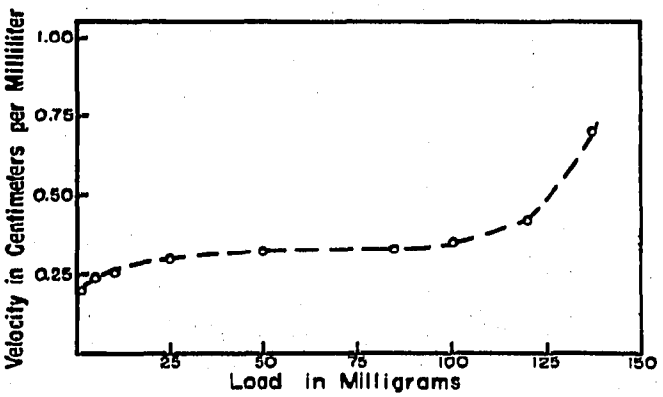

Fig. 1. The effect of the amount of hemoglobin S_{II} on the migration rate of the zone. Different quantities of hemoglobin were chromatographed with developer No. 1 on a 1 × 35 cm column.

cell hemoglobin (Hb S) was chromatographed with developer No. 1 on a 1 × 35 cm column, the peak of the main component S_{II} (see refs. 13 and 22) emerged at an effluent volume of 94 ml whereas with 10 mg of Hb S the peak emerged at 130 ml. The effect of the amount of hemoglobin S_{II} on the migration rate of the zone on the chromatographic column is depicted in Fig. 1. Similarly, the rate of movement of F_{II}, the main component in cord blood hemoglobin, is dependent upon the load on the column and this effect was confirmed by MATSUDA *et al.*[23]. Observations with other hemoglobins indicate that the influence of load on the rate of movement under the conditions described is a general phenomenon which must be considered when chromatographic movement is used to characterize an unknown hemoglobin.

Formation of double zones of single hemoglobin components

An apparent but probably false heterogeneity of hemoglobin is best illustrated in chromatograms of hemoglobin S. For example, each of more than twenty samples of hemoglobin S exhibited the formation of two zones in the region of the main component as depicted in Fig. 2. This heterogeneity of the main component is readily apparent as the zone moves down the column. If an excessively large amount of hemoglobin (200 mg on a 1 × 35 cm column) is chromatographed, the main zone appears to be homogeneous. The formation of double zones of component F_{II} has not been observed although both the amount of hemoglobin and conditions of development have been varied. The main components of hemoglobins A, C, D, H, as well as S and some minor components such as A_{IC} (ref. 6) have been observed to form double zones.

Since the initial report of this apparent heterogeneity[22], further experiments indicate that this phenomenon may be similar to the "double zoning" which occurs

with simpler compounds[24] and which has recently been reviewed by KELLER AND GIDDINGS[25]. This conclusion is based in the main on experiments with hemoglobin S_{II}. Although the formation of two zones of hemoglobin S_{II} was observed on a 1 × 35-cm column when 5–200 mg were chromatographed with developer No. 1, the detection of two peaks in the effluent fractions was generally found only when 40–100 mg were

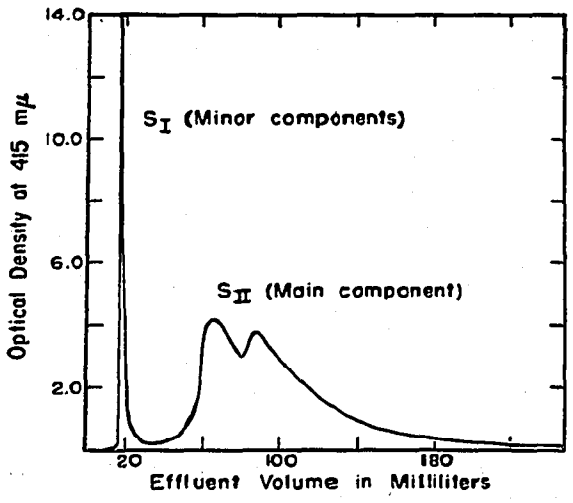

Fig. 2. Double zoning of hemoglobin S_{II}. A 50 mg sample of hemoglobin from a patient with sickle cell anemia was chromatographed on a 1 × 35 cm column of IRC-50 at 6° with developer No. 1. A slowly moving minor component, S_{III}, is not shown.

used. The more rapidly moving of the two zones proceeded down the column at an essentially constant velocity (depending upon the load), but the more slowly moving zone proceeded at a continually decreasing velocity. In a few experiments, the more rapidly moving zone appeared to divide into two new zones. Conversely, when small amounts were chromatographed, the demarcation between the more rapidly and the more slowly moving zones often disappeared before the front of the faster zone had been eluted from the column. A complete separation of the two zones has never been observed. These observations indicate a possible conversion of the main component from one chromatographic form to another (see ref. 25 for discussion of interconverting species).

When portions from each peak of the two zones of hemoglobin S_{II} were rechromatographed separately, each again produced two zones. Although a reversible change in solution is thus probable, the conversion which takes place during chromatography appears to be dependent upon interaction between hemoglobin and the resin and occurs principally during chromatographic development. For example, when the development of a chromatogram of hemoglobin S was interrupted for several hours and then continued, the behavior of the two zones was at least grossly similar to that of chromatograms in which the development had been continuous.

The N-terminal peptides from each zone of hemoglobin S_{II} as examined by the DNP-procedure[26] showed the normal ratio of α to β chain in both zones. Thus, dissociation into asymmetric subunits such as α_2, β_2 or individual α and β chains with gross separation of these subunits is not an explanation for this phenomenon. Dissociation into asymmetric subunits without gross separation of the subunits also does not seem to be involved. This was concluded from an experiment in which hemo-

globin S_{II} was applied to a column and allowed to form the two zones. Radioactive hemoglobin A_{II} was then applied in such a way that it formed double zones which passed through the region of the two S_{II} zones and were eluted from the column before the S_{II} component. Because no transfer of radioactivity to the hemoglobin S was detected, dissociation into subunits which are free to transfer between the hemoglobins[17] apparently did not occur. Because the ferrihemoglobin cyanide forms of S_{II} and A_{II} likewise produce two zones, the partial conversion of oxyhemoglobin to this form can be eliminated as an explanation of the effect.

Thus, although many possible causes for the double zone phenomenon may be eliminated, the basic cause has not yet been identified. Nevertheless these studies do indicate that the two zones are probably a single molecular species in primary structure which undergoes a reversible change from one chromatographic form to another.

Radioactive hemoglobins as chromatographic references

Differences in the chromatographic behavior of hemoglobins such as A, C, F, H, and S are so great under appropriate conditions of development that they may be easily differentiated from one another and identified simply by inspection of the column during development or by gross comparison of the elution diagrams. However, when two hemoglobins from different sources are very similar or identical in chromatographic behavior, proof of their identity or non-identity may be difficult to obtain. Small differences in chromatographic movement between an "unknown" and a reference hemoglobin cannot be detected with confidence by comparing two different chromatograms because of variations due to differences in load, temperature, and other factors which effect the chromatographic behavior of hemoglobins. Such factors obviously cannot influence the comparison if the hemoglobins are chromatographed together in a single or mixed chromatogram. Such a comparison is very sensitive if one of the two hemoglobins contains a radioactive label which does not significantly influence its chromatographic behavior. Thus, if a radioactive hemoglobin and a non-radioactive hemoglobin are mixed and chromatographed, chromatographic identity requires that the specific radioactivity be constant throughout the zone. If it is not, the two obviously are different, and the slope of the curve of specific activity through the zone, whether positive or negative, will tell the relative movement of the two.

This principle has been applied in several instances to determine whether or not a hemoglobin component was identical with an appropriately labelled known hemoglobin. For example, the chromatographic behavior of the original hemoglobin D[27,28] (hemoglobin $D_{Los\ Angeles}$) has been compared with that of hemoglobin S. This hemoglobin D is indistinguishable from hemoglobin S in its chromatographic movement when separate chromatograms of each hemoglobin are examined. This result was anticipated from the electrophoretic identity of these hemoglobins as reported by ITANO[27]. However, a detectable difference in the chromatographic movements of hemoglobins D and S became apparent when samples containing hemoglobin D were chromatographed with a small amount of radioactive hemoglobin S. Figs. 3, 4, and 5 illustrate three chromatograms which bear on this problem. Fig. 3 depicts the results from the chromatography of a mixture of radioactive hemoglobin S_{II} and non-radioactive hemoglobin S. The specific activity should be constant throughout the hemoglobin S_{II} zone of this chromatogram. However, these experiments were per-

formed before it was recognized that the ferrihemoglobin cyanide migrates more slowly than the oxyhemoglobin (see following section); the fact that the radioactive hemoglobin S_{II} was present partly as ferrihemoglobin S_{II} cyanide probably accounts for the small positive slope in the specific activity curve. Fig. 4 represents a chromatogram of a mixture of radioactive S_{II} and hemoglobin from an individual who is

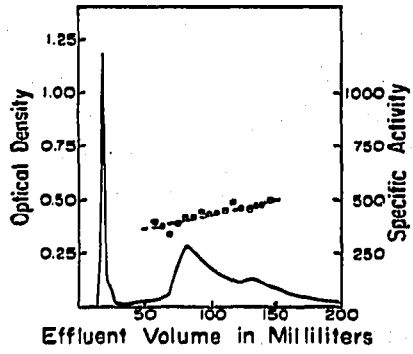

Fig. 3. Chromatogram of a mixture of 25 mg of hemoglobin S and 2.5 mg of radioactive hemoglobin S_{II} on a 1 × 35 cm column of IRC-50 at 6° with developer No. 1. — optical density at 542 mμ; —O—O— specific activity in counts per min per mg of hemoglobin.

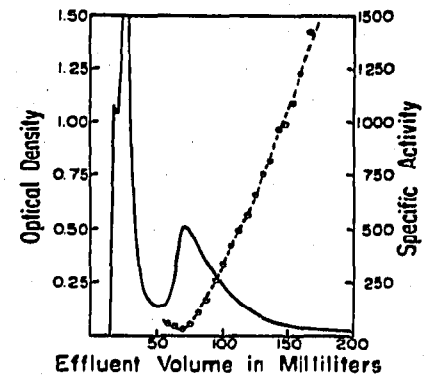

Fig. 4. Chromatogram of a mixture of 50 mg of hemoglobin from a patient heterozygous for hemoglobin A and D genes and 2.5 mg of radioactive hemoglobin S_{II}. Conditions and representation as in Fig. 3.

heterozygous for hemoglobin D and hemoglobin A genes[28]. Roughly equal amounts of hemoglobins D and A were present. The slope of the specific activity curve in this chromatogram is far different than that in Fig. 3 and indicates that hemoglobin D migrates more rapidly than S_{II}. Fig. 5 illustrates the chromatographic results from a mixture of radioactive hemoglobin S_{II} and hemoglobin from an individual with sickle cell-hemoglobin D disease (heterozygous for hemoglobin D and hemoglobin S genes[28]). The slope of the specific activity curve is intermediate between those shown in Figs. 3 and 4, and one may conclude that roughly equal amounts of hemoglobins S and D are present. The method clearly is a sensitive way to determine whether two hemoglobins are actually identical chromatographically. In this instance, other methods had showed that the two hemoglobins differed. When a radioactive reference was used, they were also shown to differ chromatographically.

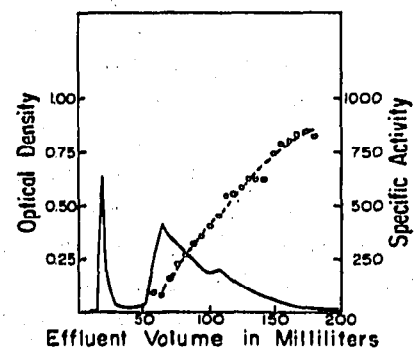

Fig. 5. Chromatogram of a mixture of 25 mg of hemoglobin from a patient with sickle cell-hemoglobin D disease and 2.5 mg of radioactive hemoglobin S_{II}. Conditions and representation as in Fig. 3.

Radioactive reference hemoglobins have been useful in showing the chromatographic similarity of the "adult" hemoglobin from an individual with thalassemia-hemoglobin H disease with that of normal adult hemoglobin[13], in investigating several minor components from hemoglobins A and S[14], and in comparing a gorilla hemoglobin with human adult hemoglobin[14].

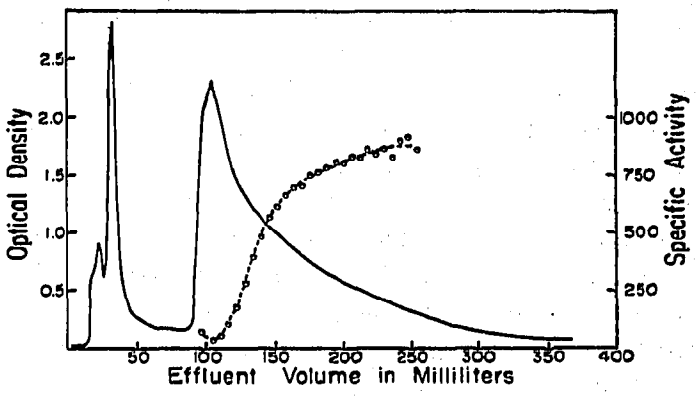

Fig. 6. Chromatogram of a mixture of 29 mg of umbilical cord blood oxyhemoglobin and 7 mg of radioactive ferrihemoglobin F_{II} cyanide on a 1 × 35 cm column of IRC-50 at 6° with developer No. 4. — optical density at 415 mμ; —○— —○— specific activity.

Differences in the chromatographic behavior of oxyhemoglobin and ferrihemoglobin cyanide

The introduction of cyanide ion into chromatographic developers in order to eliminate the formation of slowly moving extraneous zones due to the presence or formation of ferrihemoglobin (methemoglobin) during the chromatography of oxyhemoglobin was first used by ALLEN, SCHROEDER AND BALOG[6]. During the course of the present study, it became evident that the chromatographic movements of the ferrihemoglobin cyanide forms of hemoglobins A_{II}, S_{II}, and F_{II} are detectably different from their oxyhemoglobin forms. Although mixtures of these two forms of any one of these hemoglobins cannot be resolved into two separate zones and therefore appear to be the same in chromatographic behavior, small differences in their chromatographic movement can be demonstrated by radioactive tracers. Figs. 6 and 7 illustrate the results of two experiments with fetal hemoglobin. Fig. 6 depicts a chromatogram of a mixture of the oxyhemoglobin and ferrihemoglobin cyanide forms of hemoglobin F_{II}. Clearly, ferrihemoglobin F_{II} cyanide was not uniformly distributed throughout the

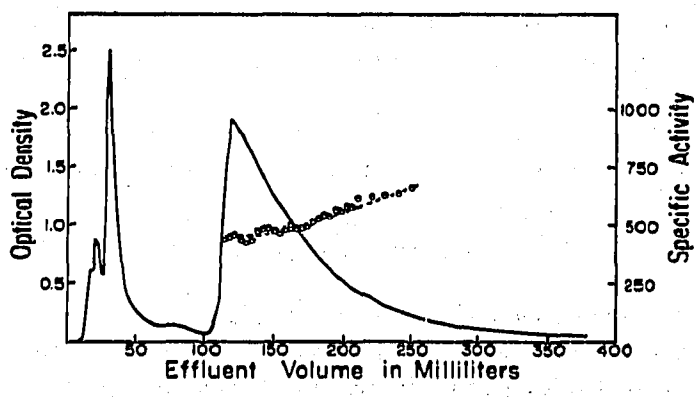

Fig. 7. Chromatogram of a mixture of 29 mg of umbilical cord blood ferrihemoglobin cyanide and 7 mg of radioactive ferrihemoglobin F_{II} cyanide. Conditions and representation as in Fig. 6.

F_{II} zone. Fig. 7 represents a control experiment in which the same mixture was first oxidized completely to ferrihemoglobin cyanide before chromatography. The distribution of the radioactivity is essentially uniform throughout the main portion of the F_{II} zone in this control. Although the migration rates of the oxyhemoglobin and ferrihemoglobin cyanide forms in the experiment shown in Fig. 6 may have been influenced by the difference in the load of the two forms, the experiments do indicate a definite difference in the chromatographic behavior of these two forms of fetal hemoglobin.

Analogous experiments have been made with hemoglobin A_{II} and Developer No. 2 and with hemoglobin S_{II} and Developer No. 1. In each of these experiments, equal amounts of the two forms were compared and positive slopes in the specific activity curves similar to Fig. 6 were observed. Probably the ferrihemoglobin cyanide form of each hemoglobin is more strongly adsorbed than its corresponding oxyhemoglobin form. However, because the difference in chromatographic behavior of the two forms is so small, the practice of adding potassium cyanide to the chromatographic developers in order to eliminate the extraneous zones of free ferrihemoglobin is certainly still useful.

Comments on the use of various developers

The chromatographic procedures which are under discussion here have been applied to many problems in the study of hemoglobin. They are based largely upon the premise that the chromatograms should be carried out under equilibrium conditions if at all possible because of the slowness with which the columns of IRC-50 come to equilibrium. As a result, gradient elution has never been used, and specific developers have been devised to solve specific problems. In conclusion, it seems desirable to indicate the way in which various developers may be most usefully applied.

Developer No. 1 is the strongest developer. It is useful for the separation of hemoglobins with grossly different behavior such as F, A, S, and C. It may be used to separate the minor components of hemoglobins S and C from the major component.

Developer No. 2 produces a more complete separation than developer No. 1 of F from A and also of the A_I components (the nomenclature of minor components is given in refs. 6 and 9) from A_{II}.

Developer No. 4 resolves umbilical cord hemoglobin into F_I and F_{II} while the hemoglobin A that is present is very strongly fixed. It is especially useful in the resolution of A_I and S_I into their components.

Developer No. 5 was devised for and is largely used for the separation of the minor components of hemoglobin A from the main component, A_{II}, in a single chromatogram.

Developer No. 6 is the weakest of the developers and is designed to retard rapidly moving components. It is especially useful in the isolation of hemoglobins γ_4, H, F_I, and A_{Ic}.

ACKNOWLEDGEMENTS

This investigation was carried out during the tenure of a Postdoctoral Fellowship to R. T. J. from the Heart Institute, U.S. Public Health Service and was supported in part by grants (H-2558 and CY-3374) from the National Institutes of Health, United States Public Health Service.

The authors wish to thank Drs. J. VINOGRAD, W. HUTCHINSON, H. BORSOOK, and H. LAMFROM for help in the preparation of radioactive hemoglobins. Abnormal hemoglobins and blood samples with high reticulocyte counts were procured with the help of Drs. P. STURGEON and W. BERGREN of the Childrens Hospital and Dr. S. RAPAPORT of the Los Angeles County Hospital. The technical help of Mrs. J. CORMICK, Mrs. K. MCCALLA, and Mrs. J. SHELTON is appreciated.

SUMMARY

A procedure for the chromatographic separation of human hemoglobins on Amberlite IRC-50 is reviewed. Important factors which influence the chromatographic behavior of hemoglobins with this procedure include temperature, pH and ionic concentration of developers, state of equilibrium of the resin, amount of hemoglobin, and oxidation state of the heme in the hemoglobin applied.

A procedure for the comparison of hemoglobins with similar or identical chromatographic behaviors using ^{14}C-labeled hemoglobins is presented. Differences in the chromatographic behavior of hemoglobins S and D and of the ferrihemoglobin cyanide and oxyhemoglobin forms of hemoglobin F were demonstrated by this radioactive tracer technique.

REFERENCES

1 N. K. BOARDMAN AND S. M. PARTRIDGE, *Biochem. J.*, 59 (1955) 543.
2 T. H. J. HUISMAN AND H. K. PRINS, *J. Lab. Clin. Med.*, 46 (1955) 255.
3 H. K. PRINS AND T. H. J. HUISMAN, *Nature*, 177 (1956) 840.
4 M. MORRISON AND J. L. COOK, *Science*, 122 (1955) 920.
5 J. L. COOK AND M. MORRISON, *Federation Proc.*, 15 (1956) 235.
6 D. W. ALLEN, W. A. SCHROEDER AND J. BALOG, *J. Am. Chem. Soc.*, 80 (1958) 1628.
7 T. H. J. HUISMAN, E. A. MARTIS AND A. DOZY, *J. Lab. Clin. Med.*, 52 (1958) 312.
8 F. J. GUTTER, E. A. PETERSON AND H. A. SOBER, *Arch. Biochem. Biophys.*, 80 (1959) 353.
9 M. D. CLEGG AND W. A. SCHROEDER, *J. Am. Chem. Soc.*, 81 (1959) 6065.
10 T. H. J. HUISMAN AND C. A. MEYERING, *Clin. Chim. Acta*, 5 (1960) 103.
11 A. G. SCHNEK AND W. A. SCHROEDER, *J. Am. Chem. Soc.*, 83 (1961) 1472.
12 C. A. MEYERING, A. L. M. ISRAELS, T. SEBENS AND T. H. J. HUISMAN, *Clin. Chim. Acta*, 5 (1960) 208.
13 R. T. JONES AND W. A. SCHROEDER (to be published).
14 R. T. JONES, *Ph. D. Thesis*, California Institute of Technology, Pasadena, 1961.
15 D. L. DRABKIN, *Arch. Biochem.*, 21 (1949) 224.
16 H. BORSOOK, C. L. DEASY, A. J. HAAGEN-SMIT, G. KEIGHLEY AND P. H. LOWY, *J. Biol. Chem.*, 196 (1952) 669.
17 J. VINOGRAD AND W. D. HUTCHINSON, *Nature*, 187 (1960) 216.
18 W. D. HUTCHINSON, *Ph. D. Thesis*, California Institute of Technology, Pasadena, 1960.
19 National Academy of Science–National Research Council, *Science*, 127 (1958) 1376.
20 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
21 I. SMITH, *Chromatographic and Electrophoretic Techniques*, Wm. Heinemann Medical Books, Ltd., London, 1960, p. 62.
22 W. A. SCHROEDER, *Fortschr. Chem. Org. Naturstoffe*, 17 (1959) 322.
23 G. MATSUDA, W. A. SCHROEDER, R. T. JONES AND N. WELIKY, *Blood*, 16 (1960) 984.
24 W. A. SCHROEDER, *Ann. N. Y. Acad. Sci.*, 49 (1948) 204.
25 R. A. KELLER AND J. C. GIDDINGS, *J. Chromatog.*, 3 (1960) 205.
26 H. S. RHINESMITH, W. A. SCHROEDER AND L. PAULING, *J. Am. Chem. Soc.*, 79 (1957) 4682.
27 H. A. ITANO, *Proc. Natl. Acad. Sci.*, 37 (1951) 775.
28 P. STURGEON, H. A. ITANO AND W. R. BERGREN, *Blood*, 10 (1955) 389.