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MICROCHROMATOGRAPHY OF HEMOGLOBINS

VIII. A GENERAL QUALITATIVE AND QUANTITATIVE METHOD IN PLASTIC DRINKING STRAWS AND THE QUANTITATIVE ANALYSIS OF Hb-F

W.A. SCHROEDER and LAURENCE A. PACE

Division of Chemistry and Chemical Engineering, California Institute of Technology, 164-30, Pasadena, Calif. 91125 (U.S.A.)*

and

T.H.J. HUISMAN

Laboratory of Protein Chemistry and Comprehensive Sickle Cell Center, Medical College of Georgia and Veterans Administration Hospital, Augusta, Ga. 30902 (U.S.A.)

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SUMMARY

This microchromatographic procedure for the quantitative analysis of the hemoglobin components in a hemolysate uses columns of DEAE-cellulose in a plastic drinking straw with a glycine—KCN—NaCl developer. Not only may the method be used for the quantitative analysis of Hb-F but also for the analysis of the varied components in mixtures of hemoglobins.

INTRODUCTION

Qualitative and quantitative microchromatographic procedures have been devised for the analysis of a variety of hemoglobin mixtures [1—7]. These methods have also been extended and used for the separation of complex mixtures of hemoglobins on more conventionally sized columns with gradient development [8—10]. This paper describes a miniaturization and simplification of one of these conventional procedures [9, 10]: the chromatographic column is packed in a plastic drinking straw and developed without a gradient

*Contribution No. 5626.

to the desired point of separation after which the column is sectioned with a sharp knife, each section is eluted separately, and the individual components are quantitatively estimated. Rather complex mixtures may be studied in this simple way, and the method is particularly suited for the quantitative determination of human hemoglobin F (Hb-F).

MATERIALS

Blood samples

Specimens were obtained through the Los Angeles Sickle Cell Center and the Childrens Hospital of Los Angeles. EDTA or heparin was the anti-coagulant.

For chromatograms in straws, whole blood was usually used to prepare the sample. When hemolysates were used, a previously described procedure [7] was applied. Only occasionally were samples dialyzed against a large volume of appropriate solution. For conventionally sized chromatograms [9, 10], the sample was prepared by hemolyzing saline-washed cells with four times their volume of water and 0.3 volume of carbon tetrachloride. After centrifuging to remove cell debris, an appropriate volume that contained about 40 mg of hemoglobin was applied directly to the column.

Equipment

Clear plastic drinking straws of the type that may be purchased from restaurant suppliers were used to contain the chromatographic column: their I.D. is about 0.6 cm and their length is 20 cm. It is convenient to have Lucite fittings* for the top and bottom of the straw (Fig. 1A). Only diameter A (0.572 cm; 0.225 in.) of the dimensions in Fig. 1A is critical and may have to be varied slightly, as our experience shows that about 20% of a given lot of straws have the wrong diameters for a given dimension of fitting. In place of such fittings, a 2—3 cm length of constricted glass tubing which is plugged with cotton and attached to the straw with rubber tubing is a somewhat less satisfactory bottom fitting; the other end of the straw is fixed with rubber tubing to a supply of solution. Any perforated object that fits the straw tightly can probably be used as a bottom fitting.

Developers

Two solutions, developers No. 1 and 2 of Ref. 7, are used. The ion exchanger is equilibrated with No. 1 which is 0.2 *M* glycine—0.01% KCN (15 g glycine and 0.1 g KCN per liter) and the chromatogram is developed with No. 2 which is 0.2 *M* glycine—0.015 *M* NaCl—0.01% KCN (15 g glycine, 0.88 g NaCl, and 0.1 g KCN per liter). The pH of these solutions is unadjusted.

Preparation of ion exchanger

DEAE cellulose (DE-52, microgranular and pre-swollen; Whatman, Clifton, N.J., U.S.A.) was equilibrated with developer No. 1 as previously described

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*Such Lucite fittings may be obtained by sending US$ 3.00 per pair to W.A. Schroeder, 164-30, California Institute of Technology, Pasadena, Calif. 91125, U.S.A.

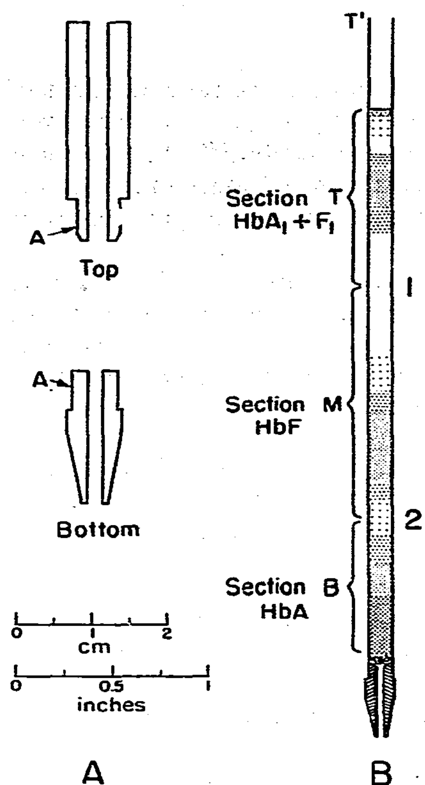


Fig. 1. (A) Dimensions of fittings. (B) Appearance of a typical completed chromatogram.

[3]. As will be discussed below, the chromatograms are equally satisfactory regardless of whether the pH of the ion exchanger is not adjusted or is adjusted as low as pH 7.1. Because of problems attendant to the adjustment of pH in stirred suspensions [3], measurement can also be made in the supernatant solution above the settled resin and adjustment made until the desired pH under these conditions is reached.

PROCEDURE

Chromatography

After a bottom fitting has been attached to a plastic straw and a small plug of cotton inserted, a 17.5-cm column is poured. The sample is approximately 10 mg of hemoglobin (5 mg if cord blood is used). This may be undialyzed hemolysate in 0.2–0.3 ml of water or a mixture of three drops of blood and 12 drops of water which is kept at room temperature for 5—10 min to allow for hemolysis. After the sample has been applied, the tube above the column is filled with developer No. 2, a top plastic fitting is inserted, and the assembly is attached by tubing to a funnel. A 60-ml portion of developer No. 2 is placed in the funnel and allowed to run through. If the initial liquid head above the bottom of the column is 20, 40, or 60 cm, the flow-rate is approximately 3, 6 or 9 ml/h. Because of the limiting amount of developer, the chromatogram may be started at any convenient time and will complete itself without attention when it runs dry.

*Elution of the zones**

The final appearance of the chromatogram will depend somewhat on the goal of the analysis. Fig. 1B depicts the typical final appearance of a chromatogram in which, for example, Hb-F is elevated in the sample. Hb-A_2 will have passed through as will part of the Hb-A. These two hemoglobins may be collected separately or together. The hemoglobins that remain on the column are eluted in the following way.

After the straw has been marked at 1 and 2 which are the midpoints of the interzones (Fig. 1B), the top fitting is removed and the straw is inserted into a 5-cm length of slightly oversize glass tubing as far as point 1. The end of the glass tubing that is positioned at point 1 should be constricted carefully so that the straw is relatively tightly held and will not flex when cut. The column is cut at point 1 and then at point 2.

After a 10-cm glass extension has been attached with rubber tubing to the top of the section B, the extension is filled with 2% KCN and the remaining Hb-A is eluted and combined with the portion that has passed through the column.

The middle section M is attached at one end with rubber tubing to a 2—3-cm piece of constricted glass tubing which has a cotton plug and at the other end to an extension which is filled then with 2% KCN for elution.

Finally, a small plug of cotton and a bottom fitting are inserted into the top section T at T′. By tapping at T′, the column will slide against the cotton plug. Then 2% KCN is added for elution.

Absorbance of all fractions is read at 415 nm and percentages are calculated as usual.

Other methods

The alkali denaturation procedure was a modification [6] of that of Betke et al. [11]; results are listed as % F_{AD}. Microchromatographic determinations of Hb-F in the absence of Hb-A used a recently described method [6]; results are given as % F_{micro}. Chromatography on conventionally sized columns of DEAE cellulose followed the method of Abraham et al. [9, 10]; results are denoted by % F_{DE}.

RESULTS AND DISCUSSION

The initial goal of this study was the devising of a quantitative microchromatographic method for Hb-F in the presence of Hb-A to complement the method for Hb-F in the presence of Hb-S and/or Hb-C [6]. As the study progressed, however, it became apparent that the complete quantitative com-

*This description is more time-consuming and difficult than the actual procedure. After the cutting has been done, an alternative procedure may be used for elution. Each section is blown separately into a centrifuge tube, and the inside of the straw is rinsed with 2% KCN. After more KCN has been added, the suspension is shaken for a few minutes. After centrifuging, the supernatant solution is removed and the procedure is repeated. The combined supernatant solutions are used for spectrophotometry. For satisfactory quantitative work, the volume of each washing with KCN solution must be at least 10 times the volume of the centrifuged ion exchanger.

position of the hemoglobin in a sample could be obtained. We discuss first the general application and then the specific use of the method for the quantitative determination of Hb-F (expressed as F_{St}).

The general nature of the chromatogram

In a mixture that might contain Hb-A_2, Hb-C_0, Hb-C_1, Hb-S_0, Hb-S_1, Hb-A_0, Hb-A_1, Hb-F_0, and Hb-F_1 in various combinations, Hb-A_2 or a mixture of Hb-A_2 and Hb-C_0 will form the first zone to move down the column. Hb-A_2 and Hb-C_0 are the hemoglobins whose rate of movement is influenced by the pH to which the ion exchanger has been adjusted. If the DE-52 is equilibrated with developer No. 1 without pH adjustment, the pH of the supernatant solution is about 7.6. If the chromatogram is developed on this ion exchanger with developer No. 2, the Hb-A_2 (or Hb-C) forms a narrow zone and moves slowly. On the other hand, if the pH is adjusted to 7.1 in the supernatant solution, Hb-A_2 (or Hb-C) moves rapidly as a somewhat diffuse zone. Thus, quantitative estimation of Hb-A_2 is most conveniently done by using ion exchanger at the lower pH. At a flow-rate of 6 ml/h, collection of the Hb-A_2 can begin after about an hour and is complete at the end of two hours.

Hb-S_0 follows Hb-A_2 (or Hb-A_2 and Hb-C_0) through the column. If the sample contains no Hb-A, then Hb-S_1 is apparent in the position of Hb-A_0 above and well separated from Hb-S_0. In the presence of Hb-S_0 and Hb-A_0, Hb-A_0 and Hb-S_1 coincide, and analogously Hb-S_0 and Hb-C_1. Above Hb-A_0 (and/or Hb-S_1) is Hb-F_0 and finally near the top of the column will be Hb-A_1 + F_1. The identity of the zones has been confirmed by comparison with other chromatographic procedures and electrophoresis.

The goal of the analysis will determine the course of the procedure. Thus, if all components of an A-S sample are to be determined, the chromatogram would be started with ion exchanger at pH 7.1 two hours before the operator would leave so that Hb-A_2 may be collected separately. Collection in the second flask is then begun and the chromatogram is allowed to go to completion (and go dry) without further attention. If 55 ml rather than 60 ml of developer No. 2 is used, the Hb-S_0 will be almost completely in the second flask but some will be at the bottom of the column so that the midpoint between Hb-S and Hb-A_0 + Hb-S_1 is apparent. Finally, the midpoints between Hb-S_0 and Hb-A_0 + Hb-S_1, between the latter and Hb-F_0, and between Hb-F_0 and Hb-A_1 + Hb-F_1 are marked, the column is sectioned, the materials are eluted, and the quantities are determined.

If, on the other hand, the percentage of Hb-F_0 is the main interest, all components that precede it can be collected in one flask and, after sectioning and eluting, Hb-A_1 + Hb-F_1 can be combined with them while Hb-F_0 alone is eluted into a second flask. Actually, the determination of the complete composition requires so little additional effort that this procedure has rarely been used.

Complete determination of hemoglobin composition

Complete quantitative analysis of the components in the hemoglobin from a variety of hematological conditions was done both by conventional chromatography [9, 10] and by the straw method. Both procedures use DE-52

TABLE I

COMPARISON OF QUANTITATIVE DATA BY CONVENTIONAL CHROMATOGRAPHY [9, 10] AND BY THE STRAW METHOD

The first of each pair of numbers is the result from conventional chromatography [9, 10] and in brackets from the straw method.

Condition	Hemoglobin composition (%)
Normal adult	A_2 = 2.4(2.6); A = 92.5(89.0); F = 1.2(2.2); A_1 + F_1 = 3.9(6.2)
Normal adult	A_2 = 2.3(2.5); A = 88.7(84.8); F = 2.6(3.1); A_1 + F_1 = 6.3(9.6)
Normal adult	A_2 = 2.5(2.4); A = 90.5(86.1); F = 1.2(2.2); A_1 + F_1 = 5.8(9.4)
Normal adult	A_2 = 2.5(2.7); A = 91.7(89.8); F = 1.3(1.5); A_1 + F_1 = 4.5(6.0)
βthal trait (adult)	A_2 = 5.2(5.3); A = 87.4(84.8); F = 2.4(2.9); A_1 + F_1 = 5.1(7.1)
S trait (adult)	A_2 = 4.2(3.7); S = 35.9(35.0); A = 56.0(55.4); F = 1.2(1.6); A_1 + F_1 = 2.7(3.8)
D trait (adult)	A_2 = 2.2(2.2); D = 37.4(37.4); A = 47.5(45.1); F = 7.6(8.0); A_1 + F_1 = 5.5(7.3)
E trait (adult)	A_2 + E = 32.3(28.9); A = 61.5(62.9); F = 0.7(1.8); A_1 + F_1 = 5.3(6.4)
New York trait (adult)	A_2 = 2.8(3.4); A = 53.7(51.9); NY = 39.5(39.2); ? = 4.0(5.5)
SS disease (adult)	A_2 = 2.6(2.3); S = 67.3(73.0); F = 23.4(23.6); F_1 = 6.8(10.6)
S-HPFH (adult)	A_2 = 1.8(1.6); S = 64.0(65.2); F = 28.2(26.9); F_1 = 5.9(6.4)
SC disease (adult)	A_2 + C = 37.6(41.3); S = 42.5(37.0); F = 16.6(17.3); F_1 = 3.3(7.0)
SC disease (cord blood)	A_2 + C = 8.5(8.5); S = 7.9(7.5); F = 73.2(68.1); F_1 = 10.5(15.9)

as ion exchanger and glycine—KCN—NaCl solution as developer. Table I presents the results. In the data as given, the minor components (except for A_1 and F_1) are summed with the appropriate major component. As previously noted [10] and as will be discussed further below, F_{DE} (conventional chromatography) is not a true measure of Hb-F in the normal individual or if the Hb-F is less than 2—3%. The results of the straw method are in good agreement with those by conventional chromatography under almost identical conditions. The agreement compares favorably with what one might expect from duplicates by either method. The higher value for A_1 + F_1 or F_1 by the straw method is due to the complete removal of material from the top section in contrast to the tendency for trailing of the last zone and incomplete removal from the conventional chromatogram.

Quantitative analysis of Hb-F

In most chromatographic systems, Hb-F is mixed with minor components of Hb-A. The present procedure which employs a single developer provides the same excellent separation of Hb-F that is shown by conventionally sized columns with gradient elution [9, 10]. Above Hb-F_0 in the upper two centimeters of the straw chromatogram are Hb-A_1 and Hb-F_1 as two distinct but not well separated zones. In older samples, these increase. The procedure has now been applied to a wide variety of samples: normal adults, individuals with Hb-A and increased Hb-F, as well as patients with sickle cell anemia, SC disease, etc.

Reproducibility. In a series of 12 simultaneous determinations with the same sample of adult blood to which a small amount of cord blood had been added, the F_{St} averaged 5.1% with a range from 4.8 to 5.5%. In the same series, Hb-A_2 was 2.4% (range 2.3—2.5%), Hb-A_0 was 83.3% (range 82.5—83.8%), and Hb-A_1 + F_1 was 9.2% (range 8.9—9.4%). In a large series of duplicate determinations, the two values with few exceptions agreed within 10% of each other despite wide variation in the percentage of Hb-F.

Quantitative analysis in known mixtures. The straw method has been applied to varied mixtures of hemoglobin from a normal adult hemolysate and the hemolysate from the blood of an HPFH homozygote who has 100% Hb-F. These same mixtures had been analyzed by conventional chromatography and alkali denaturation, and provide the basis for Fig. 3 of ref. 10. The data are presented in Table II. In this normal adult, F_{St} was apparently not observed to be 1—2.5% (see below) and A_1 + F_1 was higher than the commonly determined 6—9% (see below). Although the % F_{St} will be higher than actual when the % F is low, the straw method can be considered to provide an accurate measure of the % F above about 10% F. This conclusion also obtains from the comparison of results by both the straw method and conventional chromatography (Fig. 2A); the latter is considered to be the most accurate procedure for the determination of Hb-F [10].

Hb-F in normal adults. So small a percentage of Hb-F is present in the normal adult that it is not apparent as a zone on the chromatogram. However, if as little as 1—2% of cord blood is added, a definite zone of Hb-F can be detected. When the amount of Hb-F in a sample is small, it is advisable to use a simultaneous control in which Hb-F is definitely elevated. In this way, the positions at which the cuts should be made can be more precisely delineated. When the method was applied to 219 adults with normal hematological indices, the results of Fig. 3 were obtained. A slight tailing of the Hb-A_0 is responsible for the values. The % F_{AD} (alkali denaturation) from 36 of 37 randomly selected samples of the series ranged from 0.4 to 1.6%; one sample had 3.0%

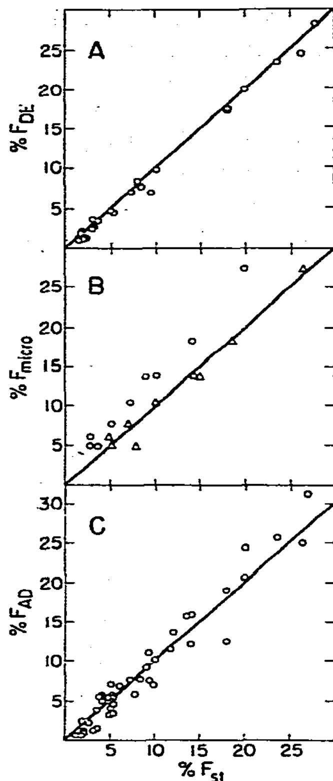
TABLE II

COMPARISON OF PERCENTAGES OF F_{St} WITH THEORETICAL PERCENTAGES IN KNOWN MIXTURES

Data provided by Dr. E.C. Abraham, Augusta, Ga, U.S.A.

$F_{Theor.}$	Determined values			Calculated values*		
	$A_0 + A_2$	F_0	$A_1 + F_1$	A_1	F_1	$F_0 + F_1$
2	82.4	4.6	13.1	12.0	1.0	5.6
5	79.3	6.3	14.4	11.6	2.8	9.1
10	75.0	9.5	15.5	11.0	4.5	14.0
15	73.5	11.0	15.5	10.7	4.8	15.8
20	68.5	13.8	17.7	10.0	7.7	21.4
25	62.8	19.6	17.6	9.2	8.4	28.0
30	59.9	22.0	18.1	8.7	9.4	31.4
40	53.4	26.0	20.6	8.0	12.6	38.6
50	48.2	31.0	20.8	7.0	13.8	44.8
60	34.5	40.1	25.4	5.0	20.4	60.5
70	28.6	45.1	26.3	4.2	22.1	67.2
80	19.8	53.3	26.9	2.9	24.0	77.3
AA	87.2		12.8	12.8	0	

*Approximate amounts of Hb-A_1 in the mixtures were calculated on the analysis of the hemolysate of the blood of the normal adult (AA). A constant proportionality 87.2 (% $A_0 + A_2$): 12.8 (% A_1) was assumed for all the mixtures.



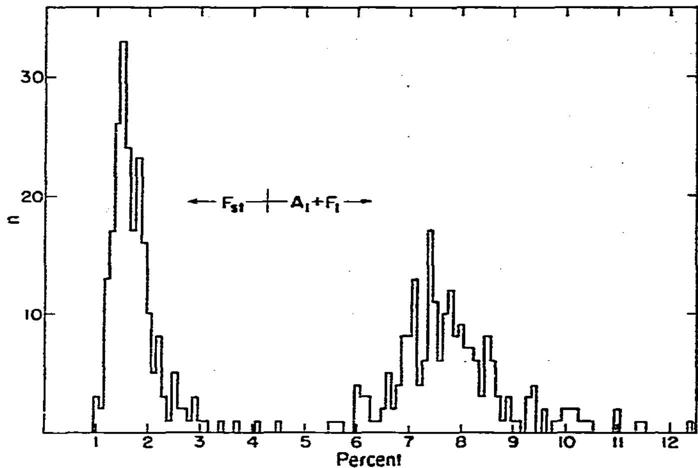


Fig. 2. Comparison of results by the straw method (F_{St}) with those from (A) conventionally sized chromatograms (F_{DE}), (B) from microchromatograms in the absence of Hb-A_0 (F_{micro}) (○ = Hb-F_{St}; △ = Hb-F_{St} + Hb-F_1), and (C) from alkali denaturation (F_{AD}).

Fig. 3. Range of values for % F_{St} and % Hb-A_1 + F_1 in samples from normal adults.

F_{St} and 3.2% F_{AD} . Although the method provides an inexact measure of Hb-F in the normal adult, the normal range is about 1—2.5% F_{St}. Thus, when Hb-A_0 is the major hemoglobin, the % F_{St} will be 1—2% higher than the actual Hb-F because of the tailing of Hb-A_0. The Hb-A_1 + F_1 tends to fall between 6 and 9% (Fig. 3).

Comparison with other methods. The straw procedure has been compared with results from conventionally sized chromatograms (F_{DE}), from alkali denaturation (F_{AD}), and from another micro procedure (F_{micro}).

A comparison of F_{St} and F_{DE} data is given in Fig. 2A. There is an excellent correlation in the results. Because the F_{DE} method has excellent accuracy at values above 10% Hb-F [10], it may be concluded that the straw method provides a valid measure of Hb-F.

When Hb-A is absent, a previously described microchromatographic method [6] may be used for Hb-F. When F_{micro} and F_{St} data were compared for SS or SC samples, the results in Fig. 2B were obtained. In Fig. 2B, the data are plotted both as F_{St} and as F_{St} + F_1 against F_{micro}. The sum of F_{St} + F_1 agrees with F_{micro} because F_0 + F_1 do not separate in the latter method.

Fig. 2C depicts a comparison of F_{St} with F_{AD} results. The excellent agreement is somewhat misleading. F_{St} does not include Hb-F_1 yet both are alkali resistant. The alkali denaturation procedure appears to be measuring a proportion of $F_0 + F_1$ that perhaps fortuitously equals the fraction $F_0/(F_0 + F_1)$ (see also below).

Nature of Hb-A_1 + F_1. When Hb-A is absent, the top zone consists of Hb-F_1 only. Although this top zone contains mainly the acetylated version of Hb-F_0, heterogeneity is apparent in the straw chromatograms as also in more conventionally sized chromatograms [10]. When fresh cord blood samples are chromatographed on the straws, the ratio of F_1 + other components to F_{St} is 1:4 to 1:3. This is comparable to previously reported data [12]. Likewise, in 18 SS samples with F_{St} above 10%, the ratio of F_1 to F_{St} averaged 1:3. Because Hb-F_1 appears to be structurally identical with Hb-F_0 except for the acetylation of its γ chains, it must be considered as part of the total Hb-F of the sample. In Hb-A-containing samples, Hb-A_1 and Hb-F_1 are eluted together in the straw method. Because the quantity of Hb-F_1 is 20—25% that of the F_{St} in cord blood samples and SS patients, the total Hb-F in an Hb-A-containing sample may reasonably be calculated to be 1.25 F_{St}.

Some samples have a yellow zone that is strongly fixed at the top of the chromatogram. Although its spectrum is not that of hemoglobin, it does have absorbance at 415 nm. Consequently, if present, it should be removed from the top before cutting and discarded prior to elution of $A_1 + F_1$.

If the sample contains electrophoretically fast-moving hemoglobins such as Hb-J or Hb-N, they will be in the region of $A_1 + F_1$ [7]. Potentially, they could be confused with an old sample with an accumulation of altered products.

Technical considerations

Concentration of NaCl. The concentration of NaCl is critical for the separation of Hb-F_0 from Hb-A_0. If it is 0.013 or 0.017 *M* instead of 0.015 *M*, the separation is unsatisfactory.

Flow-rate. Most experiments have used a flow-rate of about 6 ml/h. At 9 ml/h, the zones are more diffuse and the separations worsen. Little seems to be gained by reducing the flow-rate to 3 ml/h.

Volume of developer. This variable determines the final appearance of the chromatogram. Whether more or less than the recommended 60 ml is used will depend upon the objectives of the analysis. For example, if a complete analysis of an AS sample is desired, it is advantageous to use a few milliliters less so that the interzone between Hb-A and Hb-S is still apparent in the lower column. On the other hand, if the determination of Hb-F is of main importance, a few milliliters more might be added so that, while most of the Hb-A in in the filtrate, the Hb-A—Hb-F interzone is still obvious.

Quantity of sample. For adult samples with normal or moderately reduced packed cell volumes, the hemoglobin in three drops (about 0.07—0.08 ml) or one microhematocrit tube is adequate. If the hematocrit is very low, it is desirable to centrifuge and remove some plasma. Only one or two drops of cord blood should be used because the high percentage of Hb-F will prevent adequate separation from Hb-A if the amount of Hb-F on the column is high.

Storage of samples. If samples are refrigerated, blood may be stored for 3–4 weeks as blood or hemolysate, and the % F_{St} will not change significantly. On the other hand, increasing amounts of material fall in the $A_1 + F_1$ region in older samples. The increase is observed within a few days in hemolysates but not for about 2 weeks with blood.

Time required. This microchromatographic procedure requires more time than other microchromatographic methods. However, little attention is required of the operator. We have routinely made 12 analyses per day. The chromatograms have been started about 2 h before the operator leaves for the night so that Hb-A_2 may be collected. The chromatogram completes itself (goes dry) during the night and cutting, elution, and spectrophotometric determination then are made in the morning. If an operator devoted his time exclusively to this method of analysis, 20–25 analyses per day seem a reasonable goal.

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