the method to much harder gels may require use of a pump, such as the system described by HEITZ AND COUPEK[6], for compressive packing of the column. A subsequent publication will describe additional experiments at a wider range of gel compressions using a variety of gels, particle sizes, flow rates, and compression techniques[7].

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Received December 23rd, 1969

J. Chromatog., 47 (1970) 490–493

CHROM. 4604

Miniature column chromatography of hemoglobins

Liquid chromatography, an extremely valuable tool for the study of biological compounds, is more easily adapted to the quantitative isolation of component compounds than is electrophoresis. However, chromatography usually requires more time, labor and material than does electrophoresis. To overcome these disadvantages, a simple chromatographic method has been developed using short, narrow bore columns that allow relatively rapid flow rates because of diminished back pressure. Although such a system can be used to study many substances, the separation of hemoglobins is reported here as an illustration of the efficacy of the technique.

Methods and material

Materials. The column, called a "minicolumn" (see Fig. 1), consists of a piece of glass tubing, approximately 2 mm I.D. and 150 mm long, with Technicon polyethylene nipples, size N-5, abutted snugly against each end by the use of vinyl tubing. A tiny plug of glass wool inserted in the column against the lower polyethylene nipple supports the resin in the column.

Both diethylaminoethyl-cellulose (DEAE-cellulose) and carboxymethyl-cellulose (CM-cellulose) have been used as resins for packing the columns although CM-cellulose does not give as consistent results as does DEAE-cellulose. The DEAE-cellulose was Selectacel No. 72, type 40, purchased from Schleicher and Schüll and the CM-cellulose was Whatman CM32 or Bio-Rad Cellex CM. Since adequate removal of fines from CM32 was difficult, excessive back pressure was a problem at times. For some purposes Cellex CM, lot No. 5170, was satisfactory.

The buffers used in this work were those of HUISMAN *et al.*[1,2]. Since the buffer

gradient volumes were small (150–500 ml) it was convenient to use two chambers of a varigrad to make a linear pH gradient. 0.05 *M* trihydroxymethylaminomethane (Tris)–HCl, pH 8.7 and 7.7, was used to develop DEAE-cellulose chromatography and 0.01 *M* phosphate, pH 6.5 and 8.4, was used to develop CM-cellulose chromatography.

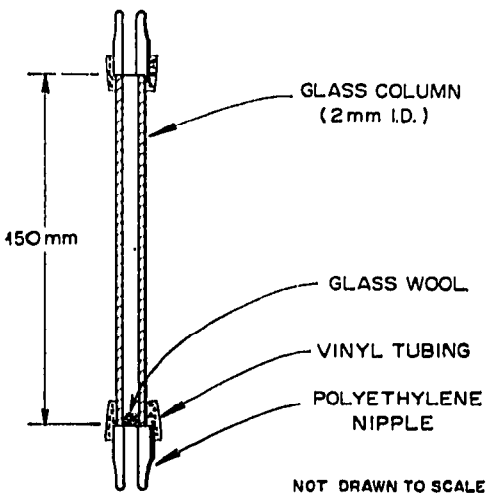

Fig. 1. "Minicolumn" for chromatography.

Procedure. The columns were loaded by introducing a thin slurry of the exchanger into a buffer-filled column as the buffer was slowly and continuously allowed to drip from the bottom of the column. The column was kept full by the addition of slurry with a pipet. Pouring a column required only a few minutes. If the flow rate at this stage was so slow that an excessive time was required for pouring, the back pressure was considered to be too high. This problem was corrected either by use of a smaller glass wool plug or removal of fines from the resin. After the columns were filled with resin, they were equilibrated with starting buffer for 15–30 min.

After equilibration, undialyzed hemolysates containing 1–5 mg of hemoglobin in 20–50 μl were applied to the columns with a Hamilton syringe. With buffer still above the resin, the tip of the Hamilton syringe needle was inserted to just above the top of the resin and the sample slowly introduced. The columns were then filled with starting buffer and the elutions started with flow rates of about 30 ml/h maintained by a Buchler peristaltic pump.

To minimize mixing of fractions, small bore (0.7 mm I.D.) Teflon tubing was used to transmit the effluents to a spectrophotometer and fraction collector. The effluents were monitored at 415 nm by use of a Model 2000 Gilford recording spectrophotometer with flow-through cuvettes having volumes of 0.125 ml and light paths of 10 mm. This arrangement permits the monitoring of up to three columns simultaneously. Fractions of 1–4 ml, depending upon the chromatogram expected, were collected. After the run, the fractions constituting a particular component of hemoglobin were pooled and the total volume (V) and the absorbance (A) at 415 nm determined. The percentage of hemoglobin in each component was calculated by multiplying the volume (V) and the absorbance (A) of each component since the product is known to be a function of the amount of hemoglobin in that component. Thus, the

product of V and A of a particular component relative to the sum of the products of V and A of all components in a hemolysate represents the relative quantity of that particular component in the hemolysate. This may be expressed as:

$$\% \text{ B in hemolysate} = \frac{V_B(A_B) \times 100}{\Sigma V(A)}$$

where B represents a hemoglobin component, V_B the volume of component B, A_B the absorbance of component B, V the volume of each component, and A the absorbance of each component.

We have also determined the relative quantity of hemoglobin in the different components by integrating areas under the peaks of the graph with a Keuffel and Esser (K & E) 620,000 compensating polar planimeter. Since the absorbance on the Gilford spectrophotometer is linear, such a method should give accurate values and the two methods of determining the percentages should yield comparable results.

Results and discussion

"Minicolumn" chromatography has been used in the study of hemoglobin in our laboratory for several years. As illustrations of the results obtained by this method, several analyses of hemoglobin solutions by "minicolumn" chromatography are presented in Figs. 2 through 5. Fig. 2 shows an analysis, using DEAE-cellulose, of a hemolysate from a normal person. As seen in this figure, Hb-A_2 is eluted first and is well separated from Hb-A_0, the major component. Hb-A_1, the so-called electrophoretically fast-moving minor fraction, is completely separated from Hb-A_0.

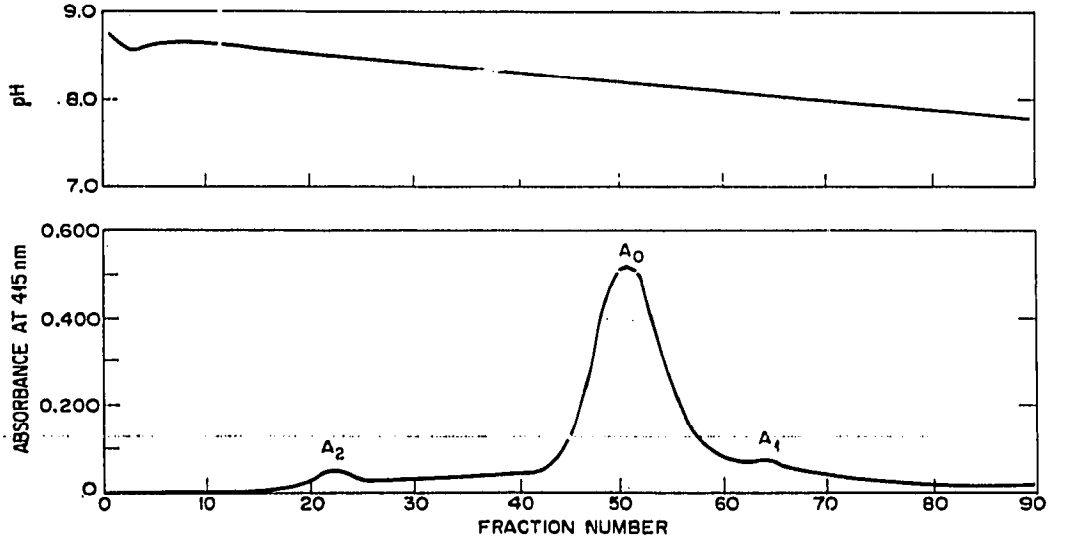

Fig. 2. DEAE-cellulose chromatogram of hemolysate containing Hb-A.

Figs. 3 and 4 illustrate a situation in which "minicolumn" chromatography was useful in clarifying the hemoglobin types of a newborn child (Fig. 4) whose mother had β chain genes for Hb-S and Hb-C (Fig. 3). The child had received a blood transfusion prior to the time at which a sample of blood for hemoglobin studies was obtained. By starch gel electrophoresis, only Hb-F and Hb-A could be seen. The presence

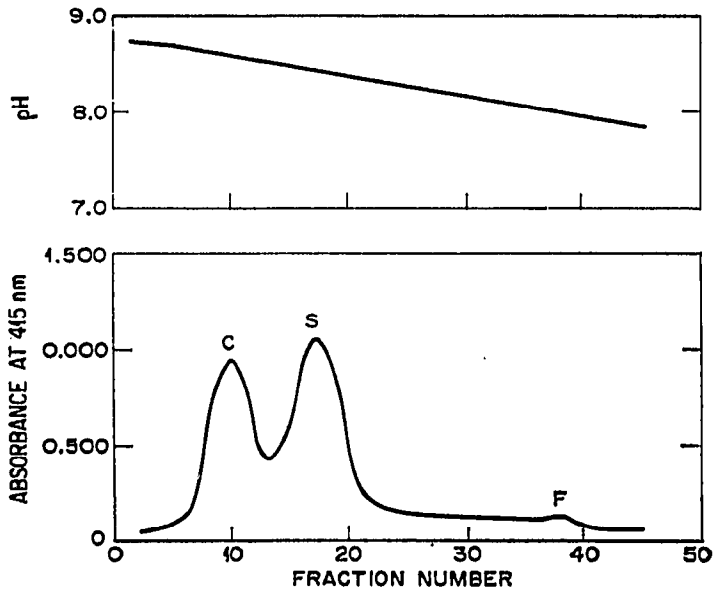

Fig. 3. DEAE-cellulose chromatogram of hemolysate containing Hb-S and Hb-C.

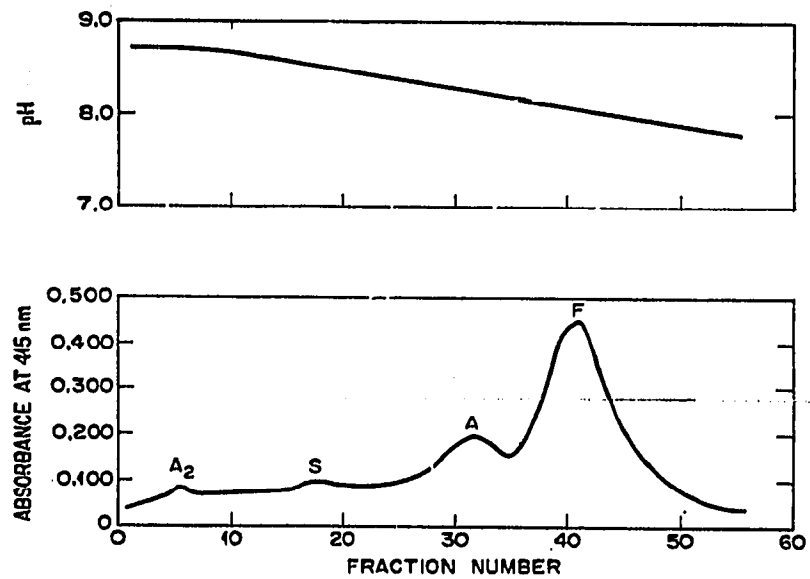

Fig. 4. DEAE-cellulose chromatogram of hemolysate containing Hb-A and Hb-S in a newborn following transfusion.

of even a tiny quantity of Hb-S or Hb-C (initially neither Hb-S or Hb-C was noted but, genetically, one of these hemoglobins had to be present) would make it quite likely that the child was heterozygous for Hb-A and either Hb-S or Hb-C. Only by "minicolumn" chromatography using DEAE-cellulose as the ion exchanger was the presence of Hb-S revealed. The high absorbance baseline and the small quantities of Hb-S and Hb-A_2 present made quantitation of Hb-S and Hb-A_2 in this graph unreliable.

In Fig. 5, a CM-cellulose "minicolumn" chromatogram is presented. This patient has Hb-A and another hemoglobin, eluted before Hb-A with the electrophoretic

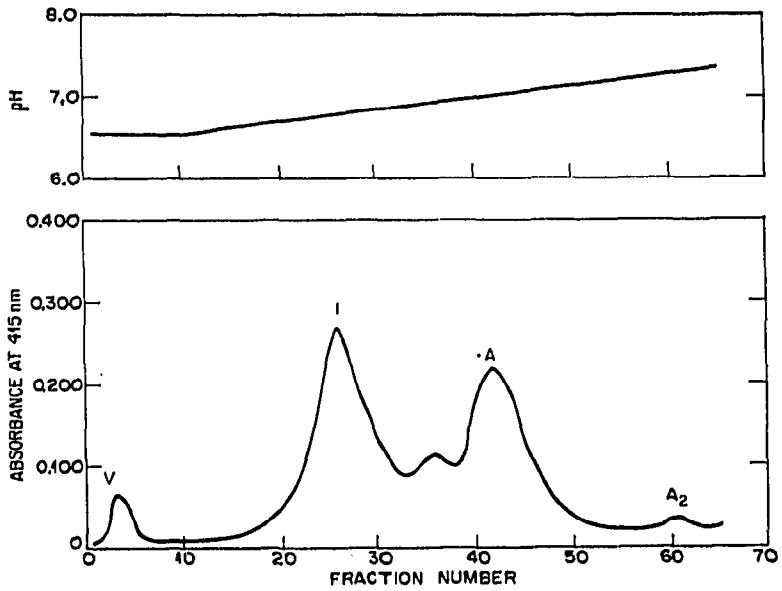

Fig. 5. CM-cellulose chromatogram of hemolysate containing Hb-A and Hb-I.

mobility of Hb-I. This abnormal hemoglobin has not been definitely identified at this time. Note early in the chromatogram a peak labeled V which contains non-hemoglobin proteins. Hb-A_2, though eluting last, can be seen easily in this chromatogram.

The analysis of a hemoglobin solution by "minicolumn" chromatography may take from 2 to 18 h although generally between 5 and 10 h are required. The most important use of the "minicolumn" technique in our laboratory has been to separate the ^{14}C-labeled hemoglobins of bone marrow cultures containing as little as 3,000,000 nucleated cells. This capability has allowed the culture of multiple samples from a single bone marrow aspiration with subsequent chromatographic separation of the hemoglobins and determination of the radioactivity in the fractions. Such studies would be impossible with ordinary chromatographic techniques.

We have also found the "minicolumn" technique useful in the study of turtle hemoglobins. The chromatographic pattern of certain turtle hemoglobins changes quite rapidly even if kept at 4°. Hemoglobins from these turtles can be studied by drawing small quantities of blood from the heart, quickly making hemolysates from the cooled samples and rapidly chromatographing the hemoglobin solutions. The "minicolumn" technique needs only small quantities of hemoglobin, eliminating the necessity of killing the turtle to get enough material for chromatography. Thus, fresh hemolysates from the same turtle can be studied repeatedly with the passage of only 3–4 h time between sampling the blood and finishing the chromatogram.

One disadvantage of the "minicolumn" is the difficulty of using Sephadex or Bio-Gel exchangers. Both have been tried in these columns but their tendency to pack prevented rapid flow rates so that their use was abandoned. If it is necessary to work with a small sample size and time is not critical, they could probably be utilized in the narrow bore tubes.

Table I compares two methods of quantitation using the results of analyses of three of the chromatograms presented here. Agreement between the two methods is

TABLE I

QUANTITATION OF HEMOGLOBIN COMPONENTS BY TWO METHODS OF CALCULATION

Chromatogram in Fig.	*Component*	*% by pooling*	*% by planimetry*
2	A_2	2.2	4.1
	A_0	90	88
	A_1	7.9	8.7
4	A	23	19
	F	77	81
5	I	47	51
	A_1	6.3	11.6
	A_0	42	37
	A_2	4.3	1.3

moderately good. Recently, mixing in the Gilford spectrophotometer cuvettes was found to be poor, *i.e.* with a solution of high absorbance and high density in the cuvette, an effluent of lesser density entering the top of the cuvette tended to flow over the high density material to the cuvette outlet without adequate mixing. This lack of mixing probably explains some of the discrepancy between the two methods of analysis. In an effort to avoid this problem, Helma Cells, No. OS76, were tried. These cells are constructed so that the inlet port is at the bottom of the cuvette chamber and the outlet port is at the top. However, again there was insufficient mixing as the low density eluent channeled up the sides of the cuvette chamber. In addition to this problem, the larger volume of the Helma Cell Chamber (0.5 ml) as compared to the Gilford cuvette chamber (0.125 ml) made it impractical to use these cells*.

The authors wish to express appreciation to Mr. JOE MILLER, Dr. WALTER FARKAS and Dr. TED MCDONALD for their helpful comments in the preparation of this article.

This research was supported by Grant T 415 from the American Cancer Society. One of us (A.I.C.) was a recipient of Career Award No. K6-GM-3750 from the National Institute of General Medical Sciences.

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Received January 12th, 1970

* *Editor's note.* Short and narrow columns have been employed and repeatedly described; however, the present method seems particularly successful.

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