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# CHROMATOFOCUSING OF HUMAN HEMOGLOBINS: APPLICATION TO THE QUANTITATION OF HEMOGLOBIN $A_2$

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## SUMMARY

Chromatofocusing of various hemoglobins was performed using standard ion exchangers and Ampholine solutions. With DEAE supports and a large difference between the adjusted pH of the ampholyte solution and the pH existing in the column, regular descending pH gradients were generated. Using CM-52 cellulose and small pH differences between the above, more complex pH profiles were obtained. Various hemoglobins generally eluted at a pH higher than their p*I* in the form of sharp peaks. The elution order was the same as with ion-exchange chromatographic procedures, including the same abnormalities with respect to the p*I* of some hemoglobins. Microchromatofocusing on DE-52 cellulose proved to be a sensitive and convenient technique for large-scale quantitation of $HbA_2$.

## INTRODUCTION

Different types of cation [1—6] or anion [7—10] exchangers have been used for the chromatographic separation of normal and abnormal human hemoglobins. Tris—HCl, glycine—KCN—NaCl and sodium phosphate solutions with different molarities and pH have been the most commonly used developers. The introduction of chromatofocusing by Sluyterman and Wijdenes

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[11—13] has allowed the development of a chromatographic system characterized by self-generated pH gradients and focusing effects which greatly improved the chromatographic resolution. Preliminary results obtained with chromatofocusing of some human hemoglobins (Hb) have been reported elsewhere [14, 15]. In this communication, we present the separation of various human hemoglobins using different supports and carrier ampholyte solutions. We also present the use of chromatofocusing for the quantitation of $HbA_2$.

## MATERIALS

### *Blood samples*

Samples were obtained from normal individuals and from patients with various hemoglobin abnormalities. Blood was collected by venipuncture in heparinized tubes or in tubes containing sodium citrate—citric acid—dextrose. Samples were stored at 4°C. The preparation of hemoglobin solutions was identical to that described elsewhere [16]. Final hemoglobin concentration in diluted hemolysate was adjusted to 4% (w/v) with distilled water. The hemolysate was dialyzed overnight at 4°C against the equilibration buffer for chromatography.

### *Developers*

Carrier ampholyte solutions used as developers were prepared from Ampholine solutions from LKB (Bromma, Sweden). Their pH was controlled and adjusted with 0.1 *N* NaOH or 0.1 *N* HCl. The solutions were carefully degassed before use.

### *Preparation of ion exchangers*

DE-52 cellulose (DE-52) and CM-52 cellulose (CM-52) were provided by Whatman (Maidstone, Great Britain) and DEAE-Sephacel by Pharmacia (Uppsala, Sweden). The equilibration buffers were 0.05 *M* Tris—HCl (pH 8.90), 0.01% KCN [16] for anion exchangers and 0.01 *M* sodium phosphate (pH = 6.80), 0.01% KCN [4] for CM-52. The equilibration procedure for the different chromatographic supports was carried out as described elsewhere [9]. Equilibration buffers and ion-exchanger solutions were carefully degassed before use.

### *Hemoglobin $A_2$ control*

Lyophilized hemolysate, used as standard for the quantitative determination of $HbA_2$ (2.85 ± 0.20% [17]), was provided by Isolab (Akron, OH, U.S.A.).

## PROCEDURE

### *Chromatofocusing*

Ion exchanger in equilibration buffer (mobile phase/stationary phase: 0.70) was packed in K 9/15 or K 9/60 columns (Pharmacia) using 10—15 volumes of equilibration buffer. Pasteur pipettes with a bed height of 10 cm were used for $HbA_2$ assay. To avoid the presence of a mixing chamber at the top of

the bed ion exchanger using K 9/15 or K 9/60 columns, the volume of the equilibration buffer was reduced and the bed heights were fixed at their maximum values. After application by pumping of 4.8 and 25 mg of hemoglobin for the K 9/15 and K 9/60 columns, respectively, the support was washed with one exchanger bed volume of equilibration buffer. Then the elution was performed with various ampholyte solutions. Fractions were read at 415 nm and their pH determined using an Ingold (Zürich, Switzerland) microelectrode, Type 403 30 M 8. Provided it was linear, the pH gradient was expressed as $\Delta pH\ ml^{-1}$.

*Other procedures*

The control of fractions was performed, after concentration, by cellulose-acetate electrophoresis following stripping of hemoglobin solution in a mixed-bed ion-exchange column (AG 50 1-X8; Bio-Rad Labs., Richmond, CA, U.S.A.) [18], or by polyacrylamide-gel isoelectric focusing [19]. Control determination of $HbA_2$ by ion-exchange chromatography was carried out according to the description of Efremov et al. [16].

## RESULTS

*Chromatofocusing on anion exchangers*

Unless otherwise stated, a 1% (w/v) Ampholine solution, pH range 6.00—8.00, adjusted to pH 7.00 [1% Ampholine (pH 6.00—8.00) pH 7.00] was

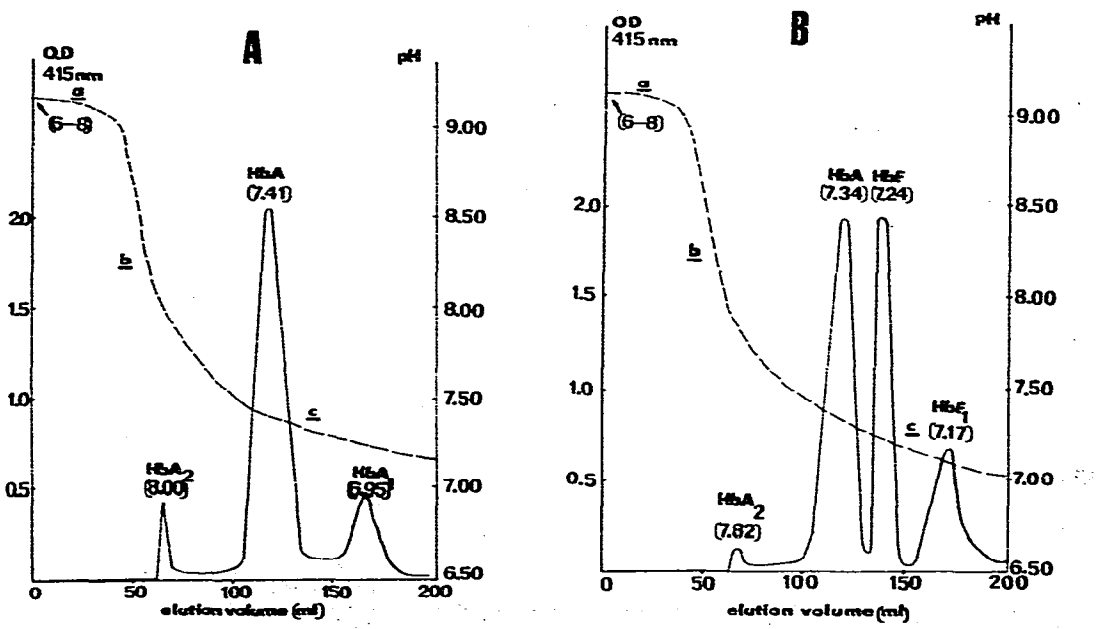


Fig. 1. Chromatofocusing on anion exchanger. Samples: (A) adult hemolysate; (B) fetal (cord blood) hemolysate. Column: K 9/60. Support: DEAE-Sephacel. Equilibration buffer: 0.05 *M* Tris—HCl (pH 8.90), 0.01% KCN. Eluent: 1% Ampholine solution (pH 6.00—8.00) pH 7.00. Flow-rate: 30 ml $h^{-1}$. In parentheses: elution pH of hemoglobins. — — —, pH profile; ——, hemoglobin profile (415 nm).

used as eluent. A typical separation using DEAE-Sephacel and a K 9/60 column is presented in Fig. 1. The pH profile contained three parts, a—c, including a self-generated descending, approximately linear pH gradient (part c) with a slope of −0.0047 pH unit per ml and a lower limit of approximately 7.00. A clear-cut separation of $HbA_2$, HbA and $HbA_1$ from adult hemolysate was obtained. In fetal hemolysate, HbA and HbF were also clearly separated. $HbA_2$,

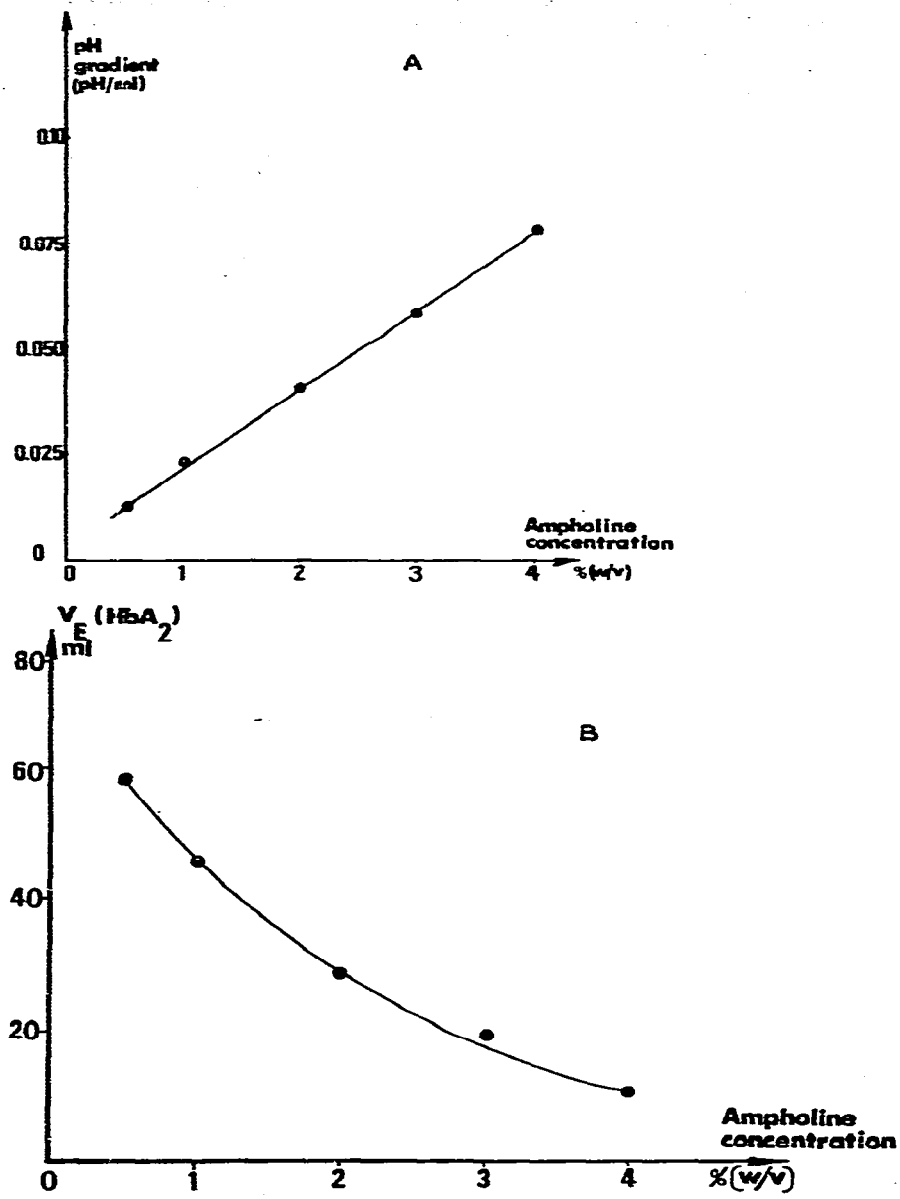


Fig. 2. Effect of Ampholine concentration on the pH gradient (A) and $HbA_2$ elution volume ($V_E$) (B). Sample: adult hemolysate. Column: K 9/15. Support: DE-52 cellulose. Equilibration buffer: 0.05 *M* Tris—HCl (pH 8.90), 0.01% KCN. Eluent: Ampholine (pH 6.00—8.00) pH 7.00 at increasing concentrations (%, w/v). Flow-rate: 30 ml $h^{-1}$.

HbA and HbF were eluted at a pH (Fig. 1) above their p*I*, i.e. 7.35, 6.95 and 7.14 [20], respectively.

The influence of several parameters of the ampholyte solution was examined using another anion exchanger, DE-52, and a K 9/15 column. Under

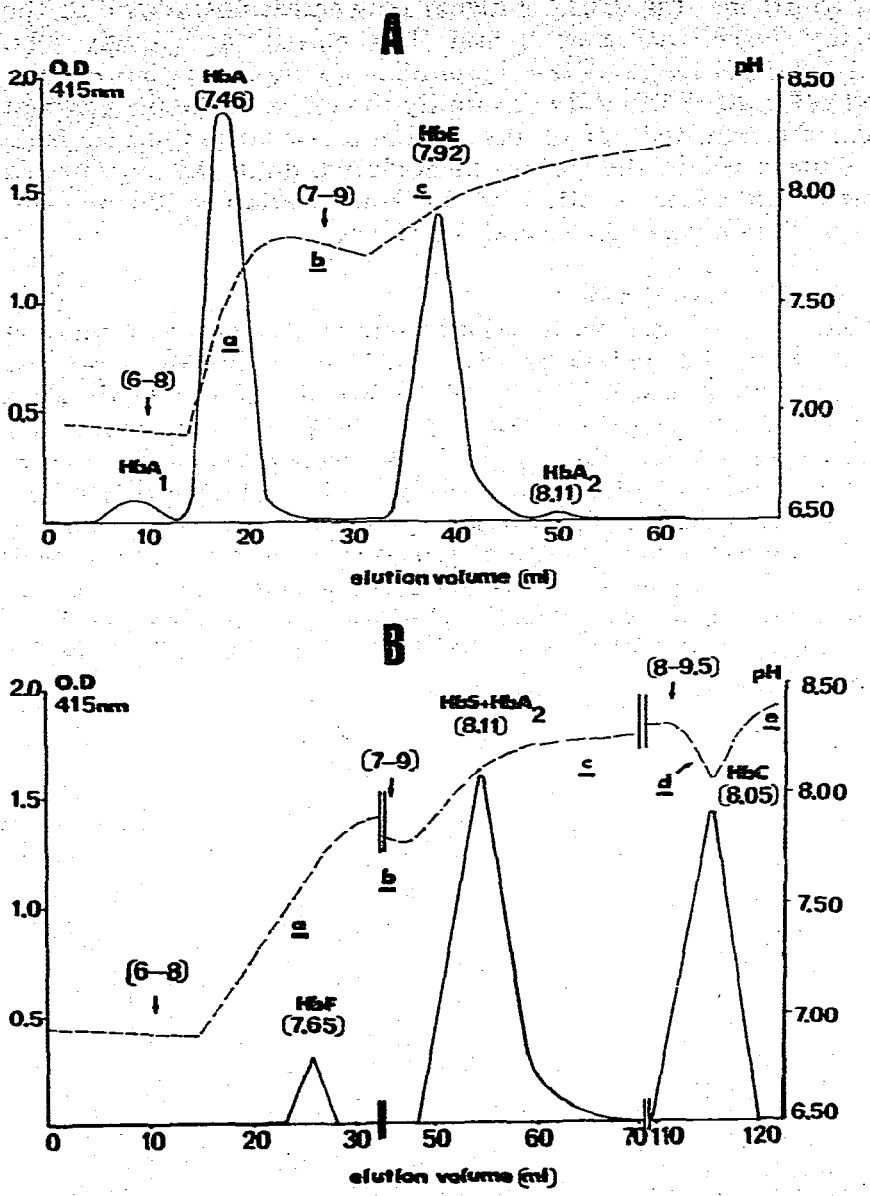


Fig. 3. Chromatofocusing on CM-52 cellulose. Samples: (A) hemolysate from a person heterozygous for HbE; (B) hemolysate from a patient double heterozygous for HbS and HbC. Column: K 9/15. Support: CM-52 cellulose. Equilibration buffer: 0.01 *M* sodium phosphate (pH 6.80), 0.01% KCN. Flow-rate: 20 ml $h^{-1}$. Subsequent eluents: 1% Ampholine (6.00—8.00) pH 7.00; 1% Ampholine (7.00—9.00) pH 8.00; and 1% Ampholine (8.00—9.00) pH 8.50. In parentheses: elution pH of hemoglobins. — — —, pH profile; ——, hemoglobin profile (415 nm).

standard conditions [1% Ampholine (pH 6.00—8.00) pH 7.00], the same general profile was obtained [slope of the pH gradient (part c) = —0.025 pH unit per ml]. When the Ampholine concentration was increased to 4%, the absolute value of the slope of part c linearly increased and the $HbA_2$ elution volume decreased to 10 ml (Fig. 2). For higher concentrations, part c of the pH profile gradually fused with part b and HbA eluted increasingly with $HbA_2$ (not shown). The pH range of the Ampholine solution (pH 5.00—8.00, pH 6.00—8.00 and pH 7.00—9.00) did not modify the slope of the pH gradient (not shown). When the adjusted pH of the Ampholine solution varied from 8.00 to 6.00, the elution pH of the various hemoglobins gradually approached their p*I* (not shown). The concentration of the equilibration buffer was not critical for the pH profile between 0.025 and 0.2 *M* Tris.

*Chromatofocusing on CM-52 cellulose*

Typical separation was obtained with CM-52 using two or three subsequent Ampholine eluents of varying pH range (Fig. 3). The pH profile contained five parts. A 1% Ampholine solution (pH 6.00—8.00) pH 7.00 yielded part a (a sharp pH increase), followed by part b (a decreasing pH gradient). A 1% Ampholine solution (pH 7.00—9.00) pH 8.00 then generated part c (an irregular ascending pH gradient). Finally, a 1% Ampholine solution (pH 8.00—9.50) pH 8.50 induced a transient pH decrease (part d), followed by a pH increase which failed, however, to be a genuine gradient. $HbA_1$ was not retained. HbA and HbF eluted in part a, HbS, HbE and $HbA_2$ eluted in part c, and HbC at the d—e junction. Again, the elution pH of the hemoglobins was above their p*I*; for example, 6.95 for HbA, 7.14 for HbF, 7.21 for HbS, 7.35 for $HbA_2$ and 7.47 for HbC [20].

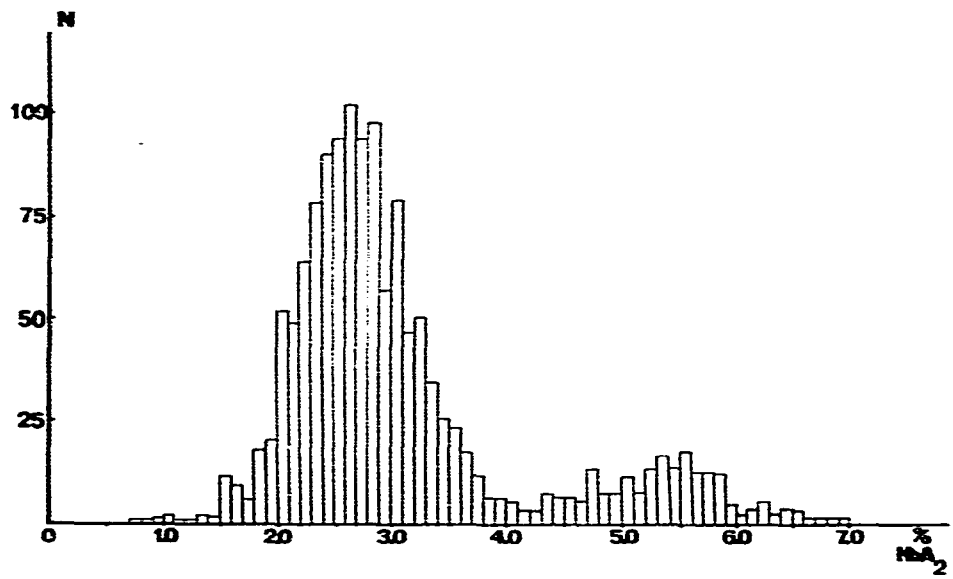


Fig. 4. General histogram of $HbA_2$ values. The lower peak corresponds to a population of patients with β-thalassemic trait.

*Quantitative determination of human hemoglobin $A_2$*

The quantitative determination of $HbA_2$ by chromatofocusing was studied over a period of one year, using DE-52 cellulose equilibrated with 0.05 *M* Tris–HCl buffer (pH 8.90) in Pasteur pipettes. Elution was performed with 1% Ampholine (pH 6.00–8.00) pH 7.00. The general histogram of percentage $HbA_2$ concerns 1334 determinations in normal subjects or in patients with various conditions involving hemoglobin (Fig. 4). The values were distributed in two peaks separated by an interval (4.1–4.2%) which included two of the patients. The correlation between chromatofocusing and ion-exchange chromatography [16] was very strong ($p < 0.01$) (Fig. 5). A linear relationship was observed between percentage $HbA_2$ and temperature (18–24° C), as is also the case with ion-exchange chromatography (Fig. 6). Finally, there was a clear-cut separation between $HbA_2$ and HbS in patients with HbS trait or disease (not shown).

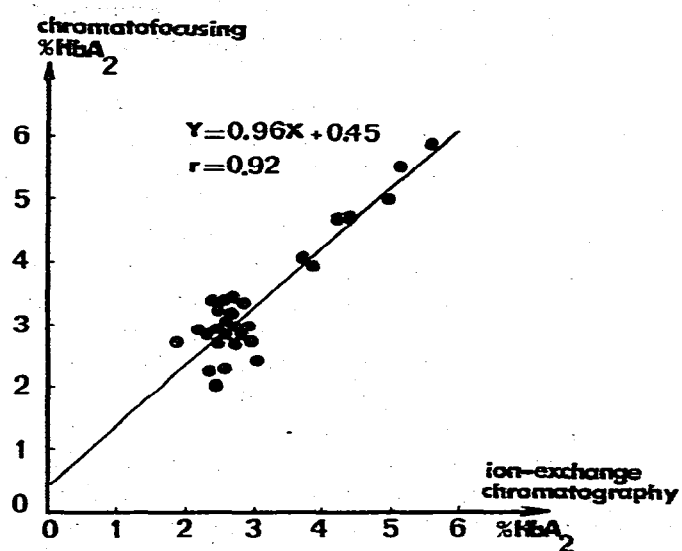


Fig. 5. Correlation between quantitative determination of $HbA_2$ by chromatofocusing (ordinate) and by ion-exchange chromatography (abscissa) ($n = 30$; duplicate determinations; $p < 0.01$). Temperature: 27° C.

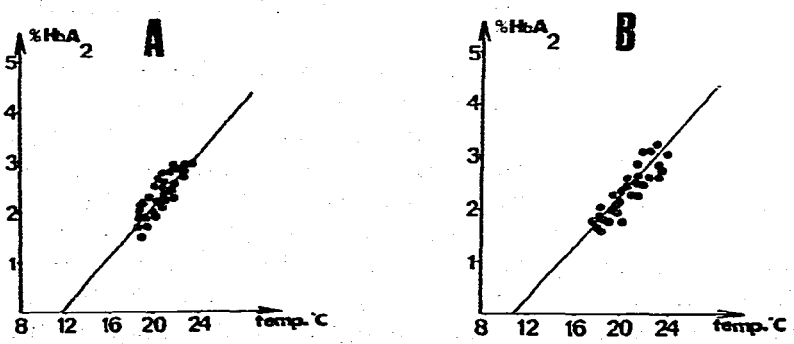


Fig. 6. Effect of temperature on $HbA_2$ determinations. (A) Chromatofocusing ($n = 30$; $y = 0.26\ x - 2.99$; $p < 0.01$). (B) Ion-exchange chromatography ($n = 30$; $y = 0.25\ x - 3.04$; $p < 0.01$). For both procedures, lyophilised hemolysate ($HbA_2$: 2.85 ± 0.20%) was used.

## DISCUSSION

The pH profiles obtained with anion exchangers are consistent with those published by Emond and Pagé [21], and Sluyterman and Wijdenes [13]. The flat part, a, reflects the chase of equilibration buffer by the incoming ampholyte solution. The sharp part, b, indicates the onset of the passage of the ampholyte solution. The chloride concentration, as measured by an automated (Technicon SMA 6-60) colorimetric method, drops to 0. At this stage, the most negatively charged species are missing because they have stuck to the support. As a result, the pH is above 7.00. Part c, the descending linear pH gradient, corresponds to the progressive saturation of the anion exchanger with ampholytes that are more and more negatively charged. The composition of the outcoming Ampholines slowly returns to normal, in such a way that the pH linearly decreases to 7.00 (the initial pH of the ampholyte solution). When the concentration of Ampholines increased, the saturation accelerated and the pH gradient became sharper (Fig. 2), reaching more rapidly its lower limit.

The pH profile obtained with CM-52 cellulose was not as simple (Fig. 3). Parts d and c (Fig. 3b) can be accounted for as parts b and c of the preceding pH profile (Fig. 1). The most positively charged ampholyte species are retained on the anionic support, resulting in a decrease in the pH of the ampholyte solution (part d). Part d is indeed descending because the pH of the Ampholine solution (pH 8.50) is approximately equal to that (pH 8.30) existing in the column at the time of application. As saturation of the support gradually takes place, the composition of the outcoming ampholyte solution returns to its initial value (pH 8.50), without generating, however, a genuine pH gradient. Part c (Fig. 3a and b), following application of Ampholine solution (pH 7.00—9.00) pH 8.00, may have an explanation roughly similar to that of part e, although the pH variation is closer to a gradient. However, it is unclear why it is not preceded by a transient decrease in pH (equivalent to part d). Parts a and b, a sharp pH increase and a descending pH gradient, respectively, occur, whereas the reverse was expected. In the absence of application of further Ampholine solution, part b would gradually return to pH 7.00 (not shown). One must assume that an entirely different type of interaction between the support and the ampholytes takes place at this early stage of the chromatographic procedure.

In both anion and cation exchangers, various hemoglobins eluted at a pH above their p*I*, as has been described by Sluyterman and Wijdenes [13]. The elution order of hemoglobins was the same as in ion-exchange chromatography on DEAE supports [9, 16] and CM supports [6]. In particular, the same abnormalities with respect to p*I* were observed: HbA (p*I* = 6.95) eluted before HbF (p*I* = 7.14) on DEAE-Sephacel. On CM-52 [2], HbE (p*I* ≈ 7.30) eluted before HbS (p*I* = 7.21). It is puzzling that HbC (p*I* = 7.47), although it required a third Ampholine solution (pH 8.00—9.50) pH 8.50 to be eluted, finally eluted at pH 8.05 (groove at the junction of parts d and e). This elution pH is lower than that (pH 8.11) of HbS with the preceding Ampholine solution (pH 7.00—9.00) pH 8.00.

Chromatofocusing separation of HbA and $HbA_2$ is simpler to monitor

than separation by ion-exchange chromatography (one buffer instead of two during the chromatographic time). Given the clinical interest of $HbA_2$ quantitation, in particular for the large-scale screening of the β-thalassemic trait, it provides a practical advantage for long series of assays.

In conclusion, chromatofocusing of hemoglobin constituents appears a sensitive and handy technique, which may improve the performance of ion-exchange chromatography for routine and research purposes.

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