CHROMATOGRAPHY ON ECTEOLA-CELLULOSE AT NEUTRAL pH VALUES

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INTRODUCTION

In a series of articles[1-3] we described the chromatographic separation of fibrinogen and the anti-haemophilic factor (AHF) from human plasma. Most of the experiments were carried out on columns of ECTEOLA-cellulose, but DEAE-cellulose has also been used. The AHF was our main interest, and it was in order to obtain better yields and a purer product that we were obliged to investigate the chromatographic process in detail. This investigation revealed several peculiarities of ECTEOLA-cellulose (and to a certain degree also of DEAE-cellulose) with regard to properties and behaviour, and about the way to handle it. It is with these peculiarities that this article will deal.

MATERIALS AND METHODS

ECTEOLA-celluloses

ECTEOLA-celluloses of various capacities were prepared as described earlier[4]. The types, most commonly used, had capacities of 0.36–0.41 mequiv./g.

Buffer systems

The following buffers were used:

(a) Imidazole–chloride buffers: 0.02 *M* imidazole, containing NaCl in various concentrations, dependent on the type of experiments, neutralized to pH 6.9 with 10% HCl.

(b) Imidazole–bromide buffer: 0.02 *M* imidazole, containing 0.5 *M* NaBr, was neutralized to pH 6.9 with 10% HBr solution, freed from Br_2 with a trace of $Na_2S_2O_3$.

pH values

These were measured with a Radiometer pH-meter, model 22, with external meter.

Preparation of fraction I

Fraction I, containing fibrinogen, AHF, and a variety of globulins, was precipitated from $BaSO_4$-treated oxalated human plasma at 8% ethanol concentration, according to COHN[5]. It was the starting product for the chromatographic purification of AHF.

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For a better understanding of this process we will summarize here the principles along which these experiments were carried out. The precipitated fraction I was dissolved in $^3/_5$ the original plasma volume of imidazole–chloride buffer. This solution was chromatographed on a column of ECTEOLA-cellulose (Cl-cycle), the column was washed with the same buffer, and, after fibrinogen and the bulk of contaminating

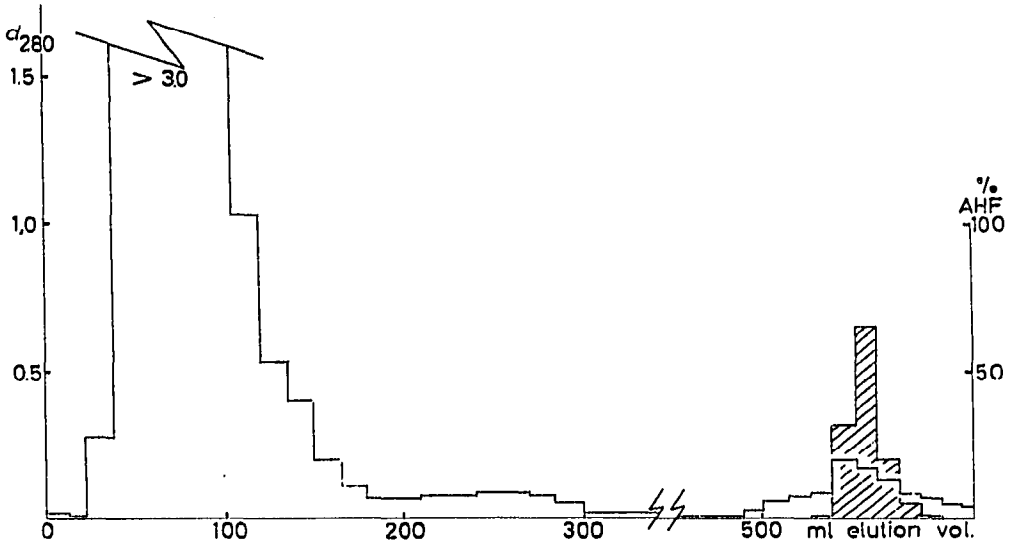

Fig. 1. Chromatography of plasma fraction I on ECTEOLA-cellulose. After 500 ml imidazole-chloride buffer had passed the column a change was made to imidazole-bromide buffer.

proteins had been washed out, the adsorbed AHF was eluted by displacement with imidazole–bromide buffer. Fig. 1 gives an example of a typical experiment. For further details see ref. 6.

EXPERIMENTAL AND RESULTS

(a) During the regeneration of the columns an important variable in the procedure, *i.e.* the reconditioning, was noted. After completion of a chromatographic run the column was washed with 0.5 *N* NaOH until the effluent was strongly alkaline. In most cases recycling to the Cl-form was carried out by passing imidazole–chloride buffer through the column until the pH of the effluent equalled that of the buffer itself. The column was then considered ready for use. Columns thus treated gave yields varying from 10 to 50 %. In some cases the regeneration was carried out with alkali followed by 0.5 *N* HCl, and the column was subsequently equilibrated with imidazole–chloride buffer. This invariably resulted in a very low yield of AHF. We therefore decided to use only 0.5 *N* NaOH for the regeneration and to wash the columns with water until the pH of the effluent was practically neutral before the equilibration was started.

(b) The equilibration proved to be one of the most important variables of the process. This is clear from the following experiments: regenerated and washed columns were equilibrated with increasing amounts of imidazole–chloride buffer and used in the chromatographic preparation of AHF. As will be seen from Table I, clear optima in the equilibration were found which gave maximum yields of AHF.

(c) From the foregoing, it can be concluded that the ECTEOLA-cellulose should not be converted completely into the Cl-form, and that some of the OH-ions should remain unsubstituted, in order to obtain satisfactory isolation of AHF. This could be verified by measuring the pH of all the effluent fractions during a chromatographic run. Fig. 2 shows the rise in pH following the shift from imidazole–chloride to –bromide buffer during a regular preparative run.

TABLE I

AMOUNT OF EQUILIBRATING BUFFER *vs.* YIELD

ECTEOLA-cellulose			
Capacity 0.40 mequiv./g		*Capacity 0.78 mequiv./g*	
Buffer ml	*AHF yield in %*	*Buffer ml*	*AHF yield in %*
125	20	125	11
250	50	250	20
500	70	500	30
1000	20	800	60
		1000	80
		1600	7

Statistically it was found that a quantitative yield of AHF was only obtained when the pH rose from 6.9 to about 7.35–7.40. By titration of all the effluent fractions, the amount of alkali liberated in this "pH-peak" was calculated as 6–7 % of the total capacity of the ECTEOLA-cellulose in the column.

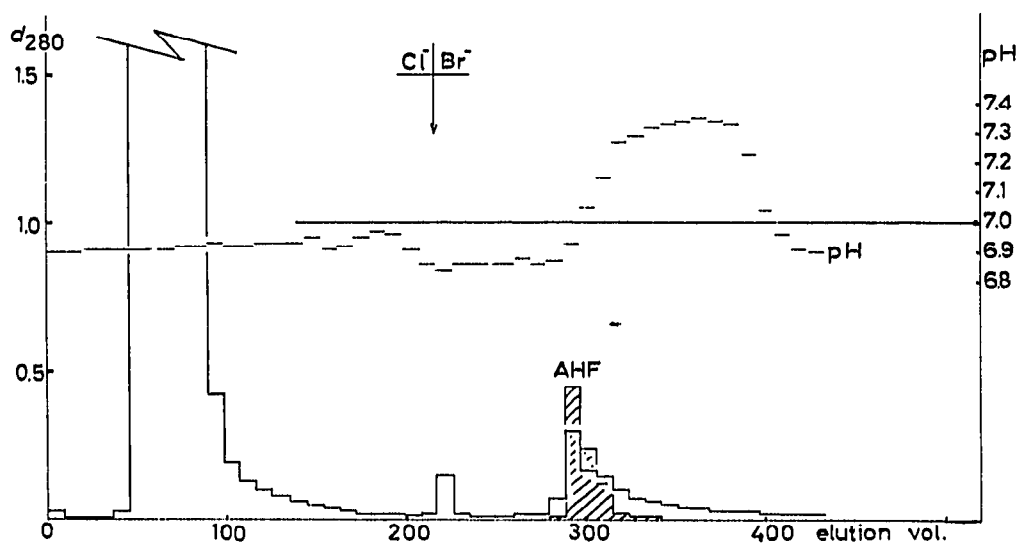

Fig. 2. Change of pH following the shift from imidazole-chloride to -bromide buffer.

The dimensions of this effect must in general be governed by:

(1) the amount of ECTEOLA-cellulose in g/column;
(2) the capacity of the ECTEOLA cellulose in mequiv./g;
(3) the total amount of buffer used for equilibration and for washing the preparation through the column;

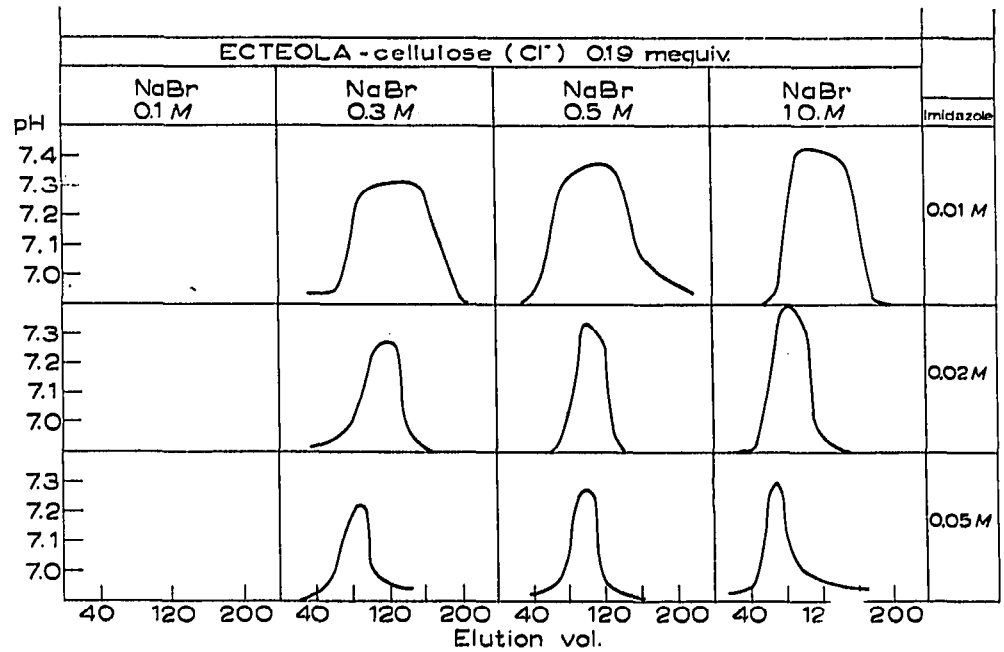

Fig. 3. Changes of pH following the shift from imidazole-chloride to -bromide buffer under various conditions of elution on ECTEOLA-cellulose of 0.19 mequiv./g.

(4) the anion concentration of this buffer;
(5) its affinity for the ion-exchanger[7];
(6) the concentration of the displacing anion;
(7) the affinity of the latter towards the ion-exchanger;
(8) the buffering capacity of the eluents;
(9) the very great affinity of OH-ions for ECTEOLA-cellulose[7].

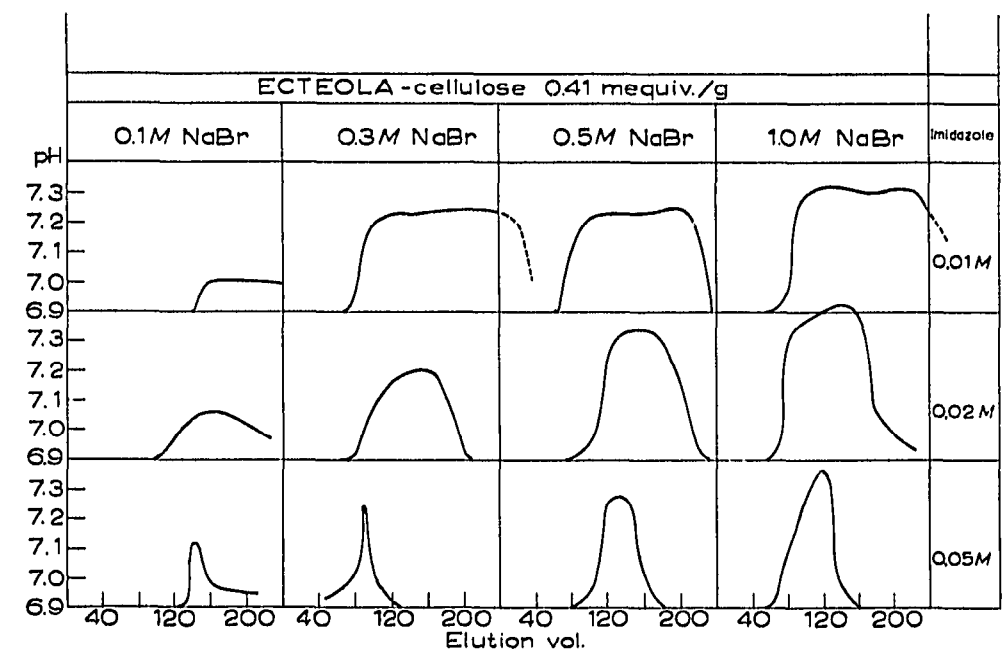

Fig. 4. Changes of pH following the shift from imidazole-chloride to -bromide buffer under various conditions of elution on ECTEOLA-cellulose of 0.41 mequiv./g.

This effect was investigated during regular chromatographic runs on two different types of ECTEOLA-cellulose for the shift of Cl- to Br-ions, at four different concentrations of the latter, and at three different buffering capacities (see Figs. 3 and 4).

(d) That some of the OH-ions of the ECTEOLA-cellulose remain in the column is due to the fact, already indicated earlier[4], that the rate of ion-exchange by ECTEOLA-cellulose in the range of pH 5–8 is very slow. This accounts for the fact that relatively large volumes of buffer of sufficient concentration must be used to convert the ion-exchanger completely from the OH-form into the desired form at neutral pH values*.

That OH-ions are tenaciously retained by the ECTEOLA-cellulose during the equilibration and chromatography was demonstrated in the following manner: a column was regenerated with alkali and washed with water until neutral in the usual way. It was then equilibrated with imidazole–chloride buffer to which phenol red was added. After the passage of 300 ml buffer solution of pH 6.91 the effluent was yellow, and had a pH of 6.92, whereas the column itself was red. After the passage of another 150 ml of buffer containing phenol red the colours were still the same. Since AHF determinations were not impaired by the dye, the column was used for the chromatography of fraction I, and washed with buffer containing phenol red; AHF was then eluted by displacement with imidazole–bromide buffer containing phenol red. Immediately after the shift to bromide, a dark red ring started passing through the column. In front of the ring the column remained red, behind it the column was yellow. Fig. 5 gives a picture of this experiment.

(e) Well-equilibrated columns could not be stored for longer periods than 1–2 days, because, even in the cold room, ion-exchange, although slow, continued to take place. In an aged column the optimum conditions for chromatography could not be attained because of liberation of the residual OH-ions, and the resulting high pH adversely affected the preparation to be chromatographed. In two experiments two columns were used, one freshly equilibrated and one one week old, but otherwise identical with regard to regeneration, washing, and equilibration. On the fresh column the yield of AHF was 85 %, on the old column 35 %. After the columns had been interchanged the experiment was repeated, and the highest yield was again obtained with the freshly prepared column.

(f) We also tried to reproduce the "pH-peak" by displacement with anions other than bromide. Most of these experiments were performed with sulphate, but a variety of organic anions have also been used. Although in most cases the affinity of the ECTEOLA-cellulose for the anion used was greater than for bromide, as determined in equilibrium experiments[7], the rise in pH was always smaller than was expected (see Table II).

(g) Efforts to elute AHF with "synthetic" pH-peaks, produced with dilute alkali or with buffers of higher pH, failed, because no sharp boundaries could be obtained.

(h) The pH-peaks could also be reproduced on columns of DEAE-cellulose, and by proper equilibration of the columns quantitative yields of AHF could be obtained with this material as well. Because of the greater protein-binding capacity of this anion-exchanger the ratio of biol. activity/d_{280} was, however, rather poor.

What has been said above about ECTEOLA-cellulose generally holds true also

* At low concentrations no complete conversion will occur, owing to hydrolysis of the ECTEOLA-cellulose-anion salt, *cf.* ref. 7.

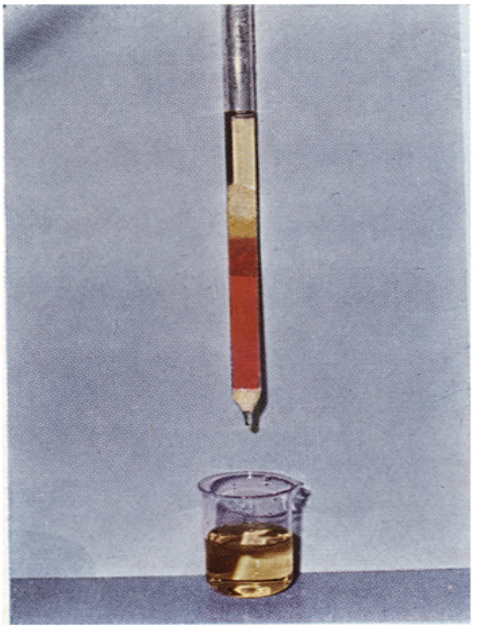

Fig. 5. Demonstration with phenol red of the increased OH-ion concentration in a column of ECTEOLA-cellulose.

TABLE II

EFFECT OF VARIOUS DISPLACING ANIONS ON pH-CHANGE

Column equilibrated with ml Cl-buffer	*Displacing anion*	*Mol. concn.*	K_c	*Rise of pH*	*ΔpH*
450	sulfate	0.25	12.1	6.91–7.08	0.17
200	sulfate	0.25		6.90–7.12	0.22
450	sulfate	0.50		6.85–7.13	0.28
500	succinate	0.10	9.8	6.92–7.06	0.16
500	succinate	0.20		6.89–7.00	0.11
480	formate	0.20	3.5	6.92–6.94	0.02
500	formate	0.50		6.93–7.07	0.14
500	glutamate	0.10	2.3	6.92–6.45	—0.47
500	glutamate	0.20		6.92–6.28	—0.64
500	propionate	0.20	1.2	6.84–6.90	0.06
500	pyruvate	0.20	1.1	6.90–6.96	0.06
500	chloride	0.50	1.0	6.88–7.16	0.28

for DEAE-cellulose except that the rate of exchange of the latter is greater, and hence fresh columns tend to deteriorate more rapidly than those of ECTEOLA-cellulose.

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SUMMARY

In the course of experiments on the chromatographic purification of the antihaemophilic factor, it was found that the cellulosic anion-exchangers, when regenerated

with alkali, tend to retain tenaciously a certain amount of hydroxyl-ions during their equilibration with neutral buffers. On shifting from one anion to another, *e.g.* in displacement chromatography, a rise in pH may result. The factors governing this phenomenon were investigated, and are discussed in the present article.

REFERENCES

1 S. VAN CREVELD, H. A. VEDER, C. N. PASCHA AND W. F. KROEZE, *Thromb. Diath. Haemorrhag.*, 3 (1959) 572.
2 S. VAN CREVELD, H. A. VEDER AND C. N. PASCHA, *Thromb. Diath. Haemorrhag*, 4 (1960) 211.
3 S. VAN CREVELD, C. N. PASCHA AND H. A. VEDER, *Thromb. Diath. Haemorrhag.*, 6 (1961) 282.
4 H. A. VEDER, *J. Chromatog.*, 10 (1963) 507.
5 E. J. COHN, L. E. STRONG, W. L. HUGHES, Jr., D. J. MULFORD, J. N. ASWORTH, M. MELIN AND H. L. TAYLOR, *J. Am. Chem. Soc.*, 68 (1946) 459.
6 C. N. PASCHA, *Een bijdrage tot de zuivering van de antihaemofilie factor*, Thesis, Amsterdam, 1963.
7 H. A. VEDER AND C. N. PASCHA, *Biochim. Biophys. Acta*, 47 (1961) 408.