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Continuous-flow electrophoresis: a separation criterion applied to the separation of model proteins

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ABSTRACT

The resolution of continuous-flow electrophoretic separations is determined by the relative importance of the migration of the products and of the widening of the corresponding peaks, both of which depend on the operating conditions and on the chamber geometry. This paper provides a separation criterion that gives a relationship between these two parameters that should be obeyed for the products to be recovered pure at the outlet of the chamber. From previous theoretical work this criterion is expressed as a function of all the operating conditions and of the chamber geometry. The theoretical calculations are first compared with experimental results for single protein samples. Both the theoretical and experimental results are found to be in good agreement in terms of migration and of peak width. The separation criterion is then further used for some model proteins in order to predict the conditions under which a complete separation could be achieved. Again, the experimental results for the separation of different proteins show that the conditions under which a total separation is achieved are very close to those expected from the calculations.

INTRODUCTION

Continuous-flow electrophoresis is a process that enables any products, such as proteins or cells, to be purified. This process has undergone considerable development owing to the possibility of converting electrophoresis, which is a high-resolution separation method, from an analytical to a preparative scale. The separation is carried out in a thin-film flow of carrier buffer, into which the sample containing the species to be separated is continuously fed. Using an electric field in a direction perpendicular to that flow, the charged particles move as a combination of two main velocities, the carrier buffer flow velocity and the electrophoretic migration velocity. Particles having different electrophoretic mobilities should therefore have different trajectories inside the chamber. Using a fraction collector at the outlet of the separation chamber, one can then recover the separated fractions.

Unfortunately, the first trials dealing with the use of continuous-flow electrophoresis to purify biological products were very disappointing. The resolution was found to be very poor compared with that which was expected from extrapolating the results obtained on an analytical scale. Among other results, it was observed that increasing the operating conditions, *e.g.*, the electric field or the residence of the products inside the electrophoretic chamber, does not necessarily lead to an improved separation. Consequently, many theoretical studies have been undertaken in order to investigate the different phenomena involved in that process, which could be responsible for the observed results. Such studies have dealt either with hydrodynamic and thermal problems [1–3] of with transport phenomena [4–8].

Very few experimental results have been published recently compared with the large amount of theoretical work that has been done in the last few years. Most of them have dealt with the separation of cell suspensions [9–11] or latex particles [12,13] and only few have concerned the purification of protein samples [6,14]. Moreover, usually it is very difficult to interpret or compare the results owing to

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a lack of information concerning the products investigated and the electrophoretic apparatus used.

In this work we were interested in searching for the optimum operating conditions for the purification of protein samples. Starting from previous work on hydrodynamic or dispersive effects involved in continuous-flow electrophoresis, we define a separation criterion that gives a relationship between all the relevant parameters to be fulfilled for a total separation to be achieved.

An experimental study was performed with two chambers and three different protein samples, the electrophoretic mobilities of which were chosen to be increasingly closer. In a first step single protein samples were investigated in order to compare the experimental results with those obtained from theoretical calculations. In a second step, separation experiments were performed and the results were compared with those predicted by using the resolution criterion.

EXPERIMENTAL

Electrophoretic apparatus

The electrophoresis apparatus used for the experimental study was designed and constructed in-house. The electrophoretic chamber is a rectangular channel between two polycarbonate plates. Two electrode compartments are located on each side of the chamber and are separated from it by ion-exchange membranes. At the outlet of the chamber, a collection port allows the flow to be divided into different fractions, collecting it every 1 mm along the chamber width. A multi-channel peristaltic pump located at the outlet of the separation chamber ensures the circulation of the carrier buffer fluid. The difference in flow-rate between the fractions does not exceed 3%. The sample containing the species to be separated is continuously fed at the top of the chamber through a needle, the diameter of which is about 1 mm, using a peristaltic pump. A power supply is used to apply a voltage between the two electrodes, thus creating an electric field in a direction perpendicular to the carrier buffer flow.

Two different chambers were used: chamber A, 600 mm long, 60 mm wide and 1.5 mm thick, and chamber B, 300 mm long, 40 mm wide and 3 mm thick.

A cooling system was used during the experimental runs to remove the heat dissipated by Joule heating in order to reduce the temperature gradients inside the chamber, which would otherwise give rise to convection and disturb the flows. To do that, the walls of the electrophoretic chamber were refrigerated by a cooling fluid, in order to keep the average temperature constant along the length of the cell. On the other hand, the electrode buffer was refrigerated in order to remove the heat dissipated in the region near the membranes, where the conductivity of the fluid was higher than that in the middle of the chamber, thus allowing the temperature gradients along the width of the chamber to be minimized. Some temperature sensors incorporated in the circuits allowed the efficiency of the thermal regulation to be controlled. For each set of operating conditions the temperatures of the cooling fluids were adjusted so as to maintain constant the temperature everywhere inside the chamber within $\pm 0.5°C$.

Fluids

The buffer used as the carrier fluid and electrode buffer was a Tris–borate buffer with an electrical conductivity of 140 $\mu S\ cm^{-1}$. Different values pH in the range 6.5–8.5 were used.

The protein solutions were prepared by dissolving in the carrier buffer lyophilized products, purchased from Sigma. The proteins used were bovine haemoglobin (Hb), bovine serum albumin (BSA) and α-lactalbumin.

Analytical methods

The experimental results were obtained by measuring the protein concentrations in the different fractions collected at the collection port. For samples containing only one kind of protein, the concentration was obtained by absorbance measurements (406 nm for the haemoglobin samples and 280 nm for the other proteins). In the separation experiments, the concentrations of the different proteins were obtained by gel permeation chromatography using a TSK G3000 SW column with Tris–borate buffer (pH 6.5) as the eluent at a flow-rate of 0.5 ml min^{-1}.

THEORETICAL APPROACH

Let us consider, for instance, the case of a sample containing two kinds of proteins, A and B, the elec-

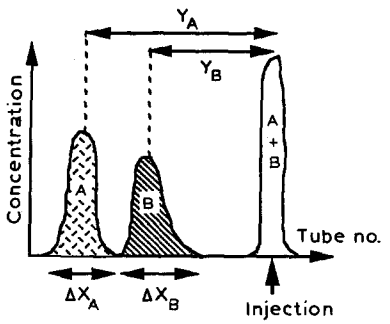

Fig. 1 Schematic representation of the separation of two proteins A and B.

trophoretic mobilities of which are u_A and u_B, respectively. This is illustrated in Fig. 1, which shows the concentration profiles at the outlet of the chamber. After flowing through the separation chamber, the two proteins have migrated distances Y_A and Y_B from their positions in the injection plane. On the other hand, considering that the widths of the corresponding peaks are ΔX_A and ΔX_B, respectively, one can define a "separation criterion" which gives the relationship between the values of Y_i and ΔX_i to be fulfilled for the two proteins to be collected completely separated:

$$Y_A - Y_B > (\Delta X_A + \Delta X_B)/2 \qquad (1)$$

assuming that A is the more mobile species.

Some previous studies have considered the different transport phenomena involved in continuous-flow electrophoresis. From these studies one can define ranges of operating parameters in which one or the other of these phenomena becomes predominant. For this work, the operating conditions were chosen in order to minimize both free convection [2,3] and electrohydrodynamic effects [7]. This is discussed in further detail later. Under these conditions, the mass transport is then determined by a combination of the Poiseuille flow of the carrier buffer, electroosmosis and electrophoretic migration [4,8], so that one can write for the migration distance of one charged particle.

$$Y_i = E\tau[(u_i + u_{os})/(1 - z^2/d^2) - 3/2\, u_{os}] \qquad (2)$$

where z represents the coordinate in the thickness of the chamber, d the half-chamber thickness, E the electric field, τ the average residence time inside the chamber and u_{os} the electroosmotic mobility. Assuming that at the outlet of the chamber the maximum of the concentration profile corresponds to the proteins located in the centre plane of the chamber, *i.e.*, to the position $z = 0$, as it is in the injection plane, one obtains for the migration distance of the maximum of the peak the expression.

$$Y_i = E\tau(u_i - u_{os}/2) \qquad (3)$$

On the other hand, owing to the Poiseuille flow of the carrier buffer, the residence time varies along the thickness of the chamber so that the particles distributed over the section of the sample do not reach the same position. This gives rise to the so-called "crescent effect". The greatest distance will be covered by the particles located at the position $z = \pm R$, where R is the radius of the sample stream. The width of the peak can therefore be written using the following expression:

$$\Delta X_i = [Y_i\,(z = \pm \mathrm{R}) - Y_i\,(z = 0)] + 2R \qquad (4)$$

Combining eqns. 2 and 4, we have

$$\Delta X_i = \{E\tau \mid u_i + u_{os}|[1/(1 - R^2/d^2) - 1]\} + 2R \qquad (5)$$

Using eqn. 1 and combining with eqns. 3 and 5, one can rewrite the resolution criterion as

$$(u_A - u_B)E\tau > E\tau/2\{(|u_A + u_{os}| + |u_B + u_{os}|)\,[1/(1 - R^2/d^2) - 1]\} + 2R \qquad (6)$$

This relationship enables different regions to be distinguished depending on the values of $E\tau$, as illustrated in Fig. 2. The relative positions of the curves that represent the variations of the left- and right-hand sides of eqn. 6 determine whether or not a complete purification can be expected. The slope of the left-hand side of eqn. 6 depends only on the protein and on the pH of the buffer, as they both determine the value of the electrophoretic mobilities, whereas depending on the values of R/d and of $|u_i + u_{os}|$ different curves can be obtained for the right-hand side of eqn. 6. For instance, in case (1), a

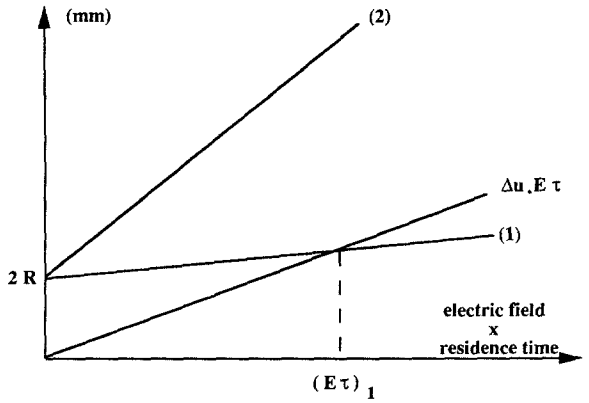

Fig. 2. Graphical interpretation of the separation criterion. (1) Right-hand side of eqn. 6 for $R/d = a1$; (2) right-hand side of eqn. 6 for $R/d = a2 > a1$.

complete separation of the two proteins should achieved for $E\tau$ values exceeding $(E\tau)_1$ whereas in case (2), which corresponds to a higher value of R/d, no purification is expected over the whole range of $E\tau$.

RESULTS AND DISCUSSION

Electrophoresis of single protein samples

In a first step, an experimental study was performed with single protein samples in order to determine the operating conditions to be used for further separation experiments.

Fig. 3 shows an example of results concerning the variation of the migration distance with $E\tau$. These variations are straight lines, the slope of which depends on the protein and on the pH of the carrier buffer. This linearity shows that for the operating conditions used, the influence of convective phenomena remains negligible. For a given protein, the curves obtained with the two chambers were found to be superimposed. These findings are in good agreement with eqn. 3. For each protein sample we then calculated the value of the "apparent" mobility, which is given by the slope fot the plot of the migration distance *versus* $E\tau$. The values obtained are reported in Table I for the three proteins and three pH values of the carrier buffer.

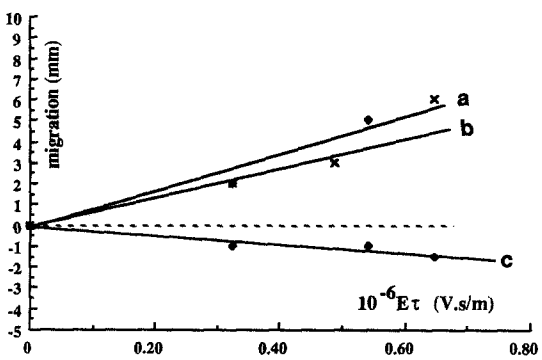

Fig. 3. Example of variation of the migration distance with $E\tau$. Operating conditions: Tris–borate buffer (pH 6.5); sample concentration, 0.3%. a = Albumin; b = α-lactalbumin; c = haemoglobin.

TABLE I

CALCULATED VALUES OF THE "APPARENT" MOBILITIES FOR VARIOUS pH VALUES OF THE TRIS–BORATE BUFFER ($\times 10^{-9}$ m^2 V^{-1} s^{-1})

Protein	pH		
	6.5	8.0	8.5
Haemoglobin	+2	−9	−9.6
α-Lactalbumin	−8.6	−13	
Albumin	−9	−16	−19

To obtain the values of the electrophoretic mobilities of the proteins, one needs to know the value of the electroosmotic mobility. To obtain that value, we used a haemoglobin sample at pH 7, which is very close to its p*I* so that its electrophoretic mobility can be assumed to be zero. As a result, the slope

TABLE II

CALCULATED VALUES OF THE ELECTROPHORETIC MOBILITIES ($\times 10^{-9}$ m^2 V^{-1} s^{-1})

Electroosmotic mobility = $10.3 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}.

Protein	pH		
	6.5	8.0	8.5
Haemoglobin	+7.1	−3.9	−4.5
α-Lactalbumin	−3.5	−7.9	
Albumin	−3.9	−10.9	−13.9

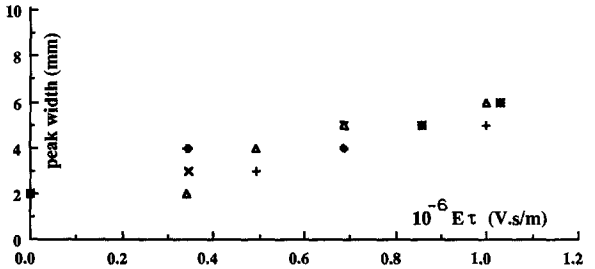

Fig. 4. Variation of the peak width with $E\tau$. Operating conditions: chamber B; Tris–borate buffer (pH 8.0); sample concentration, 0.3%; sample flow-rate = 1 ml h^{-1}. E varied: × = BSA; ◆ = α-lactalbumine. τ varied: △ = BSA; + = α-lactalbumin.

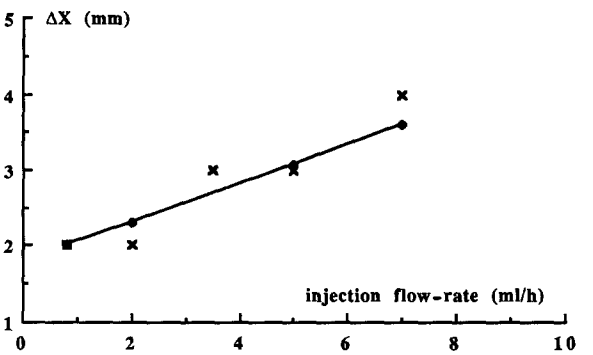

Fig. 5. Influence of sample flow-rate on the peak width: comparison between (×) experimental and (◆) calculated values. Operating conditions: chamber B; haemoglobin sample; Tris-borate buffer (pH 8.0); sample concentration, 0.3%; residence time, 120 s.

of the plot of Y *versus* $E\tau$ provides the value of $u_{os}/2$ (see eqn. 3). In this way we obtained for the electroosmotic mobility a value of about $10.3 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}, which is very close so that which had been determined by another method in a previous study [6]. Assuming that it is constant in the pH range investigated, we then calculated for the different pH values the values of the electrophoretic mobilities of the three proteins, which are reported in Table II.

Fig. 4 shows the results concerning the peak widths that were obtained for different values of $E\tau$ with two kinds of proteins. The peak width increases as $E\tau$ increases and in the range of operating conditions investigated the points obtained by changing either the electric field or the residence time are located on a single straight line. These linear variations show that the influence of electrohydrodynamic phenomena is negligible compared with that of other transport phenomena, as the peak width should otherwise be proportional the square of the electric field [7]. This means that the assumptions that were made to write eqn. 5 are correct. From that equation another important parameter appears to be the value of R/d. Its influence has already been discussed in details from a theoretical point of view by many workers [4,6,8]. In a previous paper [15], we presented a visualization system that can provide a measurement of the dimensions of the sample stream inside the separation chamber. That system was used to determine the value of R/d for different operating conditions.

Table III gives some values that were obtained with chamber B for different sample flow-rates. Using these values and those of the electrophoretic and elecroosmotic mobilities (see Table II), one can then calculate from eqn. 5 for a given value of $E\tau$ the variations of the peak width as a function of the injection flow-rate. Some calculated values are plotted in Fig. 5 together with the corresponding experimental values. There is good agreement between the calculated and experimental results.

Dealing with the influence of the chamber thickness, we have plotted in Fig. 6 the experimental results obtained with the two chambers for a given residence time and different electric field values. The width of the peaks is very sensitive to the thickness of the chamber, as halving the thickness makes the peaks about three times larger. Using the visualization device we obtained the values of R/d, which were found to be 0.9 for chamber A and 0.4 for chamber B under the operating conditions used during the experiments reported in Fig. 6. From these values we then calculated the peak width using eqn. 5 and the values were plotted in Fig. 6.

TABLE III

MEASUREMENTS OF THE VALUES OF R/d FOR DIFFERENT SAMPLE FLOW-RATES

Operating conditions: chamber B; residence time, 120 s; sample, haemoglobin (0.6%).

Injection flow-rate (ml h^{-1})	2	5	7	11
R/d	0.4	0.6	0.65	0.73

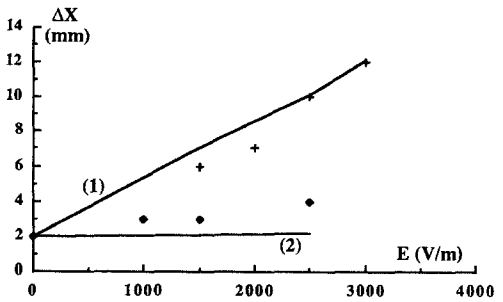

Fig. 6. Influence of chamber thickness on the variation of peak width with electric field: comparison between experimental and calculated values. Operating conditions: Tris–borate buffer (pH 8.0); BSA sample; sample concentration, 0.3%; injection flow-rate, 2 ml^{-1}; residence time, 220 s. Experimental: + = chamber A; ◆ = chamber B. Calculated: 1 = chamber A; 2 = chamber B.

These calculated values are very close to the experimental values.

The comparison of the widths of the peaks obtained for different proteins under given operating conditions also shows good agreement with eqn. 5, considering the influence of $|u_i + u_{os}|$. For instance, BSA and α-lactalbumin, the electrophoretic mobilities of which are very close to each other (see Table II), provide peaks having almost the same width (see Fig. 4), those obtained with Hb being wider. Finally, the influence of the pH was found to be much more sensitive with Hb, owing to a larger variation in its electrophoretic mobility.

Separation of proteins

The experimental results for single protein samples were found to be in good agreement with the theoretical considerations leading to eqn. 6. We therefore used that equation in order to determine the conditions for which a separation of two proteins was expected. This has already been discussed with respect to Fig. 2 in general.

Fig. 7 shows an example concerning the separation of Hb and BSA. With chamber A no total separation of the two proteins is to be expected under these operating conditions. In contrast, a total sep-

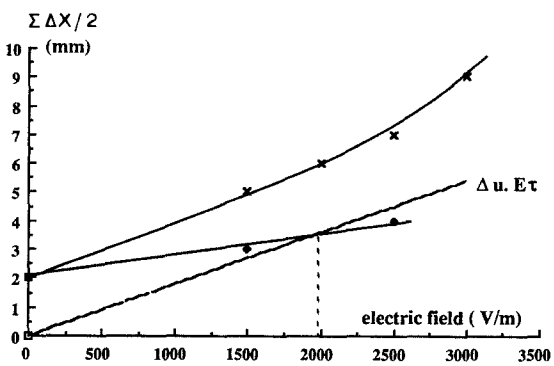

Fig. 7. Use of the separation criterion in the separation of haemoglobin and albumin. Operating conditions: as in Fig. 6; 0.3% of each protein. × = Chamber A; ◆ = chamber B.

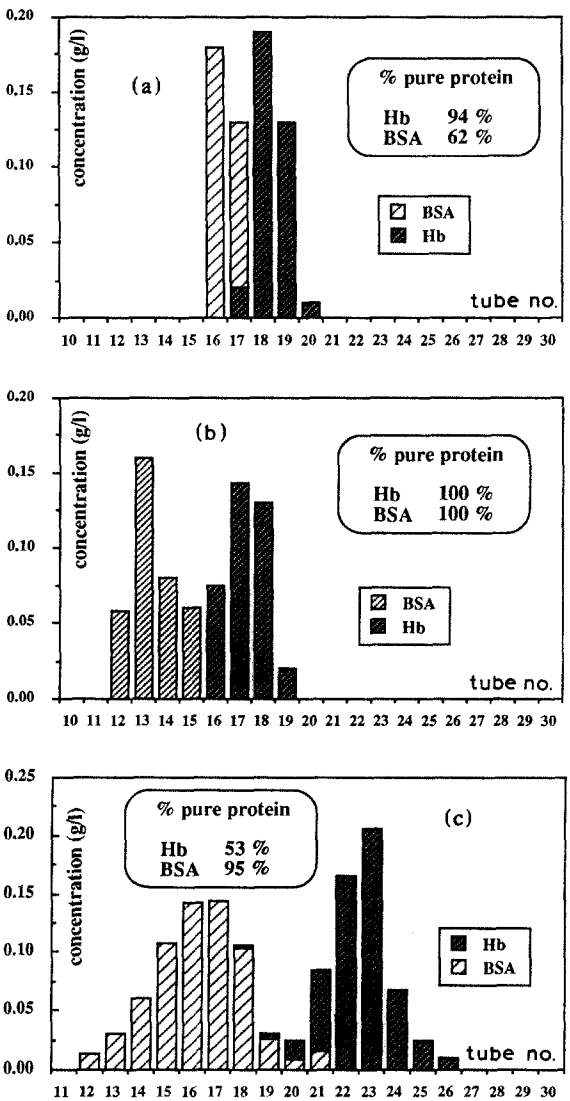

Fig. 8. Separation of Hb and BSA: experimental results. Operating conditions: as in Fig. 7. (a) Chamber B for E = 1500 V m^{-1}; (b) chamber B for E = 2500 V m^{-1}; (c) chamber A for E = 3000 V m^{-1}.

aration is expected to be possible with chamber B as soon as the electric field exceeds a given value, in this instance *ca.* 2000 V m^{-1}. Fig. 8 shows the concentration profiles obtained under these conditions during separation experiments performed at different voltages. On the same plots we give for each protein the percentage purity, which is defined as

% of A =
= (amount of A recovered pure/total amount of A) · 100.

In this instance for a pH of 8.0 the difference in electrophoretic mobility between Hb and BSA is about $7 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}. Working with chamber B a total separation of the two proteins is obtained for 2500 V m^{-1} whereas they are only partially separated at 1500 V m^{-1}. Working with chamber A only partial purification was obtained for electric fields up to 3000 V m^{-1}. These results are in very good agreement with those which were expected from Fig. 7.

Concerning the influence of the pH of the carrier buffer the difference in electrophoretic mobilities between Hb and BSA is about $11 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} at pH 6.5 whereas it is only about $7 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} at pH 8.0 (see Table III). Consequently, provided that there is no dispersive effect, the separation should be achieved more easily, *i.e.*, for lower voltages, at pH 6.5 than at pH 8.0. In Fig. 9 were plotted for the two pH values calculated variations of the left- and right-hand sides of inequality 6 *versus* the electric field strength. The value above which a separation is expected to be achieved is almost the same for the two pH values (*ca.* 2000 V m^{-1}). This is due to the fact that the variation in the difference in electrophoretic mobility is accompanied by a variation in the peak width, as discussed earlier. Again, the separation experiments have demonstrated that a total separation was obtained for the two pH values for an electric field above 2000 V m^{-1}.

As a total separation of Hb and BSA was achieved with the 3-mm thick chamber under low voltage operating conditions, we then investigated the separation of proteins with closer mobilities, such as BSA and α-lactalbumin (see Table II). The experiments were performed with chamber B at pH 8.0, where the difference in electrophoretic mobility between the two proteins is about $3 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}.

Fig. 10 shows the concentration profiles obtained for two values of the electrical field, the other operating parameters being kept constant. In addition, we have plotted in Fig. 11 the percentage purity *versus* the electric field strength. The percentage of

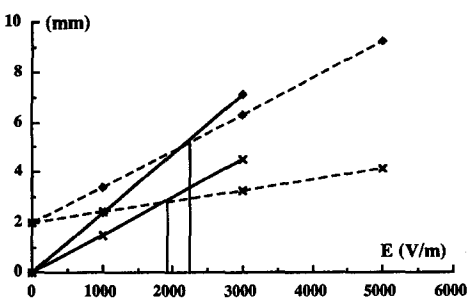

Fig. 9. Influence of the pH of the carrier buffer on the separation of Hb and BSA. Calculated variation of ΔY (solid lines) and ΔX (dashed lines) with E. Operating conditions: chamber B; R/d = 0.6; sample, BSA and Hb, 0.3% of each; injection flow-rate, 2 ml h^{-1}; residence time, 220 s. ◆ = pH 6.5; × = pH 8.0.

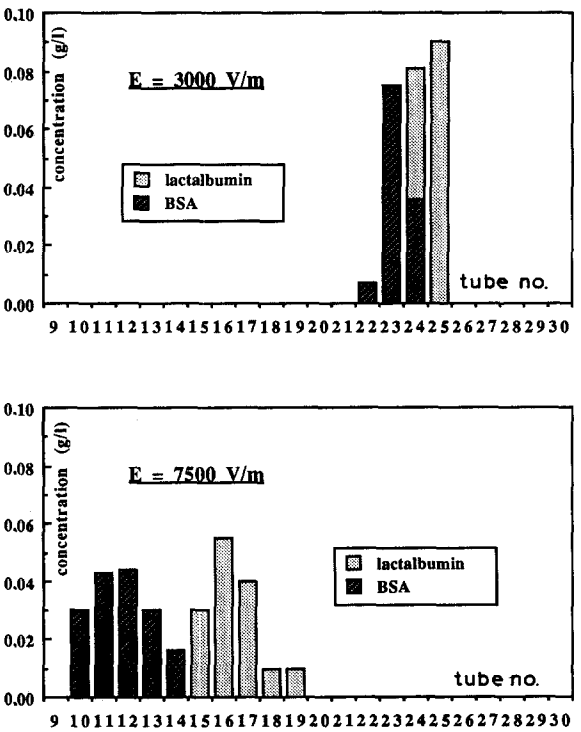

Fig. 10. Influence of the electric field on the concentration profiles obtained during the separation of albumin and α-lactalbumin. Operating conditions: chamber B; Tris–borate buffer (pH 8.0); sample concentration, 0.3% of each protein; injection flow-rate, 1 ml h^{-1}; residence time, 123 s.

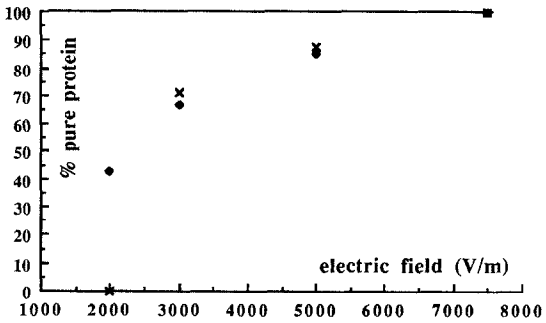

Fig. 11. Influence of electric field on the percentage of pure protein. Operating conditions: as in Fig. 10. × = BSA; ◆ = α-lactalbumin.

pure protein increases as the electric field is increased and reaches 100% at *ca.* 7000 V m^{-1}. This is in good agreement with the theoretical predictions. Indeed, under these conditions, for which R/d was measured to be 0.3, eqn. 6 predicts that a total separation of these two proteins is reached as soon as the electric field exceeds 6500 V m^{-1}.

CONCLUSION

Any electrophoretic separation is based on the difference between the electrophoretic mobilities of the compounds concerned. When performed in the continuous-flow mode, the migration is generally accompanied by a widening of the corresponding peaks. In this work we have defined a separation criterion that gives a relationship between the migration distance and the peak width that should be met for the products to be recovered pure at the outlet of the chamber.

From previous theoretical work, we have used an analytical relationship that relates both the migration distance and the peak width to the operating conditions and to the chamber geometry. In a first step an experimental study was carried out with single protein samples. It was found that in the range of operating conditions investigated both the migration and the peak width follow linear variations with $E\tau$. Using an appropriate protocol, the electroosmotic mobility was estimated, thus making it possible to obtain the values of the electrophoretic mobilities of the proteins. For each set of conditions the value of R/d was measured using a previously developed visualization system. The experimental results were then compared with the calculated values and good agreement was obtained.

In a second step, the separation criterion was used in order to predict the operating conditions to be used to perform separations of proteins of known mobilites. Again, the operating ranges for which a total separation was expected were found to be very close to those given by the experimental results. Under some operating conditions a total separation of BSA and α-lactalbumin, with a difference in mobility of $3 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}, was obtained.

To achieve the purification of proteins of closer mobilities one has to operate either with more severe conditions, *i.e.*, higher voltages or higher residence times, or with lower values of R/d. When working under severer operating conditions, some other dispersive effects, *e.g.*, convection or electrohydrodynamic phenomena, can become predominant. In that event no prediction can be made directly from the results presented in this paper as the operating conditions were intentionally restricted to those making both convection and electrohydrodynamic phenomena negligible. On the other hand, whereas using lower values of R/d should improve the purity of the collected products, it will also result in a decrease in the production rate, which is another important parameter to consider for this process to remain on the preparative scale.

Further work will concern the choice of the most suitable compromise between production and purity and the study of the purification of proteins from real protein mixtures.

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