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EXPERIMENTAL AND THEORETICAL DYNAMICS OF ISOELECTRIC FOCUSING

III. TRANSIENT MULTI-PEAK APPROACH TO EQUILIBRIUM OF PROTEINS IN SIMPLE BUFFERS

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SUMMARY

Transient states in the isoelectric focusing of proteins in simple three-component buffer systems were examined by (i) analyzing the collected fractions from continuous flow electrophoresis at a range of operating conditions, (ii) photographing the behavior of colored proteins in capillaries, (iii) monitoring the electric field dynamics along a capillary with a potential gradient array detector and (iv) computer simulation. The good agreement between simulation and experimental data clearly reveals how the separation dynamics of the buffer system, *i.e.*, the formation of a natural pH gradient, produces the observed peaks and boundaries of protein during the approach to the steady state. The protein focusing dynamics are different, but characteristic, for each buffer system, with both transient double, and multiple, peaks being observed.

INTRODUCTION

Characterization of the transient processes in isoelectric focusing (IEF) is of interest in order to establish when the steady state is reached. A variety of procedures have been developed by a number of investigators for examination of these transient states. Repetitive optical scanning has been employed with rotating[1] and density gradient-stabilized[2] free fluid columns as well as gel filled columns[2]. Gels have also been segmented at various times during the course of an experiment and measure-

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ments of pH, conductivity, absorbance, radioactivity and biological activity made on segment eluates (see ref. 3 for a review). Additionally, Weiss *et al.*[4] have developed a theoretical description of sample behavior in the presence of pre-established, linear pH gradients.

Our prior studies of the dynamics of IEF have employed an amalgam of experimental and computer simulation data. In a first publication on the subject, a general separation mechanism was elucidated[5]. It was found that the focusing process proceeds in two phases, a relatively fast separation phase followed by a slow stabilizing phase during which a steady state is reached. The latter phase provides an explanation of the plateau phenomenon in IEF[6]. A second paper reported the impact of various electrode assemblies on the focusing process and the decay of the focusing pattern caused by cathodic, anodic or symmetrical drifts[7]. Those studies utilized low-molecular-weight components with the experimental investigations conducted in free solution in capillaries. In this third contribution on focusing dynamics, computer simulation and experimental data are employed to describe transient protein distributions during focusing in three-component buffer mixtures. Experiments were carried out in free solution using both continuous flow and capillary instruments.

MATERIALS AND METHODS

Instrumentation

The CapScan capillary-type apparatus with a linear potential gradient array detector along the focusing column has been described in detail elsewhere[5,8]. This instrument allows the electric field profile along the focusing axis to be recorded within 20 min, fully controlled by a desk top computer. Two different troughs of rectangular cross-section and 10 cm length were used, which have channel widths of 1 or 15 mm and a height of about 0.4 mm (ref. 8). Dialysis membranes with a molecular mass cut-off of 12 000–14 000 (Spectrapore No. 132709; Spectrum Medical Industries, Los Angeles, CA, U.S.A.), were used to isolate the focusing capillary from the electrode compartments. Small amounts of NaOH and phosphoric acid, 0.1 *M* each, were the respective cathodic and anodic electrolytes. A Kepco power supply APH 2000 M provided either constant voltage or current. Experiments were performed at room temperature.

Some of the continuous flow experiments were done with the Elphor VaP 22 (Bender and Hobein, Munich, F.R.G.). The instrument has a vertical, rectangular separation chamber (50 cm × 10 cm × 0.05 cm). Five buffer and four sample inlets are provided at the top of the chamber. A computer controlled reflectance scanner records absorbance at 280 nm along the separation axis just prior to the exit. Ninety fractions are collected. Experiments were performed at 4°C. Other experiments utilized a laboratory-made continuous flow device with chamber dimensions of 27 cm × 22 cm × 0.05 cm. Eight inlet ports and ninety outlet ports are provided. Experiments with this device were also performed at 4°C.

Computer simulations

The dynamic computer model of Mosher *et al.*[9] was used to simulate the behavior of proteins and buffer constituents. The one-dimensional approach assumes the absence of convective flows and thermal gradients. The specified initial conditions

TABLE I

ELECTROCHEMICAL PARAMETERS USED FOR COMPUTER SIMULATION

Component	pK_1	pK_2	*Mobility* · 10^4 ($cm^2 \cdot v \cdot s$)
Glutamic acid	2.16	4.29	2.97
Cycloserine	4.40	7.40	3.42
Histidine	6.02	9.17	2.85
Arginine	9.04	12.48	2.26
H_3O^+			36.27
OH^-			19.87

included the uniform distribution of all constituents, the diffusion coefficient and net charge–pH relationship of the proteins, the pK and mobility values of the buffer constituents, the current density and the electrophoresis time. The program predicts concentration profiles and pH and conductivity gradients as a function of time. Input data are summarized in Tables I and II.

Experimental procedures

Analytical grade chemicals were used. Human hemoglobin was prepared from fresh, washed red blood cells by standard techniques. Bovine serum albumin was

TABLE II

NET CHARGE *vs.* pH RELATIONSHIPS FOR HEMOGLOBIN AND ALBUMIN

Ionization data were adopted from ref. 10 for hemoglobin and from ref. 11 for albumin. The diffusion coefficient used for hemoglobin is[12] $6.8 \cdot 10^{-11}$ m^2/s and for albumin is[13] $5.94 \cdot 10^{-11}$ m^2/s.

pH	*Net charge*	
	Hemoglobin	*Albumin*
3.0	68.5	58
3.5	43.5	35.5
4.0	–	13
4.5	25.5	–
4.8	–	0
5.56	–	−6.1
6.0	10.25	–
6.56	–	−12.2
7.0	0	–
7.56	–	−18.3
8.0	−10.25	–
8.56	–	−24.4
9.0	−20.5	–
9.56	–	−30.5
10.0	−30.75	–
10.06	–	−33.55
11.0	−50	−44
11.5	−63.5	−64
12	–	−84

obtained from Pentex as a 35% solution. In the CapScan the progress of the experiment was followed by monitoring the dynamics of the electric field and/or photographically recording the distribution of hemoglobin and bromophenol blue-stained albumin. Data from the continuous flow instruments represent either measurements on collected fractions or were obtained with the scanner on the VaP 22. Buffer constituents were analyzed by thin-layer chromatography (TLC) using a mobile phase of ethanol–water–concentrated ammonia (70:25:5, v/v). Kieselgel 60 (0.2 mm) aluminum TLC plates and ninhydrin (0.1%) spray for chromatography were from Merck (Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

The glutamic acid–histidine–arginine system

This system is comprised of 10 m*M* each of glutamic acid (Glu), histidine (His) and arginine (Arg) as the background buffers with 0.5 mg/ml albumin as the sample, which focuses in the boundary between Glu and His. Simulation, continuous flow and capillary data are shown in Figs. 1, 2 and 3, respectively, which collectively present the dynamics of this mixture. The three buffer components behave according to the focusing mechanism described previously[5], thus the presence of the protein at this concentration does not alter the behavior of the buffers, or the development of the pH gradient. Two schematic representations of the amino acid distributions are included above time points 10 and 30 in Fig. 1A. The boundaries referred to in the following discussion are indicated there.

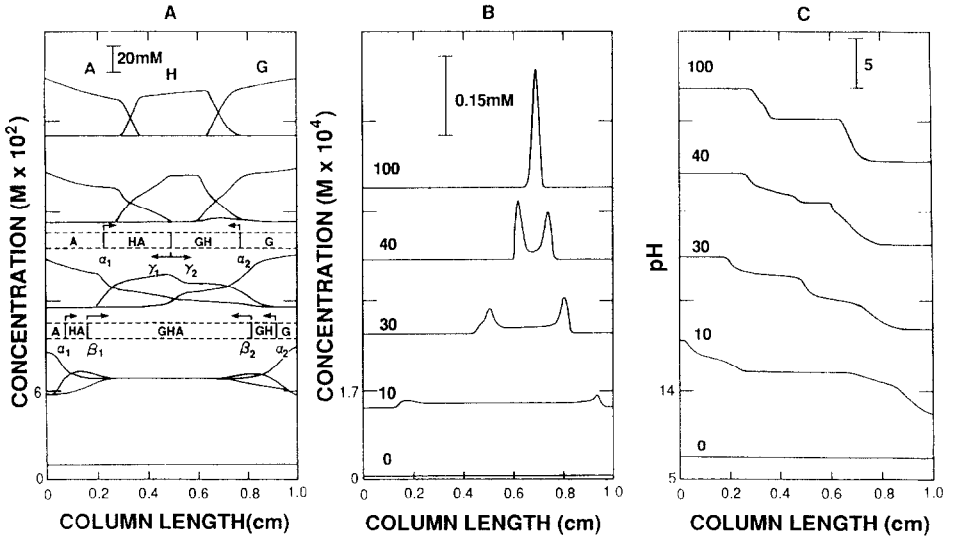

Fig. 1. Computer simulation data showing the focusing behavior of 0.5 mg/ml albumin in a buffer system composed of 10 m*M* each of glutamic acid (G), histidine (H) and arginine (A). (A) The transient behavior of the amino acids; (B) the albumin dynamics; (C) the development of the pH gradient. Profiles are shown after 0, 10, 30, 40 and 100 min of current flow (5 A/m^2) and are offset vertically by a constant amount to facilitate presentation. The focusing mechanism of the ampholytes is displayed schematically above the 10- and 30-min profiles. Migrating boundaries are indicated by vertical solid lines (5). Vectors indicate direction and relative velocity. The anode is to the right.

Fig. 1B depicts the computed albumin distribution at 0, 10, 30, 40 and 100 min after current application. On the anodic side, albumin accumulates in the boundary which demarcates the production of the pure Glu zone. On the cathodic side, the protein migrates with the boundary which marks the trailing end of the Glu distribution. These boundaries correspond to α_2 and β_1, respectively of Fig. 1A. The sharp anodic peak grows as it migrates toward the position of final focus. A sharpening of the cathodic protein peak occurs about 30 min after current application, at the time of the meeting of the two faster boundaries (β_1 and β_2), *i.e.*, the beginning of the establishment of the pure central histidine zone. During the evolution of this zone the cathodic protein peak continues to migrate with the same velocity as that of Glu (now the γ_2 boundary). The magnitudes of the two migrating protein peaks increase until they ultimately merge. This coalescence corresponds to the conclusion of the separation phase of the three buffer constituents. Thus, the protein and the buffer reach their final positions at the same time.

The experimental data shown in Figs. 2 and 3 qualitatively validate the computer predicted focusing scheme for continuous flow and capillary devices. The most obvious discrepancy is found in the continuous flow data, where the cathodic protein

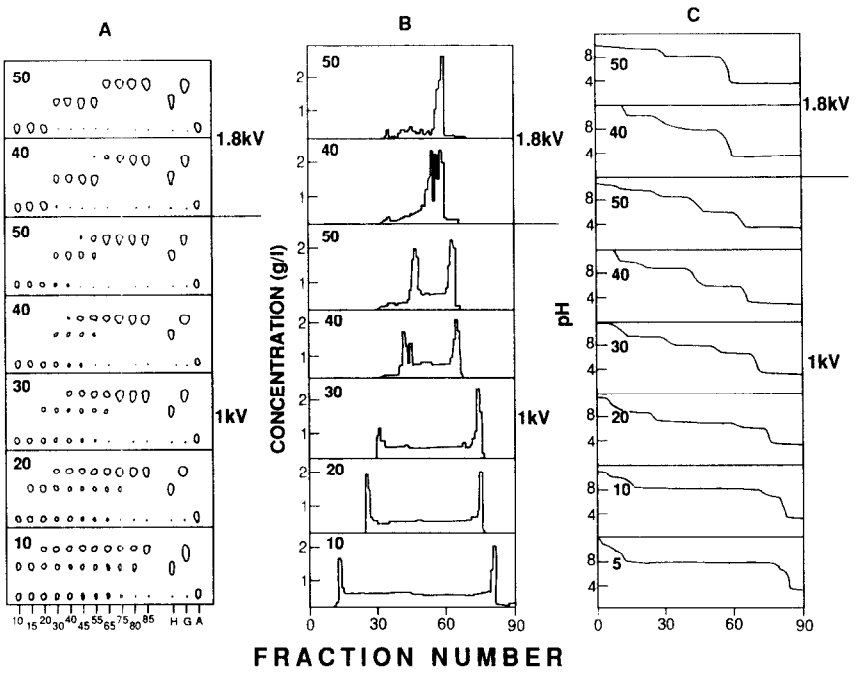

Fig. 2. Experimental data which show the focusing of 0.5 mg/ml albumin in a buffer system composed of 10 m*M* each of glutamic acid, histidine and arginine. These data were obtained with the laboratory made continuous flow instrument, utilizing cellulose acetate membranes, and 0.1 *M* phosphoric acid and 50 m*M* NaOH as anolyte and catholyte, respectively. (A) The distribution of the amino acids as a function of the residence time, indicated in minutes, and the applied voltage, 1 kV for the lower six time points and 1.8 kV for the upper two time points. These data are schematic representations of TLC separations. For each time point the first three lanes from the right contain the pure amino acids applied as references. Measurements on collected fractions provided the corresponding protein distributions shown in (B) and the pH distributions of (C). The anode is to the right.

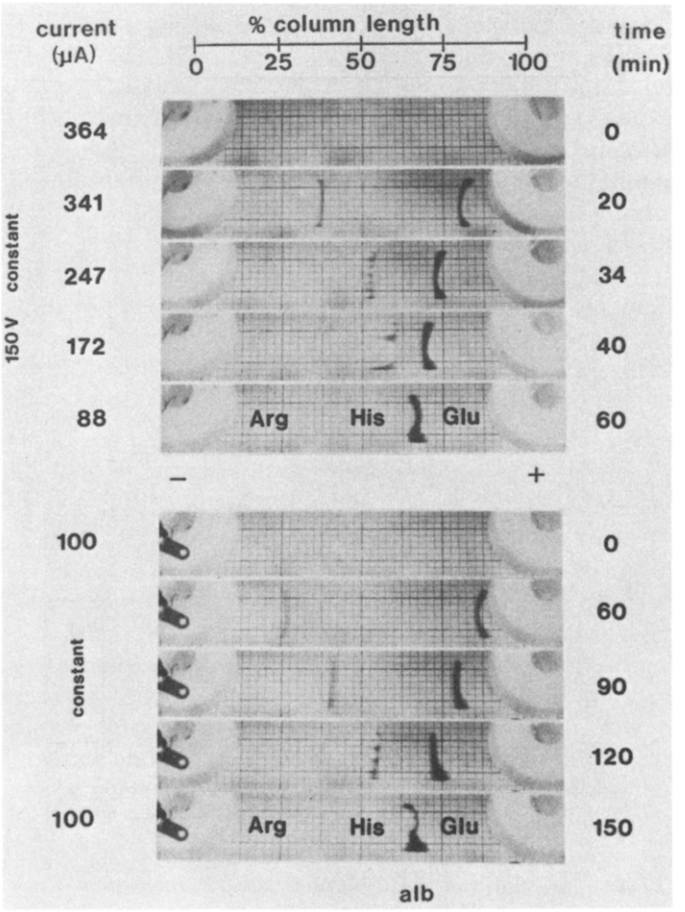

Fig. 3. The focusing of bromophenol blue-stained albumin in a ribbon-like capillary is presented at constant voltage (150 V) in the upper half of the figure and at constant current (100 μA) in the lower half. The initial buffer composition is the same as that in Figs. 1 and 2. The anode is to the right. Note that the weaker (cathodic) transient peak is interrupted by an organized, unidentified flow pattern at 34 and 40 min at constant voltage and at 120 min at constant current. The approximate steady state positions of the focused buffers are indicated by their respective abbreviations.

peak is relatively larger than predicted by the simulation. The reason for this is unclear. However, this peak in the capillary data, at both constant current and constant voltage, is much fainter than its anodic counterpart. In general, the theoretical predictions and experimental data correlate well and exhibit the focusing mechanism found in Ampholine-based configurations and referred to as the transient double peak approach to equilibrium[14]. The prediction by Dishon and Weiss[15] that nonlinear pH changes are necessary to produce that mechanism is consistent with this data.

The glutamic acid–cycloserine–arginine system

Figs. 4–6 present the detailed focusing process of albumin (about 0.5 mg/ml) in a system consisting of Glu, cycloserine (Cser) and Arg (10 m*M* each). Panel A of Fig.

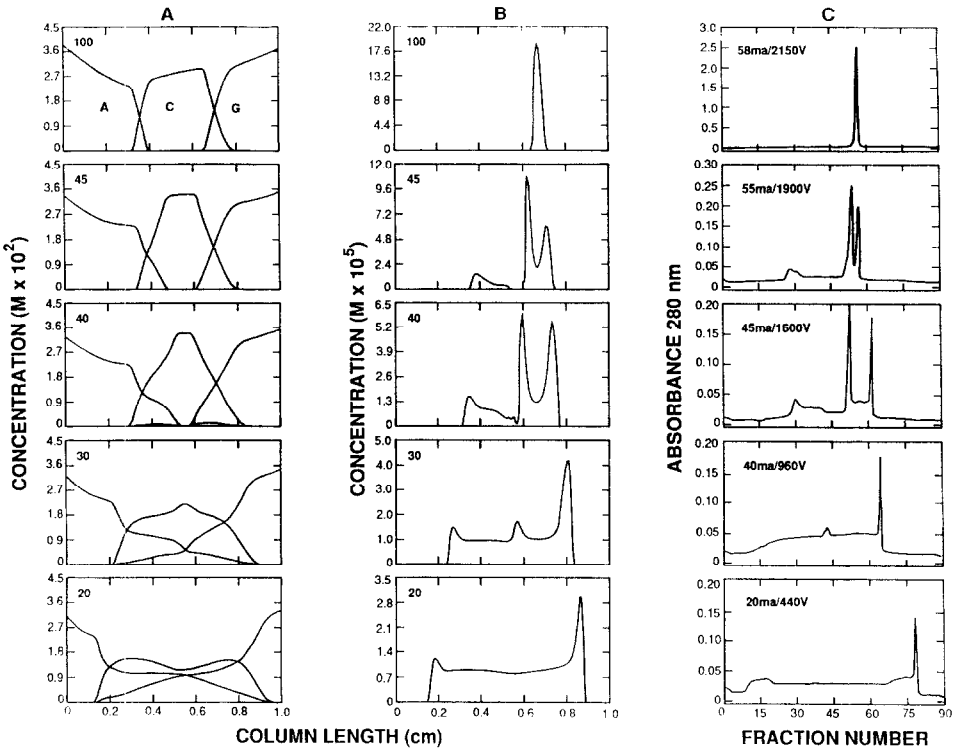

Fig. 4. Simulation and continuous flow experimental data which describe the focusing of albumin (0.5 mg/ml) in a buffer system composed of 10 m*M* each of glutamic acid (G), cycloserine (C) and arginine (A). (A) The simulated behavior of the buffer components after 20, 30, 40, 45 and 100 min of current flow (5 A/m^2). The anode is to the right. (B) The simulated behavior of albumin at the same time points. (C) Corresponding experimental data taken with scanner on the Elphor VaP 22, with a residence time of 11 min. The current and applied voltage when the scans were recorded are indicated in each panel. The upper three distributions were obtained in the presence of 0.3% hydroxypropylmethylcellulose (HPMC), whereas the lower two were taken in the absence of any additives.

4 shows the computer predicted behavior of the electrolytes which focus by the mechanism presented in Fig. 1A. The focusing of albumin (Fig. 4B and C) begins with a strong peak from the anode and a very weak counterpart at the cathode. These peaks are moving within boundaries α_2 and α_1, respectively. This is a different mechanism to that displayed in the Glu–His–Arg system, with the protein moving relatively more slowly in the anodic direction. The weak cathodic peak (α_1 boundary) is not visible in the capillary data (Fig. 5), either at constant voltage or at constant current.

The emergence of the pure zone of Cser marks the appearance of a new peak of albumin which migrates with the γ_2 boundary. This peak grows rapidly as it migrates toward the strong peak coming from the anode, surpassing it in size before the two meet. The focusing of albumin in this system thus displays three peaks or areas of increased protein concentration, which constitutes a second difference in comparison to the Glu–His–Arg system. Only a trace of albumin is present within the emerging Cser zone. The protein migrating within the α_1 boundary and in the transient zone

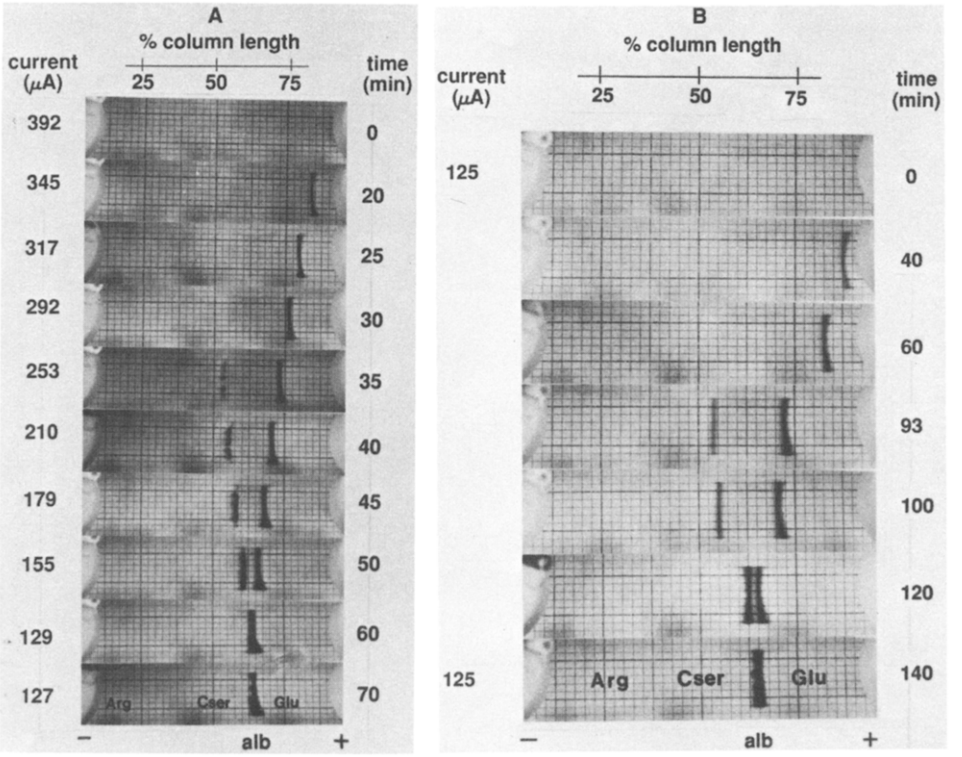

Fig. 5. The focusing behavior of 0.5 mg/ml bromophenol blue-stained albumin in a ribbon-like capillary in the Glu–Cser–Arg buffer system at constant voltage (150 V) (A) and at constant current (125 μA) (B). A strong band of protein appears suddenly between 30 and 35 min at constant voltage and between 60 and 93 min at constant current. The approximate steady state positions of the focused buffers are indicated by their respective abbreviations.

between boundaries α_1 and γ_1 slowly joins the major peak, the focusing of albumin being completed after the focusing of the ampholytes, a third difference from the Glu–His–Arg system. The data in Fig. 4C clearly confirm that the experimental behavior in the Elphor VaP 22 follows, in detail, the theoretical prediction. The capillary data (Fig. 5) also show the sudden appearance of the protein peak (in boundary γ_2) which appears upon the emergence of the pure, colorless Cser zone. This occurs between 30 and 35 min at constant voltage and 60 and 93 min at constant current.

Fig. 6 presents the computed (panel A) and experimental (panel B) behaviors of the electric field profiles for this system. The major boundaries (α_1 and α_2) which migrate away from each electrode are clearly visible as is the appearance of the pure central zone of Cser, which is just beginning at 30 min in the simulation data. In panel B the albumin makes a clear contribution to the steady state profile, in the boundary between Glu and Cser. Under the simulation conditions used, the protein makes no significant contribution to the potential gradient data. The current density in the experiment is about three-fold higher than that assumed for the simulation.

Fig. 7 presents simulation data, and experimental data obtained with the rib-

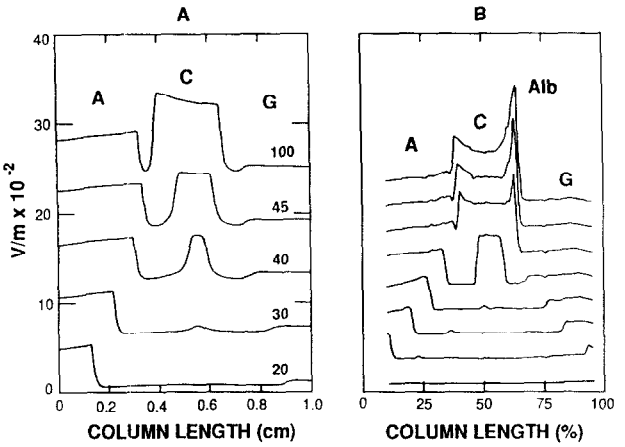

Fig. 6. Simulated (A) and experimental (B) voltage gradient profiles for the Glu–Cser–Arg system containing 0.5 mg/ml albumin. Successive time points are offset vertically to facilitate presentation. The anode is to the right. In (A) insufficient protein is present to have a visible impact on the profiles. In (B) the steady state concentration of the focused protein is high enough to affect the electric field in the Glu–Cser boundary. The positions of the focused buffers are indicated. Simulation conditions are the same as for Fig. 4. The CapScan experiment was performed at a constant current of 10 μA. The scans represent the electric field distribution at intervals of 40 min.

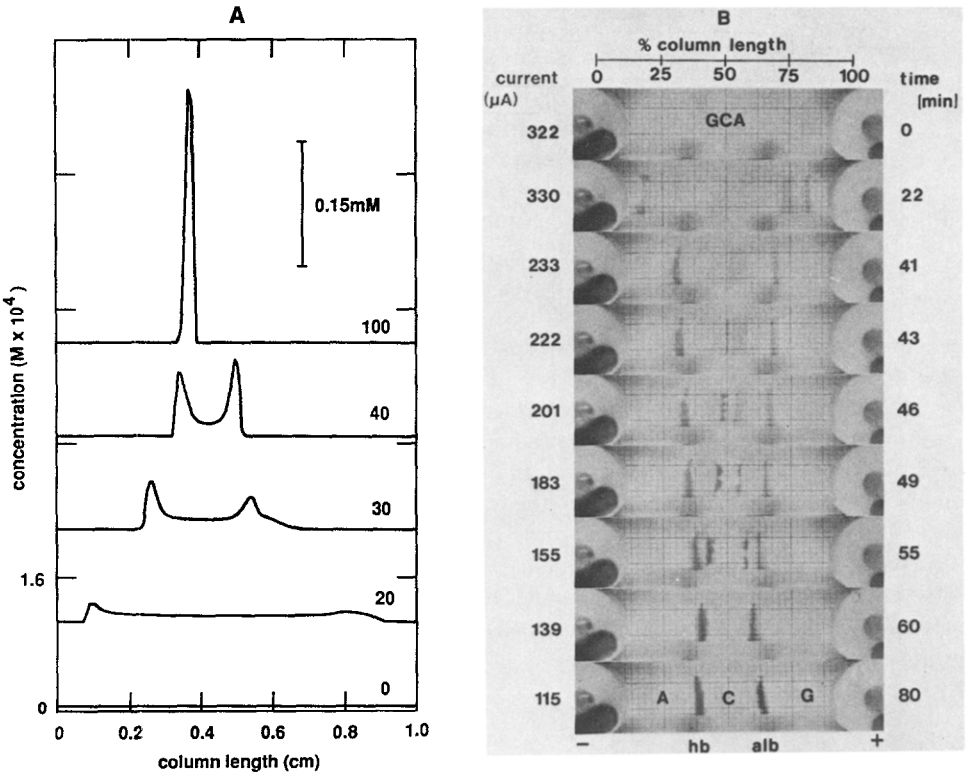

Fig. 7. The simulated focusing of hemoglobin in the Glu–Cser–Arg system (A). The time points are labelled in min of current flow (5 A/m^2). The anode is to the right. (B) The focusing of hemoglobin and bromophenol blue-stained albumin in the ribbon-like capillary. The focusing was accomplished under a constant 150 V.

bon-like capillary device, which show the focusing behavior of hemoglobin and albumin in the Glu–Cser–Arg system. Hemoglobin behaves much differently than albumin, showing a simpler, double-peak approach to equilibrium, as is the case for albumin in the Glu–His–Arg system. The peak which migrates away from the cathode is sharper than its counterpart from the anode, and moves within the α_1 boundary. The broad peak which leaves the anode migrates within the faster β_2 boundary. It undergoes a substantial sharpening when the pure zone of cycloserine begins to emerge (the appearance of the γ boundaries), after 30 min of current flow. It is clear from the simulation data that hemoglobin focuses faster than does albumin in this system, reaching its equilibrium position at approximately the same time as do the background buffers.

CONCLUSIONS

The model used to represent the electrophoretic behavior of proteins[9] accurately predicts the IEF behavior of hemoglobin and albumin in two different buffer systems. This model should prove valuable for the continued study of the fundamental behavior of proteins in this electrophoretic mode. It confirms that proteins will focus as rapidly, or nearly so, as do the background buffers in free solution. However, this will not be true in gels, because of the sieving effect of even the low %T gels commonly used for IEF. This means that the focusing mechanisms in gels will likely be different than those in free solution. The correspondence of the experimental results from continuous flow instruments and capillaries indicates the high degree of fluid stability present in these devices[16] and that the mechanisms presented hold for static as well as flowing solutions. However, a careful inspection of some of the migrating protein peaks in the capillary results in Figs. 3, 5 and 7 reveals a patterned disruption, *e.g.*, the fainter albumin line in the 120-min time point in Fig. 3. These are observed when a system-dependent potential gradient is exceeded, and are presumably due to an organized, non-uniform, electrohydrodynamic flow. This effect has been observed in continuous flow instruments and is a function of the conductivity and dielectric gradients present[17]. Electroosmosis can be ruled out as the source of the instability because these patterned states can be observed in the presence of a.c. fields[18].

Focusing mechanisms are dependent upon the protein and the buffer system and independent of whether the experiment is performed at constant current or constant voltage. Hemoglobin and albumin display completely different focusing mechanisms in the Glu–Cser–Arg system, and albumin focuses differently when cycloserine is replaced with histidine. The multiple transient peaks displayed by albumin in the Glu–Cser–Arg buffer are quite unusual; such multiple peaks are only likely to be observed in simple buffer systems. More complex buffers will promote the more common mechanism characterized by two peaks and called the transient double peak approach to equilibrium[14].

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