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AN EXPLANATION FOR THE PLATEAU PHENOMENON IN ISOELECTRIC FOCUSING

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SUMMARY

Isoelectric focusing of mixtures of simple ampholytes occurs in two phases, an initial rapid separation phase and a second relatively slow stabilizing phase. Transient and steady-state computer simulation data are shown to predict the development of pH plateaus around neutrality during the stabilizing phase of the focusing of such mixtures. This occurs because a non-zero electrophoretic flux is present in a pure zone of focused ampholyte, which is a function of both its isoelectric point (pI) and its ΔpK value. For an ampholyte with a pI > 9 or < 5 this flux causes the development of a significant concentration gradient within its focused zone which is accompanied by a contraction of this zone along the focusing axis. Acidic and basic ampholytes are thereby displaced toward the anode and cathode respectively, creating a pH plateau in the neutral region. Thus, there will be regions of the focusing column, closer to the electrodes and containing more acidic or basic pH values, within which the resolution of samples will reach a maximum and then decrease.

INTRODUCTION

Isoelectric focusing (IEF) is an exceptionally valuable technique for the fractionation of complex mixtures of proteins. Its widespread popularity was achieved with the introduction of synthetic carrier ampholyte mixtures[1] which produce reasonably linear pH gradients that are stable for several hours. In extended experiments, however, these gradients exhibit a progressive flattening around neutrality which has been referred to as the "plateau phenomenon"[2]. This behavior is characterized by a progressive loss of stainable ampholyte species near the center of the gel and a concomitant increase in such species closer to its ends[3]. Similar results have been observed when the pH gradient is formed with mixtures of simple ampholytes[4]. Several characteristics associated with the plateau phenomenon have been described by Miles *et al.*[5]. These include: (a) displacement of protein bands away from the center with no change in pI; (b) the rate of change of pH along the neutral region decreases with time; (c) the rate of plateau formation is proportional to the applied field; (d) plateau formation does not require loss of ampholytes; (e) there is a decrease in conductivity in the neutral region and an increase in conductivity in the acidic and

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basic regions; (f) increasing viscosity and ionic strength decrease the rate of plateau development.

In a previous paper[6] we have presented experimental and computer simulation data describing the dynamics of the potential gradient during the IEF of mixtures of simple buffers including amino acids, dipeptides and monovalent acids and bases. These data show focusing to occur in two phases, an initial rapid separation phase and a second relatively slow stabilizing phase which is most evident when the pH gradient encompasses regions significantly removed from neutrality, *i.e.* above pH 9 and below pH 5. The first phase involves the condensation of each buffer into a pure zone. The second phase is much longer and constitutes the approach to the final steady-state distribution. During this phase the boundaries between the buffer zones drift toward the electrodes; those below pH 7 toward the anode and those above pH 7 toward the cathode. This behavior appears to provide an explanation for the events which occur during the formation of the pH plateaus referred to above. We have used our computer models for the steady state in IEF[7,8] and for transient electrophoretic processes[9–12] to examine this phenomenon. We show the plateau phenomenon to be an integral aspect of the focusing process.

MATHEMATICAL MODELLING

Our mathematical models have been described in detail elsewhere[7–12]. The inputs required for a simulation of the steady state in IEF include the concentrations of each component (ampholytes or monovalent weak buffers) at one end of the focusing column, pK and mobility values for each component, the current and column length. The outputs include the concentration profile of each component along the column length and a variety of data which can be derived therefrom including the pH, conductivity and buffer capacity profiles. The inputs required for a transient simulation include the pK values and mobilities of each component, current, column length, the initial distribution of each component within the electrophoretic column and the amount of electrophoresis time to be simulated. The outputs include the data mentioned for the steady-state program at specified time points. All simulations were performed with a column length of 0.01 m with the solution computed at 101 evenly spaced grid points.

RESULTS

The drift which occurs after focusing ampholytes have condensed into pure zones but before the steady-state distribution has been reached is most clearly shown by conductivity profiles. Fig. 1A presents four conductivity profiles from the computer simulation of the focusing of three hypothetical ampholytes. Each was uniformly distributed throughout the column at a concentration of 10 mM prior to applying a current of 10 A/m^2. The time points shown are after 25, 50, 100 and 150 min of current flow. This system is very similar to the glutamic acid, histidine and arginine system presented previously[6], the pI values being 3, 7 and 11. The boundary shapes change very little during this time span but their positions clearly shift towards the electrodes. The boundary between the basic ampholyte and the neutral component shifts somewhat more than does the acidic boundary. Fig. 1B and C present the

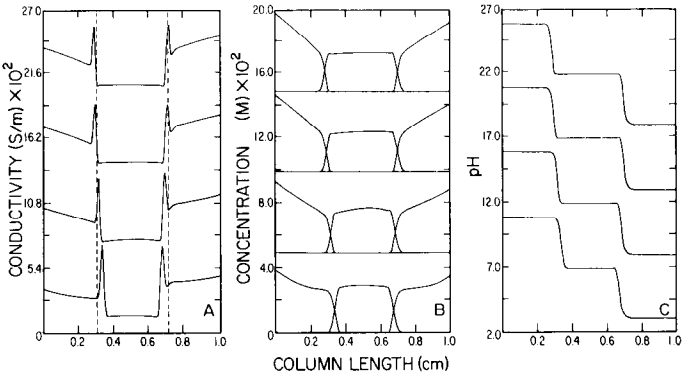

Fig. 1. Data derived from a simulation of the focusing of three hypothetical ampholytes with pI values of 3, 7 and 11. All ΔpK values are 2 and all ionic mobilities $3.0 \cdot 10^{-8}$ m²/Vs. The anode is to the right. The time points displayed are after 25 (bottom), 50, 100 and 150 (top) min of current flow. In panel A each successive conductivity profile is offset from the previous one by $6.5 \cdot 10^{-2}$ S/m for purposes of presentation. The cathodic boundary clearly drifts more than the anodic one. Panel B displays the corresponding concentration profiles plotted with an offset of $5.0 \cdot 10^{-2}$ M and panel C the corresponding pH profiles plotted with an offset of 5 pH units.

corresponding concentration and pH profiles. The 150-min time point is very close but not identical to the steady-state distribution. The experimental data for the glutamic acid, histidine and arginine system have been presented elsewhere[6].

This drift occurs because the zones of pure ampholytes which have formed during the separation phase have not yet reached the steady state. Those ampholytes with pI values other than 7 are not isoelectric, in the sense that the concentrations of the positively and negatively charged species are equal. A pure zone of acidic ampholyte has an excess of negatively charged ampholyte species, equal to the concentration of hydrogen ion present, to satisfy the requirement of electroneutrality. Thus, even after an acidic ampholyte has condensed into an "isoelectric zone" it retains an electrophoretic flux toward the anode. Similar statements apply to basic ampholytes which show a flux toward the cathode. The slow boundary drift occurs as the concentration profile across a zone of acidic or basic ampholyte acquires a slope. This generates a diffusional flux in a direction opposite to the electrophoretic flux and establishes the steady state[13]. The stabilizing phase is much longer than the separation phase[6] because the net mobilities of the focusing components are much smaller when in pure zones than when mixed with other buffers.

Systems of ampholytes with pI values closer to neutrality display a smaller drift. The pH dependence of this boundary migration is shown in Fig. 2. These conductivity data are derived from a simulation of the focusing of a hypothetical system of five ampholytes with pI values of 5, 6, 7, 8 and 9. Each was initially uniformly distributed throughout the column at a concentration of 10 mM. The applied current was 10 A/m². The times shown are 50, 100, 150 and 200 min after current application. These boundaries do not appreciably drift. The zones of the focused ampholytes display flat concentration profiles (data not shown) because the difference in the concentrations of the oppositely charged species of each is quite small. Therefore

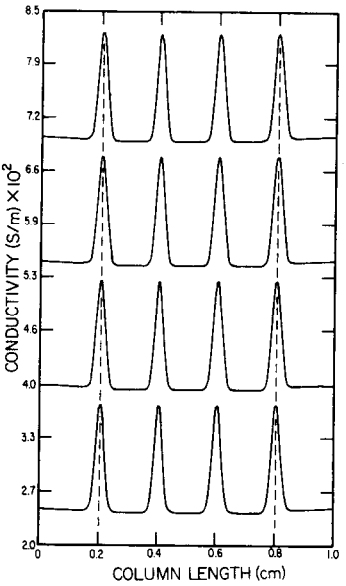

Fig. 2. Conductivity data derived from a simulation of the focusing of five hypothetical ampholytes with pI values of 5, 6, 7, 8 and 9. All ΔpK values were 2 and all ionic mobilities $3.0 \cdot 10^{-8}$. The anode is to the right and the current density was 10 A/m^2. Each successive time point is offset from the previous one by $1.5 \cdot 10^{-2}$ S/m. The time points shown are after 50, 100, 150 and 200 min of current flow.

there is a very low net electrophoretic flux of these components and no significant concentration gradient is required to establish the steady state.

The concentration gradient across a zone of focused ampholyte is a function of the pI of the ampholyte. This is shown in Fig. 3 which presents the steady-state concentration profiles of three different ampholyte mixtures. These systems are identical with respect to molar amounts of each ampholyte present, component mobilities and current. The only difference lies in the isoelectric points. The three components displayed in Fig. 3A have pI values of 3, 7 and 11. This profile is the steady-state distribution for the system presented in Fig. 1. The central component is the same in each panel and has a pI of 7. The other components have pI values of 4 and 10 in Fig. 3B and 5 and 9 in Fig. 3C. It is clear that the slopes of the zones increase as the isoelectric point recedes from pH 7. With an acidic and a basic ampholyte which have pI values equidistant from neutrality, the basic ampholyte zone has a greater slope than the acidic one. This is due to the greater electrophoretic mobility of the hydrogen ion as compared to the hydroxyl ion. The zones of focused basic ampholytes thus possess lower conductivities and greater voltage gradients than do zones of equivalent acidic ampholytes. Equivalent in this case means equal ionic mobilities and equal differences between the pK values which bracket the isoelectric point (ΔpK), as well as isoelectric points equidistant from neutrality. Given equal initial amounts, an ampholyte zone with a greater concentration slope will occupy less distance along the focusing axis. It is this contraction of the ampholyte zones along the focusing axis which causes the movement of the boundaries and the creation of

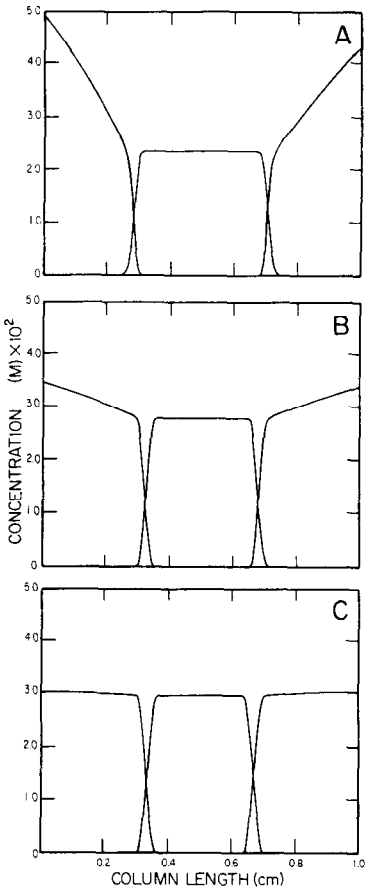

Fig. 3. Steady-state IEF profiles for three different three-component systems. The same molar amount of each ampholyte is present in all cases. The current density in each simulation was 10 A/m². The ΔpK value for each component is 2; all ionic mobilities are $3.0 \cdot 10^{-8}$. The cathode is to the left in each case. The only differences between the simulations are the pI values of the components involved. Panel A shows the profiles with pI values 3, 7 and 11; panel B with pI values of 4, 7 and 10; panel C with pI values of 5, 7 and 9. The concentration gradient across a zone of focused ampholyte is dependent upon its pI. A basic ampholyte displays a greater concentration gradient than an acidic ampholyte which has a pI equidistant from neutrality.

the pH plateau. The position of each boundary and the net electrophoretic flux of each acidic and basic component in Fig. 3 is shown in Table I.

The ΔpK value of an ampholyte also has an effect on the steady-state concentration profile. The amount of the ampholyte present in charged form in a focused zone decreases logarithmically as the ΔpK increases[14]. Thus, the conductivity of the zone decreases and the voltage gradient increases as does the slope of the steady-state concentration profile. The system shown in Fig. 4 is the same as that presented in Fig. 3A except that the ΔpK values are 3 instead of 2. The slopes of the focused zones of acidic and basic ampholytes become greater as ΔpK increases. The electrophoretic fluxes and boundary positions are shown in Table I.

TABLE I

STEADY-STATE ELECTROPHORETIC FLUX AND BOUNDARY POSITIONS AS FUNCTIONS OF ISOELECTRIC POINT AND ΔpK

pI	ΔpK	*Flux**	*Boundary position**
3	2	5.38	70
3**	3	6.75	71
4	2	1.55	68
5	2	0.200	67
9	2	0.202	33
10	2	1.67	32
11	2	7.24	28
11**	3	10.2	26

* Flux is the net electrophoretic flux of the given component from the simulations in Figs. 3 and 4 (mol/m^2 s $\times$ 10^6). Calculated for components with p*I* values 3, 4 and 5 at segment 90; for components with p*I* values 9, 10 and 11 at segment 10. The position of the center of the boundary between the component listed and the one focused adjacent to it (p*I* = 7) is given as the segment number (% column length). All simulations were performed with 101 grid points (100 segments) defining the column. The mobility of all ampholyte species was $3.0 \cdot 10^{-8}$ m^2/Vs.

** From Fig. 4.

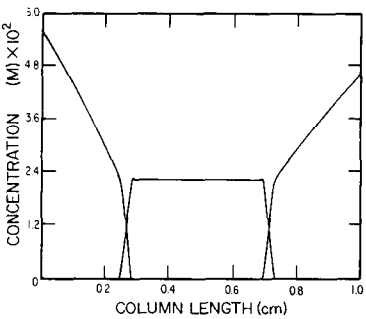

Fig. 4. The effect of ΔpK on the steady-state concentration gradients within zones of focused ampholytes can be seen by comparing this figure to Fig. 3A. The p*I* values of the components are identical in both figures (3, 7, 11). These profiles were computed using $\Delta pK = 3$ for each ampholyte. In Fig. 3A the ΔpK values are 2. All other parameters are identical.

DISCUSSION

Mixtures of ampholytes focus in two phases[6]. In the first of these, which has been termed the separation phase, individual ampholytes condense into pure zones. During the second, stabilizing phase of focusing those ampholytes with isoelectric points above 9 or below 5 will develop significant concentration gradients across their zones. This creates a diffusional mass flux to balance the non-zero electrophoretic flux and produces the steady state. The acquisition of this slope causes the ampholyte zone to contract along the focusing axis. This compaction of the acidic and of the basic ampholytes results in a depletion of the neutral region and the formation of the pH plateau.

The non-zero electrophoretic flux is a function of both the pI and the ΔpK of the ampholyte. The pH in the zone of focused ampholyte controls the imbalance in the concentrations of the positively and negatively charged species due to the requirements of electroneutrality. The more removed the pI from neutrality the greater the electrophoretic flux of the ampholyte and the greater the concentration gradient across the focused zone. Under equivalent conditions, which include equal ΔpK values, ionic mobilities, molar amount present and current density, the steady-state length of an ampholyte zone along the focusing axis is a function of its pI. Therefore, the extent of the plateau phenomenon is a function of the extremes of pH encompassed by the gradient.

The concentration of charged species in a zone of focused ampholyte has a direct influence on the voltage gradient within the zone, and thus on the electrophoretic flux. The ΔpK of the ampholyte affects the concentration gradient across the focused domain as a result of its influence on the amount of charged species present at the isoelectric point[14]. As the ΔpK increases, the amount of the ampholyte possessing a net charge decreases, and the voltage gradient increases as does the concentration gradient at the steady state.

Basic ampholytes tend to have a greater slope at the steady state than do acidic ampholytes due to the greater mobility of the hydrogen ion as compared to the hydroxyl ion. While this means that the drift in basic systems is greater than that in acidic systems it is unlikely that this provides a complete explanation for cathodic drift[15]. This also does not explain the frequent observations of the progressive acidification of the anodic end of the gradient. Righetti (see ref. 15, p. 300) has suggested that the plateau phenomenon and cathodic drift are different terms for the same instability. However it appears likely that these are different phenomena. This is supported by the observation of Chrambach *et al.*[16] that a pH gradient spanning the range 3–6 drifted toward the cathode. The mechanism presented here would predict that this gradient would drift toward the anode. It is difficult to apply this interpretation of the plateau phenomenon to synthetic mixtures of carrier ampholytes with any precision because they are so poorly characterized. There is little information pertaining to ionic mobilities, ΔpK values and the relative amounts of acidic, basic and neutral ampholyte species present. That these mixtures exhibit the plateau phenomenon is qualitatively explained by the stabilizing phase of IEF, during which there is a progressive loss of ampholytes from the neutral pH region. However, the often observed cathodic shift of the position of lowest conductivity in extended experiments[17] is apparently not based solely on this mechanism. Other factors, such as the design of the electrode assemblies and electroosmosis, can contribute to pH gradient instability.

Svendsen and Schafer-Nielsen[18] have proposed that the imbalance in the concentration of the positively and negatively charged species of a focused ampholyte is the cause of pH gradient decay. In their computer modelling system the ends of the focusing column were open to the ampholytes and the decay of the pH gradient proceeded by an isotachophoretic mechanism with the components migrating out of the ends of the column. In most IEF experiments, however, the ends of the column are defined by small volumes of relatively high concentrations of strongly acidic and basic electrolytes. Ampholytes will not migrate isotachophoretically into these solutions. Our results show that the plateau phenomenon can occur without the loss

of any ampholyte. This has been confirmed experimentally by others[5]. The type of decay reported here proceeds by an IEF mechanism, not an isotachophoretic one. This means that natural pH gradients, formed with ampholytes and spanning neutrality, will always exhibit the plateau phenomenon. Thus, there will be regions of the focusing column, closer to the electrodes and containing more acidic or basic pH values, within which the resolution of samples will reach a maximum and then decrease.

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