DEFINED BUFFER pH GRADIENTS
FOR ISOELECTRIC FOCUSING

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The members of the Committee appointed to examine the thesis of NOAH ISAAC TRACY find it satisfactory and recommend that it be accepted.

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Chair

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DEFINeD BUFFER pH GRADIENTS
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Abstract

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Isoelectric focusing (IEF) requires a pH gradient, which is typically formed using synthetic carrier ampholyte mixtures (SCAMs). Researchers have studied defined buffers as an alternative to SCAMs because they cost less and do not have the adverse interactions with proteins that SCAMs can have. In addition, it has been shown that defined buffers can be used to form shallow pH gradients, which may be useful for separating protein isoforms by IEF. This thesis investigates whether shallow pH gradients formed by defined buffers can be used in IEF to separate protein isoforms.

Three defined buffer systems were tested in a vortex-stabilized electrophoresis device to determine which ones would have pH gradients that are useful for separating protein isoforms. The defined buffer pH gradients were formed naturally by applying an electric field or by pouring a concentration gradient of two-components. Only the two-component poured pH gradients were smooth and reproducible. One of the two-component poured pH gradients was used to focus 20 mg of cytochrome c. A second two-component system separated glucose oxidase and amyloglucosidase. These results showed that two-component poured pH gradients
can focus and separate proteins. However, the wide amyloglucosidase bands suggest that isoforms may only be partially resolved.

A mathematical model to predict the behavior of two-component poured pH gradients during IEF was developed so that computer simulation could be used to test whether or not a particular buffer pair would form a desired gradient for separating proteins. The model used electro-transport theory and equilibrium between the charged states for each component to describe the behavior of the components during IEF. The model was applied to predict pH gradients using the same components as in the first investigation. The model’s predictions fell within the 95% confidence interval of a least-squares fit to the experimental pH gradients except in the regions near the ends of the pH gradients. The model could not predict changes at the ends of the pH gradients because constant composition boundary conditions were assumed. The effects of temperature and concentration on the equilibrium constants, which were neglected for simplicity, may also contribute to the discrepancy between the model and the data.
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To Jenney, Marcus, and Lars.
Chapter 1: Introduction

The ability of preparative isoelectric focusing (IEF) to separate and concentrate proteins at the same time makes it a very desirable purification step as manifest by the many different devices designed to perform preparative IEF [1]. Typically, synthetic carrier ampholyte mixtures (SCAMs [2]) are required to perform IEF. These ill-defined chemicals are expensive and difficult to remove from target proteins at the end of a separation and can cause proteins to precipitate, ruining the separation [3]. Previous researchers [2,4-10] replaced SCAMs with defined buffers to minimize interactions of gradient-forming components with proteins. Bier et al. [10] also showed that defined buffers could generate shallow pH gradients useful for separating proteins and possibly protein isoforms by IEF. The prospect of being able to separate proteins and their isoforms by IEF in defined buffers led to the work in this thesis.

The five chapters of this thesis describe the use and modeling of defined buffers at the preparative scale in a vortex-stabilized electrophoresis apparatus. The second chapter of this thesis starts with a brief history of IEF and an explanation of how it works. A literature review of defined buffer IEF and its modeling concludes the chapter.

Chapter three, a paper prepared for submission to the journal *Electrophoresis*, describes the preparative-scale experiments conducted to test defined buffer pH gradients for stability and ability to separate proteins in a vortex-stabilized electrophoresis apparatus. One defined gradient was able to focus 20 mg of cytochrome c, a naturally colored protein. A second pH gradient separated glucose oxidase and amyloglucosidase. The wide band width of amyloglucosidase suggested that the shallow pH gradients will only be able to partially separate isoforms with very similar isoelectric points (pIs), i.e., $\Delta pI = 0.2$ pH units or less.
A simple model was developed after the experimental work in order to better understand the behavior of the defined systems and provide predictive capabilities for other buffer systems in the vortex-stabilized electrophoresis apparatus. The fourth chapter of the thesis (also a paper prepared for submission to *Electrophoresis*) describes a mathematical model for the defined buffer pH gradients tested in the third chapter. The model was based on electro-transport theory rather than numerically fitting data. The validity of the model was checked by comparing its predictions to the experimental pH gradients measured in the third chapter. The model’s results reasonably matched the experimental behavior of defined pH gradients except at the ends of the gradients. The model did not account for the discrepancy between the simulated pH gradients and the data in the regions near the electrodes because the model assumed constant concentrations at the ends of the pH gradients. Additionally, the differences between the model and the data suggest that the effects of temperature and concentration on the equilibrium of the components may be important.

The thesis closes with chapter 5, summarizing the conclusions from the two papers, chapters 3 and 4, and suggests further avenues of research related to the work done.

**References**


Chapter 2: Background

Proteins, especially enzymes, are now common industrial products. Enzymes are used in household goods such as detergents [1] and toothpaste [2] to enhance cleaning, as prescription drugs [3], food additives to aid in digestion and increase nutritional value [4], and food processing to increase efficiency and yield [3,4]. Before a protein is ready for full-scale commercial production, it usually has to be produced in preparative quantities, e.g., mg-gram range, for testing. Often, it is synthesized in a host cell by means of recombinant DNA technology [5]. This results in a protein with varied post-translational modifications that can cause the protein to have different levels of activity or efficiency [6]. Researchers would like to separate these different isoforms so that they can be individually characterized, since some may perform better or worse than the others or, in the case of drugs, have adverse side effects.

Currently, separating the different forms of the target protein from the thousands of other proteins in the host cell can be a complicated process. Column chromatography, a common method of protein purification, is not well suited to discerning between the subtle differences in post-translational modifications of proteins, since the various forms are all so similar. The amounts and types of post-translational modifications, such as phosphorylation and acetylation, tend to alter the net charge of the proteins. Alterations to the net charge of the proteins change their isoelectric points (pIs), the pH where the proteins have no net charge. Isoelectric focusing (IEF) could serve as a useful tool to separate and concentrate these many forms at the preparative scale since it separates proteins based on their pIs.
1 History of IEF

Electrokinetic separation of proteins by an IEF-like process first appeared in a 1912 article by Ikeda and Suzuki [7]. They used a chamber divided by three ion-permeable membranes to produce sodium glutamate via electrolysis. Subsequent researchers used that idea as a starting point for developing their own equipment, often modifying the chamber for their experiments in separating amino acids and ampholytes. In 1941, Tiselius realized that, during electrolysis in a multi-compartment device where each compartment is at a different pH, an ampholyte is repelled by both the cathode and the anode and concentrates in the compartment where the pH most closely approximates that of the ampholyte’s pI [7].

The shift from using compartments to a continuous pH gradient was made in 1954 by Kolin [8]. In the early 1960’s, Svensson developed the basic theory of IEF and found the requirements for a stable pH gradient [9]. The components of the gradient needed to be amphoteric, good conductors of electrical current, and good buffers. Such molecules were named “carrier ampholytes”. There are, however, few natural compounds that satisfy Svensson’s requirements [10], so making stable pH gradients was not possible. It was not until Vesterberg’s work in 1969, that such molecules were synthesized [11].

From that point, IEF became a useful analytical tool for determining the purity of protein solutions and the pI of a protein has become one of the measured characteristics of proteins. IEF’s two primary features, separation and concentration of proteins, inspired researchers to develop equipment suitable for preparative IEF. In 1992, the majority of preparative IEF techniques and equipment were reviewed by Righetti et al. [12]. A more recent preparative IEF device not covered in that review is a vortex-stabilized electrophoresis apparatus designed by
Ivory and Gobie [13]. This device was used during the research for this thesis to perform preparative scale IEF and will be described in brief detail in the third chapter of the thesis.

2 How IEF Works

Isoelectric focusing uses a pH gradient and an electric field to focus proteins at their pIs. Applying voltage to a solution of carrier ampholytes forms the pH gradient. This is done in an anti-convective medium, e.g., a density gradient or polyacrylamide gel, since natural convection would hinder the focusing of proteins and ampholytes. Each species of carrier ampholyte has some surface charge based on the pH of the surrounding solution. The carrier ampholytes begin to migrate because of their surface charges when the electricity is applied. Positively charged species move towards the cathode, and those with net negative charges move towards the anode. The composition of the solution becomes heterogeneous as the carrier ampholytes begin to move and regions of differing pH form. The lowest pH is at the anode, where the more acidic components are found. pH increases towards the cathode where the most basic components collect. The pH gradient will only exist while the voltage is applied. A continuous pH gradient can be formed by carefully choosing a large number carrier ampholytes with closely spaced pIs. A pH gradient that forms spontaneously in the electric field is referred to as a natural gradient, whereas a mechanically generated gradient is described in the literature as being artificial.

A protein, like the carrier ampholytes, also migrates according to its surface charge. When inserted into a pH gradient in the presence of an electric field, a protein with a negative net charge starts moving towards the anode, or acidic end of the gradient. As it migrates, the pH of the solution steadily decreases. The protein’s basic side chains protonate and the net charge becomes less negative so the protein moves progressively slower in the electric field. This process continues until the protein reaches its pI and quits moving, or focuses.
Mixtures of proteins with different pIs can be separated from each other by making the slope of the pH gradient shallow enough so that the proteins do not focus right next to one another. If the proteins have pIs far apart from each other, i.e., bovine serum albumin (BSA) and hemoglobin (Hb), the separation is relatively easy. Proteins with closely spaced pIs, i.e., the glycoforms of a protein, are more difficult to separate and require very shallow pH gradients.

3 The Problem with IEF

Separating proteins on a preparative scale using IEF has two major drawbacks. First, the synthetic carrier ampholytes mixtures (SCAMs) are expensive, $231 for a 25mL bottle from AmershamBioscience’s 2002 catalog or $375 from Sigma’s 2002 catalog. Second, the SCAMs tend to interact with highly charged proteins, especially in pH ranges far from neutrality. The interactions lead to altered pIs, precipitation, and difficulty in removing SCAMs from target proteins [7]. Other methods of generating the pH gradient necessary for IEF needed to be found in order to avoid the undesired protein-SCAM interactions and the high cost of SCAMs.

A literature search revealed two possibilities for forming pH gradients. One option was to immobilize the SCAMs in a gel matrix [12]. Alternatively, the SCAMs could be replaced with defined buffers, such as amino acids, dipeptides, weak acids or bases, or Good’s buffers [14-17]. Working with defined buffers was the preferred choice for this thesis since the vortex-stabilized electrophoresis apparatus to be used in this work performs IEF in free solution.

There are many chemicals and combinations thereof that could be used in a defined buffer [16-18]. Mathematically modeling the defined buffer pH gradient and its behavior in the electric field could provide a means of predicting whether a given set of components might form a suitable pH gradient. This could reduce the amount of guesswork involved in preparing a defined buffer pH gradient. This thesis tests and models defined buffers in an effort to determine
if defined buffers can be used to separate proteins, and possibly their isoforms, by IEF at the preparative scale.

4 Previous Work with Defined Buffer pH Gradients

In 1954, Kolin formed the first continuous defined pH gradient in free solution by mixing the interface of two different electrolyte solutions [8]. Hemoglobin variants focused after only 6 minutes at 220 V and 9 mA in that system. Pettersson [19] coupled a pH gradient with a sucrose density gradient to stabilize the defined pH gradient. He used a combination of strong, weak, and amino acids to focus plant extracts in the pH 1-3 range.

One of the biggest proponents of defined buffer IEF was Chrambach. He and Nguyen did IEF-polyacrylamide gel electrophoresis (PAGE) with electrolytes composed of amphoteric compounds or Good’s Buffers. These naturally forming gradients were able to separate BSA and Hb [14]. In 1979 they patented their “buffer electrofocusing” system [20]. Prestidge and Hearn took Chrambach’s defined buffer concept to the preparative level, separating 280 mg of protein, using flatbed polysaccharide gels [15] and patented their preparative process [21]. Cuono and Chapo used the concepts developed by these previous researchers to put together a 47-component buffer, which they used for IEF-PAGE [16]. Their gradients were reproducible, stable for hours, and successful in separating proteins.

In 1983, Bier et al. showed that a two-component poured gradient was sufficient to separate Hb A and C in free solution [22]. The initial gradients produced by Bier and co-workers were limited to near neutrality, i.e. pH 5-9, since they said hydronium and hydroxyl concentrations interfered with the stability of the gradient far from neutrality [23]. Additionally, these early gradients were limited to low current density to keep the gradient from becoming skewed and unusable.
Then in 1993, Bier developed a method for choosing gradient components to make the poured gradient more stable in the electric field and at pHs far from neutrality. The method revolved around a concept they called “electrophoretic reserve capacity” [17]. The idea was that the components should be largely (90-99%) neutral for the pH gradient to be relatively stable to the passage of current. This way, when a small portion of the component migrated away from the position at which it was poured, there was more of that component in reserve at that location to ionize and take the place of the part that left. Their method also required that the buffer pairs forming the gradient have overlapping pKs such that the components titrate each other. The best buffer pairs were patented as the Optifocus™ buffers [18]. It is interesting to note that Bier’s group did not publish any experimental data for defined buffer pH gradients outside the pH 5-9 range or at high currents since these buffers were supposed be stable at high current and or far from pH 7.

Work done by Tulp et al. [24] used a buffer pair from Bier’s work to successfully pour a defined buffer pH gradient in the pH 5-6 range. Their gradient was poured with a density gradient for stabilization. Their test mixture consisted of three proteins, Transferrin (Tf) with a pI in the middle of the gradient, and two with pIs outside the gradient, Hb (pI=7.0) and BSA (pI=4.71). Their results showed that Tf focused in the middle and the other two proteins ran to the ends of the gradient. The Tf did separate into different bands, indicating multiple isoforms, but analytical IEF-PAGE showed that none of the isoforms were individually resolved.
5 Previous Mathematical Modeling of Defined Buffer pH Gradients

Svensson developed the mathematical theory behind steady state IEF for a single compound, i.e., a protein, in a stable pH gradient [9]. His model worked fine for pH gradients produced by SCAMs. However, there was no model to predict the behavior of the pH gradient, or species concentration profiles for IEF of a defined buffer until Almgren developed an improved model [25]. While it was the most advanced model at the time, it assumed that all mobilities (except hydronium and hydroxyl) were equal and that the current density was independent of diffusion.

According to Palusinski et al., Almgren’s simplifications limited the usefulness of the model, particularly since the latter assumption did not hold for steep gradients in dilute solutions at low or high pH values [26]. Thus, they developed a model for steady state IEF of defined buffers that included the diffusive contribution to current as well as mobilities of individual ions and equilibrium between ionic species. Their steady state model could predict pH, current, and concentration profiles for a multi-component system [26]. That model was improved upon by adding time dependence. By adjusting the initial and boundary conditions, they could observe the formation of pH gradients under different circumstances [27]. This model was the starting point for many papers by Bier, Mosher, Saville, and Thormann, which were later combined into an extensive book on electrophoresis [28]. Later, Bier used this model to successfully predict the pH gradient formed by using MOPS and GABA, one of the Optifocus™ buffer pairs [17].
References


Chapter 3: Focusing Proteins in Defined Isoelectric Focusing Buffers

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Defined Isoelectric Focusing Buffers

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Abbreviations:  EACA,  e-amino-n-caproic acid;  IEF,  isoelectric focusing;  GABA,  ?-amino-n-butyric acid;  MOPS,  3-[n-morpholino]propanesulfonic acid;  SCAMs,  synthetic carrier ampholyte mixtures;

Keywords:  IEF, defined buffer
Abstract

Recombinant proteins are often produced as isoforms with different kinds and amounts of post-translational modifications that alter their function. Isoelectric focusing in shallow pH gradients, about 1 pH unit wide, might be capable of separating isoforms. The synthetic carrier ampholyte mixtures (SCAMs) typically used to generate the pH gradients are expensive and may adversely interact with proteins. Using defined buffers instead of SCAMs reduces those problems.

This study tested three defined buffer systems in a vortex-stabilized electrophoresis device to see if they could form shallow pH gradients useful for separating isoforms. These pH gradients were formed naturally by an electric field or by pouring a two-component concentration gradient. In free solution, the naturally formed gradients failed to produce useful pH gradients, contrary to work done in gels. The poured gradients were smooth, reproducible, and stable for about 1.5 hours at 5 kV. One poured gradient focused 20 mg of cytochrome c. A second poured gradient separated glucose oxidase and amyloglucosidase. Shallow poured pH gradients might not be capable of completely separating isoforms, despite these successful results, because the focused protein bands are wide. Isoforms with pIs less than 0.2 pH units apart will probably overlap in the shallow poured pH gradients.
1 Introduction

Preparative isoelectric focusing (IEF) is an attractive protein purification technique since it combines separation and concentration into one step. Typically, IEF uses synthetic carrier ampholytes (SCAMs [1]), such as Ampholines™ or Pharmalytes™, to form the pH gradient required for separating proteins with different isoelectric points (pIs). However, the SCAMs have two drawbacks that limit the application of preparative IEF. They are expensive and interact with highly charged proteins. These interactions lead to altered pIs, precipitation, and difficulty in removing the SCAMs from target proteins [2]. Using inexpensive defined buffers instead of SCAMs to generate pH gradients could reduce the protein-SCAM interactions and make preparative IEF more economical.

An added benefit of IEF is that it can distinguish between isoforms of proteins. Protein isoforms are proteins with the same primary structure, but different post-translational modifications, i.e., glycosylation and phosphorylation. The differences in quantity and placement of the post-translational modifications can give isoforms different pIs and alter how the protein functions [3]. In IEF, proteins focus in bands, or zones, approximately centered at the protein’s pI. Isoforms with different pIs can be separated using IEF if the slope of the pH gradient is shallow, or narrow, enough so that the zones of focused protein do not overlap. The high resolution these narrow pH gradients could provide would be very useful in industry for the preparative-scale separations necessary to characterize and test therapeutic protein isoforms.

Hence, this paper considers the suitability of defined buffers to generate narrow range pH gradients and separate proteins in a vortex-stabilized electrophoresis apparatus. Although using defined buffers to generate a pH gradient is not a new idea, before now it had not been done in a preparative scale vortex-stabilized electrophoresis apparatus.
The first use of a defined buffer pH gradient dates back to Kolin in 1954 [4]. He formed the first continuous defined pH gradient in free solution by mixing the interface of two different electrolyte solutions. Hemoglobin variants focused after only 6 minutes at 220 V and 9 mA in that system. Pettersson [5] used a combination of strong, weak, and amino acids to form a pH gradient stabilized by a sucrose density gradient in order to focus plant extracts in the pH 1-3 range. These and other mechanically generated pH gradients were called “artificial” gradients in the literature, while gradients formed by the electric field were referred to as “natural” gradients.

Chrambach and Nguyen used electrolytes composed of either amphoteric compounds or Good’s Buffers to perform IEF-polyacrylamide gel electrophoresis (IEF-PAGE). These naturally forming gradients were able to separate bovine serum albumin (BSA) and hemoglobin (Hb) [6]. At the preparative level, Prestidge and Hearn used Chrambach’s defined buffer concept to separate 280 mg of protein in a polysaccharide flatbed gel [7]. In 1982, Cuono and Chapo [1] prepared a 47-component buffer as an alternative to pH 3-10 ampholytes for IEF-PAGE by combining the work of the previously mentioned researchers [1,3-6].

One year later, in contrast to Cuono and Chapo’s 47-component buffer, Bier et al. showed that proteins could be separated in a two-component poured gradient [8]. They said this technique was limited to near neutrality, since hydronium and hydroxyl concentrations supposedly interfered with the stability of the gradient outside the pH 5-9 range [9]. Low current density was another limitation that had to be imposed to keep the gradient from becoming skewed and unusable.

To alleviate the pH and current density limitations of the initial poured pH gradient, Bier and associates developed a method for choosing components that they claimed increased the gradient’s stability. A concept they called “electrophoretic reserve capacity” formed the heart of
their methodology [10]. Essentially, this meant the components needed to be largely (90-99%) neutral for the pH gradient to be relatively stable in an electric field. Thus, when a small amount of component migrated away from where it was poured, there would be more of that component to replace it. The buffer pairs forming the gradient also had to have overlapping pKs such that the components would titrate each other. A set of buffer pairs forming narrow pH gradients, e.g., 1 pH unit wide, between pH 3 and 10 were patented as the Optifocus™ buffers [11]. Bier’s group never published any poured gradient work with Optifocus™ buffers outside the pH 5-9 range where they said their original poured gradient work was stable [7,8].

Tulp et al. successfully used an Optifocus™ buffer pair to pour a two-component pH gradient in the pH 5-6 range [12]. Similar to Bier’s work in 1983, they used a density gradient to stabilize the pH gradient in a glass column. Then, they tried to separate a mixture of proteins containing Hb, transferrin (Tf), and BSA by IEF using the pH 5-6 gradient. Coomassie stained IEF-PAGE of the fractions from Tulp’s experiment showed that the Hb and BSA migrated to the ends of the pH gradient and were easily separated from the Tf. The Tf separated into several different bands, but despite the shallow pH gradient, none of the fractions contained an isolated protein band.

This paper describes the suitability of defined buffers to generate pH gradients and separate proteins in a vortex-stabilized electrophoresis apparatus. Two different methods for generating defined buffer pH gradients were used. The first method involved naturally forming a pH 3-5 gradient from a 10-component buffer. In the second method, the pH gradient was poured using components chosen from the list of pairs given by Bier [10] so that either a pH 3-5 or 9-10 gradient was poured. These gradients were of interest since they fall in a range where protein-SCAM interactions are more likely [2]. In addition, the shallow slopes of these pH gradients
could be useful for separating protein isoforms at a preparative scale in the vortex-stabilized electrophoresis apparatus.

As will be shown, both methods would yield reproducible pH gradients. Only the poured gradients were further tested for stability and ability to focus as well as separate proteins since the 10-component buffer never formed the pH 3-5 gradient expected. Stability of the poured gradients decreased as applied voltage and run-time increased. Poured pH 9-10 gradients consistently focused 20 mg of cytochrome c and pH 3-5 gradients served to separate glucose oxidase and amylloglucosidase.

2 Materials and Methods

2.1 Proteins & Buffer Components

The proteins used in these experiments, cytochrome c (bovine heart), amylloglucosidase (Aspergillus niger), and glucose oxidase (Aspergillus niger) were purchased from Sigma (St. Louis, MO, USA) as were the following buffer components: picolinic acid, isonicotinic acid, nicotinic acid, malic acid, lactic acid, anthranilic acid, taurine, DL-serine, ε-amino-caproic acid (EACA), and Trizma base (Tris). Glacial acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Propionic acid and glutamic acid came from Aldrich (Milwaukee, WI, USA). Nanopure water was obtained from a Barnstead Thermolyne (Dubuque, IA, USA) Nanopure Infinity UV/UF system.

2.2 Defined Buffer Systems

The defined buffer systems consisted of three separate solutions. Two 500 mL electrode buffers purge electrolysis products during the experiment; an anode buffer, or anolyte; and a
cathode buffer, or catholyte. The third buffer was 24 mL and formed the pH gradient in the separation chamber.

Altogether, three different defined buffer systems were used to test the functionality of the poured and natural pH gradients. The naturally forming pH gradient was tested using a 10-component buffer. The buffer components were chosen from Cuono and Chapo’s list of components [1] in an effort to replicate the pH 3-5 range of their gradient in the vortex-stabilized electrophoresis apparatus. Then, poured gradients were tested using two of the Optifocus™ buffer pairs from Bier’s patent [11]. The first poured gradient was tested using an EACA/Tris buffer to see if it would focus protein in the vortex-stabilized electrophoresis apparatus. The poured gradient was also tested using a propionic acid/DL-Serine buffer to see if it could resolve a pair of test proteins.

2.2.1 10-Component System

The composition of the 24 mL buffer used to form the pH gradient is shown in Table 3-1. The anolyte and the catholyte compositions are listed in Table 3-2. All components were dissolved in boiled Nanopure water.

2.2.2 EACA/Tris System

The anolyte and catholyte compositions of the EACA/Tris system are listed in Table 3-2. The 24 mL solution used to create the pH gradient consisted of two separate 12 mL solutions, one basic and the other acidic. They were combined using a gradient maker to form pH gradients in the separation chamber. The two 12 mL solutions used to form the gradients for this system had the same composition as the anolyte and catholyte solutions used with this buffer system. All the components were dissolved in boiled Nanopure water. Cytochrome c, a
Table 3-1. Component Concentrations of the 10-Component Buffer System.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthranilic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Isonicotinic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Malic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>10 mM</td>
</tr>
<tr>
<td>Taurine</td>
<td>20 mM</td>
</tr>
</tbody>
</table>

Table 3-2. Anolyte and Catholyte Composition for Defined Buffer Systems

<table>
<thead>
<tr>
<th></th>
<th>10-Component</th>
<th>EACA/Tris</th>
<th>Propionic acid/DL-Serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anolyte</td>
<td>110 mM Acetic acid</td>
<td>90 mM EACA/10 mM Tris</td>
<td>100 mM Propionic acid</td>
</tr>
<tr>
<td>Catholyte</td>
<td>110 mM Tris</td>
<td>10 mM EACA/90 mM Tris</td>
<td>100 mM DL-Serine</td>
</tr>
</tbody>
</table>
naturally colored protein added to the buffer solutions for focusing tests, was dissolved in the basic solution used to pour the gradients. Adding the protein to the basic solution rather than the acidic solution was an arbitrary choice. However, distributing the cytochrome c with the gradient was done on purpose. Injecting the test protein after the gradient was poured would have disturbed the gradient.

2.2.3 Propionic Acid/DL-Serine System

The anolyte and catholyte compositions of the propionic acid/DL-Serine system are shown in Table 3-2. The compositions of the two 12 mL solutions used to form the gradient were the same as the anolyte and catholyte. All the components were dissolved in boiled Nanopure water. The test proteins added to this system, glucose oxidase and amyloglucosidase, were chosen because they were inexpensive and had pI's in the range of the propionic acid/DL-Serine pH gradient. The two proteins were dissolved in the 12 mL acidic solution or the 12 mL basic solution as noted in Table 3-3, so that they would be injected into the separation chamber simultaneously with pH gradient. This avoided injecting them after the gradient was poured, which would have perturbed the pH gradient.

2.3 pH Gradient Formation

As mentioned previously, one of the methods for generating a pH gradient using defined buffers was to form the gradient naturally. The 10-component system was expected to naturally form a pH gradient upon application of voltage, based on results reported in the literature [1,4-6,13-19]. The EACA/Tris and propionic acid/DL-Serine systems required that the components be poured in linear concentration gradients so that as the concentration of the first component, c₁, linearly increased, the concentration of the second component, c₂, was linearly
<table>
<thead>
<tr>
<th>Run No.</th>
<th>Defined Buffer System</th>
<th>Voltage (kV)</th>
<th>Run Time (hrs)</th>
<th>Membrane Type</th>
<th>Protein Load and Initial Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10-Component</td>
<td>2.5</td>
<td>1</td>
<td>6000 MWCO Dialysis</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>10-Component</td>
<td>2.5</td>
<td>1</td>
<td>Ion Exchange</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>EACA/Tris</td>
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<td>0</td>
<td>6000 MWCO Dialysis</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>EACA/Tris</td>
<td>0</td>
<td>0</td>
<td>6000 MWCO Dialysis</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>EACA/Tris</td>
<td>5</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>EACA/Tris</td>
<td>5</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>20 mg Cytochrome c Basic Reservoir</td>
</tr>
<tr>
<td>7</td>
<td>EACA/Tris</td>
<td>5</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>20 mg Cytochrome c Basic Reservoir</td>
</tr>
<tr>
<td>8</td>
<td>EACA/Tris</td>
<td>5</td>
<td>2</td>
<td>6000 MWCO Dialysis</td>
<td>20 mg Cytochrome c Basic Reservoir</td>
</tr>
<tr>
<td>9</td>
<td>Propionic acid/ DL-Serine</td>
<td>0</td>
<td>0</td>
<td>6000 MWCO Dialysis</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>Propionic acid/ DL-Serine</td>
<td>5</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>Propionic acid/ DL-Serine</td>
<td>5</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>5.5 mg ea. of Glucose Oxidase and Amyloglucosidase Basic Reservoir</td>
</tr>
<tr>
<td>12</td>
<td>Propionic acid/ DL-Serine</td>
<td>5</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>1.3 mg ea. of Glucose Oxidase and Amyloglucosidase Basic Reservoir</td>
</tr>
<tr>
<td>13</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>None</td>
</tr>
<tr>
<td>14</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>1.3 mg ea. of Glucose Oxidase and Amyloglucosidase Basic Reservoir</td>
</tr>
<tr>
<td>15</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>1.5</td>
<td>6000 MWCO Dialysis</td>
<td>1.3 mg ea. of Glucose Oxidase and Amyloglucosidase Acidic Reservoir</td>
</tr>
<tr>
<td>16</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>5.5</td>
<td>6000 MWCO Dialysis</td>
<td>1.3 mg ea. of Glucose Oxidase and Amyloglucosidase Acidic Reservoir</td>
</tr>
<tr>
<td>17</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>5.5</td>
<td>500 MWCO Dialysis</td>
<td>1.4 mg ea. of Glucose Oxidase and Amyloglucosidase Acidic Reservoir</td>
</tr>
</tbody>
</table>
decreasing according to \( c_1 + c_2 = 100 \text{ mM} \). These linear concentration profiles with opposite slopes produced a linear pH gradient. The total concentration of buffer components was kept at 100 mM to keep in line with Bier’s suggestions for using the Optifocus™ buffers, although he says that the total buffer concentration may be increased if the user desires [11]. The linear pH gradients were pumped into the separation chamber using the apparatus shown in Figure 3-1.

The gradient former (Bio-Rad, Hercules, CA, USA) sat on a magnetic stir plate (Barnant Corp., Barrington, IL, USA) that mixed the contents of the basic reservoir using a magnetic stir bar. A peristaltic pump (Buchler Instruments, Lenexa, KS, USA) was used to withdraw the contents of the gradient maker and fill the separation chamber from the bottom.

Before the gradient was pumped into the separation chamber, the chamber was filled with water. This prevented bubbles from being trapped in the chamber while the gradient was being poured. Then 10 mL of the basic solution was added to the basic reservoir and pumped into the separation chamber. This filled the tubing and the bottom of the chamber with basic buffer so that as the gradient was created and pumped into the chamber it would not be diluted by the solution it was displacing. Next, 12 mL of acidic solution was added to the rear compartment and 12 mL of basic solution was added to the front compartment of the gradient former. A stir bar was placed in the front compartment and the magnetic stirrer was switched on. The valve between the two compartments was opened and the pump turned on and set to a flow rate of 0.64 mL/min. A pH gradient was created as the mixture from the gradient former filled the separation chamber from the bottom to the top. The separation chamber is only one piece of a larger device, which is not shown completely in Figure 3-1. The rest of the device is described in the following section.
Figure 3-1. A schematic of the apparatus used to pour a defined buffer pH gradient. Pumping the contents of the reservoirs into the annular separation chamber formed between the rotor and the stator forms a linear concentration gradient. The components in the linear concentration gradient titrate each other to form a pH gradient.
2.4 Vortex-Stabilized Electrophoresis Chamber

The apparatus used for preparative IEF was a vortex-stabilized electrophoresis device designed by Ivory and Gobie [20]. Thome and Ivory [21] had previously used this apparatus for continuous electrophoresis of enantiomers and a more detailed description of it can be found in that paper. Only a brief description of the apparatus (Figure 3-2) as used for the IEF experiments in this work is provided here.

The annular separation chamber was formed between a rotor and a stator. The grooved, hollow, boron nitride rotor was 12 inches tall by 1 inch in diameter and formed the inner wall of the separation chamber. A complimentarily grooved Plexiglas stator surrounded the rotor to form the outer wall of the separation chamber. The volume of the separation chamber was approximately 24 mL. Two March 1A-MD-1 pumps (March Mfg. Inc., Glenview, IL, USA) circulated electrode buffers from 500 mL tanks through the pairs of electrode housings located at the top and the bottom of the separation chamber to purge hydrolysis gases. A motor with speed control circuitry spun the rotor at 50 rotations per minute.

The matching grooves in the rotor and stator created a vortex pattern in the annular fluid, which allowed for better heat and mass transfer between the rotor and stator. The Taylor-like vortices stabilized the axial concentration profile, preventing natural convection from disturbing the concentration profile that resulted from the electrophoretic separation. These vortices also provided nearly an order of magnitude higher power dissipation within the apparatus compared to running without vortex formation.

A Syltherm XLT coolant oil (Dow Chemical, Midland, MI, USA) was recirculated through the inside of the rotor to dissipate the Joule heat generated during electrophoresis. This
Figure 3-2. The vortex-stabilized electrophoresis apparatus used is on the left. The white vertical cylinder is the rotor, which forms a 24 mL annular separation chamber with the stator. The high-voltage power supply that provides the electric field for the separation process is located above and behind the separation chamber. To the right of the chamber are the off-take valves. One of the electrode buffer tanks can be seen to the right of the valve actuators. The 96 well plates for sample collection are placed in the vacuum chamber seen in the bottom right foreground.
oil was cooled using a VWR 1197 recirculating cooler (VWR, S. Plainfield, NJ, USA). A static mixer (Omega, Stamford, CT, USA) was used inside the rotor to promote internal heat transfer.

The ends of the stator on the electrophoresis chamber were machined to accept Plexiglas electrode compartments that were partitioned from the separation chamber by membranes. The cathode was at the top of the column and the anode at the bottom. For these experiments, either 6000 molecular weight cut off (MWCO) or 500 MWCO dialysis membranes (Spectrum Laboratories, Houston, TX, USA) or ion exchange membranes were used to prevent bulk mixing between the electrode buffers and the contents of the separation chamber. Dialysis membranes of the same size were used at all electrodes. When ion exchange membranes were used, DuPont Nafion 117 cation exchange membranes (The Electrosynthesis Co., Lancaster, NY, USA) were placed at the two anode housings and AMH anion exchange membranes (The Electrosynthesis Co., Lancaster, NY, USA) at the two cathode housings.

High-voltage power for separations in the vortex-stabilized electrophoresis apparatus was provided by a Spellman SL30PN1200 power supply (Spellman High Voltage Electronics, Hauppauge, NY, USA) and controlled via a PC running LabVIEW 6.0 (National Instruments, Austin, TX, USA) with a PCI-DAS 1002 multifunction A/D I/O board (Measurement Computing, Middleboro, MA, USA). The LabVIEW power supply control program also recorded voltage and current measurements from the power supply during the course of the experiment.

At the end of an experiment, an automated fraction collection system (Figure 3-3) was used to remove the contents of the separation chamber. The fractions were collected through 53 ports. The ports started 2.5 cm below the cathode, with a port every 0.47 cm, and finished 2.5 cm above the anode. These ports were connected to a pair of custom electro-actuated multi-
Figure 3-3. The fraction collection system consists of six electronically actuated valves, a vacuum chamber that holds a microtiter plate, a pressure gauge and a pressure regulator. Vacuum is set to 20 inches Hg. A LabVIEW program controls the valve positioning so 250-300 µl samples are taken starting at the top and progressing to the bottom of the column. The samples are withdrawn from the separation chamber (upper left corner) and into one of the two large valves to the right of the chamber. Then the sample flows through the small valve next to the bottom of the separation chamber and into the small valve shown in the stack of three valves between the vacuum chamber and the pressure gauge. The sample is then directed to one of the two large valves shown between the vacuum chamber and pressure gauge. From these two valves, the sample is directed to a well in the microtiter plate.
position valves (Valco Instrument Co. Inc., Houston, TX, USA) located directly behind the separation chamber. These two valves were attached in parallel to a third electro-actuated multi-position valve (Valco Instrument Co. Inc., Houston, TX, USA) below them, which was used to select between them. This smaller third valve was connected to another identical electro-actuated multi-position valve (Valco Instrument Co. Inc., Houston, TX, USA) at the top of the fraction collector. Below this valve was another pair of custom electro-actuated multi-position valves (Valco Instrument Co. Inc., Houston, TX, USA), connected in parallel to the small valve above them. Tubing connected these two larger valves to a vacuum chamber held at -20 inches Hg. A 96-well microtiter plate was placed in the vacuum chamber to hold the samples. A program written in LabVIEW controlled the valve positions so that 250-300 µL fractions were taken progressively from the top to the bottom of the column and deposited in the microtiter plate in that same order.

2.5 Focusing Procedure

2.5.1 10-Component System

The 10-component focusing buffer was injected by syringe into the separation chamber. The anolyte and catholyte used are shown in Table 3-2. Other run conditions, such as voltage, time, and types of membranes used are listed in Table 3-3 as runs 1 and 2. At the end of the specified run times, voltage was turned off and fractions were withdrawn to a 96-well microtiter plate for analysis.

2.5.2 EACA/Tris system

The anolyte and catholyte for the EACA/Tris system are shown in Table 3-2. The pH gradient was poured using 12 mL of the anolyte as the acidic solution and 12 mL of the catholyte
for the basic solution, pouring the gradient as previously described. The gradient was poured and extracted twice without applying an electric field (runs 3 and 4) to verify that the pH gradient was linear and reproducible, as shown in Figure 3-5. Next, voltage was applied to test the stability of the pH gradient in the electric field. This was run 5 shown in Table 3-3. The gradient proved to be relatively stable (see Figure 3-5) in the electric field and so was tested for its ability to focus cytochrome c in runs 6-8. At the end of all the runs, voltage was turned off and fractions were withdrawn to a 96-well microtiter plate for analysis.

2.5.3 Propionic acid/DL-Serine system

Table 3-2 shows the anolyte and catholyte used for the propionic acid/DL-Serine system. The pH gradient was formed using 12 mL of the anolyte as the acidic solution and 12 mL of the catholyte for the basic solution, pouring the gradient as previously described. Run 1 was performed to make sure that the pH range of the propionic acid/DL-Serine gradient was desirable. The propionic acid/DL-Serine system was also tested (see run 10 in Table 3-3) to make sure that it would be stable in the electric field. Then, the gradient’s suitability for separating proteins was tested using glucose oxidase and amyloglucosidase as model proteins. These proteins were chosen because they were inexpensive and had pIs in the range of the propionic acid/DL-Serine pH gradient. The various run conditions used for separating the two proteins are listed as runs 11-17 of Table 3-3. The protein loads were decreased in an effort to improve resolution over the first run, number 8. Similarly, run times and voltages were increased in an attempt to enhance the resolution of the proteins. Additionally, tighter membranes were used and the compartment where the proteins were added was changed to try improving separation of the proteins. At the end of the specified run times, the voltage was turned off and the fractions were withdrawn to a 96-well microtiter plate for analysis.
2.6 Fraction Analysis

The shapes of the pH gradients were found by measuring the pHs of the 53 fractions taken from the separation chamber. The pH of the fractions was measured using an Orion micro probe (Orion Research, Inc., Beverly, MA, USA) and Denver pH meter (Denver Instrument Co., Arvada, CO, USA). The uncertainty in the pH meter’s readings was ± 0.002 pH units and the uncertainty in the probe’s readings was ± 0.02 pH units. The probe and meter were calibrated at pH 4, 7, and 10 before measuring the pH of the 53 fractions collected from each experiment.

Fractions containing the test proteins were located by visual inspection, in the case of the naturally red colored cytochrome c, or absorbance. The non-colored proteins, glucose oxidase and amyloglucosidase, were detected using the difference between 280 nm absorbance of the fractions and 280 nm absorbance of a 50/50 mixture of the acidic and basic components used to form the gradient. Absorbance readings were taken using a UV-compatible microtiter plate loaded with 200 µL of each fraction and then scanning the plate of fractions with a Perkin Elmer HTS 7000 Plus UV plate reader (Perkin Elmer, Norwalk, CT, USA).

Fractions were selected for IEF-PAGE analysis based on 280 nm absorbance or pH. Novex pre-cast pH 3-7 IEF gels (Invitrogen, Carlsbad, CA, USA) were used to assess the purity of proteins in the fractions selected. Gels were run according to the IEF protocol found in the Novex instruction manuals accompanying the gels and were developed using a silver-stain protocol [22].
3 Results

3.1 10-Component System

The pH gradient from the first run with the 10-component system extended from pH 2.5 to pH 3.25, as shown in Figure 3-4. Run 2, which used ion-exchange membranes, looked quite similar to the first run. This set of components did not form a smooth pH 3-5 gradient in the vortex-stabilized electrophoresis apparatus, although it did so in Cuono and Chapo’s research in gels [1]. As will be discussed later, the components themselves were probably to blame for the lack of gradient formation.

The stair-stepped pH gradients formed by the 10-component system are not useful for separating proteins. A protein whose pI is equivalent to the pH of the flat portion of the pH gradient will stay spread out, rather than concentrating to a tight band, negating one of IEF’s advantages. Any protein with a pI in the steeply sloped regions of the gradient will focus to a tight band, but the band will probably overlap with the bands of other proteins that have similar pIs. Consequently, the 10-component buffer was not pursued further as a method for separating proteins in this research.

3.2 EACA/Tris system

The first EACA/Tris gradients were poured as a test to see if linear pH gradients could really be poured in the vortex-stabilized electrophoresis apparatus. The triangles in Figure 3-5 show the average pH gradient of runs 4 and 5, listed in Table 3-3, obtained by pouring the EACA/Tris gradient and withdrawing it immediately on two separate occasions. Between the two gradients, the standard deviation of the pH in any particular fraction averaged ± 0.03 pH
Figure 3-4. Step-wise pH gradients are produced by the 10-component defined buffer system after 1 hr at 2.5 kV. The gradient marked by squares shows the results using ion-exchange membranes. The triangles show the gradient obtained using 6000 MWCO dialysis membranes.
Figure 3-5. Three EACA/Tris pH gradients at different running conditions are shown. The triangles represent the average of two poured gradients extracted without applying any voltage. The standard deviation of the two gradients averaged 0.03 pH units and had a maximum of 0.05 pH units. The squares are the average of three runs at 5kV for 1.5 hours. The standard deviation among the three 5 kV 1.5 hour runs averaged 0.05 pH units and had a maximum of 0.15 pH units near the cathode. The circles represent a 5 kV 2 hr run. Its gradient is not very different from the 5 kV 1.5 hour average. The double arrowhead bar at the top represents the fractions where cytochrome c focused.
units and was at most ± 0.05 pH units from the average pH. The poured EACA/Tris pH gradient was reproducible and approximately linear.

Once it was established that linear pH gradients were being poured, power was applied to test the stability of the pH gradient in the electric field. The average EACA/Tris pH gradient of the three 1.5 hour runs at 5 kV (runs 5-7, Table 3-3) is shown by the squares in Figure 3-5. The standard deviation of those runs averaged ± 0.05 pH units from the average fraction value with a maximum of ± 0.15 pH units near the cathode. The average pH gradient of runs 5-7 was shifted about 10 fractions toward the anode compared to the average of the two non-power gradients. Accompanying the shift was an increase in the pH at the cathodic end of the gradient up to about pH 9.9. The EACA/Tris pH gradient after 2 hours at 5kV (run 8) is shown by the circles in Figure 3-5. It looked a little bit compressed compared to the average gradient of runs 5-7.

Testing whether or not the gradient would focus protein was the next task since the gradient seemed stable after 1.5 hours in the electric field. The 20 mg of cytochrome c added to the basic solution of the system focused during two of the 1.5 hour runs and the 2 hour run. Figure 3-6 shows the cytochrome c before and after focusing in the EACA/Tris gradient. The cytochrome c consistently concentrated in the fractions of pH 9.7-9.98, as shown by the double arrowhead bar in Figure 3-5.

3.3 Propionic Acid/DL-Serine system

The encouraging results from the EACA/Tris buffer prompted the testing of a poured defined buffer pH gradient in the acidic range, the propionic acid/DL-Serine system. The solid triangles in Figure 3-7 mark the poured propionic acid/DL-Serine gradient extracted without application of current (run 9, Table 3-3). No additional tests at this condition were made since
Figure 3-6. 20 mg of cytochrome c spread throughout the separation chamber at the start of a run, image A), and at the end of a 1.5 hr 5 kV run, image B), using the EACA/Tris system.
Figure 3-7. Various pH gradients produced using the propionic acid/DL-Serine poured gradients. The solid triangles are the gradient before applying power. The average of the three 5 kV runs after 1.5 hours is shown by the solid squares. The solid circles show the average of three 10 kV runs after 1.5 hours. The gradient after 5.5 hours at 10 kV using 6000 MWCO membranes is marked with hollow triangles. The hollow circles represent the gradient after 5.5 hours at 10 kV with 500 MWCO dialysis membranes.
the results of pouring the EACA/Tris system indicated the gradients were being poured linearly
with little variation.

A plot of the average pH gradient of three 1.5 hour runs at 5 kV (runs 10-12, Table 3-3) is shown in Figure 3-7 by the solid squares. The standard deviation from the average pH in a given fraction averaged ± 0.08 pH units with a maximum of ± 0.5 pH units in the fractions closest to the cathode. The average pH gradient of runs 10-12 had a steeper slope than the non-powered gradient and an unexpected steep increase in pH, or excursion, in fractions 1-8. As will be shown later, glucose oxidase and amylglucosidase were not satisfactorily separated during a 1.5 hour 5 kV run. This led to runs with higher voltages and longer run times.

Voltage was stepped up to 10 kV to try to improve the resolution of the proteins. Three 1.5 hour runs at 10 kV (runs 13-15, Table 3-3) yielded an average pH gradient shown by the solid circles in Figure 3-7. The standard deviation from the average pH in a fraction for runs 13-15 averaged ± 0.1 pH units with a maximum of ± 0.66 pH units in the area of the excursion. The excursion started at fraction 1 and went to fraction 16, six fractions closer to the anode compared to the average at 5 kV.

Extended run times were tested at 10 kV in hopes of better resolving the proteins. Run time was increased to 5.5 hours at 10 kV for runs 16 and 17, as shown in Table 3-3. The hollow triangles in Figure 3-7 show the data from the run with 6000 MWCO dialysis membranes and the hollow circles mark the gradient when 500 MWCO dialysis membranes were used. The only difference between the two 5.5 hour runs was that the excursion had a slightly shallower slope when 500 MWCO membranes were used. Otherwise, the two pH profiles were nearly identical, with the excursions starting at fraction 1 and extending to fraction 18 for both gradients. The excursion for these runs started about 10 fractions closer to the anode than did that of the 5 kV
1.5 hour run. The excursion extended up to pH 5.5 and tended to occupy more of the gradient, as the voltage increased. From the fraction 18 to the anode, the runs matched the 5 kV 1.5 hour average very well.

Glucose oxidase and amyloglucosidase were added to the propionic acid/DL-Serine gradient to test whether or not it was suitable for separating proteins. The performance of the gradient and run conditions was assessed using IEF-PAGE to determine the purity of a fraction. Gel A of the fractions from a 1.5 hour 5 kV run (run 11) (top of Figure 3-8) showed that glucose oxidase (pI=4.2 [23]) and amyloglucosidase (pI=3.6 [23]) were not separated in the fractions between their pIs. Both proteins were found widely throughout the chamber, rather than only in bands centered at their pIs. The proteins had not focused to their pIs, possibly because the protein load was too large. Decreasing the protein load from 5.5 mg each to 1.3 mg each did not increase resolution, so the voltage and run times were increased.

In run 14, voltage was increased to 10 kV for a 1.5 hour run while adding approximately 1.3 mg of each protein to the basic solution. This resulted in only trace contamination of glucose oxidase at its pI, as shown in gel B of Figure 3-8. Glucose oxidase was no longer so widely spread and definitely more concentrated at its pI. Amyloglucosidase, however, remained spread throughout the chamber.

A possible explanation for amyloglucosidase not focusing in the 1.5 hour run time was that the protein migrated slowly and had to travel a relatively large distance to reach its pI. Alternatively, approximately 1.3 mg of each protein was added to the acidic, rather than the basic solution used in pouring the gradient. This way more of the slow migrating amyloglucosidase would start closer to its pI and the run should finish in a shorter amount of time. Voltage was set
Figure 3-8. IEF-PAGE of fractions from propionic acid/DL-Serine gradients run at the following conditions: A) 1.5 hr 5 kV run with 5.5 mg each of glucose oxidase and amyloglucosidase added to the basic solution. B) 1.5 hr 10kV run with 1.3 mg each of glucose oxidase and amyloglucosidase added to the basic solution. C) 1.5 hr 10 kV run with 1.3 mg each of glucose oxidase and amyloglucosidase added to the acidic solution. D) 5.5 hr 10 kV run with 500 MWCO dialysis membranes and 1.3 mg each of glucose oxidase and amyloglucosidase added to the acidic solution. For all four gels, the anode is at the bottom and the lanes marked G and A contain crude glucose oxidase and amyloglucosidase, respectively.
to 10 kV for 1.5 hours (run 15) and fractions analyzed by IEF-PAGE, resulting in gel C of Figure 3-8. Under these conditions, glucose oxidase was pure and relatively concentrated at its pl, but spread throughout the column. Amyloglucosidase was still spread throughout the lower 2/3 of chamber and did not appear very concentrated in any of the fractions analyzed. It seemed like more time might be needed to focus the proteins.

Thus, run times of 5.5 hours were tested. Run 15 showed that the glucose oxidase and amylglucosidase were separated from each other, as shown in gel D of Figure 3-8. The gel showed that amylglucosidase was free of glucose oxidase and more concentrated in the fractions closer to its pl than in the other fractions, although it remained spread throughout the lower half of the separation chamber. Glucose oxidase was focused in the region surrounding its pl. Only a slight trace amount of amylglucosidase was apparent at glucose oxidase’s pl and no amylglucosidase appeared present in the pH 4.53 fraction.

4 Discussion

4.1 10-Component System

According to Cuono and Chapo’s work [1], a combination of weak acids and buffers could be used to form a pH gradient. A gradient similar to theirs was expected to form in the vortex-stabilized electrophoresis apparatus, since the components used for the pH gradient were primarily those taken from Cuono and Chapo’s list spanning the range of pH 3.2-5.1. The natural gradient formed by the 10-component system when 6000 MWCO dialysis membranes were used was not smooth between pH 3.2 and 5.1, like Cuono and Chapo’s gradient. Instead, a step-wise pH gradient from pH 2.5-3.25 was formed. A similar pH gradient was formed when ion-exchange membranes were used as well.
The step-wise pH gradient (Figure 3-4) obtained using dialysis membranes probably resulted from cathodic drift, the migration of buffer components through the membrane and into the cathode reservoir [24]. Cathodic drift, however, could not account for the behavior of the pH gradient in the experiment performed with ion-exchange membranes, since ion-exchange membranes should have prevented the buffer components from escaping.

A more plausible explanation for why the rest of the gradient was absent was that it simply never formed. Svensson [25] classified most of the amphoteric components used in the buffer as poor ampholytes because of their wide ?pKs, meaning that they would not focus into tight bands during IEF. Thus, a smooth pH gradient would not be formed. Mosher et al. [24] found in their modeling work that weak acids, such as those used in the 10-component buffer, can take as long as 3.33 hrs to focus, three times longer than the tested run time. According to these authors [24,25], it seems the 10-component pH gradients may not have formed yet and were not likely to form at all, despite the high voltage applied.

Unfortunately, the naturally forming pH gradient did not provide a gradient suitable for separating the model proteins glucose oxidase and amyloglucosidase in the vortex-stabilized electrophoresis apparatus. Hence, the method was not used to separate proteins. Further research lead to modest success with the two component defined systems.

4.2 EACA/Tris System

The EACA/Tris system was chosen to test the concept of poured gradients in the vortex-stabilized electrophoresis apparatus since the components were on hand, as was a protein, cytochrome c, which would focus in the expected pH range. In Figure 3-5, the average of the two gradients poured and extracted without power showed that poured gradients in the vortex-stabilized electrophoresis apparatus were reproducible and nearly linear. The average gradient
from 1.5 hour runs at 5 kV (Figure 3-5) showed that the pH gradients at the end of the experiment were similar to the ones poured initially.

The increase in pH at the anode and shift of the gradient after 1.5 hours of applied voltage looked similar to anodic drift, although that would not account for the excursion at the cathode. Bier claimed that these alterations at the ends of the gradients were due to polarization [11]. Possible causes for the alterations at the ends of the gradients will be discussed in the next section since it was during the work with the propionic acid/DL-Serine gradient that the reasons for the alterations became clear.

The consistent focusing of cytochrome c in the EACA/Tris gradients (Figure 3-6) and the stability of the gradients showed that the EACA/Tris gradient was useful for focusing mg quantities of protein in 1.5 hours at 5 kV in the vortex stabilized electrophoresis apparatus.

4.3 Propionic Acid/DL-Serine System

Similar to the EACA/Tris system, the propionic acid/DL-Serine gradients were repeatable and relatively stable for 1.5 hours at 5 kV (Figure 3-7). Like the EACA/Tris system, the propionic acid/DL-Serine gradients had excursions at the cathode. The excursion occupied more of the gradient as run times and voltage were increased. The cathodic end of the gradient seemed to be drifting towards the anodic end of the chamber. Interestingly, the anodic end seemed relatively stable in the pH 3.1-3.3 range over all of the runs, regardless of time or voltage. As the gradient compressed, the fractions nearest the cathode developed a pH very nearly equal to that of 100 mM DL-Serine in the buffer tank, about pH 5.5. That suggested that the content of cathode buffer tank was entering the separation chamber. The use of 500 MWCO rather than 6000 MWCO dialysis membranes did not alter the amount of compression after 5.5 hours at 10 kV, eliminating the porosity of the membranes as a cause for the compression.
Work by Mosher et al. [24] in tube IEF-PAGE suggested that gradient drift was related to the buffer tank concentrations. Thus, the buffer tank concentration could be responsible for the compression seen in EACA/Tris and propionic acid/DL-Serine pH gradients, although that idea has not yet been tested in the vortex-stabilized electrophoresis apparatus.

The wide distribution of amyloglucosidase seen in gel D of Figure 3-8 is partially due to the shallow slope of the pH gradient. The effect of the pH gradient on the width of the focused protein band could be estimated using the following two relationships. First, the width of a band was \( s \) [26], if the protein concentration in the focused band was assumed to be a Gaussian distribution. Second, the standard deviation, \( s \), for such a distribution was given by Righetti [2] as

\[
\sigma = \pm \sqrt{\frac{D}{E} \left( \frac{d\mu}{dpH} \right)^{-1} \left( \frac{d\mu}{dpH} \right)^{-1}},
\]

where \( D \) was diffusion coefficient in \( \text{cm}^2 \text{s}^{-1} \) and \( E \) was the electric field in \( \text{V cm}^{-1} \). The slope of the mobility near the pI was \( \frac{d\mu}{dpH} \), with units of \( \text{cm}^2 \text{V}^{-1} \text{s}^{-1} \). The slope of the pH gradient near the pI, \( \frac{dpH}{dx} \), had units of \( \text{cm}^{-1} \). The estimated band widths of glucose oxidase and amyloglucosidase in run 17 are shown in Table 3-4, along with the values used to calculate them. The value for \( \frac{d\mu}{dpH} \), \( 0.1 \cdot 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1} \), was estimated from data for albumin and hemoglobin [24]. The estimated band width showed that amyloglucosidase should have been spread out over about 12 fractions, over three times the breadth of the estimated glucose oxidase band that
Table 3-4. Estimated Band Widths of Proteins at the End of Run 17

<table>
<thead>
<tr>
<th>Protein</th>
<th>$D$ (cm$^2$ s$^{-1}$)</th>
<th>$E$ (V cm$^{-1}$)</th>
<th>$\frac{d\mu}{dpH}$ (cm$^2$ V$^{-1}$ s$^{-1}$)</th>
<th>$\frac{dpH}{dx}$ (cm$^{-1}$)</th>
<th>Band Width (cm, No. Fractions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Oxidase</td>
<td>$2 \cdot 10^{-4}$</td>
<td>333.33</td>
<td>$0.1 \cdot 10^{-4}$</td>
<td>0.345</td>
<td>1.67, 3.55</td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>$2 \cdot 10^{-4}$</td>
<td>333.33</td>
<td>$0.1 \cdot 10^{-4}$</td>
<td>0.03</td>
<td>5.65, 12.03</td>
</tr>
</tbody>
</table>
focused in a steeper portion of the pH gradient. That means the amyloglucosidase would have occupied about 23% of the separation chamber.

Another contribution to the wide distribution of amyloglucosidase came from the fraction withdrawal process. Preliminary work indicates that the sample collection system causes tailing in the direction of the anodic end of the separation chamber. The pressure on the membranes changes as the fractions are withdrawn and causes the membranes at the bottom of the chamber to flex. The flexing of the membranes moves the fluid in the separation chamber up and down, which mixes the protein bands with the solution around it (work is underway to test supported membranes to prevent flexing). The tailing skews the protein concentration distribution, increasing the focused protein’s apparent band width. Bands that focus at the top of the column are withdrawn sooner and show less tailing than those at the bottom. Large bands at the bottom of the chamber increased in band width by about 7-9 fractions during sample collection, whereas small bands at the top of the separation chamber only increased 3-5 fractions. Hence, the 12-fraction wide amyloglucosidase band in the separation chamber at the end of the run would end up in the 96-well plate as a skewed band approximately 20-fractions wide. The dispersion during sample collection and the shallow pH gradient seem like the principal causes of the wide amyloglucosidase distribution since they accounted for about 2/3 of amyloglucosidase’s 33-fraction band width and all of glucose oxidase’s 6-fraction band width, based on the estimated band widths.

4.4 Conclusions

Separating therapeutic protein isoforms is important because of the effects that post-translational modifications can have on the protein’s function. Different post-translational modifications to the protein’s primary structure can alter the protein’s pI. Using shallow defined
buffer pH gradients to perform preparative-scale IEF in the vortex-stabilized electrophoresis apparatus could potentially separate protein isoforms in the quantities necessary for characterizing and testing the different isoforms. This study aimed to determine if defined buffers could be used to generate shallow pH gradients suitable for separating protein isoforms at a preparative scale in the vortex-stabilized electrophoresis apparatus.

In this work, defined buffers were tested for stability, reproducibility, and their ability to focus and separate proteins. The results showed that the naturally forming pH gradient only produced step-wise pH gradients in the vortex-stabilized electrophoresis apparatus. Step-wise pH gradients are not useful separating proteins, especially ones with similar pIs. The poured concentration gradients resulted in pH gradients that were reproducible and stable long enough to separate proteins. The EACA/Tris buffer system consistently focused 20 mg of cytochrome c in 1.5 hours at 5 kV. This showed that the poured gradients could focus preparative-scale protein loads in a reasonable amount of time. The propionic acid/DL-Serine system separated glucose oxidase and amyloglucosidase in 5.5 hours at 10 kV with the proteins loaded in the acidic solution used to pour the gradient. This demonstrated that the shallow pH gradients could separate a pair of test proteins, but not necessarily isoforms.

Complete separation of protein isoforms with pI differences less than 0.2 pH units may not be possible in the vortex-stabilized electrophoresis apparatus using the two-component defined buffers, even though glucose oxidase and amyloglucosidase were separated. The reason for the limitation is the large band width of a protein in a shallow pH gradient. Protein bands would overlap in the column if the pIs of the two test proteins were less than 0.2 pH units apart, according to the estimate used for the amyloglucosidase band width. Dispersion during sample collection would only increase the overlap, decreasing the resolution of the isoforms.
Partial separation or enrichment of protein isoforms may be the best that can be done with shallow, poured gradients. This idea fits the results seen by other researchers using poured two-component gradients. Bier’s early work [8,9] with a defined gradient did not completely resolve Hb A (pI=7.2) and Hb C (pI=7.8). Although there were focused proteins, there was protein smeared between the two bands. Tulp et al. [12] also saw similar results with a MOPS/GABA pH gradient, spanning pH 5.2-6.1, used to separate BSA, Tf, and Hb. The pIs of BSA and Hb were outside the range of the gradient and so those proteins focused at the anode and the cathode, respectively. The Tf remained in the middle of the column. The Coomassie stained IEF gel of the fractions from Tulp’s separation showed 5 different Tf bands with pIs around 5.5 (Tulp specifically identified bands with pIs of 5.51 and 5.52). While some fractions were enriched in a particular form of Tf, none of the fractions contained an individual band.

Two-component gradients were unable to separate protein isoforms in the work presented by other groups [8,9,12] and it looks doubtful that the gradients could separate isoforms in the vortex-stabilized electrophoresis apparatus at the tested run conditions. This seems to indicate that the problem is with the two-component gradients, particularly the large band widths they cause, rather than their implementation in the different instruments. The results from this work also lead to some interesting possibilities for future experiments.

Equation 1 suggests that the limitations of the large band widths might be overcome by increasing the electric field strength, the length of the separation chamber, or decreasing the dispersion of the system. Increasing the length of the chamber reduces the percentage of the separation chamber a band occupies. However, the increased chamber length would prolong the required run-times for the proteins to separate and focus at their pIs. Increasing the voltage or chamber length cannot be tested yet because the gradient’s stability decreases with increasing
run-time and voltage, as shown in Figure 3-7. A series of tests examining the effect of the buffer concentration on the pH gradient might lead to more stable gradients. Then, perhaps increased voltage and chamber length could be tested to see whether the shallow poured gradients might be able to separate isoforms under those conditions.

The separations using glucose oxidase and amyloglucosidase could be performed using SCAMs, rather than the defined buffers. Results from that experiment could be compared to the defined buffer results. Any substantial differences between the two sets of results, such as band widths and time required to separate the proteins, might suggest that there are differences in the protein focusing mechanism between the SCAMs and the two-component buffers. A better understanding of the protein focusing mechanism in the two-component gradients might elucidate their suitability for separating isoforms.

Another experiment might be to enzymatically cleave all the post-translational modifications from the amyloglucosidase to create a more homogenous sample and try to focus it. If the more homogenous sample focused into a tight band, it would suggest that the poured gradients separated isoforms in the work presented here. These follow-up experiments may help clarify whether or not two-component defined buffers really can separate protein isoforms.

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5 References


Chapter 4: Modeling Two-Component Isoelectric Focusing Buffers in a Vortex-Stabilized Electrophoresis Apparatus

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Modeling Two-Component Isoelectric Focusing Buffers

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Abbreviations:  EACA, e-amino-n-caproic acid; IEF, isoelectric focusing; GABA, ?-amino-n-butyric acid; MOPS, 3-[n-morpholino]propanesulfonic acid; SCAMs, synthetic carrier ampholyte mixtures;

Keywords: model, IEF, defined buffer
Abstract

Defined buffer systems have been considered by researchers as replacements for synthetic carrier ampholyte mixtures in order to reduce costs and their deleterious interactions with proteins. In this paper, a mathematical model for the behavior of two-component pH gradients during IEF in a vortex-stabilized electrophoresis apparatus is developed. The model is based on theory and predicts experimental results well. It solves equations for mass conservation, molar fluxes, and the electric field to describe the behavior of the components in an electric field. Equilibrium constants are used in the model to account for interconversion between the positive, negative, and neutral states for each component.

The model was applied to predict pH gradients using three different defined buffer systems. The model’s predictions fell within the 95% confidence interval of a least-squares fit to the experimental pH gradients except in the regions near the ends of the pH gradients. The model could not predict changes at the ends of the pH gradients because it used constant composition boundary conditions at the ends of the gradients. Other discrepancies between the model and the experimental results could be due to variations in the buffer component’s pKs caused by concentration and temperature effects.
1 Introduction

Replacing synthetic carrier ampholyte mixtures (SCAMs [1]) with defined buffers has been studied as a means to alleviate unfavorable protein-SCAM interactions and reduce the cost of preparative isoelectric focusing (IEF). These unwanted interactions lead to altered isoelectric points (pIs), precipitation, and difficulty in removing SCAMs from target proteins [2]. Bier and associates [3,4] developed a particularly simple replacement for the SCAMs, which needed only two components to generate shallow gradients about 1 pH unit wide, while Cuono and Chapo [1] used up to 47-components to naturally form a pH 3-10 gradient. Natural pH gradients are those which form spontaneously in an electric field [2]. The literature refers to pH gradients formed by other means as artificial.

The shallow pH gradients are of particular interest because they may be useful for separating protein isoforms, proteins with the same primary structure but different post-translational modifications. The differences in post-translational modifications between proteins can alter their function [5] and give them slightly different pIs. IEF separates proteins based on differences in pIs and focuses the proteins into bands centered on their pIs. Separating isoforms by IEF requires pH gradients shallow enough to prevent the focused isoform bands from overlapping.

Bier [3,4] and Cuono’s [1] work with naturally forming pH gradients from defined buffers led to testing defined buffers with 2 or 10 components in a vortex-stabilized electrophoresis apparatus to determine if they could produce shallow pH gradients for separating isoforms. The natural pH gradients formed from defined buffers tested in the vortex-stabilized electrophoresis apparatus only produced step-wise pH gradients. The problem with step-wise pH gradients is that the protein bands will stay spread out in the flat parts of the gradient and overlap.
in the steep sections. The smooth useful gradients seen by Bier and Cuono never formed in the vortex-stabilized electrophoresis apparatus. Unlike the SCAMs, defined buffers failed to naturally form useable pH gradients in the vortex-stabilized electrophoresis apparatus.

Artificially forming the gradient by pouring a concentration gradient using two components was tried next since the defined buffers did not spontaneously form pH gradients. It turned out that Bier et al. [6,7] had tried this technique in free solution several years before turning to work with naturally forming pH gradients. They showed that hemoglobin (Hb) variants could be separated in a two-component poured pH gradient coupled with a density gradient [6,7]. Initial tests with e-amino-n-caproic acid (EACA) and Trizma base (Tris) (runs 1-3 of Table 4-2) showed that the pH gradients poured in the vortex-stabilized electrophoresis apparatus were shallow, reproducible, and relatively stable in the electric field (Figure 4-4 and Figure 4-5).

An obstacle to the practical use of these two-component buffers was that the measured pH gradients did not necessarily span the pH ranges expected, based on the pH of the solutions used to pour the gradients. That made it difficult to predict the pH gradient that a pair of buffers would form in the vortex-stabilized electrophoresis apparatus. The many possible buffer pairs that could be used increased the problem of choosing components to form a desired pH gradient. Bier’s paper [3] listed 68 different buffer pairs from 46 different compounds among the many possibilities that surely exist. It would be impractical to experimentally test every buffer pair of interest in order to find out what pH gradient would be formed. Mathematically modeling the defined buffer pH gradient and its behavior in the electric field could provide a means of predicting whether or not a given set of components might form a shallow pH gradient in a
particular range. This would greatly reduce the amount of guesswork involved in trying to pour a shallow, defined-buffer, pH gradient for separating isoforms by IEF.

Svensson [8] developed the first mathematical model for steady state IEF of a single compound, i.e., a protein, in a stable pH gradient, such as those produced by SCAMs. However, this model did not include the behavior of the pH gradient so it would not serve to describe the pH gradient formed by the defined buffer systems.

Almgren developed an improved IEF model that predicted pH and concentration profiles of a defined buffer system [9]. Although it was an advanced model in 1971, it assumed that all mobilities (except hydronium and hydroxyl ions) were equal and that the current density was independent of diffusion.

In an effort to eliminate the assumptions made by Almgren, Palusinski et al. developed a model in 1980 for steady state IEF of defined buffers that included the diffusive contribution to current as well as mobilities of individual ions and equilibrium between ionic species [10]. This steady state model predicted pH, current, and concentration profiles for multi-component systems. Bier et al. [11] improved the Palusinski et al. [10] model by adding time dependence. Then, they could observe the formation of pH gradients under different circumstances by adjusting the initial and boundary conditions. Bier et al. [12] used that model as the starting point for several papers, that were combined into an extensive book on electrophoresis. Bier’s model successfully predicted the pH gradient formed from 3-[n-morpholino]propanesulfonic acid (MOPS) and ω-amino-n-butyric acid (GABA) [3], one of the buffer pairs from Bier’s defined buffer work.

This paper presents a useful mathematical model for the behavior of defined pH gradients preformed in a vortex-stabilized electrophoresis apparatus. The model’s predictions are
compared to experimental data to assess the model’s accuracy. Two pH ranges were explored, pH 3-5 and 9-10, using components chosen from the list of pairs given by Bier [4]. These gradients were of interest since they were expected to form shallow pH gradients that could be used to separate isoforms. Another reason for choosing pH 3-5 and 9-10 ranges was that the model would likely be used in those ranges, because SCAM-protein interactions are more likely in those pH ranges [2]. As will be shown, the model and the experimental data matched well at different applied voltages, run times, and buffer compositions, suggesting that the model developed in this study reasonably predicts the pH gradients formed by two-component poured concentration gradients.

2 Materials and Methods for Experiments

2.1 Buffer Components

DL-serine, ε-amino-n-caproic acid (EACA), and Trizma base were purchased from Sigma (St. Louis, MO, USA). Propionic acid came from Aldrich (Milwaukee, WI, USA). Nanopure water came from a Barnstead Thermolyne (Dubuque, IA, USA) Nanopure Infinity UV/UF system.

2.2 Defined Buffer Systems

The defined buffer systems in this work consisted of four separate solutions. Two 500 mL electrode buffers purged electrolysis products during the experiment: an anode buffer, or anolyte; and a cathode buffer, or catholyte. The other two solutions were 12 mL each, one acidic, and one basic. These were combined using a gradient maker to form the pH gradient in the separation chamber.
Defined buffers were chosen to form shallow pH gradients in the ranges where SCAM-protein interactions are most likely, in the very acidic and very alkaline pH ranges. This would test the model in the ranges where its predictions would most likely be applied. Altogether, three different defined-buffer systems selected from Bier’s work [4] were used to pour pH gradients for comparison to the mathematical model that was developed. Two EACA/Tris buffer systems at different concentrations were used to form pH gradients in the basic pH range and a propionic acid/DL-Serine buffer was used to form pH gradients in the acidic range.

The anolyte and catholyte compositions for the three defined buffer systems are listed in Table 4-1. The two 12 mL solutions used to form the gradients for these systems had the same composition as the anolyte and catholyte solutions used. All components were weighed out and dissolved in boiled Nanopure water.

2.3 pH Gradient Formation

The EACA/Tris and propionic acid/DL-Serine buffers were used to form pH gradients in the alkaline and acidic pH regions for comparison with the mathematical model. The components were poured in linear concentration gradients so that as the concentration of one component, \( c_1 \), linearly increased, the concentration of the second component, \( c_2 \), was linearly decreasing according to \( c_1 + c_2 = 100 \text{ mM} \). These linear concentration profiles with opposite slopes produced a linear pH gradient. The total concentration was set at 100 mM, as suggested in Bier’s patent [4].

The linear pH gradients were pumped into the separation chamber using the apparatus shown in Figure 4-1. The gradient former (Bio-Rad, Hercules, CA, USA) sat on a magnetic stir plate (Barnant Corp., Barrington, IL, USA) that mixed the contents of the basic reservoir using a magnetic stir bar. A peristaltic pump (Buchler Instruments, Lenexa, KS, USA) was used to
Table 4-1. Anolyte and Catholyte Composition for the Defined Buffer Systems

<table>
<thead>
<tr>
<th></th>
<th>EACA/Tris System 1</th>
<th>EACA/Tris System 2</th>
<th>Propionic acid/DL-Serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anolyte</td>
<td>90 mM EACA/10 mM Tris</td>
<td>100 mM EACA</td>
<td>100 mM Propionic acid</td>
</tr>
<tr>
<td>Catholyte</td>
<td>10 mM EACA/90 mM Tris</td>
<td>100 mM Tris</td>
<td>100 mM DL-Serine</td>
</tr>
</tbody>
</table>
Figure 4-1. A schematic of the apparatus used to pour a defined buffer pH gradient. Pumping the contents of the reservoirs into the annular separation chamber formed between the rotor and the stator forms a linear concentration gradient. The components in the linear concentration gradient titrate each other to form a pH gradient.
pump the contents of the gradient maker into the separation chamber, filling the chamber from the bottom.

First, the separation chamber was filled with water to prevent bubbles from being trapped in the separation chamber while the gradient was being poured. Then 10 mL of the basic solution was added to the basic reservoir and pumped into the separation chamber. This filled the tubing and the bottom of the chamber with basic buffer so that as the gradient was formed and pumped into the chamber, it would not be diluted by the solution it was displacing. Then, 12 mL of acidic solution was added to the rear compartment and 12 mL of basic solution was added to the front compartment of the gradient former. A stir bar was placed in the front compartment and the magnetic stirrer was switched on. The valve between the two compartments was opened and the pump turned on and set to a flow rate of 0.64 mL/min. A pH gradient was created as the mixture from the gradient former filled the separation chamber from the bottom to the top. The separation chamber is only one piece of a larger device, which is not shown completely in Figure 4-1. The rest of the device is described in the following section.

2.4 Vortex-Stabilized Electrophoresis Apparatus

The apparatus used for preparative IEF separation was a vortex-stabilized electrophoresis device designed by Ivory and Gobie [13]. Thome and Ivory [14] had previously used this apparatus for continuous electrophoresis of enantiomers and a more detailed description of it can be found in that paper. Only a brief description of the apparatus (Figure 4-2) as used for IEF is provided here.

The annular separation chamber was formed between a rotor and a stator. The grooved, hollow, boron nitride rotor was 12 inches tall by 1 inch in diameter and formed the inner wall of the separation chamber. A complimentarily grooved Plexiglas stator surrounded the rotor to
Figure 4-2. The vortex-stabilized electrophoresis apparatus used is on the left. The white vertical cylinder is the rotor, which forms a 24 mL annular separation chamber with the stator. The high-voltage power supply that provides the electric field for the separation process is located above and behind the separation chamber. To the right of the chamber are the off-take valves. One of the 500 mL electrode buffer tanks can be seen to the right of the valve actuators. The 96 well plates for sample collection are placed in the vacuum chamber seen in the bottom right foreground.
form the outer wall of the separation chamber. The volume of the separation chamber was about 24 mL. Two March 1A-MD-1 pumps (March Mfg. Inc., Glenview, IL, USA) circulated electrode buffers from 500 mL tanks through the pairs of electrode housings located at the top and the bottom of the separation chamber to purge hydrolysis gases. A motor with speed control circuitry spun the rotor at 50 rotations per minute.

The matching grooves in the rotor and stator created a vortex pattern in the annular fluid that allowed for better heat and mass transfer between the rotor and stator. The Taylor-like vortices stabilized the axial concentration profile, preventing natural convection from disturbing the concentration profile that resulted from the electrophoretic separation. These vortices also provided nearly an order of magnitude higher power dissipation within the apparatus compared to running without vortex formation.

A Syltherm XLT coolant oil (Dow Chemical, Midland, MI, USA) was recirculated through the inside of the rotor to dissipate the Joule heat generated from electrophoresis. This oil was cooled using a VWR 1197 recirculating cooler (VWR, S. Plainfield, NJ, USA), and a static mixer (Omega, Stamford, CT, USA) was used inside the rotor to promote internal heat transfer.

The ends of the stator on the electrophoresis chamber were machined to accept Plexiglas electrode compartments that were partitioned from the separation chamber by membranes. The cathode was at the top of the column and the anode at the bottom. For these experiments, either 6000 MWCO or 500 MWCO dialysis membranes (Spectrum Laboratories, Houston, TX, USA) or ion exchange membranes were used to prevent bulk mixing between the electrode buffers and the contents of the separation chamber. Dialysis membranes of the same size were used at all electrodes.
High-voltage power for separations in the vortex-stabilized electrophoresis apparatus was provided by a Spellman SL30PN1200 power supply (Spellman High Voltage Electronics, Hauppauge, NY, USA) and controlled via a PC running LabVIEW 6.0 (National Instruments, Austin, TX, USA) with a PCI-DAS 1002 multifunction A/D I/O board (Measurement Computing, Middleboro, MA, USA). The LabVIEW power supply control program also recorded voltage and current measurements from the power supply during the course of the experiment.

At the end of each experiment, an automated fraction collection system was used to remove the contents of the separation chamber. The fractions were collected through 53 ports by a series of custom, electro-actuated, multi-position, valves (Valco Instrument Co. Inc., Houston, TX, USA) and house vacuum. The ports started 2.5 cm below the cathode, with a port every 0.47 cm, and finished 2.5 cm above the anode. A program written in LabVIEW controlled the valve positions so that 250-300 µL fractions were taken progressively from the top to the bottom of the column and deposited in the microtiter plate in that same order.

2.5 Experimental Conditions for Testing pH Gradients

Poured pH gradients for each buffer system were tested according to the run conditions shown in Table 4-2. The anolyte and catholyte for each buffer system in Table 4-2 is specified in Table 4-1. The gradients were poured as previously described using 12 mL each of the anolyte and catholyte, for the acidic and basic solutions. The gradients poured and extracted without applying an electric field verified that the pH gradients were linear and reproducible, as shown in Figure 4-4. Voltage was applied to the pH gradients to generate data that could be compared to the model. At the end of each run, voltage was turned off and fractions were collected for analysis.
<table>
<thead>
<tr>
<th>Run No.</th>
<th>Defined Buffer System</th>
<th>Voltage (kV)</th>
<th>Run Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EACA/Tris System 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>EACA/Tris System 1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>EACA/Tris System 1</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>EACA/Tris System 1</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
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<td>EACA/Tris System 1</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>EACA/Tris System 1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>EACA/Tris System 2</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>Propionic acid/ DL-Serine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Propionic acid/ DL-Serine</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>Propionic acid/ DL-Serine</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>11</td>
<td>Propionic acid/ DL-Serine</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>Propionic acid/ DL-Serine</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>13</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>14</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>15</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>16</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>17</td>
<td>Propionic acid/ DL-Serine</td>
<td>10</td>
<td>5.5</td>
</tr>
</tbody>
</table>


2.6 Analysis of pH Gradients

The shapes of the pH gradients were found by measuring the pHs of the 53 fractions taken from the separation chamber at the end of each run. Fraction pH was measured using an Orion micro probe (Orion Research, Inc., Beverly, MA, USA) and Denver pH meter (Denver Instrument Co., Arvada, CO, USA). The error in the pH meter’s readings was ± 0.002 pH units and the error in the probe’s readings was ± 0.02 pH units. The probe and meter were calibrated at pH 4, 7, and 10 before measuring the pH of the 53 fractions collected from each experiment.

3 Mathematical Model Development

3.1 General Equations

The purpose of mathematically modeling the defined buffer system is to predict the behavior of the pH gradient over time in an electric field, specifically the component concentrations and the pH profile. This way computer simulation can test whether or not a particular buffer pair will form a desired pH gradient.

The model is based on electro-transport theory uses no fitting parameters. The molar flux includes a term for the electrophoretic mobility to describe the motion of the species in the electric field. The equation for mass conservation includes generation rates for species. Interconversion between charged states for each component is included in the model by using equations for the equilibrium constants to relate the positive, negative, and uncharged states. The defined buffer solutions in the separation chamber are considered electrically neutral.

Radial mixing in the separation chamber is assumed to be uniform so the model dimensions are time (s), width (cm), and axial length (cm). The length of the annular separation...
The chamber is 30 cm and its cross sectional area is 2.97 cm$^2$. The dispersion in the system, guessed to be about 2x10$^{-4}$ cm$^2$ s$^{-1}$, is used in place of the diffusivities in the flux equations because of the axial mixing induced by the vortices. The actual diffusivities, $D_i$ (cm$^2$ s$^{-1}$), of the components are only used to calculate their mobilities, $\mu_i$ (cm$^2$ V$^{-1}$ s$^{-1}$) by the Einstein expression $D_i = \frac{RT\mu_i}{F}$. $F$ is Faraday’s constant, 96485 C/mol, and $R$ is the ideal gas constant.

The temperature, $T$, is in Kelvin. Properties of the components are given in Table 4-3. Electro-osmosis and temperature effects are neglected to keep the model from being unduly complicated.

The model starts with a mass conservation equation for each species, $i$.

$$\frac{\partial c_i}{\partial t} + \nabla \cdot N_i = R_i$$

The concentration, $c_i$, is in terms of mol L$^{-1}$. The generation rate of species $i$ is $R_i$ (mol L$^{-1}$ s$^{-1}$). $N_i$ (mol cm L$^{-1}$ s$^{-1}$), the molar flux of $i$, includes diffusion and electro-migration and is defined as

$$N_i = -D_i \nabla c_i + \mu_i E c_i.$$  

(2)

The electric field, $E$ (V cm$^{-1}$), is derived from the following equation for current, $I$ (A cm$^{-2}$), in terms of the molar flux of all $m$ species.

$$I = F \sum_{i=1}^{m} z_i N_i$$

(3)

$z_i$ is the $i^{th}$ valence charge. The net charge balance is

$$\sum_{i=1}^{m} z_i c_i = 0,$$

(4)

since the defined buffer solution is electrically neutral.
Table 4-3. Defined Buffer Component Properties at 25 C.

<table>
<thead>
<tr>
<th>Component</th>
<th>$P_kA_1$ [15]</th>
<th>$pK_a_2$ [15]</th>
<th>$D \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ [15]</th>
<th>$\mu \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionic acid</td>
<td>4.86</td>
<td>-</td>
<td>0.953</td>
<td>4.8</td>
</tr>
<tr>
<td>DL-Serine</td>
<td>2.21</td>
<td>9.15</td>
<td>1.5*</td>
<td>4.6</td>
</tr>
<tr>
<td>EACA</td>
<td>4.37</td>
<td>10.8</td>
<td>-</td>
<td>3.11 [12]</td>
</tr>
<tr>
<td>Tris</td>
<td>8.1</td>
<td>-</td>
<td>-</td>
<td>2.41 [12]</td>
</tr>
<tr>
<td>$H_3O^+$</td>
<td>-</td>
<td>-</td>
<td>9.3011</td>
<td>36.2</td>
</tr>
<tr>
<td>OH</td>
<td>-</td>
<td>-</td>
<td>5.273</td>
<td>20.5</td>
</tr>
</tbody>
</table>

Note: Mobilities are calculated from D unless marked otherwise.
* indicates an estimate.
The reaction expression for the $j^{th}$ ampholyte is

\[ A_j^+ \rightleftharpoons K_{j1} A_j^0 + H^+ \rightleftharpoons K_{j2} A_j^- + H^+ , \]  

(5)

where $K_{j1}$ and $K_{j2}$ are the equilibrium constants, $A_j$ is the $j^{th}$ ampholyte and the superscript on $A_j$ represents the charge state. The equilibrium between the various charge states is assumed to be fast in comparison to transport rates, with the equilibrium constants for each component written as

\[ K_{j1} = \frac{c_{A_j^0} c_{H^+}}{c_{A_j^+}} = 10^{-pK_{a1,j}} \]  

(6)

and

\[ K_{j2} = \frac{c_{A_j^-} c_{H^+}}{c_{A_j^0}} = 10^{-pK_{a2,j}} . \]  

(7)

The equilibrium equation for water is also used.

\[ K_w = c_{H^+} c_{OH^-} = 10^{-14} \]  

(8)

### 3.2 Combination of Equations

The goal of combining the above equations is to obtain a simplified set of equations that can be solved for the concentrations of ampholyte species throughout the system at any time and position in the separation chamber based on known initial values and boundary conditions. Once the ampholyte concentrations are known, the pH can be calculated as shown below in equation 13.
The preceding equations are combined for a general three-component system: water, an acidic ampholyte \((A_1)\), and a basic ampholyte \((A_2)\). Each component has multiple charge states, two for the water and three each for the ampholytes. Each charge state is considered one of the \(i\) species, for a total of eight species: \(A_1^+, A_1^0, A_1^-, A_2^+, A_2^0, A_2^-, H^+, \) and \(OH^-\).

Simplification starts with the mass conservation equations for the ampholyte species since the model should give the concentration of the ampholytes as a function of time and position. Substituting equation (2) into equation (1) for each ampholyte species gives

\[
\frac{\partial c_i}{\partial t} = D_i \nabla^2 c_i + \nabla \cdot (\mu_i \mathbf{E} c_i) = R_i. \tag{9}
\]

These equations include unknown reaction rates. The reaction rates for the acidic ampholyte are eliminated by summing the above equation for all the charge states of that ampholyte, likewise, the reaction rates for the basic ampholyte are eliminated.

Then by assuming the diffusivity is the same for all charge states of a component, the following equation can be written for each ampholyte.

\[
\frac{\partial}{\partial t} (c_{A_1^+} + c_{A_1^0} + c_{A_1^-}) = D_i \nabla^2 (c_{A_1^+} + c_{A_1^0} + c_{A_1^-}) - \nabla \cdot (\mathbf{E}(\mu_{A_1^+} c_{A_1^+} + \mu_{A_1^0} c_{A_1^0} + \mu_{A_1^-} c_{A_1^-})) \tag{10}
\]

The equilibrium constants for the ampholytes, equations (6) and (7), show that both of the charge states in equation (10) are functions of the neutral state. Thus, the equilibrium constants for each ampholyte are used to write the charged ampholyte species in terms of the neutral species of ampholytes. This results in
\[
\frac{\partial}{\partial t} c_{A_i} \left( 1 + \frac{c_{H^+}}{K_{H^+}} + \frac{K_{j2}}{c_{H^+}} \right) = \\
D_i \nabla^2 c_{A_i} \left( 1 + \frac{c_{H^+}}{K_{H^+}} + \frac{K_{j2}}{c_{H^+}} \right) - \nabla \cdot \left( \mu_{A_i} \left( \frac{c_{H^+}}{K_{H^+}} + \mu_{A_i} \frac{K_{j2}}{c_{H^+}} \right) \right)
\]

which becomes a function of the hydronium ion concentration as well.

The hydronium ion concentration is also a function of the neutral ampholyte species.

This can be seen from the equation for electro-neutrality, equation (4), with the charged ampholytes written in terms of the neutral ampholyte species.

\[
c_{H^+} + \frac{c_{H^+} c_{A_i}^o}{K_{11}} + \frac{c_{H^+} c_{A_i}^o}{K_{21}} - \frac{K_{12} c_{A_i}^o}{c_{H^+}} - \frac{K_{22} c_{A_i}^o}{c_{H^+}} - K_W = 0
\]

This polynomial can be solved for the hydronium ion concentration in terms of the neutral ampholyte species.

\[
c_{H^+} = \sqrt{\frac{K_{12} c_{A_i}^o + K_{22} c_{A_i}^o + K_W}{1 + \frac{c_{A_i}^o}{K_{11}} + \frac{c_{A_i}^o}{K_{21}}}}
\]

At this point, the only part of the mass conservation equations still not in terms of the concentrations is the electric field. Substituting equation (2) into equation (3) gives

\[
E = \frac{I + F \sum z_i D_i \nabla c_i}{F \sum z_i \mu_i c_i}.
\]

Equation (14) is then expanded and put in terms of hydronium ion and neutral ampholyte concentrations. The diffusive contribution to current density is neglected because it is canceled.
out by the dispersion of the vortices generated in the separation chamber. The electric field is
assumed to vary only axially and written as

\[
E = \frac{I}{F \left[ c_{A_1^0} \left( \mu_{A_1^0} \frac{c_{H^+}}{K_{11}} - \mu_{A_1^i} \frac{K_{12}}{c_{H^+}} \right) + c_{A_2^0} \left( \mu_{A_2^0} \frac{c_{H^+}}{K_{21}} - \mu_{A_2^i} \frac{K_{22}}{c_{H^+}} \right) + \mu_{H^+} c_{H^+} - \mu_{OH^-} \frac{K_w}{c_{H^+}} \right]}.
\] (15)

From this point, further manipulation is unnecessary. Equations (11), (13), and (15) describe the necessary relations in terms of the same variables \(c_{H^+}, c_{A_1^0}, c_{A_2^0}\) and can be numerically solved as they are.

### 3.3 Solution Procedure

The solution of equations (11), (13), and (15) can only be obtained numerically since they result in two partial differential equations for the neutral species of each ampholyte and two non-linear algebraic expressions, one for the electric field and one for the hydronium ion concentration. The computer program FlexPDE 3 (PDE Solutions, Inc., Antioch, CA, USA), which is a scripted finite element builder and solver [16], successfully solves the system of equations for \(c_{A_1^0}\) and \(c_{A_2^0}\), the variables for the neutral species of the acidic and basic ampholytes. The other ampholyte concentrations and pH can then be calculated from \(c_{A_1^0}\) and \(c_{A_2^0}\).

For this simple model, the boundary conditions are: constant composition at the electrodes, as maintained by the electrode buffers; and no flux along the length of the separation chamber, since the walls were solid. The boundary conditions at the ends of the separation chamber are

\[
c_{A_j^0} \bigg|_{x=0} = c_{A_j^0}^0
\] (16)
and

$$c_{A_2^0}(x) = \frac{c_{A_2}^o}{l}x + c_{A_2}^o,$$  \hspace{1cm} \text{(20)}

where superscript $o$ indicates an initial concentration, and $l$ the length of the separation chamber.

The conditions along the length of the chamber are

$$\left. \frac{\partial c_{A_1^0}}{\partial y} \right|_{y=0} = 0$$ \hspace{1cm} \text{(18)}

and

$$\left. \frac{\partial c_{A_2^0}}{\partial y} \right|_{y=w} = 0,$$ \hspace{1cm} \text{(19)}

where $w$ is the width of the area being modeled, essentially the circumference of the annulus. In the actual calculations, the width is scaled down so the program runs faster. This seems acceptable since the contents of the annular separation chamber are assumed to be angularly symmetric (the width is only included because the program used to solve the equations requires two or three dimensions).

The initial conditions for the simulation are one ampholyte linearly increasing in concentration as the other linearly decreases, as shown in equations (20) and (21),

$$c_{A_1^0}(x) = \frac{c_{A_1}^o}{l}x + c_{A_1}^o,$$ \hspace{1cm} \text{(20)}

and

$$c_{A_2^0}(x) = -\frac{c_{A_2}^o}{l}x + c_{A_2}^o.$$ \hspace{1cm} \text{(21)}
In equations (20) and (21), \( c_{A_i}^{a} \) and \( c_{A_i}^{b} \) denote the concentrations of component \( i \) in the acidic and basic solutions used to pour the pH gradient.

The electric field in the model is applied at constant current, rather than at constant voltage like the experiments, to simplify the model. An average current from the experimental data is used in the models to make the pH gradient from the computer simulation comparable with the experimental data. The equilibrium constants in the model are calculated from the pKs of the ampholytes listed in Table 4-3.

4 Results

The results are shown as figures comparing the simulated pH gradients from the mathematical model with the experimental ones. The simulated pH gradients are compared to 95% prediction intervals derived from experimental data to determine how well the simulations correlate with the experiments. The simulations are considered acceptable if the model is in the same range as 95% of the experimental pH gradients.

The 95% prediction interval gives the range that 95% of the future pH gradients should fall within based on a least squares fit of the experimentally measured pH gradients. Prediction intervals are a type of confidence interval that account for the random variability in the experimental data as well as the error involved in estimation of the mean [17].

Mathematica 4.1 (Wolfram Research, Inc., Champaign, IL, USA), a mathematical software package, was used to calculate a least-squares fit of the pH gradient data to the curve \( ax^3 + bx^2 + cx + d \). Then, Mathematica was used to calculate the 95% prediction intervals. In the instances where there were multiple runs performed at the same run time and voltage, all the pH gradients at those conditions were treated as one data set. In some cases, data at the ends of the
experimental pH gradients was not used in curve fitting. Only enough data was used in order to show where the simulations and experiments started to diverge.

Average currents have been used in the simulations, as mentioned previously. This way the simulated gradient will experience the same amount of current flow as the experimental gradient. The average current from an experiment is calculated by dividing the area under the current vs. time curve by the total time for a run. An example of the voltage and current data collected during a run is shown in Figure 4-3. The voltage and current were recorded by the computer program written to control the high voltage power supply. The current vs. time plots for all the runs are not shown because they had much the same trend as the one shown in Figure 4-3.

In the following figures, the model line represents the results of the mathematical simulation for the run conditions specified in the caption. The thinner solid is the regression line, or fit to the data points. Each set of data points represents a pH gradient and has a different style of point. Dashed lines indicate the 95% prediction interval about the fit to the data. In the upper right corner of each of these figures is a single point with error bars to show the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.

4.1 EACA/Tris Systems

Computer simulations of defined buffer IEF in an alkaline pH region using FlexPDE were performed with EACA/Tris systems as the gradient components. The properties for EACA and Tris from Table 4-3 and the average current from the experimental runs were used in the simulations of runs 1-7 (run conditions in Table 4-2). Then, the pH gradients predicted by the mathematical model and the experimentally observed pH gradients were compared.
Figure 4-3. The current and voltage were recorded during the experimental runs, as illustrated here by the data collected during Run 5. The area under the current vs. time curve was divided by the total time to find an average current. The average of the average currents for a given set of run conditions was used in the model to simulate the same run conditions.
Runs 1 and 2 were the first runs performed to verify that poured pH gradients were linear. As shown in Figure 4-4, the data and model had the same shape from about 10-27 cm, but were distinctly offset. More importantly, the model was within the 95% prediction interval for most of the pH gradient, signifying that the model was coming close to predicting where the real pH gradient would be 95% of the time.

A 1.5 hour run at 5 kV was modeled by setting the current to 5.92 mA, the average current of runs 3-5. In Figure 4-5, the model’s predicted gradient matched the data well in the middle of the gradients, but the two diverged at the ends of the gradients. However, more of the model was within the 95% prediction interval than outside of it, meaning the simulated pH gradient was reasonable.

The run time was extended to 2 hours for run 6. The average current from run 6, 5.84 mA, was used to simulate the 5 kV, 2 hour run. Figure 4-6 compares the simulation to the measured pH gradient of run 6. The model fit the data from run 6 reasonably well from 8-24 cm, staying in the 95% prediction interval in that region. As in the previous experiments, the simulation and experiment diverged at the ends of the pH gradient.

In run 7, the concentrations of the EACA/Tris system were changed to try to extend the linear region of the pH gradient. The average current from the 1.5 hour run at 5 kV using EACA/Tris system 2 was 5.28 mA. The comparison of run 7 with the model’s prediction, shown in Figure 4-7, does not look favorable. The model never falls within the 95% prediction interval, signifying that the real pH gradient would probably not be predicted by the model. The model showed the same trend as the data, but it did not predict the right pH values.
Figure 4-4. Runs 1 and 2 are compared to a simulation with the same run conditions: no voltage with EACA/Tris system 1. The model line represents the results of the mathematical simulation. The data points from the experimentally observed pH gradients are shown as points with each gradient having a different style of point. The thinner solid line is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.
Figure 4-5. Runs 3-5 are compared to a simulation at the same run conditions: 1.5 hrs at 5 kV with EACA/Tris system 1. The model line represents the results of the mathematical simulation. The data points from the experimentally observed pH gradients are shown as points with each gradient having a different style of point. The thinner solid curve is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.
Figure 4-6. Run 6 is compared to a simulation at the same run conditions: 2 hrs at 5 kV with EACA/Tris system 1. The model line represents the results of the mathematical simulation. The data points from the experimentally observed pH gradient are shown as circles. The thinner solid curve is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.
Figure 4-7. Run 7 is compared to a simulation at the same run conditions: 1.5 hrs at 5 kV with EACA/Tris system 2. The model line represents the results of the mathematical simulation. The data points from the experimentally observed pH gradient are shown as circles. The thinner solid curve is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.
4.2 Propionic Acid/DL-Serine System

Computer simulations of two-component poured pH gradients were also performed in the acidic region using the propionic acid/DL-Serine system, in addition to the those done in the alkaline region. The properties for propionic acid and DL-Serine from Table 4-3 and the average current from the experimental runs were used in the simulations of runs 8-17 (run conditions in Table 4-2). Then, the pH gradient predicted by the mathematical model and the experimentally measured pH gradients were compared.

The propionic acid/DL-Serine gradient was only poured and extracted without power once, since the resulting pH gradient was linear (see Figure 4-8) and the previous work with the EACA/Tris gradients showed that the gradients were reproducible and linear. Figure 4-8 shows the simulated result of the poured pH gradient using the propionic acid/DL-Serine system without applying an electric field compared to the measured pH gradient. The data for the actual experiment was fairly close the model’s prediction, although the disparity between the model and data increased towards the anode. While the model showed the right trend, it was outside the 95% prediction interval.

Next, runs 9-11 were simulated. The average current of the three 1.5 hour runs at 5 kV using the propionic acid/DL-Serine system was 3.75 mA. The gradient produced by the simulation at that average current for 1.5 hours was compared to the experimental pH gradients in Figure 4-9. Except from 2.5-7 cm, the data and the model matched very closely.
Figure 4-8. Run 8 is compared to a simulation at the same run conditions: no voltage with the propionic acid/DL-Serine system. The model line represents the results of the mathematical simulation. The data points from the experimentally observed pH gradient are shown as circles. The thinner solid curve is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.
Figure 4-9. Runs 9-11 are compared to a simulation at the same run conditions: 1.5 hrs at 5 kV with the propionic acid/DL-Serine system. The model line represents the results of the mathematical simulation. The data points from the experimentally observed pH gradients are shown as points with each gradient having a different style of point. The thinner solid curve is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.
When the run time was increased to 2.5 hour at 5 kV in run 12, the average current dropped to 3.16 mA. Simulating the 5 kV 2.5 hour run with that average current gave the pH gradient shown in Figure 4-10 with the experimentally measured pH gradient for comparison. Similar to the 1.5 hour run, the data corresponds very well with the model except in the cathodic region from 2.5-7.5 cm. The fit and 95% prediction interval are difficult to see because they sit so close together.

Decreasing the run time back to 1.5 hours and increasing the voltage to 10 kV for runs 13-15 gave an average current of 4.27 mA. In Figure 4-11, the simulated gradient for the propionic acid/DL-Serine system at these conditions compared moderately well with the experimentally observed pH gradient. The model showed the same trend as the data in the 10-27 cm range, but was shifted down somewhat from the data and fell right on the 95% prediction interval.

Increasing both the voltage and the run time, as in runs 16 and 17, led to an average current of 1.83 mA. Run 18, marked by the triangles Figure 4-12, was performed with 500 MWCO dialysis membranes rather than the 6000 MWCO ones used in runs 1-17. The membrane’s pore size seemed to make little difference. Both pH gradients were fit as one data set because they were so similar. The data in Figure 4-12, from a run at 10 kV and 5.5 hours, agreed closely with the model over the range of 13-27 cm. The steep region near the cathode of the experimental pH gradients seems to have grown larger as time and voltage were increased, cutting down on the usable length of the gradient.
Figure 4-10. Run 12 is compared to a simulation at the same run condition: 2.5 hrs at 5 kV with the propionic acid/DL-Serine system. The model line represents the results of the mathematical simulation. The data points from the experimentally observed pH gradient are shown as circles. The thinner solid curve is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings. Distinguishing between the different lines is difficult because of the close fit.
Figure 4-11. Runs 13-15 are compared to a simulation at the same run conditions: 1.5 hrs at 10 kV with the propionic acid/DL-Serine system. The model line represents the results of the mathematical simulation. The data points from the experimentally observed pH gradients are shown as points with each gradient having a different style of point. The thinner solid curve is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.
Figure 4-12. Runs 16 and 17 are compared to a simulation at the same run conditions: 5.5 hrs at 10 kV with the propionic acid/DL-Serine system. The model line represents the results of the mathematical simulation. The data points from the experimentally measured pH gradients are shown as points with each gradient having a different style of point. Run 17, marked by the triangles, was performed with 500 MWCO dialysis membranes rather than the 6000 MWCO ones used in all the previous runs. The thinner solid curve is the regression line, or fit to the data points. Dashed lines indicate the 95% prediction interval derived from the fit to the data. The single point with error bars in the upper right corner shows the pH probe’s ± 0.02 pH unit uncertainty in the experimentally measured pH readings.
5 Discussion

5.1 EACA/Tris Systems

The results from simulating runs 3-6, the 1.5 kV runs with EACA/Tris system 1, were within the 95% prediction interval from 8-25 cm, nearly half the separation chamber. This showed that the mathematical model, based on theory and chemical properties, could reasonably predict two-component pH gradients in the vortex-stabilized electrophoresis apparatus. Even the model’s worst predictions, like run 7, showed the same trend as the experimental pH gradients, albeit offset by about 0.15 pH units.

Slight variations in the pKs of the components could have been responsible for the offset between the simulated pH gradients and the experimental ones (Figure 4-4 and Figure 4-7). The model showed that changing the pK of Tris from 8.1 to 8.3 caused the predicted gradient to match nearly perfectly with the experimental gradient shown in Figure 4-7, but caused the simulated pH gradient shown in Figure 4-4 to fall outside the 95% prediction interval. Such small changes in the pK could have stemmed from temperature or concentration effects in the separation chamber.

Temperature and component concentration both affect the component pKs. Tris, in particular, is known to be temperature sensitive with a 3 degree C temperature decrease changing the pK by -0.084. The pH of the fractions was measured at room temperature, 22-23 C. Part of the offset between the simulations and the data was due to measuring the pH gradients at room temperature, but modeling them at 25 C. Daily temperature fluctuations probably caused part of the shift among the measured pH gradients from experiments with identical conditions. The activity coefficients of the components also could affect the pKs as much or more than the
temperature dependence, if the activity coefficients are significantly different from unity, which is not uncommon for ions. Temperature and activity coefficient corrections were not made to the model since the activity coefficients and temperature dependence of the pKs could not be found for all the components. Such properties could be measured, although the equipment required to make the measurements was unavailable and the work involved would constitute a paper in its own right.

Other factors in addition to temperature and concentration effects could have caused the offset between experimental pH gradients at the same run conditions (Figure 4-4 and Figure 4-5). Variation in the defined buffer solutions used to pour the gradients could have caused the offset. If the solutions used were significantly different from one run to the next, then the gradients poured would not be the same. A review of the data showed that the solutions used in forming the pH gradient did not have exactly the same pH from run to run or day to day. However, no correlation was found between the small differences (0.25 pH units on average) in pH of the solutions used to pour the gradients and the actual pH gradients observed at the ends of the runs. For example, when comparing runs with acidic solutions at different pHs, the gradient with the more acidic anolyte did not necessarily produce the pH gradient with the lowest pH at the anodic end. Hence, the small variations in the buffer solutions did not seem to account for the offset.

The accuracy of the pH measurements also should be factored into the offset. As shown in Figure 4-5, the pH gradient marked by the triangles is shifted down (about 0.07 pH units, on average) from the other two. The uncertainty in the measurements, ± 0.02 pH units, could have contributed to the offset. The shift between the measured pH gradients and the model in Figure 4-4 did not seem so unreasonable when considered in terms of the ± 0.02 pH unit uncertainty in the pH probe’s readings. However, the error in the pH probe’s readings was not large enough to
account for the offset between the simulation and experiment in Figure 4-7, although the combination of temperature and concentration effects probably could explain that difference.

### 5.2 Propionic Acid/DL-Serine System

The model’s simulated pH gradients were on or within the 95% prediction intervals for the linear portions of the experimental pH gradients from runs 9-17. The model still predicted the right shape of the pH gradients even when the simulation and data were offset. These results, like those from the EACA/Tris comparisons, showed that the model could reasonably predict two-component pH gradients in the vortex-stabilized electrophoresis apparatus without any fitting parameters. The steep increase in pH exhibited at the cathodic ends of the experimentally observed pH gradients presented a new problem, and the offset seen between the data and simulations, as seen before in the EACA/Tris comparisons, was also present.

The offset between the model and data in Figure 4-8 and Figure 4-11 was noticeable. In Figure 4-11, the simulation of the 1.5 hour, 10 kV runs barely fell on the 95% prediction interval. The shift seen in Figure 4-8 was not much larger than that seen in Figure 4-11. It is possible that another data set or two would have given a more realistic 95% prediction interval that would probably contain the model. The shifts seen in the propionic acid/DL-Serine system were likely rooted in the accuracy of the pH probe, and the temperature and concentration effects.

The steep increase at the cathodic end of the gradient seemed to be moving toward the anode as time and voltage increased. The model could not describe this migration because constant concentrations were assumed at the ends of the gradients. The experimental data suggests that constant concentrations were not the correct boundary conditions for the system, although they simplified the problem. Mathematical modeling and tube gel IEF results presented in *Dynamics of Electrophoresis* [12] suggested that the volume and composition of the buffer
tank solution were responsible for anodic and cathodic drift, which particularly altered the ends of pH gradients. The model used in this paper did not include the buffer tanks and failed to predict the alterations at the cathodic end of the gradient. The experiments and model presented in this paper seem to agree with the results from *Dynamics of Electrophoresis*, indicating that the size and composition of the buffer tank solutions may have been responsible for the alterations at the ends of the pH gradients.

### 5.3 Conclusion

This paper presented a mathematical model and experimental correlation of defined pH gradients poured in a vortex-stabilized electrophoresis apparatus. Two pH ranges were explored; pH 3-5 using propionic acid/DL-Serine, and pH 9-10 using EACA/Tris. These gradients were of interest since they fall in a range where undesired protein-SCAM interactions were more likely [2] and defined buffer pH gradients might be used to replace them.

The predicted results from the model were within the 95% prediction interval of the least-square fits to the data for nearly all buffers and run conditions tested. Of course, the model had some limitations. One problem with the mathematical model seemed to be a shift between the data and the model caused by error in the pH probe and alterations to the buffer components’ pKs due to temperature and or activity coefficients. Another problem was that choosing constant concentration boundary conditions in the model prevented it from predicting the migration of the cathodic end of the pH gradient toward the anodic end of the chamber. Additionally, the model used constant current rather than constant voltage for simplicity. What limitation, if any, was imposed by that simplification could be found by a more complicated solution to the problem using constant voltage.
The combination of this work with the results from Dynamics of Electrophoresis [12], suggests two things. First, altering the buffer tank concentrations may reduce the compression of the pH gradient. Second, including the buffer tanks in the model might enable it to predict the compression of the pH gradient. A model that correctly predicts gradient compression could be used to optimize the concentrations in the buffer tanks and minimize gradient compression. In its current condition, the model could reasonably be expected to predict at least the right trend and possibly the right pH gradient for other two-component poured gradients in the vortex-stabilized electrophoresis apparatus.

A worthwhile task would be to modify the mathematical model to include the buffer tanks, since they might account for the deviations between the model and the data at the ends of the gradients. If adding the buffer tanks still doesn’t correct the model, then it would seem that the end effects must be due to something else, possibly a mechanical effect of the system, like the membranes charging up and pumping fluid across themselves. Testing various cathode buffer concentrations with the modified model might be a solution to reducing the compression of the propionic acid/DL-Serine gradient. The reliability of the model could be further tested by examining the correlation between model and experimental data for other buffer systems.

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6 References


Chapter 5: Conclusions and Recommendations

1 Conclusions

This thesis set out to study whether defined buffers could separate proteins and develop a mathematical model that would predict the pH gradient formed by a defined buffer in the vortex-stabilized electrophoresis apparatus.

The work presented in chapter three focused on testing naturally formed and poured pH gradients using defined buffers. The gradients were tested for reproducibility, stability, and ability to focus proteins. Contrary to earlier work done in gels [1-11], the 10-component defined buffer did not form the expected pH gradient in the vortex-stabilized electrophoresis apparatus. This was probably because the components were all poor ampholytes [12] and would not focus to the tight band required to form a pH gradient.

Data from multiple experiments in the vortex-stabilized electrophoresis apparatus showed that the gradients poured using the EACA/Tris system were reproducible and relatively stable at 5 kV for up to two hours. Experiments showed that 20 mg of cytochrome c focused in roughly the same pH region of the EACA/Tris pH gradients after about 1.5 hours at 5 kV in the vortex-stabilized electrophoresis apparatus.

Poured pH gradients using propionic acid and DL-Serine were used to separate glucose oxidase and amyloglucosidase since initial tests showed the gradient was stable and reproducible after 1.5 hours at 5 kV. Analytical IEF-PAGE showed that glucose oxidase and amyloglucosidase were not completely separated in the poured propionic acid/DL-Serine pH gradients after 1.5 hours at 5 kV in the vortex-stabilized electrophoresis apparatus. Runs conducted for 5.5 hours at 10 kV separated the two proteins. Glucose oxidase focused around its
isoelectric point, but amyloglucosidase remained widely distributed throughout the lower half of
the separation chamber. These results showed that defined buffer pH gradients can separate
proteins with $\Delta pI = 0.6$ pH units in the vortex-stabilized electrophoresis apparatus.

Increasing the voltage and the length of the run separated the proteins, and revealed that
the cathodic end of the gradient was drifting towards the anodic end of the separation chamber.
Work by Mosher et al. [13] suggested that gradient drift was related to the buffer tank
composition. The modeling work presented in chapter four seemed to confirm this because the
model neglected the buffer tanks and consequently failed to predict the compression.

The mathematical model developed in chapter four for the two-component poured
gradients predicted the general shapes and about the right pHs of the gradients except at the very
ends of the gradients. The model did not predict the offset between the simulations and the
experimental data. This was likely due the accuracy limit of the pH probe and exclusion of
temperature and concentration effects on the pKs used in the model. As mentioned previously,
the model did not predict the appropriate end effects on the gradient since the buffer tanks were
excluded and constant concentration boundary conditions assumed. Except for the drift and
shifts, the model predicted the right shape for the gradient and very close to the right pH at the
tested voltages and run times.

The large band width of amyloglucosidase in the propionic acid/DL-Serine system
described in chapter three could have a serious implication for poured pH gradients. Partial
separation or enrichment of a particular protein isoforms with $\Delta pI \leq 0.2$ pH units may be the best
that can be done with shallow poured gradients. This idea fits the results seen by other
researchers. Bier’s early work [14,15] with a defined gradient could not completely resolve
hemoglobin (Hb) A (pI=7.2) and Hb C (pI=7.8). Even though there were focused proteins, there
was protein smeared between the two bands. Tulp et al. [16] also saw similar results with a MOPS/GABA pH gradient spanning pH 5.2-6.1 used to separate bovine serum albumin (BSA), transferrin (Tf), and Hb. The pIs of BSA and Hb were outside the range of the gradient and so those proteins ran off to the anode and the cathode, respectively. The Tf remained in the gradient, and while some fractions were enriched in a particular form of Tf, as shown by Coomassie stained gels, none of the fractions contained an individual band. Since the work presented here and that of the other two groups [14-16] was performed in different instruments, it seems that the difficulty in completely resolving the test proteins resides with shallow pH gradients themselves.

2 Future Work

In order to get better resolution from the shallow defined buffer pH gradients the problem of large protein band widths needs to be solved. Decreasing the dispersion in the separation chamber by adding a viscosity enhancer may be the easiest option. Alternatively, the length of the separation chamber or the voltage could be increased. Testing the latter two ideas requires that the problem of gradient migration be solved first, since migration increase with run-time and voltage. The effect of the electrode buffer composition on the migrations of the pH gradients could be tested by repeating the propionic acid/DL-Serine experiments with electrode buffers at different concentrations and looking for changes in the pH profiles.

Deciding if amyloglucosidase’s large band width was protein-specific could be tested by replacing it with a different protein that focuses in the pH 3.3-3.7 range. If the new protein were to focus, it might be reasonable to conclude that something about amyloglucosidase, possibly a small electrophoretic mobility, was responsible for the lack of focusing. Another experiment along the same line of thinking might be to enzymatically cleave all the post-translational
modifications from the amyloglucosidase to create a homogenous sample and try to focus it again at previously tested conditions. If the homogenous protein focused into a tight band, it would indicate that the poured gradients may have worked and separated isoforms of amyloglucosidase in the work presented in chapter 3. These follow up experiments could help clear up whether or not two-component defined buffers really can separate protein isoforms.

A worthwhile task, would be to modify the mathematical model to include the buffer tanks to see if they account for the deviations between the model and the data at the ends of the gradients. If adding the buffer tanks still does not correct the model, then it would seem that the end effects must be due to something else, possibly a mechanical effect of the system, like membrane pumping.

The modified model might also serve to test various cathode and anode buffer concentrations in order to reduce the effects of drift on the pH gradients, especially the compression of the propionic acid/DL-serine gradient. Testing various cathode buffer concentrations might illuminate the way to reduce the observed compression of the propionic acid/DL-serine gradient. Testing the correlation between model and data for other buffer systems would also be a good way to further test the reliability of the model.

Poured two-component pH gradients seem to have been largely ignored or overlooked by the electrophoresis community based on the paucity of literature available on the subject. This thesis showed that defined buffers could separate proteins by IEF. The mathematical model developed in this thesis seems to predict the experimental defined buffer pH gradients well enough for computer simulations to be used to choose the components that will form a desired pH gradient. Solutions to the band width and migration problems would enable defined buffers
to separate isoforms by IEF at the preparative scale and that would definitely draw some attention to defined buffers.

References