Spring 1993

Equilibrium electrophoresis: Results from the second prototype

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*University of New Hampshire, Durham*

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Equilibrium electrophoresis: Results from the second prototype

Hayes, David Barton, Ph.D.

University of New Hampshire, 1993
EQUILIBRIUM ELECTROPHORESIS:
RESULTS FROM THE SECOND PROTOTYPE

BY

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A.B. Liberal Arts, Magdalen College, 1984
M.S. Biochemistry, University of New Hampshire, 1990

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Biochemistry

May, 1993
This dissertation has been examined and approved.

Dissertation director, Thomas M. Laue, Associate Professor of Biochemistry

Jonathan Chaires, Professor of Biochemistry

Charles W. Walker, Professor of Zoology

Donald M. Green, Chairperson and Professor of Biochemistry

Andrew P. Laudano, Assistant Professor of Biochemistry

January 5, 1993
DEDICATION

I would like to mention the people who generously helped in this project. Thank-you, Tom Laue, the inventor of the machine and my paradigm of the scientist. Thank-you Terry Ridgeway, Tom's ideas are incarnated through your hands (blood, sweat, and tears); it's good to have the sympathy of someone else who knows what it feels like to try to push out that last bubble for the hundredth time.

I am also grateful for the faculty in the department of biochemistry, especially Dr. Green for his integrity managing the department and his concern for the good of each student and the common good of the whole department.

Magdalen College has paid the bills and given me the motivation to continue my academic career.

And most of all, thanks to Mom and Dad. As a twenty-nine year old I can appreciate even more that you gave me life and instilled in me a love of learning. Your unselfish love for me and support in my life is invaluable.

And at this milestone in my life, I look back and see the plan of God's providence that is much better than anything I could have planned or dreamed. Jesus, Mary, and Joseph, pray for me.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$e=1.60291\times10^{-19}$ Coulombs (C)</td>
<td>The charge on a proton</td>
</tr>
<tr>
<td>$e=4.8032\times10^{-10}$ esu</td>
<td>The charge on a proton (cgs)</td>
</tr>
<tr>
<td>$k$ measured in units of cm$^{-1}$</td>
<td>The inverse Debye radius</td>
</tr>
<tr>
<td>$N_A = 6.0221\times10^{-23}$ mol$^{-1}$</td>
<td>Avogadro's number</td>
</tr>
<tr>
<td>$k_B = 1.38066\times10^{-16}$ erg K$^{-1}$</td>
<td>Boltzmann's constant</td>
</tr>
<tr>
<td>$Z$ measured in units of $e$</td>
<td>The molecular charge on an ion</td>
</tr>
<tr>
<td>$\psi$ measured in units of $e$ or C</td>
<td>The apparent charge on a molecule</td>
</tr>
<tr>
<td>$T$ measured in $\circ$K</td>
<td>Absolute temperature</td>
</tr>
<tr>
<td>$D$ (dimensionless)</td>
<td>Dielectric constant</td>
</tr>
<tr>
<td>$I$ measured in mol C$^2$</td>
<td>Ionic Strength</td>
</tr>
<tr>
<td>$E$ measured in volts/cm</td>
<td>Electric Field</td>
</tr>
<tr>
<td>$Q$ measured in Coulombs</td>
<td>Charge</td>
</tr>
<tr>
<td>$\sigma$ measured in cm$^{-1}$</td>
<td>A parameter in a NONLIN fit</td>
</tr>
<tr>
<td>$\sigma=E[\psi/k_BT]$</td>
<td>For equilibrium electrophoresis only</td>
</tr>
</tbody>
</table>
ABSTRACT

EQUILIBRIUM ELECTROPHORESIS: RESULTS FROM THE SECOND PROTOTYPE

by

David B. Hayes
University of New Hampshire, May, 1993

Equilibrium electrophoresis is a new method that determines the apparent charge on macroions. The theory of equilibrium electrophoresis is summarized. The second generation device is described. Apparent charges, at various ionic strengths, measured by equilibrium electrophoresis are reported for pd(A)_{20}, pd(T)_{20}, pd(A)_{20} \cdot pd(T)_{20}, cytochrome c from horse heart, TA lysozyme mutant, TA(119/135) charge mutated lysozyme, and EDTA. The apparent charge of plasmid pBR322 from electrophoretic mobility and sedimentation velocity is reported. The results are compared to those expected according to the Record-Manning counterion condensation theory and the Debye-Hückel shielding theory. The apparent charges of the two ss DNA samples and the cytochrome c sample were equivalent to theoretical values within the expected experimental error. The validity of the basic equilibrium electrophoresis theory is argued. The range of usable experimental conditions for the present device is discussed. The sources of error in the current device are discussed, and suggestions for future models are given.
INTRODUCTION

The Importance of Net Electrostatic Charge in Molecular Association

Four elementary forces are defined in physics: the strong nuclear force, the weak nuclear force, the electrical force, and the gravitational force. The electrical force is the cause of all molecular and particulate interactions studied in chemistry. Due to the quantum nature of matter, the strongest electrical attractions and repulsions (covalent bonds, hydrogen bonds, and van der Waals repulsion) occur at very short distances between particles and have complicated geometrical properties. The energies of other electrical interactions are more significant at larger distances between particles and have simpler geometrical properties. Mathews and Van Holde\(^1\) list these non-covalent forces and the distance dependence of the energy for each interaction (Table 1).

<table>
<thead>
<tr>
<th>Type of Interaction</th>
<th>Relation of Energy to Distance</th>
<th>Geometry of Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dispersion</td>
<td>(1/r^6)</td>
<td>non directional</td>
</tr>
<tr>
<td>Dipole-induced Dipole</td>
<td>(1/r^5)</td>
<td>non directional</td>
</tr>
<tr>
<td>Charge-induced Dipole</td>
<td>(1/r^4)</td>
<td>non directional</td>
</tr>
<tr>
<td>Dipole-Dipole</td>
<td>(1/r^3)</td>
<td>both dipoles aligned</td>
</tr>
<tr>
<td>Charge Dipole</td>
<td>(1/r^2)</td>
<td>dipole aligned</td>
</tr>
<tr>
<td>Charge Charge</td>
<td>(1/r)</td>
<td>non directional</td>
</tr>
</tbody>
</table>

Net electrostatic charge results in the strongest interaction between particles at long distances. Many biological molecules, particularly the nucleic acids, have a considerable net charge. Therefore, the charge on biological molecules, and the interactions of that charge with solutions and other molecules, are important basic phenomena that call for scientific investigation.

The charge of particles has a natural method of study: charged particles experience a force in an electric field regardless of its source. The electrical force
influences the strength and kinetics of molecular interactions. For instance, molecules of opposite charge will be accelerated towards one another along the path of the field between them and will be bound more tightly to one another than identical neutral particles. An external electrical field will accelerate a particle along the path of its field (if the particle is free to move). In general, the study of the motion of charged particles embedded in a liquid exposed to an external electric field is called electrophoresis.

**Equilibrium Electrophoresis**

If the velocity of charged particles in an external field is considered directly, as it is in polyacrylamide gel electrophoresis, the force on the particle cannot be computed directly without measuring the molecular frictional coefficient in the medium through which the particle is flowing. In equilibrium electrophoresis, the velocity of the charged particles is not measured directly. Rather, in an enclosed space, the flow caused by the movement of charged particles is allowed to reach an equilibrium with the flow towards a uniform concentration resulting from diffusion. Assuming that the frictional coefficient is identical for both types of flow, it is possible to calculate the force on the molecule since the flow due to diffusion is defined. If the force on the particle is known, the apparent charge can be calculated from the strength of the electric field.

**Previous Prototypes and Other Devices**

Jamie Godfrey independently designed and built an instrument to perform equilibrium electrophoresis, to which he gave the name: "steady state electrophoresis device." This instrument was based on the same theory as the device used here, but differed sufficiently in mechanical details that separate patents were issued. The experimental device used for equilibrium electrophoresis in these studies was developed in the laboratory of Tom Laue at the University of New Hampshire. It is fully described in a forthcoming paper. The present device is based, in part, on one described previously. Work is continuing at the University of New Hampshire on another generation of equilibrium electrophoresis devices.
The present device is described in the materials and methods section. The details of the previous devices are not mentioned; however, suggestions for future devices are included.

**Purpose of the Project**

**Test the Range of Molecules Appropriate for Electrophoresis**

The test molecule used during the initial development of equilibrium electrophoresis was horse heart ferri-cytochrome c.\(^4\) Result from other proteins and from DNA of various sizes was desired. Very simply, interesting experiments with various molecules were designed and attempted, but the experiment would be terminated if it became obvious that the present device was incapable of providing reliable information. The molecules upon which experiments were attempted are: EDTA, T4 lysozyme, pd(A)\(_{20}\), pd(T)\(_{20}\), pd(A)\(_{20}\)*pd(T)\(_{20}\), the pBR322 plasmid, and further experiments with cytochrome c.

**Obtain Data on the Charge of Well Characterized Molecules**

The basic information available from equilibrium electrophoresis is the apparent charge on molecules. This information is valuable for a better understanding of the molecule. The apparent charge was found for all of the above molecules except pBR322.

**Find Usable Experimental Conditions for the Device**

The apparent charge on molecules varies according to the conditions of the experiment. For the molecules tested, the buffer concentration and the electric field were varied. The operating limits of the device and of the process of analysis were investigated for these variables. The composition of the buffer and the concentration of the subject molecule also were varied in order to investigate the reproducibility of determining the apparent charge, though the limits of these variables were not investigated.

**Identify Problems with the Device**

Especially when no results were obtained, or when the range of experimental conditions that produced acceptable results was severely limited, the cause of the failure
was investigated. The experimental conditions would be varied to try to alleviate and identify problems. For instance, if convection was the suspected reason for experimental failure, a non-ionic viscous substance could be added to the solution to reduce turbulence.

Suggest Improvements to the Device

Finally, the experiments performed in this project have great value as part of the continuing effort to develop a widely usable equilibrium electrophoresis device. Limitations often become evident only upon the actual use of a device. Even experiments that fail to produce a reliable charge estimate are of use in providing information concerning the device. Many details of practical concern are best investigated through experience.
THEORY

Molecular Charge

Molecular charge is caused by the presence of ionized groups on a molecule. At any instant, a molecule possesses an exact molecular charge that is an integer equal to the total number of excess protons or electrons. Since equilibrium electrophoresis is concerned with the movement of the molecule as a whole, only the total net charge on the molecule is considered. The charge distribution is important only in determining the local interaction of the charged molecule with the solution.

Charge Reduction in Solution

Macromolecules of biological interest typically exist in a solution of water and dissolved ions. Equilibrium electrophoresis is focused primarily on macromolecules in these aqueous systems. However, the ionic constituents of the solution interact with the molecule being examined. The particle that forms a gradient may include not only the molecule being examined but also bound or condensed ions. It is useful to model non-random distributions of loosely bound ions surrounding the charged macromolecules as a reduction in its point charge. There are three major categories of charge reduction due to interaction with the solution.

Titrable Groups

Ionized groups on a molecule are in rapid equilibrium with the solution. These charges and interactions are associated with a particular site on the macroion, and the binding of the counterion is considered an ionic bond. When bound, the ion does not contribute to the osmotic potential of the solution. The net molecular charge measured at equilibrium will be a time-average.
Condensed Ions

If the charge density on a molecule is particularly high, as in the case of DNA, ions in the solution will be tightly constricted to an area close to the molecule; these ions are labeled "condensed counterions" by Record, Lohman and Anderson. These ions can exchange with those in the solution. They are free to diffuse over the surface of the macromolecule, and thus are not associated with any particular site on it. However, the free range of motion of such ions is so restricted to the vicinity of the macroion that they do not contribute in any significant degree to the osmotic potential of the solution. Because they are not site associated, condensed counterions reduce the net charge in a non-integral fashion when the particle is considered as a non-point charge.

Ion Clouds

"There must, on the average, be a tendency for a given ion to be surrounded in its immediate vicinity with an excess of ions of the opposite charge." The effect of this excess of ions is called Debye-Hückel shielding. The excess of counter ions in this "cloud" will reduce the net overall free motion of ions in the solution and thus lower the osmotic potential of the ionic solute. Even though individual ions are not bound to the molecule or restricted to the boundaries of the "cloud", the effect of their average interaction with the molecule is that the charge density in and beyond the "cloud" is less than the total net charge on the molecule. If the microscopic charge density could be measured, even at the surface of the central ion, it is the apparent charge that actually exists. The molecular charge is theoretical; it is the portion of the apparent charge contributed by the central ion.

However, the apparent charge, \( \psi \), will be related to the molecular charge, \( Z \). Eisenberg and Crothers give a summary of Debye-Hückel shielding. In an ideal solution with no other components, \( \psi = Ze \), where \( e \) is the charge on a proton. Assuming an insulating sphere in a solution, the apparent charge will be:

\[
\varphi = \frac{Ze}{1 + ka}
\]
where \( k \) is the inverse Debye radius and \( a \) is the radius of the central ion. The variable \( k \) depends only on the ionic strength of the solution, and \( 1/k \) is the distance from the central ion that describes the peak of the charge due to the ion atmosphere. The value of \( k \) is:

\[
    k^2 = \frac{8\pi N_A e^2}{Dk_B T} I
\]

where \( I \) is the ionic strength defined by \( I = \frac{1}{2} \sum_{i=1}^{n} C_i Z_i^2 \), \( N_A \) is Avogadro's number, \( D \) is the solvent dielectric constant, \( k_B \) is Boltzmann's constant, and \( T \) is the temperature in degrees Kelvin.

**The Apparent Charge: a Measured Quantity**

**The Basic Idea**

The purpose of equilibrium electrophoresis is to provide information about molecular charge. The expected net molecular charge (the excess of electrons or protons on the macromolecule) often can be calculated from the chemical composition of the molecule and the pH of the buffer. Using the Poisson-Boltzmann equation, it is possible to model and calculate the apparent charge of a molecule in a solution containing ions. Yet, the apparent charge molecules exert in solution has not been investigated by direct experimentation.

**The Equations Describing the Equilibrium**

A rigorous derivation, based on non-equilibrium thermodynamics, of the equations that describe the steady state achieved by ions in the presence of an external electrical field is given by Godfrey. The same results are obtained by Laue et al. in a simpler derivation that is repeated here.

An electric field, \( E \), is established across the cuvette in the \( x \) direction, which creates a flux, \( J_e(x) \), of macroions \( J_e(x) = [c(x)\psi/f_e]E \), where \( J_e(x) \) is the flux of macroions in \( g/cm^2 \) sec at the point \( x \) along the \( x \) axis in the cuvette and in the direction of the field, \( c(x) \) is the mass concentration of macroion, \( \psi \) is its apparent, or effective net charge in coulombs, and \( f_e \) is the translational frictional coefficient as measured in the electric field. Diffusion is used as the counter flux and is described by Fick's first law, using the Einstein-Sutherland
formulation of the diffusion coefficient and assuming thermodynamic ideality is \( J_D(x) = -\left[ k_B T / f_t \right] \frac{dc(x)}{dx} \), where \( J_D(x) \) is the flux of macroion in \( g/cm^2 \) sec at point \( x \), \( dc(x)/dx \) is the concentration gradient at that point, \( k_B \) is Boltzman's constant (1.3807 \( \times \) \( 10^{-23} \) J/K), \( T \) is the absolute temperature, and \( f_t \) the translational frictional coefficient.

Since the macromolecule is confined, a concentration gradient develops along the axis of the electric field until a steady state is reached at each point, \( x \), with the flux from electrophoresis and from diffusion becoming exactly equal and opposite. Equating the magnitudes of the two fluxes and rearranging:

\[
\frac{d[\ln c(x)]}{dx} = E \frac{f_t}{f_e} \left[ \frac{\psi}{k_B T} \right].
\]

For the purpose of nonlinear least-squares fitting, the equation may be rewritten \( c(x) = c_0 \exp\left[ \sigma (x-x_0) - 2B (c(x)-c_0) \right] \), where \( c(x) \) is the concentration at point \( x \) along the axis of the electric field, \( c_0 \) is the concentration at \( x_0 \), and arbitrary reference position in the cuvette, \( \sigma = E \frac{\psi}{k_B T} \), and \( B \) is the second virial coefficient. The ideal case is modeled by holding \( B = 0 \). Analysis of the results from equilibrium electrophoresis for non associating systems, including those that are heterogeneous, should be nearly identical to that for sedimentation.\(^4\)

Since the second virial coefficient is set to 0, the linear least squares fitting program NONLIN uses a form of this equation to fit equilibrium electrophoresis data:

**Equation 3**

\[ c(x) = c_0 e^{\sigma (x-x_0)} \]

where \( c(x) \) is the concentration at position \( x \); \( c_0 \) is an arbitrary concentration at \( x_0 \), the first data point; and \( \sigma \) is the parameter proportional to the apparent charge defined above.

**Comparison with Sedimentation Equilibrium**

Electrophoresis and ultracentrifugal sedimentation are theoretically analogous techniques. Since centrifugation is a familiar experimental technique, it is useful to compare the two methods.

In centrifugation, a gravitational field (which is generated by the spinning rotor) accelerates particles through a medium in an enclosed cell. In electrophoresis an electrical field accelerates particles through a medium in an enclosed cell. The difference between the two is that the centrifugal field increases according to the square of the distance from the center of the rotor and does not depend on the medium, while the electrical field
depends only on the conductivity and cross-sectional area of the conducting medium. The centrifugal field is uniform; its direction depends only on the center of rotation, and its apparent force on the sedimenting particle depends only on the buoyancy of the medium (which may vary due to compressibility and solution composition). The electrical field interacts to some extent with all matter exposed to it; and the field may be very non-uniform depending on conductivity, dielectric constant, the shape of the container, and any uneven distribution of charged components.

In sedimentation velocity, the rate at which a boundaries of sedimenting particles move is measured. The sedimentation coefficient, S, is equal to the buoyant weight of the particle divided by the frictional coefficient of the particle. In standard gel electrophoresis experiments, the velocity of a charged particle is measured. This electrophoretic mobility is based on the apparent charge on the particle divided by the frictional coefficient of the particle. However, the electrophoretic mobility also depends on variations in the buffer, variations in the electric field, and on the particle's interaction with the gel substrate. These variables are difficult to determine.

In equilibrium sedimentation, analysis of the concentration gradient caused by the balance of sedimenting flow and diffusional flow yields the reduced molecular weight of the particle. The reduced molecular weight takes into account the buoyancy of the medium and any repulsive or attractive forces between the sedimenting particles. In equilibrium electrophoresis an analysis of the concentration gradient caused by the balance of electrophoretic flow and diffusional flow yields the apparent charge on the molecule. The apparent charge on the molecule takes into account the shielding of the surrounding medium and any other attractive or repulsive forces between the particles.

In equilibrium sedimentation, all components are sealed in the cell and the entire system comes to a true thermodynamic equilibrium. Every component will form a gradient based on its buoyant molecular weight and the centrifugal force applied. Because of this, the molecule being studied must be distinguishable. This may be done using
methods sensitive to a particular molecule's concentration or by subtracting the sedimentation gradient formed in an identical cell without the component or components being studied. The selection of a single gradient out of many components is justified so long as components in one gradient do not interact with components in other gradients.

In equilibrium electrophoresis a completely sealed cell is not practical for three reasons. First, the electrical field depends on ions continually entering and leaving the cell. Second, there will necessarily be a charge-charge interaction between the particles of one gradient with the particles of any other gradient. Third, the pH gradient that would form in a completely sealed cell is inconvenient due to the chemical interactions it may cause. For these reasons, the equilibrium electrophoretic cell is bounded by semi-permeable membranes that confine only the particle being studied. Due to a constant flow of components through the cell, a true thermodynamic equilibrium is not reached, and the system is described more accurately as a thermodynamic steady state. However, the steady state equations for equilibrium electrophoresis are derived with the assumption that moving buffer components do not affect the macroion gradient; therefore, the gradient takes on a form identical to that described in sedimentation equilibrium.

With the proper experimental setup, the analysis of equilibrium sedimentation and equilibrium electrophoresis is essentially the same: an exponential gradient must be analyzed to compute the force experienced by a particle in a field.

Non-linear Least Squares Analysis

To describe and analyze an exponential function is the last step of an equilibrium sedimentation or equilibrium electrophoresis experiment. This step ultimately limits the complexity of experiments that can be performed usefully. The data points must be fit to the exponential function describing the equilibrium, and this fit determines the precision of the information obtained. To deconvolute multiple exponential gradients, the proper mathematical model must be chosen. The information for this choice may not be available from an analysis of the data from the experiment; therefore, such an analysis is ill-posed.
Practically, curve fitting is performed by the program NONLIN,\textsuperscript{10} which also is used to analyze equilibrium sedimentation data. Therefore, the reliability and precision of the information calculated depends on the characteristics of the type of fitting done by NONLIN. The residuals of the fit by NONLIN are used to judge the applicability of the model chosen and to identify problems such as non-equilibrium and convection.

**Indirect Measurement**

Equilibrium electrophoresis measures the charge on a particle by balancing the flow induced by the electric field with the flow due to diffusion. By definition, the magnitude of an electrophoretic flux is set by the apparent charge of a particle in an electric field. The apparent charge is a parameter of the whole system: macroion, buffer, and external field. The "particle" on which the apparent charge rests would be very hard to describe in individual molecular terms.

It must be remembered that, at equilibrium, the flow resulting from the electric field and the flow due to diffusion do not stop; only the net flow is zero. The velocity impressed on individual macroions by the electric field at equilibrium is exactly as large as the velocity these ions reach quickly after the initial application of the electric field; therefore, the flow at any given concentration of macroion remains constant throughout the experiment. At the start of the experiment, the flow due to diffusion was equal in all directions since the concentration was equal in all directions. At equilibrium, the flow due to diffusion in the direction opposite the electric field exceeds the flow due to diffusion in the same direction as the electric field. The net flow due to diffusion becomes equal and opposite to the flow due to the electric field. Consequently, macroions at equilibrium in an external field have a significantly different velocity distribution than they would have in a solution without an external field.

For this case, where a uniform electric field impresses motion on charged particles, Edsall and Wyman describe two effects resulting from the presence of Debye-Hückel counterion shielding.\textsuperscript{6} The first is the electrophoretic effect. The charged particle is
surrounded by an excess of oppositely charged ions. These excess ions move in the direction opposite to that of the charged particle, and transfer this motion to the nearby solution. This flow retards the motion of the charged particle, making it seem as though it had less charge. The retardation is equal to the square root of the ionic strength of the solution, and is taken into account in the equation 2 dealing with Debye-Hückel shielding. The second is the time relaxation effect. As an ion moves through a solution, it moves away from the excess counterions surrounding it. New ions tend to move towards the central ion and reform a Debye-Hückel spherical distribution around it. But the finite time needed for this process distorts the spherical distribution of counterions leading to a drag in the direction opposite to the central molecule's motion. These two effects will apply to equilibrium electrophoresis measurements. They are depicted in figure 1.

The electrophoretic effect will depend on the size of the particle and the ionic strength of the solution. The derivation of Debye-Hückel shielding assumes that an external electric field is applied to the central ion, and that the lines of force run between the cathode and anode. However, if the particle is large, or if the ionic strength is high
(and therefore the Debye radius 1/k is small), the electric field near the particle is
distorted, and the electrophoretic effect will not retard the macroion's motion as much as
equation 2 states. For an insulating sphere, Henry's function calculates this decrease in
shielding.\(^7\) The apparent charge of a macroion is given by the formula

\[
\varphi = \frac{Qf_1(ka)}{1 + ka},
\]

where \(a\) is the effective radius of the macroion, \(k\) is the inverse Debye radius, and the
function \(f_1(ka)\) is Henry's function which varies from 1 to 1.5. When \(f_1(ka)=1\), the
electric field runs between the anode and cathode with no distortion; when \(f_1(ka)=1.5\), the
local electric field is everywhere parallel to the macroion and electrophoretic retardation is
at a minimum.

The time relaxation effect is not significant when \(ka<5\),\(^7\) and is not considered
here. In the absence of a uniform electric field it has even less effect, since the random
diffusional motion of the central ion will cause less distortion in the Debye-Hückel
spherical distribution than the directed electrophoretic motion.

Because the apparent charge is measured indirectly, its interpretation must take
into account the assumptions of theory and the conditions of the experiment. First, theory
assumes a uniform electric field running from the anode to cathode. This is not a very
good model for a cell in which various charged bodies exert electrostatic fields in a
complex geometry. Second, if instead of asking what will be the effect of an external field
on a macroion, one asks what is the strength of the electrostatic field set up by a
macroion, the reduction of the apparent charge due to the electrophoretic effect and time
relaxation would not come into play. This is probably the case with large pieces of DNA,
where various smaller ions are attracted to the DNA, but the DNA is not significantly moved.
The Electric Field in Solution

The central particle being studied in equilibrium electrophoresis is considered to be a small insulating sphere in a solution with homogenous conductance. With this assumption, the electric field acting upon the particle can be considered to be homogeneous around the molecule.

According to theoretical modeling of DNA, the local details of dielectric constant and ion distribution are not expected to affect the overall charge of the macroion greatly, but only affect the charge distribution very close to the macroion. However, in the analogous case of electrorheological fluids, the overall charge and motion of large particles is affected by the dielectric constant and by the conductance of the particle. The much larger size of particles in electrorheological fluids seems to be the main factor accounting for the differing importance of local structure. Yet, there is no higher theory by which the assumptions made in any model can be judged independently; only the success of the whole model in making predictions can be judged.

Some theoretical questions arise concerning the electrostatic modeling of macroions. Could the presumed mobility of condensed counterions surrounding DNA act to form a "particle" with a finite conductance? The concentration of condensed counterions near the DNA is much greater than the bulk solution. What will be the effects of the Donnan potential on the conductivity of the buffer, and how will this affect the concentration gradient?

These questions go well beyond the experimental nature of this project. They are raised here to point out the simplicity inherent in the theory used for calculations done here. The best method to address these questions would seem to be numerical finite differences methods such as that used by Jayaram, Sharp, and Honig. Here, experimental data may give clues to what parameters ought to be investigated further in theoretical studies.
MATERIALS AND METHODS

Physical Description of the Device

Since the purposes of this project not only focus on biophysical measurements but also focus on device design, a complete description of the device will be given. The design of an equilibrium electrophoresis device has two major objectives. First, an analysis chamber in which a stable concentration gradient can be formed must be constructed. Second, an optical system to measure the gradient must be constructed.

Figure 2 presents the detailed exploded view of the present equilibrium electrophoresis device. In the figure, the electric field is vertical, from the top to the bottom of the analysis chamber(7). The optical path is along a line perpendicular to the surface of the page.
The design of the analysis chamber and buffer flow in the present device. The electric field runs vertically through the analysis chamber, and the optical path is perpendicular to the page.

(The legend of part numbers is on the next page.)
**Figure 2 Legend**

*Note that 2,3,8,10 are 2" round Delrin. They have four evenly spaces 1/8" holes around their perimeters for positioning on the alignment pins (11).

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Locking Screw, 1/2&quot; -13\textsuperscript{th} thread (Delrin)</td>
</tr>
<tr>
<td>2</td>
<td>Locking Screw Plate 1/2&quot; -13\textsuperscript{th} tapped center hole (Delrin)</td>
</tr>
<tr>
<td>3</td>
<td>Top Buffer Chamber (Delrin)</td>
</tr>
<tr>
<td>3a</td>
<td>Buffer Access Holes</td>
</tr>
<tr>
<td>4a &amp; b</td>
<td>Membrane Hoop</td>
</tr>
<tr>
<td>5a &amp; b</td>
<td>Semi-permeable Membrane (types used described below)</td>
</tr>
<tr>
<td>6a &amp; b</td>
<td>Membrane Hoop O-ring (buna-N)</td>
</tr>
<tr>
<td>7</td>
<td>Cuvette Frame (Fused Silica Quartz, Precision Glass Products Company)</td>
</tr>
<tr>
<td>7a</td>
<td>Cuvette Access Channel, 2 mm diameter by 5 mm length</td>
</tr>
<tr>
<td>8</td>
<td>Cuvette Holder (Delrin)</td>
</tr>
<tr>
<td>8a</td>
<td>Cuvette Holder 10-32 tapped hole for Filling and Assembly Screws</td>
</tr>
<tr>
<td>9</td>
<td>In flowing buffer line, male luer connector (Biorad 732.8304)</td>
</tr>
<tr>
<td>10</td>
<td>Bottom Buffer Chamber (Delrin)</td>
</tr>
<tr>
<td>10a</td>
<td>Buffer Access Holes</td>
</tr>
<tr>
<td>11</td>
<td>Alignment Screws, 6-32 stainless steel screw, 4&quot; long, four required</td>
</tr>
<tr>
<td>12</td>
<td>Assembly Screw 10-32 nylon cap screw</td>
</tr>
<tr>
<td>12a</td>
<td>O-ring</td>
</tr>
<tr>
<td>13</td>
<td>Filling Screw, 10-32 nylon cap screw with two 0.25 mm diameter channels</td>
</tr>
<tr>
<td>14</td>
<td>Out flowing buffer line (Teflon, 1.6 mm ID Biorad 732.8204)</td>
</tr>
<tr>
<td>15</td>
<td>Gold plated headphone jack with drilled tip. (Radio Shack 274.877)</td>
</tr>
<tr>
<td>16</td>
<td>Phone jack wire (Radio Shack 274.284)</td>
</tr>
<tr>
<td>17</td>
<td>Two hole amber gum stopper size 0 (VRW 59.585)</td>
</tr>
<tr>
<td>18</td>
<td>Electrode Chamber (acrylic)</td>
</tr>
<tr>
<td>19</td>
<td>Platinum wire, 24 gauge</td>
</tr>
</tbody>
</table>

The analysis chamber is designed according to the following considerations. The electric field depends on the cross-sectional area of the analysis chamber perpendicular to the electric field. If the concentration gradient is to be measured optically, the optical path at right angles to the electric field also should be constant. These factors dictate that the analysis chamber be rectangular, with non-conductive walls parallel to the electric field and that opposite transparent regions be available to serve as a cuvette. In the present device, the chamber (7) is a 1 cm by 1 cm square quartz enclosure, so its cross-section is 1 cm\(^2\) and its optical path is 1 cm. To avoid distortions in the electric field, the buffer above and below the analysis chamber must be contained in buffer chambers (3,10) that
have the same sized opening as the analysis chamber. To allow the flow of small buffer ions and to retard the flow of macroions, a dialysis membrane (5a,5b) must be mounted on the top and bottom of the analysis chamber. The membrane must be mounted to form a smooth surface because it is the barrier upon which the macroion concentration gradient will build up.

Electrodes are placed in contact with the solution. It is convenient to place the electrodes away from the analysis chamber so that gaseous electrolysis products do not form bubbles in the electrical path between the electrodes and the analysis chamber, and so that electrolysis products do not contaminate the analysis chamber. To prevent an overall gradient in ionic components of the buffer between the electrodes, fresh buffer is pumped into each buffer chamber and out past the electrodes. This direction of flow prevents electrode by-products from approaching the analysis chamber.

The optical system of the present device is based on visible and UV absorbance. It is diagrammed in figure 3.

![Figure 3](image)

The optical path of the present device.

An Oriel 400 watt xenon arc lamp (1) provides illumination. An Oriel 1/4-meter monochromometer (2) selects a narrow wavelength band and an output lens collimates the beam along the horizontal axis. Mirrors (4 and 6) and the camera lens (7) are mounted on a sliding carriage (3) to guide the beam through the analysis chambers (5). The camera
lens, a Spindler & Hoyer (part #03 3510) air-spaced 100 mm focal length achromat for the UV region (7), focuses the image of the cell onto a Reticon RL512Q linear photo diode array (9) mounted on an RC1001 mother board (9a). The signal from the array is digitized by a 12 bit A/D and absorbances are computed and stored on an IBM PC compatible computer (10). Different cells are viewed by sliding the carriage along the optical track (3). Absorbances are computed using intensity measurements made at a position adjacent to the cell; therefore, air is the reference for 100% transmission. Unfortunately, beam divergence along the vertical axis results in significant differences in light level at different carriage positions, resulting in an offset in absorbance measurements. This is taken into account during data analysis.11

Electrical Description of the Device

Electrical Current was supplied by a Keithley 224 programmable current source. Voltage was measured by a Keithley 197A digital multi-meter connected to the leads coming out of the power supply. The leads from the power supply were connect through gold plated jacks to two 5 cm lengths of platinum wire that were immersed in electrode chambers as shown in figure 2.

Experimental Protocol Summary

The procedures for sample loading and data analysis are fully described by Ridgeway et al. 3 in a forthcoming paper. A summary of this is presented here.

First the cell is assembled. A sample is loaded into the analysis chamber through the fill hole and the cell assembly is mounted on the optical track. The buffer chambers are connected to the buffer reservoir, pumped full of buffer, and any air bubbles removed. The buffer is allowed to flow for some time to help equilibrate ion concentrations across the dialysis membranes.

Following this, experiments may be performed. An absorbance scan of the initial concentration distribution is taken prior to the application of the electric field. According to the estimated charge on the molecule, an electric field that will produce a gradient with
a $\sigma \approx 5$ is chosen. The field strength is increased in intervals until $\sigma \approx 30$ is observed. Absorbance scans are taken at each field strength at least six hours after the field has been applied. A steady state is determined by lack of change in absorbance scans taken at least one hour apart (usually they were taken at least four hours apart). Once a steady state has been reached at one field strength, the electric field is changed and scans taken until a new steady state is achieved. Since buffer constantly circulates and equilibrates freely through the dialysis membranes into the analysis chamber, the buffer may also be changed and a whole new series of experiments at different field strengths may be conducted.

Finally, data sets are analyzed. Absorbance data sets known to have reach a steady state are edited to remove regions scanned beyond the confines of the analysis chamber, regions of high absorbance that show excessive noise, regions of obvious optical noise (like scratches or smudges), and sometimes for regions that seem to have a discontinuous gradient (which is probably due to convection in the analysis chamber). The individual data sets are fit to a form of equation 3 by NONLIN, and the apparent charge computed. Combinations of data sets are also fit by NONLIN to a single $\sigma$, and the apparent charge is computed.

**Buffers.**

Buffers were prepared in 2L batches, with the components being weighed to at least three significant digits. The pH was adjusted to $\pm 0.01$ pH units on a VRW Scientific 1052 pH meter using HCl or KOH. The conductance of the buffer was measured using a Orion Research 811 conductivity meter. For experiments with short DNA oligonucleotides, the standard buffer was 20 mM KCl, 20mM Tris pH 8.00. For pBR322 plasmid DNA, 1mM EDTA was added to the standard buffer and 10-14 kD molecular weight cutoff (MWCO) membranes were used. Dilutions to 10:10 mM KCl Tris and 2:2 mM KCl:Tris were also used. For T4 lysozyme, the standard buffer was 2.5 mM KCl, 2.0 mM PO$_4$ pH 5.5.
Membranes

The top and bottom of the analysis chamber were sealed with dialysis membranes characterized by a nominal molecular weight cutoff (MWCO). The MWCO of a membrane is defined as the minimum molecular weight at which 90% of an uncharged globular solute is retained by the membrane. The membranes used in all the experiments were regenerated cellulose Spectro/Por 3 ©, 3500 MWCO and Spectro/Por 4 ©, 10-14K MWCO membranes. These membranes were the only type of commercial membranes that had the mechanical flexibility to be fitted on the membrane holder (figure 2). Other membranes were tested, but problems with cell leakage and buffer evaporation through the membranes prevented their use in experiments. These other membranes were the Elutrap BT1 © membrane from Schleicher & Schuell, Spectro/Por CE © (cellulose-ester) membranes from 500 to 40,000 MWCO, and Millipore PLHK 100,000 MWCO polypropylene backed regenerated cellulose ultrafiltration discs.

Macroions.

The first DNA sample studied was pd(A)\textsubscript{20} from Pharmacia #27-7984-01; it was diluted with the standard buffer to 0.5 A\textsubscript{260}. The second DNA sample studied was pd(T)\textsubscript{20} from Pharmacia #27-7841-01; it was diluted with the standard buffer to 0.5 A\textsubscript{260}. Equimolar samples of pd(A)\textsubscript{20} and pd(T)\textsubscript{20} (molarity determined by A\textsubscript{260}) were mixed, heated to 65°C, and then allowed to cool slowly to room temperature over a period of 2 hours in 200 mM KCl 20 mM Tris pH 8.0 buffer to form double stranded pd(A)\textsubscript{20}•pd(T)\textsubscript{20}.

The T4 lysozyme mutants were supplied by Brian Mathews and are described by Sun Dao-pin et al.\textsuperscript{11} The first mutant, labeled TA, is identical to wild type T4 lysozyme except that it contains no Cys residues. It has a calculated net molecular charge of +9. The second mutant labeled 119/135 has Arg 119 and Lys 135 mutated to glutamic acid, reducing its net molecular charge to +5. The samples supplied were diluted to ≈ 3.0 µg / ml in the buffer described above.
Cytochrome c was Sigma #C-7752 from horse heart, type IV, acetic acid extraction. It was dissolved and diluted to 0.96 A_{530} in the same buffer as that used for T4 lysozyme.

pBR322 plasmid DNA was from New England Biolabs #303-3L. It was diluted to 0.664 A_{260} with 20:20:1 mM KCl:Tris:EDTA pH 8.0.

EDTA was from Baker #8993-01. EDTA was found to form a gradient with Spectro/Por 3 membranes. Accordingly, steady-state gradients were examined

**Sedimentation Velocity**

Sedimentation velocity experiments were performed in a Beckman XLA analytical ultracentrifuge. Samples were loaded into separate sedimentation velocity cells in an An-60-ti rotor and centrifuged at 60,000 rpm at 20° C. Continuous absorbance scanning at 260 nm was performed for the duration of the experiment resulting in 80 data sets for each sample.
EXPERIMENTS AND RESULTS

Buffer Electrophoresis

Experimental Design
To obtain an accurate baseline for further experiments, equilibrium electrophoresis was performed on buffer with no macroions present in the analysis chamber. When fresh buffer was continuously pumped into the buffer chambers, it was expected that a stable pH would be maintained through the entire system. Since all buffer components were smaller than the 3500 MWCO of the dialysis membrane, it was expected that a buffer concentration steady state with no gradients would be observed.

pH Measurements
Without buffer circulation, there was a pH difference between the efflux from the two electrode chambers. An experiment was carried out with 20 mM Tris 20 mM KCl 1 mM EDTA pH 8.00 buffer. After eight hours at 200 µA, with no fresh buffer being pumped into the buffer chambers, the pH of the efflux from the negative electrode chamber was 11.7 and the pH of the efflux from the positive electrode chamber was 2.23. With a buffer flow set at a relative rate of 10 out of 1000 on the Ranin pump, and the current again set at 200 µA, the pH of the efflux from the negative electrode chamber fell to 7.94, and the pH of the efflux from the positive electrode rose to 7.67. Some of this difference in pH from the efflux was presumed to be due to electrolysis reactions at the electrodes. Accordingly, efflux pH was monitored periodically using pH indicator strips to ensure that no significant pH gradients were established in the cell.

Retardation of EDTA
Experiments were conducted with 20 mM Tris 20 mM KCl 1 mM EDTA buffer. Electric fields of 0.0132, 0.526, and 0.658 volts cm\(^{-1}\) were applied to the analysis chamber. Absorbance scans at 235 nm showed a definite concentration gradient. Since
this gradient was highest near the positive electrode, and since only EDTA has a significant absorbance at this wavelength, the gradient was presumed to be due to EDTA. The apparent charge of this gradient was determined to be $-2.93 \pm 0.19$ proton equivalents. Further experiments showed that no gradient formed when using 10,000 - 14,000 MWCO dialysis membrane. This shows that with the 3500 MWCO dialysis membrane, the passage of EDTA must have been slow in comparison with the motion of equilibrium electrophoresis. When a buffer with no EDTA was circulated through the system, the gradient and all absorbance at 230 nm disappeared, showing that EDTA was the component forming the gradient and that it was not completely impeded by the dialysis membranes.

For future experiments, either the 10,000 - 14,000 MWCO membranes were used, or no EDTA was added to the buffer.

Preliminary results with BT1 thick matrix membranes showed a buffer gradient near the negative electrode at low wavelengths, suggesting that Tris$^+$ ions were retarded by this membrane. However, the mechanical problems of membrane mounting made further tests with this membrane impossible.

**DNA Oligonucleotides of 20 Bases or Base Pairs**

**Experiment Design**

This experiment had the simple purpose of determining the apparent charge on single and double stranded DNA. The effects of ionic strength and electric field strength were also to be investigated. It was not possible to obtain stable gradients with high ionic strength buffers, so the variation in ionic strength was not as large as originally planned. The buffer used consisted of Tris KCl pH 8.00 at various concentrations.

**ss DNA in 20:20 Tris KCl Buffer**

Equilibrium electrophoresis was performed on a sample of pd(A)$_{20}$, 1.0 A$_{260}$, in 20:20 mM Tris KCl buffer. Over a period of four days, fifteen scans that were at a steady state were chosen for analysis. Twenty-one scans of pd(T)$_{20}$, 0.5 A$_{260}$, in 20:20 Tris
KCl buffer were chosen for analysis. The first seven scans were taken at intervals over a period of three days. Over the following three days, no gradients formed in the analysis chamber. The following fourteen scans were taken on the same sample over the next three days. No stable gradients formed for the next five days. Then the buffer was changed to 2:2 Tris KCl and six scans were taken over a period of three days. The apparent charges from all of the ss DNA scans are shown in figure 4.

![Figure 4: Individual apparent charge determinations for ss DNA.](image-url)
As can be seen from the figure, there was no apparent difference in charge between poly-A and poly-T oligonucleotides. The poly-A nucleotide in 2:2 mM Tris KCl buffer did have a noticeable increase in apparent charge. Hypotheses concerning the increase in apparent charge with lower field strengths for the three series of data will be given in the discussion section.

**ds DNA in Various Buffers**

The pd(A)$_{20}$ and pd(T)$_{20}$ oligonucleotides were annealed to form double stranded nucleotides according to the procedure in the material and methods section. Over a period of eight days, eleven scans of one sample in 20:20 Tris KCl buffer were chosen for analysis. Over the next three days, five scans of the same sample in 10:10 Tris KCl Buffer were chosen for analysis. Seven scans in 2:2 Tris KCl buffer from the next five days were chosen for analysis. In the next ten days, no observable gradients formed at any field strength in 20:200 Tris KCl buffer or in 6.7:66.7 Tris KCl buffer. Apparent charges computed for ds DNA in the 20:20, 10:10, and 2:2 mM buffers are shown in figure 5.
Individual apparent charge determinations for ds DNA.

No significant systematic dependence of the apparent charges on buffer composition for individual data sets was evident. As in the case of ss DNA, the apparent charge calculated for individual data sets was dependent on the electric field strength.
Overall Results

The individual least squares fits of DNA oligonucleotides showed a dependence on electric field strength. Not surprisingly, fits of data sets of each species that included all electric field strengths in single fits showed systematic error in their residual plots. The low electric field data sets with $\sigma < 8.0$ were excluded from the combined fits because the individual fits of these sets reported apparent charges much higher than theoretically reasonable and showed the most variance with electric field strength. The RMS errors of the combined fits of the remaining data sets were usually comparable to or less than the average RMS errors of the individual fits. The results of the combined fits are listed together in table 2.

Table 2: The Apparent Charge on DNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Data Sets</th>
<th>Apparent Charge in electron equivalents</th>
<th>RMS error in A$_{260}$</th>
<th>Average RMS of single sets in A$_{260}$</th>
<th>Concentration range in A$_{260}$</th>
<th>Electric Field Strengths volts/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)$_{20}$ in 20:20 buffer</td>
<td>5</td>
<td>5.89 (5.86-5.91)*</td>
<td>0.00256</td>
<td>.00413</td>
<td>0.3-0.9</td>
<td>0.284 0.568 0.852</td>
</tr>
<tr>
<td>(T)$_{20}$ in 20:20 buffer</td>
<td>7</td>
<td>5.78 (5.77-5.79)</td>
<td>.00149</td>
<td>.00426</td>
<td>0.3-0.9</td>
<td>0.404 0.539 0.809</td>
</tr>
<tr>
<td>(A)$_{20}$ in 2:2 buffer</td>
<td>4</td>
<td>6.63 (6.47-6.80)</td>
<td>.00719</td>
<td>.00520</td>
<td>0.0-1.0</td>
<td>0.367 0.611</td>
</tr>
<tr>
<td>(AT)$_{20}$ in 20:20 buffer</td>
<td>3</td>
<td>7.96 (7.84-8.07)</td>
<td>.00495</td>
<td>.00468</td>
<td>0.3-1.0</td>
<td>0.284 0.567</td>
</tr>
<tr>
<td>(AT)$_{20}$ in 10:10 buffer</td>
<td>3</td>
<td>8.52 (8.46-8.58)</td>
<td>.00289</td>
<td>.00375</td>
<td>0.3-1.0</td>
<td>0.389 0.518</td>
</tr>
<tr>
<td>(AT)$_{20}$ in 2:2 buffer</td>
<td>3</td>
<td>7.98 (7.92-8.08)</td>
<td>.00334</td>
<td>.00368</td>
<td>0.3-1.0</td>
<td>0.272 0.409 0.681</td>
</tr>
</tbody>
</table>

* The 65% confidence intervals returned by NONLIN
Comparison of ss DNA and ds DNA

Although the formal charge on the ds DNA is 2 fold higher (40 electron equivalents) than the formal charge on ss DNA (20 electron equivalents), the apparent charge on the ds DNA is only 1.3 to 1.4 fold higher than the apparent charge on the ss DNA in the same buffer. This shows that the higher charge density on ds DNA results in greater charge reduction due to condensed counterions and Debye-Hückel shielding. On the ss DNA the apparent charge is approximately 30% of the formal charge, while on ds DNA the apparent charge is approximately 20% of the formal charge. The basic prediction of Record et al. is similar, predicting ss DNA to show 30% of its formal charge, while predicting ds DNA to show only 12% of its charge.5

Cytochrome c

Experimental Design

Equilibrium electrophoresis was performed on a sample of cytochrome c for two reasons. First, since cytochrome c was the first protein studied in this particular device, it was studied again to test the consistency of the device and to test the suitability of the low ionic strength phosphate buffers that had to be used for the mutants of T4 lysozyme. Second, since the calculated apparent charge of DNA had shown a dependence on electric field strength at low electric fields in this particular device, a protein was chosen to study whether or not this dependence was peculiar to DNA.

Apparent Charge

From a period of three days, nine scans of cytochrome c that had reached a steady state were analyzed. The apparent charges determined are shown in figure 6.
An overall fit of all these data sets gave a charge of 6.44 proton equivalents, a low of 6.37 and a high of 6.51, with an RMS error of 0.00241 A<sub>520</sub>, compared to an average RMS of 0.00291 for the individual data sets. This was similar to the previous pattern in that the fit of all the data sets was very close to the lowest apparent charge of the individual fits. The calculated charge of 6.44 proton equivalents did fall in between the
previously determined charges of cytochrome c in pH 7.0 buffer (4.7 electron equivalents) and pH 4.7 buffer (9.9 electron equivalents).^T4 Lysozyme Mutants

**Experimental Design**

Mutanted forms of T4 lysozyme that differ significantly in charged residues on the surface of the protein were prepared in the lab of Brian Matthews. The effects of this change in charge on thermal stability experiments, enzyme activity experiments, and X-ray crystallographic structure have been reported. The apparent charges determined by equilibrium electrophoresis could be of use in further theoretical analysis of the above results. The purpose of the experiment was to determine the apparent charges of various mutants in the same buffer (25:20 KCl PO$_4$ pH 5.3) as that used in the thermal stability experiments.

**Unsuitability of the Chosen Buffer**

When the first experiments were attempted with the 25:20 KCl PO$_4$ pH 5.3 buffer, no stable gradients of the fully charged TA protein formed, even over a period of nine days at various field strengths. The reason for this difficulty was not evident. Therefore, the buffer was changed to the 2:2 Tris KCl pH 7.00 buffer. This buffer was chosen for three reasons: Tris buffers had worked with DNA, the low ionic strength would provide less shielding of the macroion's charge, and a low ionic strength buffer also would maximize the percentage of current carried by the macroion. The pH of the buffer was lowered to 7.00 to be two pH units from T4 lysozyme's isoelectric point. When experiments with this buffer succeeded in forming gradients, the original phosphate buffer was diluted 10X (raising its pH to 5.5) in hopes that at this ionic strength the increased apparent charge would be more conducive to gradient formation. A series of experiments with the 10X diluted buffer did form stable gradients with the fully charged TA lysozyme.

Subsequent experiments using the 10X diluted phosphate buffer with mutated TA proteins of a lesser charge were less successful. No stable gradients were observed with
proteins mutated to have a charge of +7e or +3e also provided by Brian Mathews. A few data sets with TA(119/135) were good enough to analyze, though the data collected showed higher RMS error and more systematic deviation in the residuals than the fully charged mutant.

After this, experiments were again attempted with the fully charged TA protein and the original buffer. Again, no stable gradients formed. It was decided at this point to wait for the next generation of equilibrium electrophoresis device to be developed before continuing experiments with these proteins.

**Apparent Charges**

Equilibrium electrophoresis of a sample of TA lysozyme (titrable charge of +9) resulted in five data sets that came to equilibrium in 2:2 Tris KCl pH 7.0 buffer. The buffer was changed to 2.5:2.0 KCl PO₄ pH 5.5 and fourteen data sets were chosen for analysis from the next seven days. Equilibrium electrophoresis also was performed on a protein (TA 119/135) that had a titrable charge of +5. From a period of six days, four scans that had reached a steady state were chosen for analysis. (A fifth scan, apparently at a steady state was not analyzed completely because the program NONLIN could not fit the curve.)
Figure 7

Individual apparent charge determinations for TA and TA(119/135).
A comprehensive fit was also performed on each collection of data sets.

Table 3: The Apparent Charge on Lysozyme

<table>
<thead>
<tr>
<th>Name</th>
<th>Apparent Charge in proton equivalents</th>
<th>RMS error in A₂₈₀</th>
<th>Average RMS of single sets in A₂₈₀</th>
<th>Concentration range in A₂₈₀</th>
<th>Electric Field Strengths volts/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA in 2:2 Tris KCl pH 7.00 buffer</td>
<td>5.72 (5.65-5.78)</td>
<td>0.00200</td>
<td>0.00166</td>
<td>0.0-1.0</td>
<td>0.310</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.413</td>
</tr>
<tr>
<td>TA in 2.5:2.0 KCl PO₄ pH 5.5 buffer</td>
<td>5.22 (5.16-5.28)</td>
<td>0.00240</td>
<td>0.00163</td>
<td>0.05-0.50</td>
<td>0.258</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.425</td>
</tr>
<tr>
<td>TA(119/135) in 2.5:2.0 KCl PO₄ pH 5.5 buffer</td>
<td>2.88 (2.80-2.97)</td>
<td>0.01124</td>
<td>0.00762</td>
<td>0.0-1.3</td>
<td>0.425</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.594</td>
</tr>
</tbody>
</table>

From the combined fit, TA in the phosphate buffer showed 58.0% of its titrable charge and TA(119/135) in the same buffer showed 57.6% of its titrable charge. The difference in apparent charge between TA and TA(119/135) was 2.34 proton equivalents, which is 58.5% of the change in titrable charge.

pBR322 Plasmid

Experimental Design

Equilibrium electrophoresis was to be performed on the plasmid pBR322 in two states: the super coiled state and the relaxed state. Relaxed pBR322 was expected to have a higher overall charge because it would have a lower charge density than the supercoiled form.

Lack of Gradients

Unfortunately, this simple experiment was not completed because pBR322 did not form stable steady state gradients under any experimental conditions in the present equilibrium electrophoresis device. At low electric fields, no effect of the field was evident. At high electric fields, a moving electrophoretic boundary was observed.
Boundary Observations

A single moving boundary was observed for pBR322 in a 20:20:1 mM Tris KCl EDTA pH 8.00 buffer. Six scans were taken at times 7800, 11340, 14,880, 18780, 22260, and 29580 seconds. The electrophoretic mobility computed for this boundary over a period of 8.5 hours was $3.93 \cdot 10^{-4} \text{cm}^2\text{volts}^{-1}\text{sec}^{-1}$, ± 10%.

To estimate the charge of pBR322 from this electrophoretic mobility, a value for the frictional coefficient of the molecule was needed. Therefore, a sedimentation velocity experiment was performed on the sample taken from the analysis chamber of the device after the equilibrium electrophoresis experiments were completed. The sample was undiluted, had an absorbance of 0.5 A$_{260}$, and was sedimeted at 60,000 RPM at 20° C in a Beckman XLA. A sample of fresh pBR322 with an absorbance of 0.1 A$_{260}$ was also prepared and centrifuged in the second cell in the rotor. Eighty scans of each were taken over 7,137 seconds. A single sedimenting boundary was found with each sample, both with a midpoint at 18.5 S, showing that the pBR322 was basically intact after twenty-one days in the analysis chamber at room temperature. Taking $2.9 \cdot 10^6$ for the molecular weight of pBR322, the frictional coefficient computed from S was $1.28 \cdot 10^{-6}$.

Now applying the formula $u = \frac{v}{E} = \frac{2\varepsilon}{f}$ where $u$ is the electrophoretic mobility, $v$ is the velocity of the ion, $E*$ is the electric field, $z$ is the number of charges, $e$ is the charge on an electron, and $f$ is the frictional coefficient; the charge on pBR322 is estimated to be 3,100 electron equivalents, or about 35% of the formal charge (8722 electron equivalents) that resides on this plasmid of 4361 base pairs.

Qualitatively, the shape of this boundary (seen in figure 8) as it changed with time was not as expected.
As can be seen, the boundary at the third time period had an aberrant shape. And, in all subsequent scans, there seems to be some dilution of the "plateau" region from the first scan. This dilution was more pronounced in other moving boundaries of pBR322. Also the width of the boundary at times 4, 5, and 6 did not increase as quickly as expected. The apparent buildup of material at the bottom of the cell was expected since the pBR322 cannot penetrate the dialysis membrane.

The aberrant shape of some of the boundaries of pBR322 was probably due to convection. The dilution of the plateau region likewise probably resulted from convection, since theoretically the plateaus of electrophoretic boundaries are not expected to experience dilution.

That the boundaries did not spread much with time indicates that there may have been some concentration dependence of the electrophoretic boundary. This was probably
due to the high dependence of viscosity on DNA concentration, as is the case in sedimentation velocity experiments with DNA.\textsuperscript{13} Viscosity is the limiting force in particle velocity at the molecular scale.

**Convection**

Convection was the most evident source of error in the equilibrium electrophoresis experiments conducted in the present device. A convective cell forms due to density gradients within the solution being acted upon by gravity. The density gradient is usually formed by a temperature difference caused by the heat generated by the current passing through the analysis chamber; however, bulk fluid flow cannot be ruled out as the cause of convection. The general result of convection is a turbulent mixing of the solution in the analysis chamber that destroys or perturbs the electrophoretic gradient.

**Convection Depends on Current**

Examples of current dependent mixing in the analysis chamber were apparent from the absorbance scans of the analysis chamber. In figure 9, a sample of TA lysozyme in 2.5:2.0 KCl Tris pH 5.5 buffer showed mixing suddenly appearing at high currents.
As can be seen in figure 9, the equilibrium electrophoresis gradients displayed a steeper exponential with increasing current. However, the gradient at 25 μA showed a noticeable divergence from a smooth exponential and the 35 μA gradient was almost flat except for a portion near the top of the analysis chamber. The residuals of the NONLIN fit of the 25 μA scan showed systematic error, confirming the visual appearance of error.
seen in the raw data, and, of course, the 35 \(\mu\)A scan was useless in determining the charge on the T4 lysozyme.

In this example, the dependence of mixing on increased current supported the hypothesis that convection caused by resistive heating of the sample introduced systematic error into equilibrium electrophoresis gradients. It also supported the alternative hypothesis that the bulk fluid flow due to differences in ion mobilities was the cause of convection. With an event as random as convection, it was not possible to distinguish between these two causes. The only hint was that the outside room temperature seemed to influence the presence of convective currents, but this correlation was not certain.

Convection not only prevented gradient formation or completely destroyed gradients (as shown above), but it also introduced error into the analysis of gradients. The RMS errors returned by NONLIN for the five TA data sets, in order of increasing current, were: 0.00146, 0.00143, 0.00186, 0.00555, 0.247. Systematic error in the residual plots was also dependent on current. This evidence shows that the error introduced by convection appears before the obvious destruction of the gradient. This error shows up as non-systematic distribution of residuals of the least squares fit. Thus convection not only sets the range of usable experimental conditions but also introduces error into the final results.

Another Type of Convection

Another possible source of convective flow could be the density gradient formed by the concentration of protein due to the process of electrophoresis itself. The electric field in the analysis chamber is oriented parallel to the gravitational field of the earth, so that the density gradient formed by the macroion will not tend to mix due to gravity. If the analysis chamber is not oriented parallel to gravity, there will be an orthogonal component to the density gradient that forms. But, the analysis chamber is sometimes tilted slightly to one side if there is a bubble in the chamber or if the optical path of the system is not perfectly adjusted. A sample of ss DNA showed a pattern that seems to
have resulted from such causes. The pattern was observed by imaging the UV light on a fluorescent card; it could not be scanned since the photo diode array is oriented vertically with the analysis chamber and cannot scan horizontally. The pattern observed is represented in the following diagram, figure 10.

![Figure 10](image)

A view of convection in the analysis chamber.

Since the DNA is collected on the right side of the cell in a swirling pattern, bulk fluid flow must have been acting perpendicular to both gravity and the electric field.

**Convection is Unstable**

Convection in the analysis chamber is a condition that is very difficult to characterize. While there are causes that contribute to convection, the presence and magnitude of convection show an extremely random character.

The primary cause of convection, as shown above, is related to current. This cause is presumed to be the temperature gradient induced by resistive heating of the solution by the current passing through it. Temperature variations in the space surrounding the whole device also seem to promote convection. Particularly, the 400 watt arc lamp used as the light source is capable of increasing the temperature around the device approximately 1° C per hour.
Other factors stabilize the gradient against convection. Primarily, a previously formed gradient is more stable than a gradient being formed. In a certain set of conditions, no gradient will form for hours or days, presumably due to convection. Yet once a gradient forms, it is stable for days, even with changes in current. This pattern is also seen in gradients perturbed by convection. The most sensitive area to convection is the top of the analysis chamber where there is very little gradient in concentration. Theoretically, that a gradient stabilizes itself makes sense if temperature driven convection is the major turbulent flow mixing the solution. The more concentrated solution at the bottom of the gradient is more dense than upper layers; therefore, a greater temperature differential is required to make the lower layers less dense than upper layers and start convective flow. Also, the directed dynamic flow of electrophoresis would tend to conduct heat vertically in the analysis chamber, retarding the formation of turbulent flow (convection).

There are several actions that can be taken in an attempt to form an initial gradient of a particular macroion. A constant outer temperature (as in a locked room overnight) contributes to gradient formation. Presumably, the constant outer temperature helps stabilize the flow of heat from inside to outside the chamber. The use of a refrigeration unit to lower the temperature also helps in some cases to form a new gradient. Presumably, convective vortex formation is retarded by a lower Reynolds number. However, the viscous agents, 8.0 M urea and 2% cyclohexaamylose, show no positive effect on gradient formation in the present device, even though a higher viscosity also ought to lower the Reynolds number. Turning off the current entirely for a period of time is sometimes immediately followed by stable gradient formation after days with no success. Presumably, the convective currents are stable as a current keeps generating a temperature differential or keeps producing bulk fluid flow, but their reformation is a random process.
DISCUSSION

Reliability of Results

The Basic Physical Evidence

The basic physical evidence gathered from equilibrium electrophoresis is that charged molecules do form steady state concentration gradients under the influence of external electrical fields. These gradients are reproducible within ±10%. These gradients are related to the charge on the molecule being tested and are related to the electric field. There are questions concerning the stability of these gradients. There are also questions concerning the accuracy of the charges computed by fitting these gradients to the formulae described in the theory section.

Reproducibility and Consistency

Results from individual data sets showed a deficiency in the reproducibility of the charges fit to equilibrium electrophoresis experiments; namely, the 65% confidence intervals reported by NONLIN did not consistently overlap for individual data sets. This indicates that there are significant sources of error that go beyond the random noise of absorbance scanning.

A better estimate of the true accuracy of the present device is the range of values that are given for a number of data scans of the same sample. Since the combined fit of all the data sets shows systematic error in the residuals, yet the individual fits do not show systematic error in the residuals, it is uncertain whether the combined fit is actually more accurate than any of the individual fits. The species that has the largest amount of data is pd(T)_{20}. For data sets with \( \sigma > 5.0 \) that had come to a steady state, the extreme range of charge measurements is 5.50 to 6.64 electron equivalents with a simple average of 6.13 electron equivalents. The range from the combined fit value of 5.78 electron equivalents is +15% to -5%. If the combined fit did not show systematic error, it would increase the
precision and accuracy of the determined charge. But with the systematic error, the combined fit only increases the precision of the fit because we are not assured that the fit dealt with the error properly. It is relatively certain that the true charge falls between the extremes of the individual data sets, because no systematic error is seen in individual fits. However, it is not possible to say exactly what intermediate charge is more accurate without knowing the nature of the error of the individual fits.

The 65% confidence limits returned by NONLIN (for example, a range of ±0.17% for pd(T)\textsubscript{20}) probably exaggerate the precision of the data. A better estimate of the overall precision of the present device is the difference between the charge determinations of pd(A)\textsubscript{20} and pd(T)\textsubscript{20}, a difference of only 1.4%. These two molecules are expected to have close, if not identical, charges. This comparison provides an estimate of the limits of accuracy due to experimental error of differing concentration, of different mechanical setup, of random optical variations, of buffer preparation, and of conductance and current measurements.

The Decision on Curve Fitting

The ultimate limiting factor in the accuracy and precision of charge determinations from the present device is the curve fitting procedure. NONLIN fits the best curve to a number of data points, but the statistical RMS error, residuals, and confidence intervals do not correctly represent the uncertainty of the curve fit itself. In simpler terms, the curve fitting program NONLIN cannot judge itself. A parallel indication of this is that different fits performed on various truncations of the data often give non-overlapping confidence intervals. This behavior is not peculiar to equilibrium electrophoresis; the limitation of curve fitting is also a familiar problem in sedimentation equilibrium experiments.

The limitation of the curve fitting procedure may have three causes. First, curve fitting requires sufficient information in the data for the curve fitting procedure to work. For example, in the case of low σ data sets, the slight curvature of the gradient may be indistinguishable from its slope due to the relative magnitude of the random error in the
system. Second, curve fitting requires a random distribution of error. In the case of data taken with high current, the non-random effects of convection are not modeled. Third, curve fitting requires that the correct model be chosen for the fit. A deficiency in the third requirement is not simple to diagnose. An error in the model will apply to and color all data analyzed.

Here, the consistent presence of systematic error whenever combined fits of multiple field strengths are performed, does question the model describing equilibrium electrophoresis in the present device. Yet the lack of systematic error in the majority of individual data sets argues that the model is an essentially correct description of the equilibrium electrophoresis gradient. At stake between these conflicting indications is the reliability of the entire method of equilibrium electrophoresis. For if the model is incorrect, there is no way to interpret the accuracy of the least squares fits given by NONLIN.

The Mathematical Model and Data Fitting with NONLIN

The linear least squares fitting program NONLIN uses this equation to fit equilibrium electrophoresis data:

\[ c(x) = \delta + e^{\ln(c_0) + \sigma(x-x_0)} \]

where \( c(x) \) is the concentration at position \( x \); \( \delta \) is an arbitrary concentration offset, \( \ln(c_0) \) is the natural log of an arbitrary concentration at \( x_0 \), the first data point; and \( \sigma \) is the parameter proportional to the apparent charge (the second virial coefficient discussed in the theory section is held at 0 and its term drops out of the equation). Some typical data sets with varying \( \sigma \) values are shown in figure 11.
As can be seen from the figure, the parameter $\sigma$ measures the exponential rise in concentration. There are two indications that equation 5 has the correct functional form. First, the values of the RMS error returned from various data sets are less than 0.01 absorbance units. This is well within the optical error expected for the absorbance system. Second, the individual residual error plots show no systematic error. However, there are
two indications that the formula does not have the correct functional form. First, the
dependence of calculated charge on electric field strength is not predicted by the formula.
Second, the residual plots of combined fits do show systematic error (although the RMS
errors for the combined fits are not much higher than that for individual fits).

This conflicting evidence leads us to examine the possible errors that may arise in
the process of curve fitting. Many factors will affect the RMS error and the residual plots
of a fit. Such factors lead to error introduced by the curve fitting itself.

The effects of $\sigma$. A low value of $\sigma$ indicates that the form of the data is
approaching a straight line. Because of this, a curve fitting program will encounter
difficulty in determining $\sigma$ accurately. The program will give no indication of this
difficulty since the difference in curvature between parts of a nearly straight line will
always be small. Because random noise from the device has a relatively larger magnitude
than systematic noise resulting from the difference between the slight curvature of the data
and the slight curvature of the fitted line, systematic error in low $\sigma$ will be hidden by the
high frequency noise of random error. The total RMS error of any fit for a low value of $\sigma$
will always be small because the data is approximately a straight line. In figure 7 it can be
seen that although the 50 $\mu$A curve can be seen to have a greater inflection than the 25 $\mu$A
curve, the $\sigma$'s fit to these curves indicate the opposite. To understand this, it must be
remembered that the curvature of the line is fit, not its slope. For this reason, the
combined fits of the data avoided curves with low $\sigma$ (low field strength).

Unfortunately, curves with a high $\sigma$ also have problems. These curves do not
contain as many data points in the part of the curve that has the most curvature. In a steep
exponential curve, the flat region near the top of the analysis chamber is prone to reduced
accuracy since even a little random noise in this region significantly affects a least squares
fit that weights each data point equally. The high slope region near the base of the
analysis chamber likewise is not fit accurately due to its small amount of curvature.
Moreover, with absorbance data, the measurements above 1.0 are increasingly inaccurate, and regions of high slope quickly enter this range.

**Relation of Convection to** $\sigma$. The problem of convection in the present device is discussed above. Here it is important to note that since convection depends on current, the higher $\sigma$ data sets will be expected to show more noise due to convection. For the present device, the destruction of the gradient by convection at high currents sets the experimental limit of the highest value of $\sigma$ that may be measured.

The limitation of convection also appears in low $\sigma$ data sets. Since the electrophoretic gradient stabilizes itself against convection, the very slight gradients at low field strengths are less stable. In this way convection sets the lowest usable field strength. In one experiment, the electric field applied to a previously formed stable gradient was cut in half; the gradient did not show a $\sigma$ cut in half, it disappeared entirely.

**Parameters of the System.** Finally, in the present device, some systematic error in combined fits is expected due to the variation in the parameters that describe the entire experiment.

The first parameter that describes the entire system is temperature. The NONLIN program is set up to fit a single $\sigma$ to data sets with different field strengths, but a compensation for differing temperatures is not part of the fitting program. Therefore, some systematic error is expected when fitting multiple sets with different temperatures. The extreme range of temperatures used in these experiments was 291° K to 301.2° K, a maximum divergence of 3.5%. This magnitude of error ought not to be very significant.

The second parameter that describes a whole experiment is the field strength. In the present device, the field strength is calculated from a measurement of the conductivity of the buffer and from the current flowing into the electrodes. Since the curve fitting package is specifically set up to fit data sets produced at different field strengths, varying the field is not a problem. But any error in measuring the current flowing through the
buffer, or any change in the conductivity of the solution at different field strengths will cause systematic error with multiple fits.

There is Not Sufficient Reason to Abandon the Model

The equation that describes equilibrium electrophoresis is simple and based on trustworthy physical principles. The dependence of charge on electric field for individual data sets is best explained by the limits of fitting curves with small curvature. The problem of systematic error in the residuals of combined fits can best be explained as a systematic error in the experiment itself, such as convection or inconsistent measurement of the electric field, rather than by assuming a theoretical problem with the mathematical model chosen for the fit. Furthermore, if the systematic error shown in the combined fit is due to deficiencies in the equations derived from the theory of electrophoresis, it would still be expected that the present equation would still be the best first approximation of more complicated events.

If the model for curve fitting is accepted, the charges determined by equilibrium electrophoresis in the present device are reliable and accurate to at least ±10%.

Sources of Error Other than Convection

Bulk Fluid Flow

Bulk fluid flow other than convection is another possible source of error in equilibrium electrophoresis experiments. Although there is no direct evidence that this type of flow influenced the electrophoretic gradients analyzed here, the overall instability and limited range of gradient formation suggests that there are problems with bulk fluid flow in the present device. Observations that lead me to believe that some bulk fluid flow is present in these experiments are described below.

First, changing the buffer from one ionic strength to another always destroys a gradient in the analysis chamber. This is explained by noting that the boundary between two concentrations of buffer does move in the presence of an electric field because of the "volume change taking place at the electrode and the difference in buffer ion transport
numbers at the two concentrations." Evidently, a steady state is reached in buffer ion flow when a sample gradient is produced. The K\(^+\) and Cl\(^-\) ions are used in these buffers because of their nearly equal mobilities, but the buffering component (Tris or PO\(_4\)) will always be slower than the OH\(^-\) or H\(^+\) ion with which it is in equilibrium.

Second, with no membranes in the analysis chamber, the volume of outflow from the buffer chambers shows an extreme sensitivity to the height of the output tubes. With membranes in place, a difference in height does not produce a noticeable difference in outflow volume. But the buffer chambers cannot be totally isolated from the analysis chambers simply because the membranes do allow the passage of buffer. With the membranes in place, it is difficult to determine whether the pressures in the top and bottom buffer chambers are equal; therefore, it is also difficult to prevent a small bulk flow due to pressure differences.

Third, if the buffer inlets and outlets of the present equilibrium electrophoresis cell are sealed, the buffer in the analysis chamber will evaporate and be replaced by air. In the present device, the dialysis membranes make a seal with the analysis chamber only by being compressed and their edges are exposed to the air. The amount of evaporation through wicking depends on the physical characteristics of the dialysis membrane. With the BT1 membranes, which are rigid and thick, the 0.5 ml of buffer in the analysis chamber will evaporate in 24 hours. With the Spectro/Por 2 membranes, the 0.5 ml of buffer will evaporate in 7 to 10 days. When the cell is connected to circulating buffer, the analysis chamber remains full. This shows that the evaporating buffer is replaced by buffer flowing into the analysis chamber from the buffer chambers. This bulk flow to replace evaporated buffer may perturb a gradient, especially in the regions near the membranes.

**Electrical Phenomenon**

The electric field that generates an equilibrium electrophoresis gradient is not directly measured in the present device, but is calculated from indirect measurements. Because of this, there are identifiable limitations in the accuracy of the electrical
measurements necessary for equilibrium electrophoresis. There are also some theoretical questions concerning the electrical field that arise from the setup of equilibrium electrophoresis experiments.

**The Measurement of Conductance.** The first actual measurement used to compute the electric field is the conductance of the buffer. This is measured before the experiment by testing a sample of the bulk buffer supply prepared for the experiment. Usually 2 liters of buffer were prepared in advance to avoid slight changes in conductivity apparent in individual buffer mixings and titrations.

The conductivity of buffer is dependent on temperature. Due to the physical arrangement of the present device, experiments were conducted at room temperature. The room temperature varies, and thus the conductance will be affected. Ultimately, even with better temperature control, the resistive heating due to the current passing through the buffer will cause uncertainty about the temperature inside the analysis chamber. However, since Joule heating is dependent on current squared, the power dissipated in the chamber is small for currents less than 1 mA.

The conductivity of the buffer is dependent on the concentration and activity of components in the buffer. Even with buffer circulation, the pH and conductivity of the buffer flowing into the buffer chambers is not identical with the pH and conductivity of the buffer flowing out of the chamber. The standard DNA buffer with a pH 8.00 and a conductivity of 3.73 mmhos was used in an electrophoresis experiment with and without buffer circulation. Table 4 shows the resulting pH and conductance of the outflows.
Table 4: Buffer Electrophoresis

<table>
<thead>
<tr>
<th>20mM KCl 20mM Tris Buffer</th>
<th>Original pH</th>
<th>Original conductivity in mmhos</th>
<th>Final pH</th>
<th>Final conductivity in mmhos</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Flow Negative Electrode</td>
<td>8.00</td>
<td>3.73</td>
<td>11.65</td>
<td>4.31</td>
</tr>
<tr>
<td>With Flow Negative Electrode</td>
<td>8.00</td>
<td>3.73</td>
<td>7.94</td>
<td>3.84</td>
</tr>
<tr>
<td>No Flow Positive Electrode</td>
<td>8.00</td>
<td>3.73</td>
<td>2.23</td>
<td>5.56</td>
</tr>
<tr>
<td>With Flow Positive Electrode</td>
<td>8.00</td>
<td>3.73</td>
<td>7.94</td>
<td>3.75</td>
</tr>
</tbody>
</table>

Some of this change in pH and conductivity is due to chemical reactions at the electrodes; these changes hopefully do not reach the analysis chamber. Some of the change may be due to an unequal flow of ions that conduct the current; this change will be important in the analysis chamber.

The conductivity of the buffer is dependent on the concentration and activity of ions in the solution. Here there are some theoretical questions concerning the distribution of ions in the equilibrium electrophoresis gradient and their effect on buffer conductivity and, thus, the electric field. Do counter ion condensation and Debye-Hückel screening significantly lower the activity of buffer ions that are partially associated with the macroion? What effect does the macroion have on the conductance of each layer of solution in the boundary? Is the electric field in volts/cm constant throughout the cell, or does the presence of the gradient necessarily make the field dependent on the concentration of the macroion? These questions are beyond the scope of this paper, but will need to be addressed when improved devices are available.

The Measurement of Current. The second measurement needed to compute the electric field is the current passing through the analysis chamber. The current is actually measured at the connection between the constant current source and the positive electrode.
or in the constant current source itself. However, not all the current supplied at the electrode will travel through the analysis chamber. Some current will be consumed in electrochemical reactions at the electrodes. For instance, the electrolysis of water will consume some current. A number of electrons will reduce H\(^+\) ions forming hydrogen gas at the negative electrode and an equal number of electrons will be accepted at the positive electrode by OH\(^-\) ions producing oxygen gas. The electrons involved in these two half reactions will travel through the wire (where the meter is) but will not travel through the analysis chamber.

As an example, if 0.005 ml/hour of hydrogen gas is produced by the electrolysis of water, \(5.7 \times 10^{-6}\) amperes of current are being consumed in this reaction. This would be a significant amount of current in the present device, where currents of \(1.0 \times 10^{-6}\) to \(500 \times 10^{-6}\) amperes are used.

Other electrochemical reactions show their presence in equilibrium electrophoresis as a capacitance or battery effect. When the constant current source is turned off at the conclusion of an experiment, an electric current opposite in direction to the driving current develops. The whole apparatus is charged like a capacitor or like a battery. Through a 100 kΩ resistor, a 0.1 V potential is held for over an hour. The discharge of this current is dependent on time and on the physical setup of the device more than on how long the forward current was charging the device.

Overall, a measurement of the nonideality of current transfer at the electrodes is given by the voltage drop through the electrodes that is not dependent on current. The following graph (figure 12) plots a normalized path length versus the voltage applied to the system. The total resistance of the system was computed by dividing the voltage by the current at the power supply. The normalized path length is a property of the whole system; it is computed by dividing the total resistance of the system by the specific conductance of the buffer in the device. Thus it represents the length in cm of a 1.0 cm\(^2\) cross-section buffer filled tube that would have the same resistance as the whole device.
The voltage dependence of the equivalent length (defined in the text) shows how the device does not have a constant resistance.

As can be seen in the figure, the apparent length (or resistance) of the entire system increases with decreasing voltage. This shows how the resistance at the electrodes is not a constant but is a function of voltage.
The Measurement of the Cross-section. The final parameter in calculating the electric field in the analysis chamber is the cross-sectional area of the analysis chamber itself. The precision of this parameter depends on the machining of the chamber itself. Also, air bubbles reduce the cross-section at the top of the chamber; the electric field of data taken to the side of a bubble will be higher than that computed for the analysis.

Comparison of Results to Present Theories

DNA Oligonucleotides

Fenley et al. have reported the effect, based on numerical calculations, of polymer length on counterion condensation (not covering Debye-Hückel shielding). They report the following results. Condensed counterions reduce the charge of an infinite length DNA model to 23.4% of its total charge. Condensed counterions will reduce the charge of a 20 bp fragment of ds DNA to 27.2% of its total charge in 10 mM NaCl, and to 32% of its total charge in 1 mM NaCl. The percentage of charge apparent on a finite polymer approaches the limit of the infinite model either when the polymer length is increased or the molarity of the buffer is increased. The expected charge of DNA in solution will be further reduced from these percentages due to Debye-Hückel shielding.

Record et al. have reported the expected apparent charge of ss and ds DNA taking into account both counterion condensation and Debye-Hückel shielding, but not including polymer length. The apparent charge on ds DNA, including Debye-Hückel shielding, is calculated using the this equation given by Record:

\[
\psi = 1 - (2\xi)^{-1}
\]

Using this formula and working backwards from the numerical results of Fenley, it is possible to give rough estimates of the expected charge of the short oligonucleotides used in these experiments. The polymer length effects for ss DNA are estimated to be equal to that of ds DNA. Here the effect of a polymer length of 20 will be estimated using the following values:
Table 5: DNA Polymer Length Effects

<table>
<thead>
<tr>
<th>Buffer (pH 8.00)</th>
<th>% of total charge added to apparent charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:2 mM Tris KCl</td>
<td>2.8%</td>
</tr>
<tr>
<td>10:10 mM Tris KCl</td>
<td>1.6%</td>
</tr>
<tr>
<td>20:20 mM Tris KCl</td>
<td>1.0%</td>
</tr>
</tbody>
</table>

The theoretical apparent charge on ss DNA using the infinite length model is 30% of its titrable charge. The theoretical apparent charge on ds DNA using the infinite length model is 12%. Using these figures and correcting for a polymer length of 20, the following table lists the estimated theoretical charges and compares them to the apparent charges measured here.

Table 6: DNA Charge Comparison

<table>
<thead>
<tr>
<th>Macroion</th>
<th>Buffer Tris KCl pH 8.00</th>
<th>Estimated Charge Electron Equivalents</th>
<th>Measured Charge Electron Equivalents</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pd(T)20</td>
<td>20:20 mM</td>
<td>6.2</td>
<td>5.78</td>
<td>6.8%</td>
</tr>
<tr>
<td>pd(A)20</td>
<td>20:20 mM</td>
<td>6.2</td>
<td>5.98</td>
<td>3.5%</td>
</tr>
<tr>
<td>pd(A)20</td>
<td>2:2 mM</td>
<td>6.56</td>
<td>6.63</td>
<td>1.1%</td>
</tr>
<tr>
<td>pd(A)20 • pd(T)20</td>
<td>20:20 mM</td>
<td>5.2</td>
<td>7.96</td>
<td>53%</td>
</tr>
<tr>
<td>pd(A)20 • pd(T)20</td>
<td>10:10 mM</td>
<td>5.44</td>
<td>8.52</td>
<td>57%</td>
</tr>
<tr>
<td>pd(A)20 • pd(T)20</td>
<td>2:2 mM</td>
<td>5.92</td>
<td>7.98</td>
<td>35%</td>
</tr>
</tbody>
</table>

The charges measured for ss DNA are in excellent agreement with the predicted charge. However, the charges measured for ds DNA are significantly higher than the predicted charge.

Notice that theoretically, the ds DNA ought to have a lower charge than the ss DNA oligomers of which it is made. Even if the accuracy of the present device in determining absolute charge is questioned, the comparison of the effects of the electric field on different macroions is valid. Not only in the combined fits, but also in all individual fits at the same electric field strength, the ds DNA always shows a larger σ than
either ss DNA sample. The ds DNA species must have a higher apparent charge than the ss DNA species.

Does this result challenge the counter ion condensation theory? Not while alternative explanations can be offered. The results with ss DNA shows the consistency of the theory with the measurements performed here. It is highly unlikely that the theory would work only for ss DNA since the assumptions used are identical regardless of the macroion being modeled.

A more likely explanation of the higher charge found on ds DNA presents itself: if the double stranded DNA species used here actually has single stranded portions under these conditions, the charge on these incompletely polymerized molecules would be higher than on completely annealed, double stranded DNA. If 32% of the polymer were actually single stranded (and thus had 30% instead of 12% of its titrable charge) the average of 2.3 electron equivalents of charge would be added to the estimated charge of the ds DNA. Incomplete annealing could have two causes. First, the strands of pd(A)20 and pd(T)20 may not have been perfectly aligned when annealed together; short single strands may be sticking out of both ends since there is no sequence specificity that would ensure that A1 would hydrogen bond with T1. Second, these short oligonucleotides are approaching their melting temperature in the low ionic strength buffers used here. (Unfortunately, the present device does not form stable gradients at higher ionic strengths.)

Cytochrome c.

In the phosphate buffer, cytochrome c, modeled as a 1.7 nm sphere in equation 5, is expected to show 75% of its +9e titrable charge. The measured charge of +6.44e was within 5% of the expected charge of +6.75e.

Lysozyme

The titrable charge on TA lysozyme is +9e at pH 5.3 and for TA(119/135) it is +5e. The titrable charge is expected to experience shielding and be reduced to an apparent charge. According to the Debye-Hückel theory, shielding depends only on the
ionic strength of the buffer and the size of the central ion (which is modeled as an evenly charged sphere). Qualitatively, the measurements made by equilibrium electrophoresis were consistent with this theory. If the titrable charges are assumed to be the correct molecular charge, TA and TA(119/135) both had an apparent charge of 58% in the same buffer, and TA had an apparent charge of 64% in the Tris buffer which has a lower ionic strength.

Using equation 4 to model the shielding, a sphere with a size of 3.5 nm predicts an apparent charge of 58% in the phosphate buffer and 69% in the Tris buffer. However, in lysozyme, the C-terminal domain of the protein contains most of the positive charge on the molecule, and this region has a radius of about 1.0 nm. If the 1.0 nm radius is used in equation 4, the predicted apparent charges are 83% in the phosphate buffer and 89% in the Tris buffer. These predicted apparent charges are 40% larger than the experimentally measured apparent charges.

Cytochrome c does show 75% of a +9e titrable charge in the same buffer as TA lysozyme shows 58% of a +9e titrable charge, and both are small globular proteins. Even if the absolute accuracy of equilibrium electrophoresis is in question, it is evident that TA lysozyme has a lower apparent charge than cytochrome c under identical conditions.

Due to the small number and limited range of these experiments, it is difficult to explain the discrepancy between predicted shielding and the measured apparent charge. Either the Debye-Hückel model is not appropriate for this protein and it experiences much more shielding than calculated, or the actual molecular charge is not +9e. The appropriate experiment would be to extrapolate the molecular charge from apparent charges at various ionic strengths; unfortunately this is not possible in the present device because of the limited stability of gradients depending on ionic strength.
pBR322

The apparent charge on pBR322 is expected to be 12% of its titrable charge according to the counterion condensation theory. Since pBR322 is supercoiled, it could be thought that its charge density would be higher than relaxed ds DNA. This would increase the shielding on the molecule and further reduce the percentage of titrable charge appearing in the apparent charge. However, the apparent charge estimated from electrophoretic mobility and sedimentation velocity experiments was 30% of the titrable charge. Due to the unstable nature of the electrophoretic boundaries in this experiment, it is not unreasonable to think that difference between theory and experiment here is due entirely to experimental inaccuracy.

The Experimental Range of the Present Device

Besides the particular results with each molecule, this project sought to characterize the general performance of the present equilibrium electrophoresis device. In this determination, the experiments that failed to produce results are also considered.

Electric Field.

The condition that is varied in every experiment is electric field. Varying the electric field contributes greatly to the accuracy and precision of data analysis by the fitting program.

The range of effective electric fields in the present device depends primarily on the curvature of the resulting gradient. Gradients with low curvature are very poorly fit by NONLIN. This is probably due to a low signal to noise ratio. Low curvature gradients are also tend to disappear entirely because a gradient contributes to its own stability against disruption by convection. Gradients with extremely high curvatures are also poorly fit by NONLIN. Also, convective flow increases with increasing current; this sets the high limit for electric field in some cases. Finally, device dependent limitations interfere with the accurate measurement of steep gradients. The absorbance of a gradient near the membrane is difficult to measure due to the light path in the device. There are
three reasons for this problem: it is difficult to aim the light path directly parallel to the analysis chamber, the dialysis membrane acts as a mirrored surface and creates Lloyd's mirror interference fringes, and the light beam diverges vertically. Absorbance measurements above 1.0 are subject to error. This is due to the very low absolute light signal compounded in this device by the limited dynamic range (4000:1) of the photo diode array.

The range of electric field strength is properly given by noting the range of the parameter $\sigma$ fit to the resulting gradient. Generally, $\sigma$ must be between 10 and 20 to produce accurate results in this device.

**Ionic Strength.**

It is desirable to vary the ionic strength of the buffer used in equilibrium electrophoresis in order to distinguish the charge on the macroion from the shielding of the bulk buffer. This experimental condition was very limited in the present device. In initial experiments with and without a stabilizing gel, cytochrome c formed stable gradients in 100:20 mM KCl Tris, yet these experiments could not be reproduced at this time. Cytochrome c also formed gradients in 2.5:2.0 KCl PO$_4$ buffer. With the oligonucleotides, only buffers of 20:20 mM KCl Tris formed stable gradients. Buffers with 67: 6.7 mM KCl Tris and higher molarities never formed stable gradients. With TA lysozyme, 20:20 mM buffers failed to form gradients and only the extremely low ionic strength 2.5:2.0 mM KCl PO$_4$ buffers formed gradients. The trend is that low ionic strength buffers commonly form gradients. Also, in the ds DNA experiments, the lower ionic strength buffers also had a lower average RMS error.

**Charge**

The charge of the macroion studied also seemed to affect the range of usable experimental conditions. Of the molecules studied, the overall stability and range of macroion gradient formation, from highest stability to lowest stability, forms this series:
ds DNA, ss DNA, cytochrome c, TA lysozyme, TA(119/135) lysozyme, pBR322. Except for pBR322, this series has a good correlation to macroion charge.

**Molar Concentration**

This leaves the problem of why pBR322, even with its high charge, never formed a gradient under any conditions and why EDTA, even though it has a low charge and is not completely restricted by the dialysis membrane, did form a gradient in 20:20 mM KCl Tris buffer. For these two extreme molecules, the biggest difference between them is size. It seems then that pBR322 did not form gradients because its molar concentration is low compared to that of the other molecules studied.

**The Pattern of Gradient Stability**

The basic source of the limited applicability of equilibrium electrophoresis is the device itself. The signal to noise ratio of the data collected and the stability of the environment within the analysis chamber limit the conditions under which successful experiments can be performed. Improvements to the device ought to increase the range of usable experimental conditions. The factor inherent to the experimental system itself seems to be the amount of current carried by the macroion. All the experimental ranges discussed above are summarized by saying that systems in which the macroion carries a significant amount of current will form stable gradients. Low σ boundaries in low electric fields are not stable, and the macroions carry less current because the overall current supplied is less. Boundaries in high ionic strength buffers are not stable, and the macroions carry less current because the high ionic strength buffer carries a proportionally greater part of the current. Boundaries of macroions with a low charge are not stable, and the macroions carry less current because of their low charge. Finally, boundaries of large ions are not stable, and the macroions carry less current because of their small number and lesser mobility. That the inherent stability of an equilibrium electrophoresis gradient depends on the amount of current carried by the macroion is intuitively reasonable and is
supported by the observations made in this project; however, the overall limited range of
the device itself limits the certainty of this conclusion.

Problems and Particular Suggestions

The last purpose of this project was to identify problems with the present device
through practical experience with it, and from this, to propose improvements to the
design. The sources of error noted in the discussion section are the problems in the
present device. Suggestions to improve the device are now given in response to these
problems.

Convection

Convection is the most severe source of instability in electrophoretic gradient
studied here. Efforts to limit convection are of primary importance in improving the
present device, especially since the presence of convection makes other problems with the
device hard to diagnose. Attempts to limit convection operate on two primary principles:
stabilization of the environment of the analysis chamber and adjusting the state of the
chemical system.

Stabilization of the environment of the analysis chamber. Assuming at least part of
the cause of convection is related to heat flow, stabilizing the temperature in the analysis
chamber ought to reduce convection. The present device has very poor temperature
control because the analysis chamber is exposed to the air and is not isolated from the
remainder of the device, which includes many heat generating components. The basic
solution to this problem is a mechanical redesign of the analysis chamber that includes a
water bath surrounding the analysis chamber. Preferably, the water bath should be
oriented so that if any temperature gradient forms, the bottom of the cell would receive
the most cooling.

Also important in a redesign of the cell environment is to reduce possible bulk fluid
flow. The buffer outlets ought to be placed at equal heights to prevent a pressure
difference between the top and bottom buffer chambers. The membranes ought to be sealed from the exterior of the chamber to prevent fluid evaporation through them.

It may also be useful to reduce the amount of physical vibration in the analysis chamber. During these experiments, mechanical adjustments were necessary. The cooling fan of the arc lamp had to be isolated mechanically from the analysis chamber. When filters were clogged on the peristaltic pump, the buffer could be seen to flow unevenly and this seemed detrimental to gradient stability. Physical mounting of the analysis chamber can be improved. Filters and partially air filled chambers in the buffer line could help isolate the buffer flow from pumping irregularities. It may also be possible to produce smoother buffer pressure with a pump that is powered by a compressed air bladder and is controlled by a sensitive, low volume, air pressure valve.

The size of the container also influences turbulent flow such as convection. A smaller container would tend to have less turbulence. It may be possible to reduce convection by reducing the size of the analysis chamber. However, any perturbing effects of the chamber walls on the gradient will be more significant according to surface area / volume ratio of the analysis chamber.

Adjusting the chemical state of the system. The most effective method of reducing bulk fluid flow is to introduce viscous barriers to such flow. This was done in the previous equilibrium electrophoresis prototype device by forming the gradient in a 3% polyacrylamide gel. Theoretically, if the viscous barrier is uncharged, it will not affect the final electrophoretic equilibrium, but only the kinetics of its formation. However, there remains questions about the dielectric constant and activity of the surrounding buffer in the presence of such agents. In these experiments, the viscous agents cyclohexaamylose and urea had no apparent beneficial effect on gradient formation, and in fact, the addition of 8.0 M urea to 2.5:2.0 mM KCl PO$_4$ buffer prevented TA lysozyme from forming a gradient. Since gradient formation is a kinetic process, it may be that the reduction in convection by viscous agents is offset by a proportional slowing of gradient formation.
Lower temperatures also seem to help stabilize gradients, but with the poor temperature control on the present device it was impossible to verify this observation. However, with improved temperature control, lower temperatures ought to be tested.

**Measuring the Electric Field**

In the present device, the electric field is computed by measuring the current during the experiment and measuring the conductance of the buffer before the experiment. It would be better to measure the electric field directly with high impedance reversible sensing electrodes; this would circumvent uncertainty due to non-ideality of the driving electrodes and changes in the buffer conductance. However, this improvement would complicate the mechanical design of the analysis chamber. It is not self-evident where the electrodes should be placed because introducing them into the analysis chamber itself may distort the electric field.

A way to improve the measurement of the electric field in the system without introducing extraneous devices into the analysis chamber would be to sense current actually passing through the solution with a shielded magnetic sensor and compute the field using the conductance of the buffer. Measuring current in this way circumvents the problem of non-ideality at the driving electrode, but it may be difficult to perform this measurement accurately due to the low amounts of current used in equilibrium electrophoresis. It also does not address the problem of unstable buffer conductance.

**Producing Current**

The problems caused by the non-ideality of the electrodes, inaccurate current measurements and electrolysis reactions, leads to the question, why not use reversible electrodes. The problem with reversible electrodes is that the electrode itself, usually a heavy metal like silver, must be able to dissolve into the solution. The presence of these electrode ions will disturb greatly most biological solutions. In previous experiments, silver-silver chloride electrodes were tried and abandoned; an insoluble yellow precipitate formed wherever the silver ions came in contact with the Tris buffer solution. If it is
possible to isolate the buffer chamber from the electrode chamber chemically, reversible electrodes may be preferable. One way in which this might be done is to place an osmotic membrane between an electrode chamber with saturated silver chloride and a buffer chamber with any other standard buffer. The osmotic membrane would prevent the mixing of the two solutions, yet the current could be carried by $\text{H}^+$ and $\text{OH}^-$ ions through the membrane.

Another possible way of generating an electric current for equilibrium electrophoresis would be to abandon electrodes entirely and use an electro-magnetic device to generate current in the solution. The proposed device would be essentially a dc generator with tubes of buffer replacing the copper wire conductors in which current is normally generated. Such a device would produce current without an electrode - buffer interface; this avoids the problems of both reversible and nonreversible electrodes. It also offers the possibility that buffer exchange would be unnecessary, since buffer ions would not congregate or react at an electrode, but instead would make a complete electrical circuit.

**Time to Equilibrium**

In the present system, the observed equilibrium times averages about 12 hours. Since the limiting factor in attaining equilibrium is diffusion, which depends on $\sqrt{t}$, a chamber with a height of 1 mm instead of the present 5 mm would come to equilibrium in $1/25$ of the present time. This would enhance the number of experiments that could be performed in a reasonable amount of time.

There are some disadvantages to reducing the size of the analysis chamber. The optical setup of the system limits the size of the chamber that can be accurately imaged. In the present device, about 0.5 mm of the image at both the top and bottom of the chamber cannot be imaged because of optical fringes. The height of the chamber also limits the portion of a gradient that may be observed; the possible curvature of the line is limited in a limited space.
CONCLUSIONS

The present equilibrium electrophoresis device is a useful method of obtaining the apparent charge on molecules, over a very limited range of conditions. The limiting factors are the amount of current carried by the macroion and the stability of the analysis chamber from convective currents. The most important resulting limitation is that only low ionic strength solutions will form stable gradients. Next, very large ions, like pBR322, are not suitable experimental objects in the present device. Finally, macroions with a low charge may not form stable gradients.

Decreasing convection by mechanical design improvements is expected to improve the usable range of the device. The extent of improvement is not possible to judge due to the random nature of convection.

No significant theoretical limitations with the basic idea of equilibrium electrophoresis were evident in these experiments. Practical improvements to instrument design are clearly possible, and these improvements will increase the quality and range of information provided about the electrostatic properties of molecules.
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