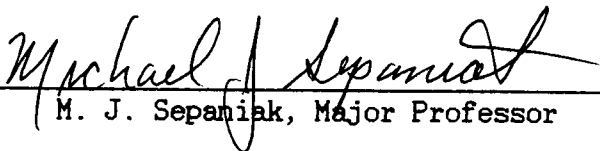
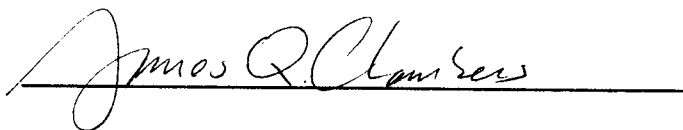


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PRELIMINARY STUDIES OF IMMUNOAFFINITY DOPED CAPILLARY ZONE
ELECTROPHORESIS (ID-CZE)

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Susan E. Schonlaw-Finniss

May 1992

DEDICATION

This thesis is dedicated to my mother, Patricia K. Schonlaw, who
always encouraged me to pursue my dreams,

and, in loving memory,

to my father, Gary E. Schonlaw, and my grandmother (BAGS), Julia A.
Krupnik, who watch me from above.

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Finally, I wish to thank my husband Gary M. Finniss for cooking me dinner on that Sunday night. His patience, understanding, humor, and love provided me with strength and determination that is indescribable. I am eternally grateful.

ABSTRACT

Preliminary experiments were performed to test the feasibility of developing a new immuno-analytical technique, Immunoaffinity Doped Capillary Zone Electrophoresis (ID-CZE). This technique combines the high sensitivity of CZE and the selectivity of immune affinity electrophoresis. Alteration of the electrophoretic mobility of an analyte by selective interaction with an affinity reagent doped into the mobile phase was the primary emphasis of this investigation. Three different systems were investigated: avidin-biotin (a non-immunogenic system), FITC-anti-FITC (a hapten-anti-hapten immunogenic system), and IgG-anti-IgG (an antigen-anti-antigen immunogenic system). The avidin-biotin system showed that the analyte's mobility could be altered in a doped mobile phase. The complex formed by the interaction of FITC with anti-FITC was presumably a FITC labeling product and not the result of an affinity interaction. No definitive IgG-anti-IgG complex was visualized. This result was most likely due to either complex and free reactant mobility similarities or poor detection limits. Experimental conditions are studied and calculations are presented that could be used to determine fundamental parameters such as K_a values.

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CHAPTER I

INTRODUCTION

When a hyphenated technique is produced, the background and theory from the two previous techniques is generally important. This introduction will briefly discuss the relevant background and theory important to the development of "Immunoaffinity Doped Capillary Zone Electrophoresis" (ID-CZE).

A. *CAPILLARY ZONE ELECTROPHORESIS (CZE)*

1. BACKGROUND

Jorgenson in his book New Directions in Electrophoretic Methods described electrophoresis as the "most powerful method available for separating complex mixtures of charged biopolymers" [1]. Transport of molecules by an electric field has been around for about 150 years; Tiselius was the first to separate a mixture of proteins by electrophoresis [2]. Electrophoresis is the migration of particles under the influence of an electric field, E . The observed current, I , and the applied voltage, V , are related by Ohm's Law ($V = IR$) where R is the resistance of the electrophoretic medium. Charged particles migrate in accordance with Stokes law such that the force generated by the effective charge of the particle and the potential gradient are balanced by the frictional resistance of the buffer medium [3]. Hence, the particle will have a unique mobility, μ , and velocity, v , in an electric

field as described by Equation 1:

$$v = \mu E \quad (1)$$

where μ is equal to ze/f (charge/ friction coefficient) [3].

Factors that influence a particle's mobility include: effective radius of the particle, pH of the buffer, ionic strength of the buffer, and viscosity of the buffer [3,4]. Ions with a small ionic radius will be extensively solvated by solvent molecules, which, in turn, results in a lower conductivity. A particle with acidic or basic functional groups (e.g., proteins) will be influenced by the medium's pH such that at a given pH, a net ionic charge will be established. The net charge on a particle will influence the magnitude and sign of μ . The ionic strength of the buffer can also be an important parameter in that it influences the amount of heat generated in an electrophoretic separation. Viscosity of the buffer will also influence the conductivity of the solution, which, in turn, affects the mobility of the solute.

There are four major electrophoretic techniques: moving boundary, zone, isotachopheresis, and isoelectric focusing (IEF) [1,3]. The two latter techniques involve focusing particles in a gradient, either an ion mobility gradient (isotachopheresis) or a pH gradient (IEF). Both techniques yield good separation but have limited use. Isotachopheresis separates either cations or anions but not both simultaneously and IEF separates according to isoelectric point (pI). Moving boundary and zone electrophoresis have wider uses because they separate particles by

differential mobility. Both moving boundary and zone electrophoresis are based on the same separation principle; however, in zone electrophoresis a narrow band of sample is applied whereas in moving boundary electrophoresis a large band of sample is applied. Thus, zone electrophoresis allows complete separation of sample components into pure zones, and, as a result, zone electrophoresis is the most widely used electrophoretic separation technique.

Electrophoretic techniques have two other classifications. Electrophoresis can be performed either in stabilizing media (e.g., gels) or in free solution. An advantage of free solution electrophoresis is that there are no adsorption or eddy migration problems. On the other hand, gel electrophoresis is more widely used because it alleviates the major difficulty in electrophoresis, zone broadening caused by temperature gradients. Unfortunately, gel electrophoresis lacks the sophisticated instrumentation associated with comparable high resolution techniques such as High Performance Liquid Chromatography (HPLC). Even still, it is the preferred separation technique among biotechnologists.

According to Jorgenson *et al.* [1, 5-7], the first attempt at developing a free solution zone instrumental apparatus began with Hjerten. His apparatus involved rotation of three millimeter inner diameter (i.d.) tubes along their horizontal axis in order to control convection problems associated with heating. Mikkers, Everaerts, and Verheggen tried a different approach [8,9]. Their apparatus consisted

of narrow bore Teflon columns having an i.d. smaller than three millimeters. By decreasing the i.d. "the drag of the stationary tube acting on the fluid," or "wall effect," reduces the dispersive effects of convection [1, 5-9]. The deleterious effects of radial temperature gradients within capillaries can also be avoided by employing microcapillaries (i.d.'s $\leq 100 \mu\text{m}$), that efficiently dissipate the heat generated in the electrophoretic process. Jorgenson and Lukacs were the first to demonstrate this effect, using capillaries of $75 \mu\text{m}$ i.d. [5,6]. As a result, zone broadening resulting from temperature differences in the capillary was minimized. Zone broadening was largely attributed to longitudinal diffusion. This form of electrophoresis is called capillary zone electrophoresis (CZE).

2. THEORY AND EQUATIONS

Both cations and anions can be separated by CZE. This separation capability is the result of a phenomenon called electroosmotic flow [1, 5-10]. Electroosmotic flow is the consequence of a "zeta potential" formed within a diffuse layer near the capillary surface. When an electric field is applied, solvated mobile ions migrate and effectively transport solvent through the capillary. The magnitude of the zeta potential, the viscosity of the solution, and the applied electrophoretic field are parameters that influence the direction and magnitude of the electroosmotic flow. The greater the zeta potential, the stronger the electroosmotic flow. As previously stated, both cations and anions can be separated in a single experiment.

Anions electrophoretically migrate toward the anode (positive electrode) while the cations electrophoretically migrate toward the cathode (negative electrode). Each solute ion's migration rate is influenced by the magnitude and the direction of the electroosmotic flow. A strong electroosmotic flow in the direction of the cathode causes anions, cations, and neutrals to be transported to a common end (cathodic end) of the capillary.

Electroosmotic flow produces a "plug-like" velocity profile [5-7]. Consequently, band broadening, due to resistance to mass transfer in the mobile phase [10], is minimized because fluid velocity at the center of a zone is approximately equal to the fluid velocity near the wall. In the absence of thermal band dispersion, the solute band width is usually limited by longitudinal diffusion. The resultant electrophoretic peak is Gaussian in shape and highly efficient (i.e., sharp solute bands are observed).

Equations describing separation performance in CZE have been derived from other chromatographic techniques which can be used to determine a solute's migration velocity and time in the capillary, the efficiency, and resolution of solute components [1,5-7]. Migration velocity, v_P , is the velocity at which a solute particle moves through a column of a certain length when an electric field is applied. It can be written as follows:

$$v_P = (\mu + \mu_{eo}) V / L \quad (2)$$

where μ_{eo} is the coefficient of electroosmotic flow; V is the total applied voltage; and L is the length of the column. Migration velocity is inversely proportional to column length and directly proportional to applied voltage. The electroosmotic flow, as discussed above, can also influence the solute's velocity, depending on its magnitude and the direction of the flow. Under a given set of operating conditions, it is generally more difficult to alter the mobility (μ) of a solute.

Migration time is defined as the time it takes for an ion to migrate the length of the column when an electric field is applied. Migration time, t , can be written as follows:

$$t = L_D / (\mu + \mu_{eo})E \quad (3)$$

where L_D is the length of the column to the detector and E , the field strength, is equal to the applied voltage divided by the total length of the column. Migration time is dependent on the field strength. Therefore, E must be kept constant in order for migration times (or mobilities) to be reproducible on a day to day basis.

Efficiency of a separation is usually described by the number of theoretical plates, N [5]. If band dispersion is limited strictly by longitudinal diffusion, the efficiency of a CZE separation is given by the following equation:

$$N = (\mu + \mu_{eo}) V / 2D \quad (4)$$

where D is the solute's diffusion coefficient [1]. The diffusion coefficient and the solute's electrophoretic mobility, which are characteristic to a solute under a set of operating conditions, cannot be manipulated to optimize N . In practice, the best efficiency is achieved at high voltages since this minimizes analysis times and, hence, longitudinal diffusion. Excessive voltages, however, can cause zonal broadening from the generation of Joule heat. Heat generated in an electrophoretic separation is only dissipated at the outer capillary surface and at the ends of the capillary. Because mobilities are temperature dependent [1, 5-7], radial temperature gradients cause band broadening. One way to prevent radial temperature gradients is to use applied voltages that do not exceed the capillary's ability to efficiently dissipate Joule heat. Attaining high efficiency usually requires optimization of the following parameters: applied voltage (discussed above), capillary diameter, pH, injected sample plug length, and buffer ionic strength.

Decreasing the inner diameter of the capillary reduces band broadening in two ways. First, radial temperature differences are minimized. Reducing the i. d. of the column also increases the column's surface to internal volume ratio, which serves to facilitate heat dissipation. As mentioned above, radial temperature gradients result from Joule heat only being dissipated at the capillary surface. When the capillary i. d. is large, molecules in the center of the capillary tend to move at a different velocity than those at the capillary surface, causing band broadening [5-9]. Second, if the column diameter

is reduced, molecules diffuse across the radius of the capillary many times while traveling down the column. By "randomizing" their position, molecules spend less time in one particular region of the capillary, and thus, their velocity is averaged, minimizing radially associated zone broadening.

Improvements in CZE efficiency can also be achieved by pH manipulation. Proper adjustment of pH can significantly reduce solute-wall interactions (adsorption problems) [11,12]. For example, in the case of proteins, which is the highlight of this research, increasing the pH above the pI of the protein imparts a net negative charge on the protein. This negatively charged protein will be electrostatically repelled by the negatively charged capillary surface, thus, adsorption will be reduced. Coating the capillary walls with a silanization reagent, e.g. chlorodimethyloctadecylsilane (C18), also reduces protein adsorption problems [11,13]. The coating covers the charged sites of the capillary so that the protein is not attracted to the surface. Adsorption of proteins to the capillary wall usually causes severe tailing [6,11], therefore, by manipulating the pH or by using coated capillaries, efficiency should increase.

The ionic strength of a buffer affects efficiency in the following ways: high ionic strength buffers can reduce tailing and fronting problems, resulting from overloading; conversely, high ionic strength buffers can exacerbate thermal dispersion. Several papers have addressed this subject [1, 5-10, 14]. The best buffers for a CZE separation, to date, include zwitterionic buffers with a small amount of

salt added for conductivity and the mixed buffer phosphate-borate. The type of buffer used depends upon the protein, the pH at which the experiment is performed, and the type of column.

Several papers have also examined the effects of sample overloading on efficiency of a CZE separation [1,15,16]. Sample overloading causes band broadening due to electric field distortions. Generally, "fronted" peaks are observed. Ideally, the initial sample plug should be no more than 1% of the length of the column [15], and the analyte concentration should be two orders of magnitude lower than that of the mobile phase electrolyte [16].

Finally, efficiency can be affected by derivitization procedures. Protein detection limits in CZE can be enhanced by derivitizing the protein with a fluorescent label. Jorgenson *et al.* [6] and, recently, Swaile *et al.* [17] have discussed the problems with labeling proteins and the techniques that are used to combat these problems. In essence, labeling proteins causes band broadening due to the formation of a complex mixture of products. Consequently, the majority of CZE protein separations utilize absorbance detection.

Resolution (R_s) of two components in a sample is another important parameter which affects separation performance in CZE. Resolution can be defined by the following equation:

$$R_s = [N^{1/2} / 4][(\mu_2 - \mu_1) / (\mu_{ave} + \mu_{eo})] \quad (5)$$

where μ_1 and μ_2 are the electrophoretic mobilities for solute 1 and 2, respectively; and μ_{ave} is their average electrophoretic mobility. Resolution depends on the differential mobility of components 1 and 2 (or μ_1 and μ_2), the electroosmotic flow, and N . In order for two components of a sample to be resolved, their mobilities must differ. If their mobilities are the same, the two components will elute at the same time. The magnitude and direction of the electroosmotic flow influences the average electrophoretic mobility. If the electroosmotic flow dominates, then the two zones will migrate at approximately the same rate--no separation will occur. In addition, if efficiency is poor then two zones will not be resolved because R_s is proportional to $N^{1/2}$.

In summary, CZE is a powerful separation technique which separates both cations and anions. The optimal separation conditions include short columns, for short analysis times, and high voltages, up to the onset of thermal problems, to insure high efficiency. High efficiency in protein separations, however, is difficult to achieve due to adsorption problems. Therefore, protein CZE separations remain a challenge to researchers.

B. IMMUNOAFFINITY ELECTROPHORESIS

1. BACKGROUND

Affinity Chromatography takes advantage of the specific attraction of proteins for chemical groups [18]. A classical example of affinity chromatography given in biochemistry texts is the purification of lectins using sugar columns. Lectins are plant proteins that have an

affinity for specific sugar residues. For example, concavalin A (con A) crude extract can be purified on a glucose column. Con A is then released either by competitive binding elution with free glucose or with chaotropic reagents. Immunoaffinity chromatography, a type of affinity chromatography, employs antigen-antibody interactions. An antigen is a foreign molecule which elicits an immune response in an organism. This immune response generates specific antibodies directed against the antigen. Antibodies recognize specific sites on antigens called epitopes.

There are five main classes of antibodies (or immunoglobulins)-- IgG, IgM, IgD, IgA, IgE-- that have similar structures but different functions. The structure of a common antibody (IgG) is shown in Figure 1. IgG is composed of four polypeptide chains linked by disulfide bonds [19]. There are two side chains having a molecular weight of about 50,000 Da (heavy chains), and two other chains of about 25,000 Da (light chains). The functional activity of the different parts of the antibody molecule was determined by enzymatic digestion [20]. In essence, the antibody molecule can be visualized as Y-shaped, consisting of two identical Fab portions and an Fc portion (Figure 1). The Fab portion consists of the amino terminus of the heavy chain and the adjacent light chain; the Fc portion consists of the carboxy terminus of the heavy chains. Furthermore, antibody molecules contain a carbohydrate moiety, attached in the IgG molecule to the Fc portion. The site of the antibody that is specific for an antigen is the amino terminus of the Fab portion. This site is called the variable region because the amino

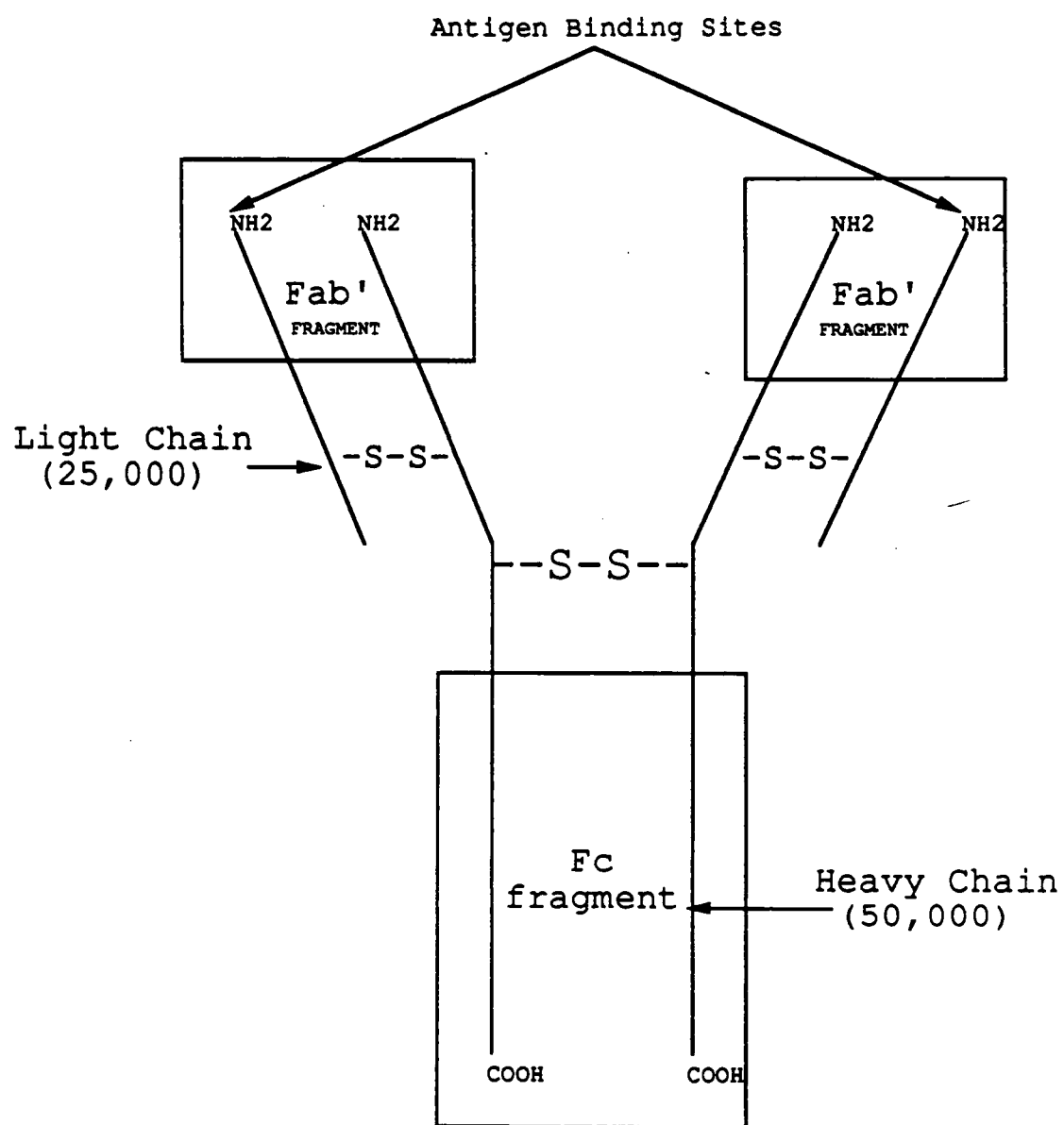


FIGURE 1 Structure of a Common Antibody (IgG).

acid sequence here varies with antibody type. The affinity interaction is non-covalent and a wide variety of antibodies can be produced for a range of antigens [21].

Immunoaffinity chromatography can be converted to an electrophoretic technique by taking advantage of the amphoteric nature of proteins. Proteins, because they are composed of amino acids with ionizable side chains, can be manipulated by pH to produce a species with a net ionic charge, which will migrate toward a specific electrode when an electric field is applied. Consequently, many important immunological diagnostic techniques have evolved [19,20,22]. There are three major techniques: a) immunoelectrophoresis; b) countercurrent electrophoresis; c) rocket electrophoresis.

Immunoelectrophoresis involves a gel electrophoretic separation of an antigen mixture followed by antibody diffusion into the gel matrix. Precipitin arcs, formed by antibody recognition of antigen, are visualized by staining the gel. A comparable technique is countercurrent electrophoresis. In this technique there is no diffusion step. Conditions are chosen such that the antibody and antigen have different charged groups, therefore, they migrate in opposite directions. The antigen is placed at the anode; the antibody at the cathode. When the electric field is applied, the antigen migrates toward the cathode; antibody, on the contrary, migrates electro-osmotically [22] toward the anode. When antibody meets antigen precipitin arcs are formed.

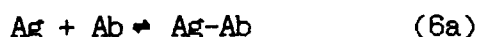
Immunoelectrophoresis and countercurrent electrophoresis are

traditionally used as qualitative techniques. Rocket electrophoresis, on the other hand, is a quantitative technique [19]. In this case, the antibody is incorporated into the gel and the antigen is dissolved in the running buffer. Again the antibody and antigen have different charges; thus, when an electric field is applied, the antigen migrates toward the cathode while the antibody remains immobile. Here a "rocket" precipitin is formed. The length of the precipitin arc formed is proportional to the antigen concentration. Therefore, unknown antigen arc heights can be compared to standards to obtain quantitative information.

The three techniques described have been in use for about twenty years. Their sensitivity and usefulness has only been matched by "crossed techniques."

2. THEORY AND EQUATIONS

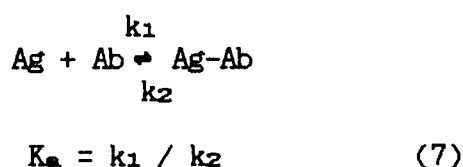
Affinity electrophoresis is based on the strength of, or the affinity of, an antigen-antibody bond. This non-covalent bond is the summation of the attractive and repulsive forces existing between specific regions of antigen and antibody [20]. The affinity of an antibody (Ab) is defined as the strength of a single antibody site (one Fab site) for an epitope on an antigen (Ag) [19,20,23]. This reaction is reversible and is characterized by an equilibrium constant, K_a . The equilibrium constant, K_a , for the association of a single antibody site with an antigen epitope can be defined as follows:



$$K_a = [\text{Ag-Ab}] / ([\text{Ag}][\text{Ab}]) \quad (6b)$$

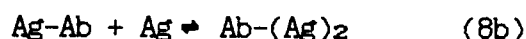
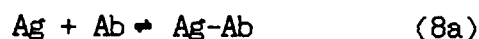
where $[\text{Ag-Ab}]$, $[\text{Ag}]$, and $[\text{Ab}]$ represent the concentrations of antigen-antibody complex, free antigen, and free antibody, respectively.

The reaction above, between antigen and antibody, can also be kinetically described in terms of the "number of molecules which will react in unit time" [24], or the rate of the forward reaction (association), k_1 , versus the rate of the reverse reaction (dissociation), k_2 . The equilibrium constant defined by the rate is then given by the following equation:



When Ab and Ag are first mixed, the forward reaction rate is high while the reverse rate is low. As the reaction proceeds, the concentration of free antibody and free antigen decrease at a rate in proportion with the forward rate constant, while the concentration of the complex increases. Eventually, a dynamic equilibrium is established with forward association and reverse dissociation rates equal. At this time, no further change in the concentrations of the species on both sides of the reaction occurs. In most reaction schemes, a set amount of time is needed for this reaction mixture to equilibrate [25].

The equilibrium presented above considers only a single antibody and antigen site. But, in fact, antibodies are bivalent (have two Fab portions). Thus, an antibody has two possible sites for binding antigens. Avidity refers to the overall strength of a polyclonal mixture of multivalent antibodies for a multivalent antigen [20,26]. Monoclonal antibodies are antibodies made from a homogenous population of antibodies (antibodies with the same chemical nature), directed against one epitope on an antigen. Hence, the equilibrium constant should be in accordance with the theory discussed for affinity. The reactions possible for a monoclonal antibody are as follows [27]:



Only two reactions are possible. Polyclonal antibodies, on the other hand, can involve complex simultaneous immunochemical equilibria.

Experimental conditions that influence equilibrium constants include temperature and pH. Generally, equilibrium constants decrease with an increase in temperature according to the Gibbs-Helmholtz equation ($[\partial(\Delta G/T)/\partial T]_P = -\Delta H/T^2$) [23,25]. The enthalpy of binding is usually negative, indicating a decrease in binding upon temperature increase. This condition varies with antibody and incubation conditions. The pH of a medium can disrupt complex formation by changing the ionizable side chains on antigen and antibody or by degrading their normal configuration. Hence, classical immunoassays

require optimization and control of temperature and pH [23]. Furthermore, the chemical nature of antibodies affect the complex formation.

C. IMMUNOAFFINITY DOPED CAPILLARY ZONE ELECTROPHORESIS (ID-CZE)

1. JUSTIFICATION

The potential merits of this technique are two-fold. First, from an analytical perspective, immuno-techniques are used extensively for analytical and immune diagnostic separation procedures. Immunoaffinity techniques are selective and versatile (e.g., antibodies can be bought or generated for a wide range of antigens [21]). Moreover, separation of coelutants could be achieved (e.g., an antigen could be selectively resolved from other compounds in the sample matrix). By manipulation of parameters, such as pH, dopant concentration, and buffer ionic strength, the operating conditions could be changed so that the antigen-antibody complex would elute at a retention time different from interferences. Additionally, only small amounts of immune reagents would be needed and the capillary column would be continually regenerated without any of the elution problems encountered in affinity liquid chromatography. Second, from a biotechnologist's perspective, fundamental information about affinity systems could be obtained. Information such as K_a 's, kinetic data, complexes formed (monoclonal vs. polyclonal), and cross reactivity could be determined rapidly, because analysis times in CZE are short (on the

order of 15 minutes or less [7]), and operating conditions could readily be changed. Furthermore, this technique could be used for identification of unknown immunological systems by comparison of fundamental information with standards.

The objective of this research is to test the feasibility of ID-CZE. In an effort to accomplish this objective, three preliminary immunological systems are investigated. The first system, Avidin-Biotin, a non-immunologic system, was chosen because of the availability of the reagents. Unfortunately, the enormous K_a for this system (approximately 10^{15} M^{-1} [28]) proved to be problematic for investigating certain aspects of the ID-CZE system. The second system, Anti-Fluorescein Isothiocyanate (FITC)-FITC, an antibody-hapten system (small molecules such as FITC must be bound to large proteins to elicit an immune response and are referred to as haptens) was also chosen for its availability and because labeling was not required. This system is an immunologic system. The third system, Anti-IgG-IgG, was chosen as an antigen-antibody ID-CZE prototype. These experiments will investigate if the mobility of an antigen (analyte) can be altered in a doped mobile phase.

2. BACKGROUND

In principle, ID-CZE should combine the high efficiency and short analysis times of CZE with the high selectivity of immunoaffinity electrophoresis. As previously mentioned (see section A.1.), CZE was developed as a means for performing electrophoresis in free solution.

Highly efficient separations can be achieved rapidly using CZE. Affinity techniques take advantage of the specificity of the interaction of affinity proteins for specific chemical groups on certain molecules. Immune interactions are highly selective, therefore, several sensitive electrophoretic techniques have evolved. These electrophoretic techniques, unfortunately, rely on stabilizing media (gel support) in order to minimize convective band dispersion caused by radial temperature gradients. Thus, detection and quantification of immune complexes is complicated. Yet these techniques are widely used by biotechnologists because no replacements have been developed. The preliminary objective of this research was to develop ID-CZE as a new immune electrophoretic technique that could replace the existing techniques, in certain applications, as well as provide better quantitative information.

ID-CZE involves doping the mobile phase with an immunoaffinity reagent (generally an antibody, Ab). A sample containing the affinity ligand (the antigen, Ag) is then injected into the column and the mixture equilibrates as electrophoresis is performed. Figure 2a depicts the interaction of Ab with Ag to form an Ag-Ab complex, having a distinct mobility intermediate between the Ab and the Ag. Figure 2b illustrates the hypothetical electropherograms of this interaction between Ab and Ag to form a complex. Notice that in this hypothetical case the Ab has a lower migration rate than the Ag, and therefore, the Ab's retention time is greater than that of the Ag and the complex. Because Ab, Ag, and the complex have distinct migration times, each

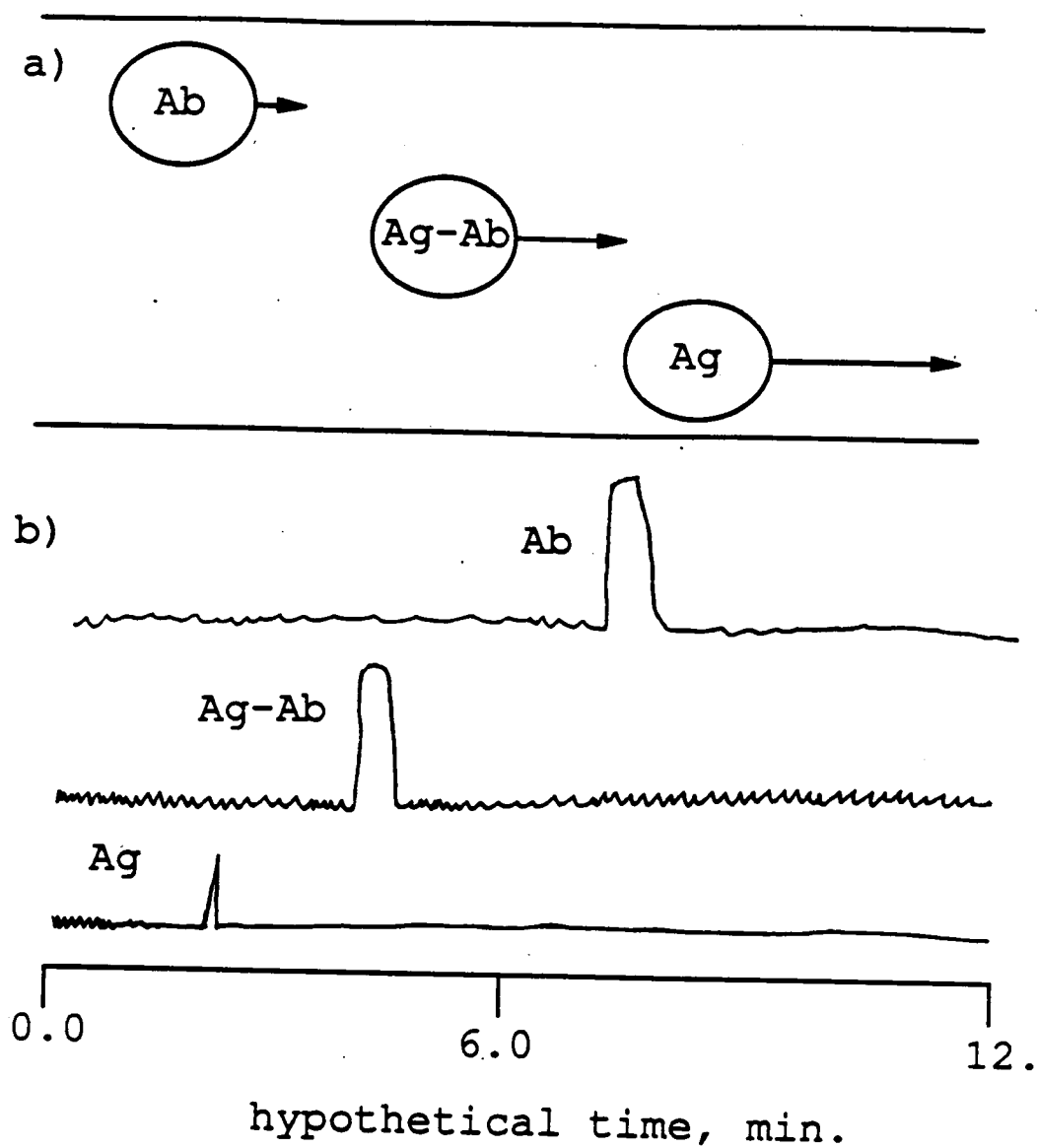


FIGURE 2 Interaction of Antibody with Antigen in the Capillary. a) formation of the antigen-antibody complex with an intermediate mobility, and b) resultant hypothetical electropherogram.

electrophoretic band profile can be distinguished from the others, assuming, in this case, that efficiency is sufficiently high and the electroosmotic flow allows mobility selectivity (see section A.2.). Consequently, the electropherograms can provide both quantitative and qualitative information. Fundamental information, such as K_a values, can also be obtained from this technique (see section C.2.), which would make ID-CZE valuable to biotechnologists. Other research groups are investigating similar free solution systems [27,29]. A group at The Eli Lilly Company incubates Ag with Ab, to "preform" a complex, before injection into the CZE apparatus. The advantages to this approach include initialing a system (seeing if an Ab and Ag will combine) and standardization of a system. The major disadvantage to this approach is that dissociation of the immune complex dominates during the separation process. In order to accurately determine K_a values and other fundamental information about Ag and Ab reactions (as discussed in section B.2.), certain conditions should be optimized. In the "preformed" technique, the Ag-Ab complex formed under one set of conditions is then transferred to another system (the capillary) with different conditions. This transition may have two effects on the equilibrium: a) the complex may be destroyed completely, leaving free Ag and Ab; b) the equilibrium may be slightly shifted, yielding partially associated Ag and Ab. The conditions in ID-CZE, on the contrary, could be optimized to enhance immune complex formation.

In the classical immune techniques, the Ab and Ag are in separate "reaction chambers" (e.g., in rocket electrophoresis, the Ab is in a gel

matrix and the Ag is in the buffer). Another advantage of ID-CZE is that no separation step would be required. Generally, in classical techniques, after the reaction, only complex can be detected. In ID-CZE, on the other hand, the Ab and Ag are in the same reaction chamber before reaction, and, after reacting, all species can be simultaneously detected, if certain reaction conditions are maintained (see section C.3). In addition, concentrated (impure) samples can be separated without a preliminary clean-up step, unlike classical techniques where samples must be relatively pure for reasonable results [26]. These two advantages can decrease the actual assay time.

Hence, ID-CZE could prove to be an important technique for biotechnologists. Fundamental parameters as well as quantitative and qualitative information could be obtained in short analysis times with little clean-up procedures and no separation steps.

3. THEORY AND EQUATIONS

a. Efficiency Considerations

Similar to CZE, both cations and anions should be separable in a single ID-CZE experiment. The electroosmotic flow, which may be altered by the presence of doped protein, is responsible for this separation capability. Once again a plug-like flow should exist and band broadening, in the absence of thermal band dispersion, could be limited by longitudinal diffusion. Unfortunately, the sorption of doped antibody on the surface of the capillary can lead to serious band dispersion. Thus, efficiency in ID-CZE is expected to be lower than in

CZE separations. In addition, "apparent" efficiency will depend upon the nature of Ag-Ab system. If an Ag is injected into a doped polyclonal Ab mobile phase, the system will be inherently heterogeneous (i.e., several different Ag-Ab complexes are formed). If these complexes have different migration velocities, it is likely that broad peaks will be observed. Rapid exchange between different Ag-Ab complexes will minimize this dispersion. On the other hand, the specificity of the ID-CZE technique is the result of large K_a values (see discussion below), and, thus, slow exchange is expected. Monoclonal antibody-doped mobile phases should produce narrower peaks because each monoclonal antibody recognizes only one antigenic epitope. Therefore, only one (or two) type(s) of complexes should exist.

When limited by longitudinal diffusion, high efficiency can be achieved with high voltage. Conversely, excessive voltage will cause band broadening due to convection from temperature gradients. As previously discussed (section B.2.), temperature affects Ag-Ab equilibrium constants. Therefore, high voltages may influence the interaction between Ab and Ag, which can degrade the separation capability of this technique. One way to maintain optimal conditions is to lower the voltage.

Efficiency can also be improved by a) increasing the pH above the pI of the Ag and Ab, b) by using high ionic strength buffers to decrease fronting and tailing effects, and c) by keeping the injected sample plug length less than 1% of the length of the capillary. In ID-CZE, however, these operating conditions may affect the formation of an Ag-Ab complex.

Increasing the pH may affect the environment around the active site of the Ab, thereby, decreasing the Ag-Ab interaction. Buffer ionic strength, on the other hand, plays a role in the solubility of Ag, Ab, and complex [30]. If the ionic strength is too high these proteins can be "salted" (precipitated) out of solution. Furthermore, sample plug length can affect the stoichiometric ratio of Ag and Ab, which, in turn, affects the amount of product formed.

Although efficiency is important in ID-CZE separations, for reasons discussed below, this technique's objectives also include the measurement of fundamental parameters. Consequently, the ability to investigate the influence of such parameters as pH and ionic strength on the formation of Ag-Ab complexes is desirable. Therefore, efficiency considerations will have to be balanced by the ability to alter the electrophoretic mobility of the complex.

b. Separation Performance

The velocity of an antigen band, V_{AG}^* , depends on the electrophoretic velocity of the free antigen, V_{AG} , the antigen-antibody complex, V_{AG-AB} , and the distribution ratio, R , which is the mole fraction of unbound antigen. The velocity of an antigen band is given by Equation 10 [31]:

$$V_{AG}^* = R(V_{AG} - V_{AG-AB}) + V_{AG-AB} \quad (10)$$

R is related to the equilibrium association expression, the K_a value, by

Equation 11a [31]:

$$R = [Ag] / ([Ag] + [Ag-Ab]) \quad (11a)$$

Assuming equilibrium is established, this equation can be rearranged to produce Equation 11b [31]:

$$R = 1 / (1 + K_a [Ab]_{Eq}) \quad (11b)$$

where $[Ab]_{Eq}$ is the equilibrium concentration of the affinity reagent in the region of the antigen band. In Equation 11b, it is assumed that equilibrium is established for the moving band, which is never strictly true in chromatography.

Combining Equations 10 and 11b, the following expression is obtained [31]:

$$V^*_{AG} = [1 / (1 + K_a [Ab]_{Eq})](V_{AG} - V_{AG-AB}) + V_{AG-AB} \quad (12)$$

Antigens (analytes) can be resolved from interfering analytes if their V^*_{AG} values are sufficiently different. Large $K_a[Ab]_{Eq}$ differences between analytes and interferants and large values of $V_{AG} - V_{AG-AB}$ are desirable. In most cases, a "non-specific" interaction will exist between the interferant (usually a protein) and the antibody. In order to separate an analyte and an interferant, it is important to optimize $[Ab]_{Eq}$. Optimal $[Ab]_{Eq}$ values will probably maximize differences in R

(hence V^*_{AG}) and minimize the dispersive effects of slow mass transfer caused by slow rate of Ag-Ab exchange. For instance, when $[Ab]_{E_0}$ is 10^{-6} M and the K_a 's for Ag-Ab interaction and interferant-Ab interaction are 10^6 and 10^4 , respectively, then R_{AG} equals 0.01 and $R_{interferant}$ equals 1.00. These R values are very different and separation should be facilitated. When $[Ab]_{E_0}$ is increased to 10^{-3} M, then the R values are both very close to zero (10^{-5} and 10^{-2} , respectively) and separation should not occur.

Resolution in an ID-CZE separation is given by the following equation [31]:

$$R_s = [(V_{AG2} - V_{AG1})/V_{AG1}V_{AG2}](V_{Ave} \times N^{1/2}/4) \\ \approx \Delta V^*_{AG} \times N^{1/2}/4V_{Ave} \quad (13)$$

where ΔV^*_{AG} is the velocity difference of Ag band one and Ag (or interferant) band two, and V_{Ave} is their average velocity. Equation 13 indicates that even with a relatively small plate count of 10,000 ($N = 10,000$) unit resolution of antigen and interferant can be achieved if their V^*_{AG} values differ by 2%. In the example given above, with $[Ab]_{E_0}$ equal to 10^{-6} , if the antigen and the interferant have identical mobilities, but their associated Ag-Ab complexes have velocities that differ by 4%, resolution will be achieved. Since it is unlikely that there will be only one interferant, the ability to continuously alter V^*_{AG} over the range V_{AG} to V_{AG-AB} via adjustments in $[Ab]$ will often be important.

c. Fundamental Parameters (calculation of K_a Values)

ID-CZE can be used to measure fundamental parameters such as K_a values, which would be useful to biotechnologists. Equation 6 (see section B.2.) defines the reaction between antigen and antibody to form an immune complex that is characterized by an equilibrium constant. Measuring equilibrium constants requires measurement of equilibrium concentrations, or any parameter that is related to concentration. In this technique, mobility differences are important, therefore, they would be used to obtain K_a values.

The experiment would involve doping the mobile phase with different concentrations of antibody, followed by the injection of antigen at a constant concentration, and the measurement of the difference in velocity of the antigen band (V^*_{AG}) in the doped mobile phase versus the undoped mobile phase. Hence, a plot of ΔV^*_{AG} (Y-axis) versus $[Ab]$ (X-axis) could be obtained, where $[Ab]$ is the concentration of antibody doped into the mobile phase. Figure 3 shows this hypothetical "titration-like" curve. At the beginning of the titration ($[Ab] = 0$) no complex is present, whereas, at the end of the titration ($[Ab]$ is large) only complex is present. The midpoint of the titration is the point where half the antigen and the antibody have reacted to form complex (i.e., $\Delta V^*_{AG} = 1/2 \Delta V^*_{AGmax}$ and $[Ag] = [Ag-Ab]$). The equilibrium concentration of antibody at this point should be equal to $1/K_a$. This antibody equilibrium concentration is then given by Equation 14 [31]:

$$[Ab]_{E_1} = [Ab]_0 - 1/2[Ag]_{inj}. \quad (14)$$

where $[Ab]_0$ and $[Ag]_{inj}$ are the concentrations of doped antibody and injected antigen, respectively. Although this equation does not take into account the dilution of the antigen as it travels through the column, it should provide a reasonable approximation of $[Ab]_{E_1}$. At extremely high or low values of K_a it may not be possible to adjust conditions to produce the curve shown in Figure 3 (see later discussion related to the avidin-biotin system).

In summary, ID-CZE has the potential of replacing classical immune electrophoretic techniques because of the fast analysis times and little preparative work. In addition, fundamental parameters could be obtained. This research will provide insight into the potentials and obstacles of this technique, which, in the future, may help other scientists to develop this technique as a practical immune diagnostic technique.

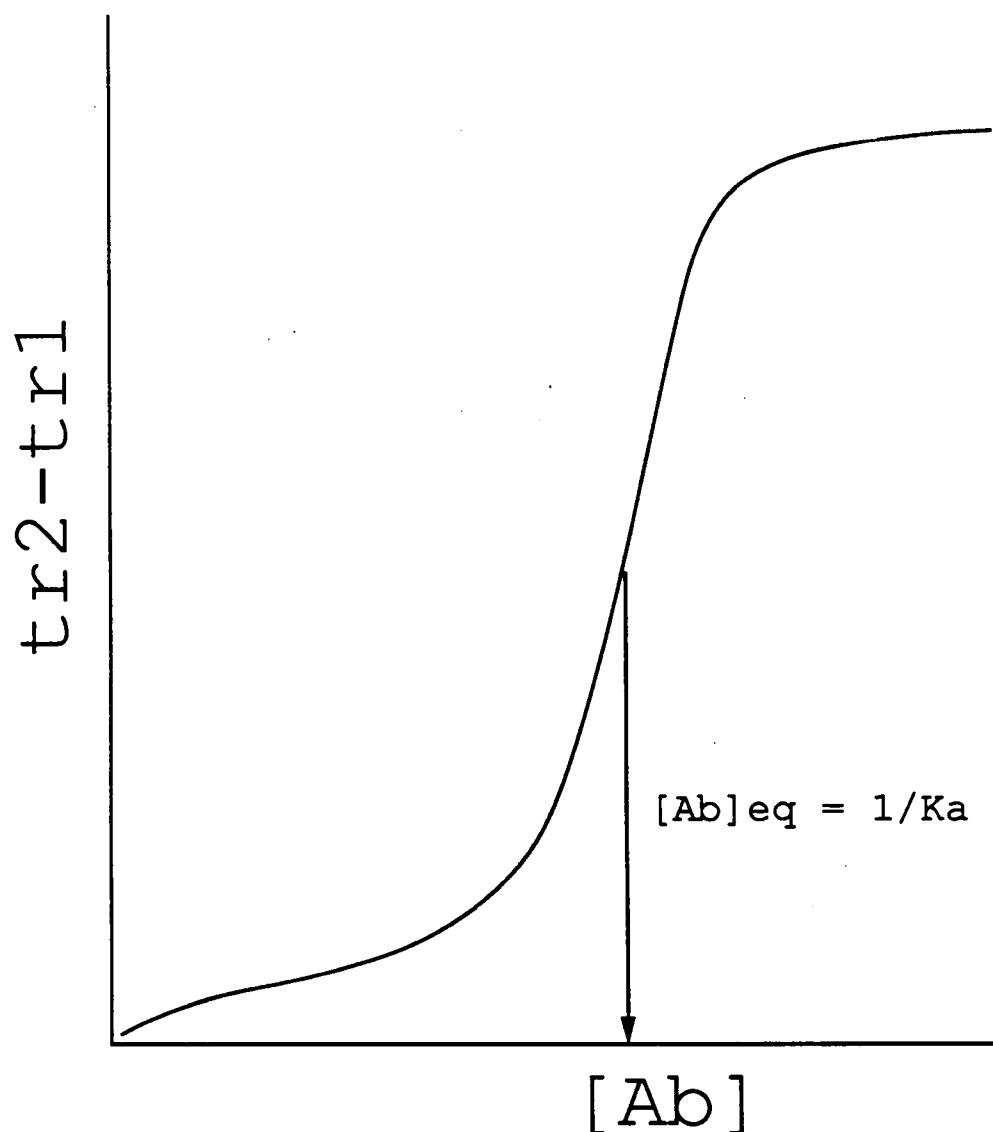


FIGURE 3 Hypothetical Titration-like Curve for Determination of K_a Values. ΔV^*_{Ag} is $tr2 - tr1$, $[Ab]$ is the concentration of antibody doped into the mobile phase, and $[Ab]_{eq}$ is where half of the antigen and the antibody have reacted to form complex.

CHAPTER II

EXPERIMENTAL

A. CZE APPARATUS**1. APPARATUS**

The CZE apparatus used in this work is shown in Figure 4. Polymicro Technologies Inc. (Phoenix, AZ, USA) fused silica capillary columns (50 μm I.D., 300 μm O.D., and approximately 50 cm in length) were arranged such that the capillary's inlet and outlet were emersed in buffer reservoirs maintained at equal heights to prevent hydrostatic flow. Electric fields were generated with a Hipotronics (Brewster, NY, USA) Model 840A high-voltage power supply. The inlet end of the buffer reservoir was maintained at a positive potential and the outlet end was at ground potential. A cell holder containing the capillary was placed in front of the detection system. The detection system was placed at the outlet end of the capillary, approximately 10 cm from the end of the column.

2. DETECTION

Two types of detection were employed. In early experiments (avidin-biotin and FITC-anti-FITC), on-column laser fluorescence detection was utilized; the later experiments (IgG-anti-IgG) employed absorbance detection. The laser detection system, shown in Figure 5, employed a Coherent (Palo Alto, CA, USA) Model 52 Regulated DC Argon Ion

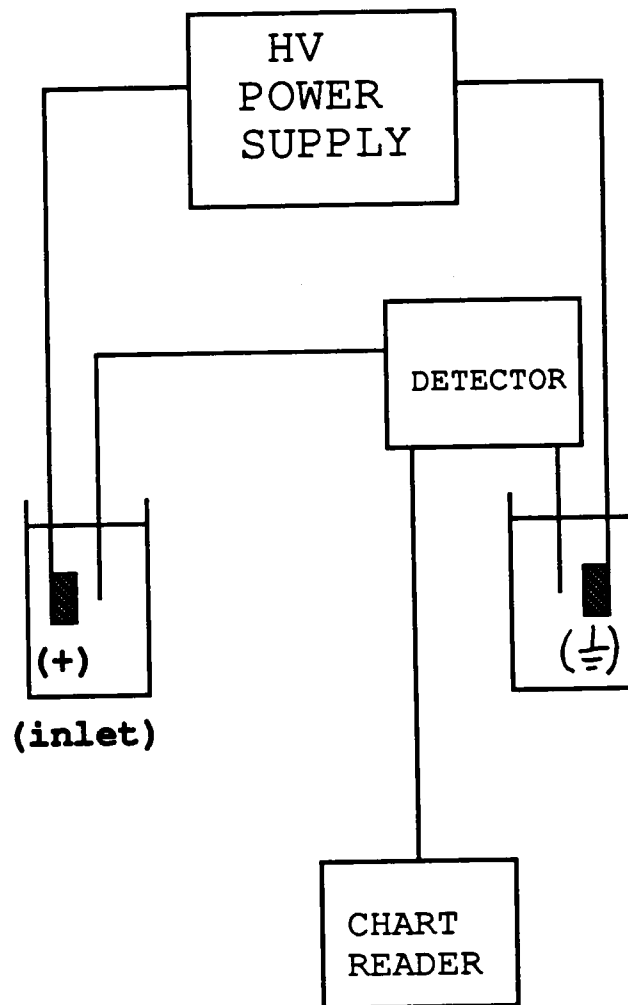


FIGURE 4 CZE Apparatus.

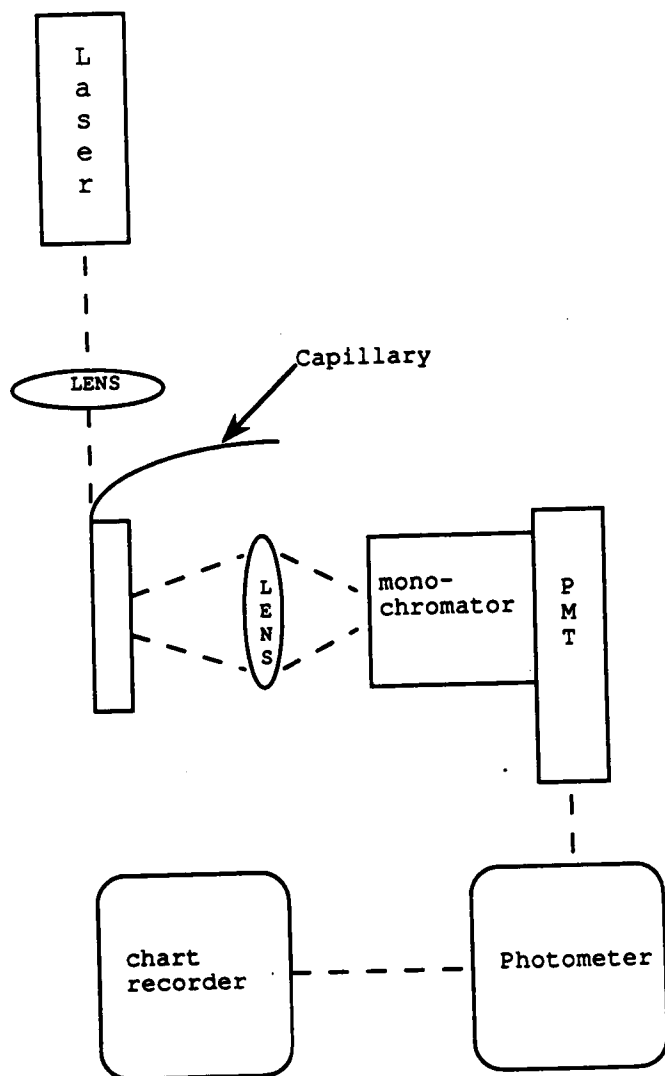
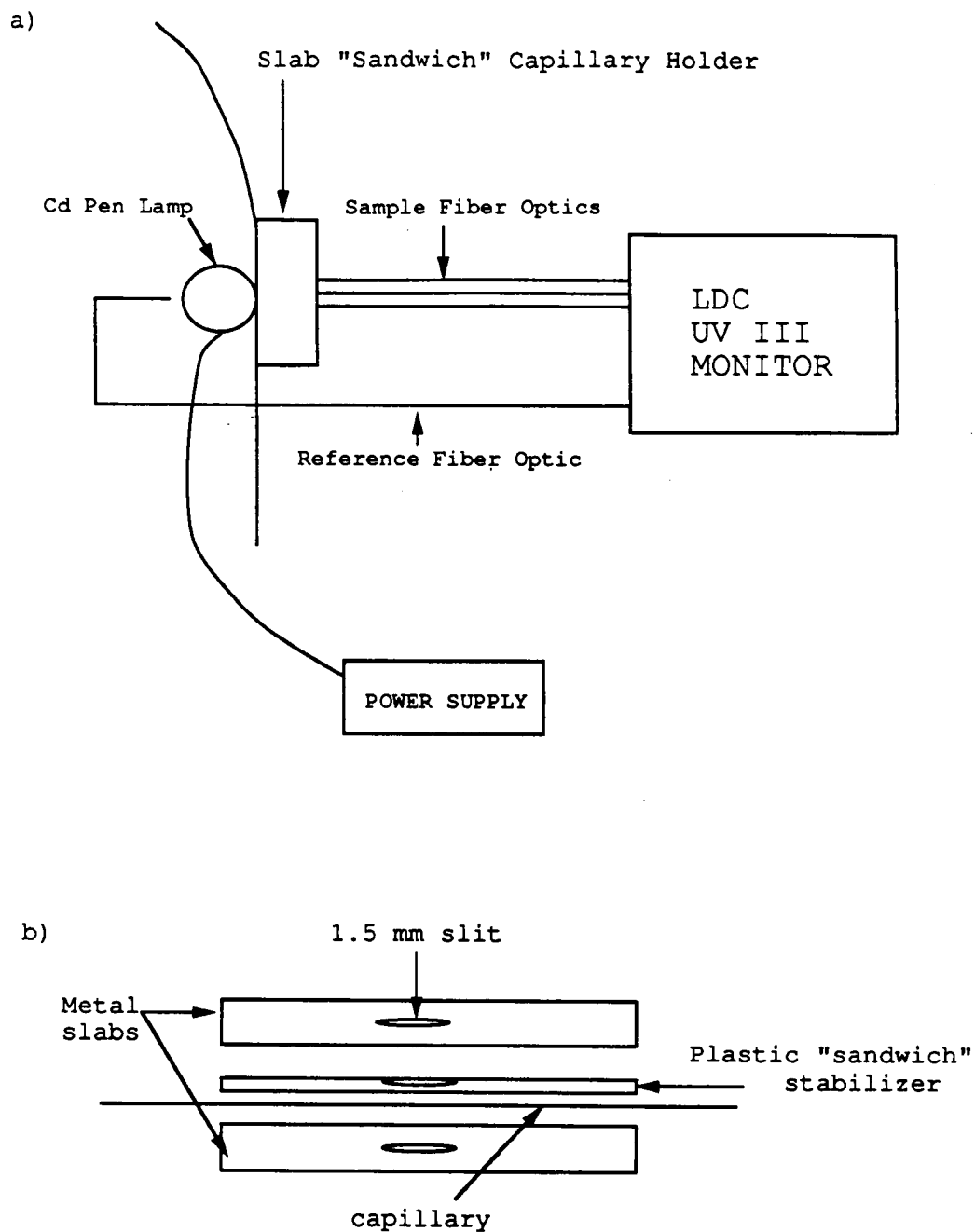


FIGURE 5 ID-CZE Laser Detection System.

Laser (488 nm, 20mW) for excitation. Fluorescence emission at 525 nm was collected and isolated with a bandpass filter (540 nm maxima, 60 nm FWHM) and an Instruments SA (Metuchen, NJ, USA) Model H-10 monochromator, set at 525 nm with a 6 nm bandpass. Signals were detected with a RCA (Lancaster, PA, USA) Model 1P28 photomultiplier tube, processed with a Pacific Precision Instruments (Concord, CA, USA) Model 126 photometer, and displayed with a Kip and Zonen BD40 (Delft-Holland) chart recorder. Two 25 mm diameter x 35 mm focal length achromat lenses (Oriel Corp.) were used to focus the laser radiation onto a flow cell (cell holder containing the capillary) and collected the fluorescence. Optical windows for detection were made by burning a short section of the polyimide coating on the fused silica near the end of the capillary. The capillary was attached to a metal cell holder with epoxy and placed in a fiber-optic positioner (Newport Corp., Model FP1). Alignment of the flow cell was accomplished according to the procedure described by Sepaniak and Cole [32].

A single channel absorbance detection system described by Sepaniak *et al.* [33] was modified by R.O. Cole (University of Tennessee, Knoxville, USA) was also used in this work. Figure 6 outlines the details of this detection system. A Laboratory Data Control (LDC) UV III monitor with a cadmium pen lamp, 229 nm output (UVP, Palo Alto, CA, USA), excitation source was utilized. The lamp was placed adjacent to the flow cell. Light passing through the flow cell's slit was collected and delivered to the sample photodiodes of the detector by three 400- μ m

**FIGURE 6**

ID-CZE Absorbance Detection System.

a) Detector (reprinted from reference 33) and b) slab-metal "sandwich" flow cell (designed by R. O. Cole, University of Tennessee, Knoxville).

-diameter UV-grade optical fibers (General Fiber Optics, Cedar Grove, NJ, USA) arranged in a line, yielding an approximate effective slit height of 1.2 mm. A 200- μ m-diameter UV-grade optical fiber was employed in the reference channel. The flow cell, designed by R.O. Cole, consisted of a slab-metal frame slit having an approximate slit width of 1.5 mm. The capillary column was "sandwiched" between the metal slabs. This type of construction provided capillary stability.

B. MATERIALS AND SPECIAL TECHNIQUES

1. ANALYTES

Antibodies, strepavidin, and Fluorescein Isothiocyanate (FITC) were purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium fluorescein was purchased from Eastman Kodak (Rochester, NY, USA). The strepavidin was purchased unlabeled and later labeled following a modified procedure (described below). Fluorescein-labeled biotin [5-((N-(5-N-(6-(biotinoyl)amino)hexanoyl)amino)pentyl)thioureidyl)fluorescein] was purchased from Molecular Probes (Eugene, OR, USA). Polyclonal IgG's were purchased in 1.0 mL portions, having 1.0 mg of total protein/mL. Monoclonal antibody to FITC was purchased as 3.3 mg of active protein in 1.0 mL of ascites fluid.

Strepavidin (m.wt. 60,000) solution was made at a concentration of 1.0×10^{-5} M by taking 1.2 mg of dry strepavidin and diluting to 1.8 mL with phosphate-buffered saline (PBS); 100 μ L aliquots were stored at -20° C. Stock fluorescein-labeled biotin (m.wt. 831) solution was made at a concentration of 3.8×10^{-4} M by diluting 1.1 mg with 3.5 mL of

PBS; the remaining fraction of dry biotin was stored desiccated at -20°C . For the experiment, $11\ \mu\text{L}$ of $3.8 \times 10^{-4}\ \text{M}$ was diluted with $88\ \mu\text{L}$ of PBS to yield a new biotin concentration of $4.2 \times 10^{-5}\ \text{M}$, which was stored in the refrigerator until used. Stock FITC (m.wt. 389.4) solution was made at a concentration of $1.5 \times 10^{-5}\ \text{M}$ and stored in the dark until used. Stock FITC solution was diluted to $1.5 \times 10^{-6}\ \text{M}$ with PBS for the experiments. Stock sodium fluorescein (m.wt. 376.3) solution was made at a concentration of $1.0 \times 10^{-2}\ \text{M}$ and stored in the dark until used. Stock sodium fluorescein solution was diluted to $1.0 \times 10^{-6}\ \text{M}$ for the experiments. Anti-FITC solution was diluted to $2.0 \times 10^{-6}\ \text{M}$ by taking $0.2\ \text{mL}$ of $3.3\ \text{mg}$ active protein/mL ($2.0 \times 10^{-5}\ \text{M}$) and diluting to $2.0\ \text{mL}$ with PBS; $100\ \mu\text{L}$ aliquots were made and stored at -20°C . Polyclonal antibodies (m.wt. 160,000) solutions were stored in $1.0\ \text{mL}$ vials at 4°C , and diluted as needed.

2. BUFFERS

All buffers were made with HPLC grade water. Two buffers were employed: $0.01\ \text{M}$ phosphate- $0.006\ \text{M}$ borate and PBS. Phosphate-borate was made by taking $6\ \text{mL}$ of $0.1\ \text{M}$ sodium tetraborate ($\text{NaB}_4\text{O}_7 \cdot \text{H}_2\text{O}$) and $5\ \text{mL}$ of $0.2\ \text{M}$ disodium hydrogen phosphate (Na_2HPO_4) and diluting to $0.1\ \text{L}$ with HPLC water. The final pH of the buffer was 9.3. Stock solutions of sodium tetraborate and disodium hydrogen phosphate had $0.002\ \%$ sodium azide (NaN_3) added as a preservative. Sodium tetraborate and disodium hydrogen phosphate were purchased from Fischer Scientific (Springfield, NJ, USA). PBS was made by taking $7\ \text{g}$ sodium chloride

(NaCl), 0.2 g potassium chloride (KCl), 1.3 g potassium dihydrogen phosphate (KH_2PO_4), and two potassium hydroxide (KOH) pellets and diluting to 1 L with HPLC water. The pH was brought to 7.4 with 0.1 M NaOH and 0.002% NaN_3 was added to the PBS stock solution as a preservative. NaCl, KCl, and KOH were purchased from Fischer Scientific; KH_2PO_4 was purchased from Mallinckrodt Chemicals (St. Louis, MO, USA).

3. MOBILE PHASES AND COMPLEXES

Doped mobile phases were made by diluting a volume of antibody or strepavidin (in PBS) to a particular concentration with 0.01 M phosphate-0.006 M borate running buffer. For the avidin-biotin system 75 μL of 1.0×10^{-5} M avidin was diluted to 2.0 mL with running buffer at a pH of 7.0, yielding an avidin concentration of 3.8×10^{-7} M. This solution was then diluted (serially) to a final concentration of 3.8×10^{-9} M. A similar series of dilutions was used for the anti-IgG-IgG system, beginning with a doped antibody concentration of 1.0×10^{-8} M and ending with a concentration of 1.0×10^{-9} M. The pH of the running buffer depended on the experiment. For the avidin-biotin and anti-FITC-FITC systems, the pH of the running buffer was 7.0. For the anti-IgG-IgG system, the pH of the running buffer was 9.3.

Preformed complexes were made by taking a volume of antigen (or analyte) at a particular concentration and adding an equal molar volume of antibody (or affinity reagent). For the avidin-biotin system, 10 μL of 1.0×10^{-5} M avidin was added to 10 μL of 4.2×10^{-5} M biotin.

Hence, the avidin to biotin molar ratio was 1:4.2. For the anti-FITC-FITC system, 10 μ L of 1.0×10^{-8} M FITC was added to 10 μ L of 1.0×10^{-8} M anti-FITC, resulting in a FITC to anti-FITC molar ratio of 1:1. With the anti-IgG-IgG system stoichiometric molar ratio solutions of 1:1 and 1:2 were used.

4. DERIVITIZATION PROCEDURES

The avidin labeling procedure was adapted from McKinney *et al.* [34]. A 1.0 mg sample of avidin was diluted with 1.0 mL of PBS; this solution was then added to 50 μ L of concentrated FITC. The mixture was stirred overnight at 4°C, followed by dialyzation for 6 hours against PBS. A spectrum was taken of the labeled avidin on a Hewlett Packard Model 845A Diode Array Spectrometer. The spectrum was kept for comparison with unlabeled avidin, if necessary. Labeled avidin was used only as a retention time marker in the laser detection system; therefore, it was not necessary to compare spectrums. The concentration of labeled avidin was assumed to be 1.0 mg/mL.

C. METHOD

1. AVIDIN-BIOTIN

For the avidin-biotin system, a new column was rinsed with 0.01 M phosphate-0.006 M borate buffer for five minutes by pushing buffer through the column with a syringe pump. The column was then equilibrated electrophoretically with the doped mobile phase for five to

ten minutes. The voltage was turned off, followed by injection of the analyte hydrostatically into the column [35]. The voltage was then turned back on and set to a level $>20\text{KV}$. After the analyte had eluted, the column was rinsed for five minutes with phosphate-borate in order to wash away excess protein. The column was then equilibrated with a new doped mobile phase for five to ten minutes. This process was repeated for each injection for a total of seven injections. The doped mobile phase concentration was decreased step-wise during the experiment. After the experiment, the column was rinsed and stored in buffer solution.

2. ANTI-FITC-FITC PREFORMED COMPLEXES

The anti-FITC-FITC system did not employ doped mobile phases. For this system, column equilibration was similar to the avidin-biotin experiments. The electrophoretic equilibration, however, was performed with phosphate-borate rather than with a doped mobile phase. Analyte injections were executed as described above. The analyte consisted of an antigen-antibody preformed complex. Storage of the column and applied voltages were the same as in the avidin-biotin system.

3. ANTI-IgG-IgG

In this system, the column was again rinsed for five minutes prior to electrophoresis. This time, however, the rinsing procedure consisted of an eight minute 0.02 M sodium hydroxide (NaOH) wash,

followed by an eight minute water wash, which was followed by an eight minute phosphate-borate buffer wash. The column was then electrophoretically equilibrated with the doped mobile phase and analyte was injected. The voltages for these experiments were <20KV. After the analyte eluted, another injection was immediately performed without rinsing the column. The capillary columns were stored as described above.

CHAPTER III

RESULTS AND DISCUSSION

A. AVIDIN-BIOTIN SYSTEM

Avidin-biotin was used as a preliminary ID-CZE system because the reported K_a value is 10^{15} M^{-1} [28,36], indicating a strong avidin-biotin interaction. This large K_a value would assume a strong interaction and facilitate the altering of biotin's mobility in an avidin-doped mobile phase, only if free and bound biotin exhibit different migration velocities. Thus, in principle, analyte could be separated from interferant and the analytical merit of this technique could be justified. On the other hand, this high K_a value suggests that formation of complex goes to completion even with very dilute solutions. Therefore, equilibrium constants could not be measured for this system using the ID-CZE technique.

Avidin (approximate molecular weight 66,000) is a basic glycoprotein, having a pI of 10 [37]. It is stable at extreme acidic and basic pH values, unlike most proteins which lose their tertiary structure (unfold) at extreme pH values [38]. *In vivo*, avidin is a competitive inhibitor of biotin. Green obtained kinetic measurements [39], finding a high forward rate constant ($7 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$) and low reverse rate constant ($9 \times 10^{-8} \text{ sec}^{-1}$) for the avidin-biotin

interaction. The dissociation constant of a single (avidin) binding site obtained from these values is $8 \times 10^{-14} \text{ M}^{-1}$.

Avidin has four subunits, each capable of binding a biotin molecule [40]. Equation 14 describes the overall interaction:



where Av, B and Av-B₄ represent avidin, biotin, and complex, respectively. The avidin-biotin complex is also stable at extreme pH values and cannot be readily dissociated.

Streptavidin is a protein produced in bacteria that has properties and biotin-binding affinity similar to avidin. However, streptavidin lacks the carbohydrate moiety found in avidin, therefore, it has an acidic pI [28,37]. This acidic pI would allow more freedom in pH manipulation than the basic pI of avidin (i.e., it is easier to make the charged groups on a protein with an acidic pI negative, such that, the protein can repel the negatively charged capillary). Hence, streptavidin was used in these experiments.

Because of its inherent high sensitivity, laser fluorescence was employed in this initial experiment. Streptavidin was labeled with FITC in order to detect it with the available laser (avidin's natural fluorescence emission wavelength is 338 nm [37]). Unfortunately, labeling proteins degrades efficiency, and tailing of the streptavidin peak was expected [17]. Thus, labeled biotin was used as the analyte (because it is not a protein, labeling should not cause extreme

degradation of efficiency, due to multiple labeling sites), and unlabeled streptavidin was doped into the mobile phase. Additionally, the pH of the electrophoretic running buffer (phosphate-borate doped with streptavidin) was maintained at a neutral pH, indicative of common immunoassays. An internal standard (electroosmotic flow marker) was added to each sample to observe and, if possible, correct for changes in the electroosmotic flow with time.

Before doping the mobile phase, labeled avidin and labeled biotin were injected into an undoped mobile phase in order to compare their retention times. These electropherograms are shown in Figures 7a and 7b. Avidin elutes before biotin (pI 3.5 [41]), indicating that avidin has a higher mobility than biotin in this buffer (phosphate-borate, pH 7.0). A preformed avidin-biotin complex at a one-to-four avidin to biotin stoichiometric proportion was also injected to see where the complex would elute. This electropherogram is shown in Figure 7c. The complex elutes at a retention time between avidin and biotin. Additionally, no free detectable biotin is present, suggesting that the K_a is large. Undetectable biotin may also be the result of CZE detection limits (approximately one femtomole injected [17]). If the reaction was in accordance to Equation 14, then the reaction should have gone to completion. Dissociation of the complex should yield only a small amount of biotin, which may be less than the CZE detection limits reported. This preformed complex also displays band broadening. The band broadening is possibly caused by: a) slow exchange rate between the forward and reverse reactions, resulting in different avidin-biotin

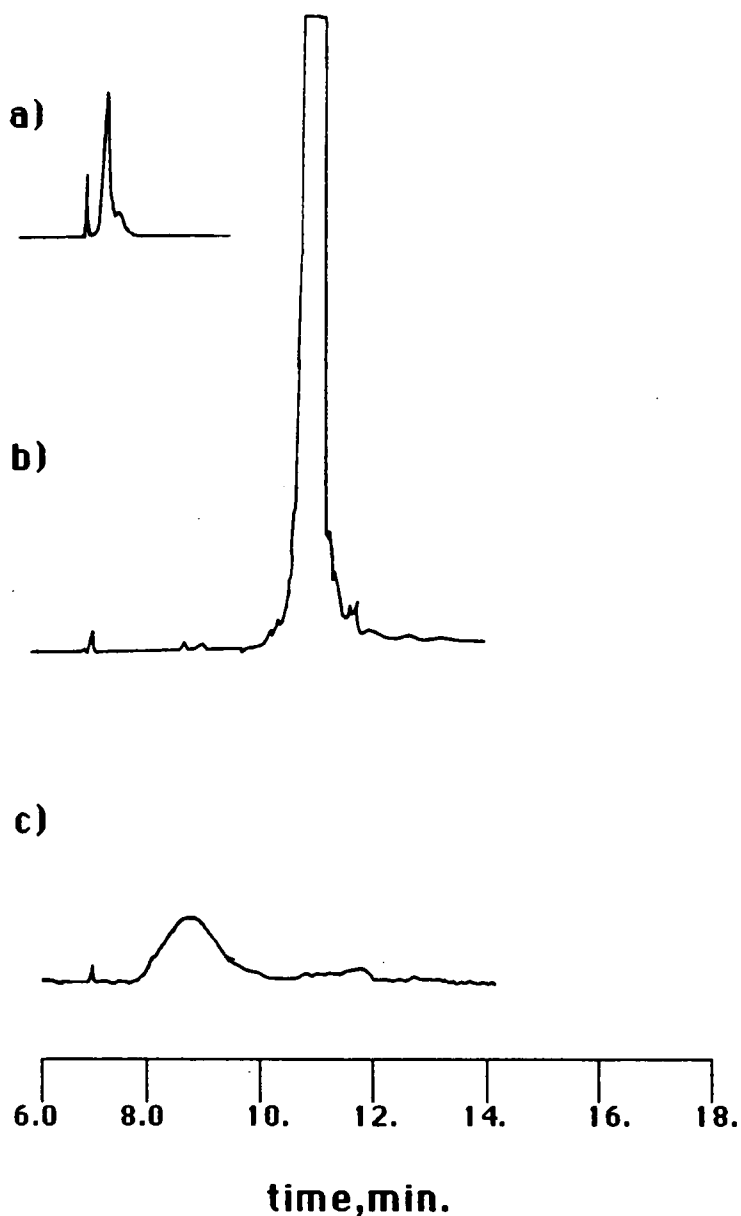
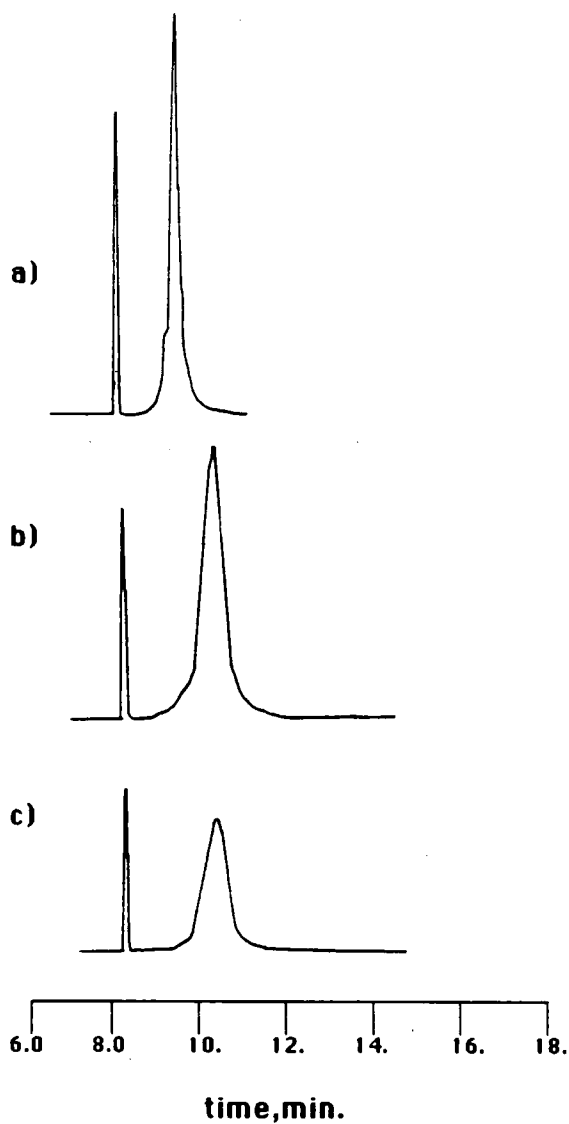


FIGURE 7 Avidin-Biotin Interactions in an Undoped Mobile Phase. a) avidin, 1.0×10^{-5} M, b) biotin, 4.2×10^{-5} M, and c) avidin-biotin "preformed" complex (4 moles of biotin to one mole of avidin). The internal standard in each electropherogram is methylamine (retention time 7.8 minutes).

complexes with different velocities, b) by decomposition of the preformed complex by introduction into an environment different from that in which the complex was formed (discussed in chapter I, section C. 2.), and c) adsorption of the complex to the capillary walls. Furthermore, a loss of fluorescence signal was observed. Loss of fluorescence may be due to a change in the absorption maxima of the complex as compared to the labeled biotin [37], which would change the fluorescence of the complex.

The next step in the experimental plan involved doping the mobile phase with decreasing concentrations of avidin to see if the mobility of the biotin could be manipulated within a range as defined by Equation 12. Figures 8 and 9 show the results of this experiment. A constant biotin concentration (4.2×10^{-5} M) was injected in each of the experiments. The avidin concentration in each of the experiments was as follows: Figure 8a, 3.8×10^{-7} M; Figure 8b, 1.5×10^{-7} M; Figure 8c, 7.5×10^{-8} M; Figure 9a, 3.0×10^{-8} M; Figure 9b, 1.5×10^{-8} M; Figure 9c, 7.5×10^{-9} M.

In each of the electropherograms different stoichiometries of avidin and biotin exist. In Figure 8a, avidin is in excess (approximately two moles of avidin to one mole of biotin). The mobility and peak profile of the "complex" is similar to avidin. Because the peak is efficient and the retention time is the same as that observed for the preformed complex (refer to Figure 7), it is assumed that the complex is rapidly formed in this excess avidin mobile phase and remains intact during the experiment. In Figures 8b and 8c biotin is slightly

**FIGURE 8**

Avidin-Biotin Interactions in a Doped Mobile Phase with Biotin as the Limiting Reagent. The complex is formed in a a) 3.8×10^{-7} M, b) 1.5×10^{-7} M, and c) 7.5×10^{-8} M streptavidin doped-mobile phase. The biotin concentration injected in each electropherogram is 4.2×10^{-5} M. Methylamine is the internal standard in each electropherogram (retention time approximately 8.1 minutes).

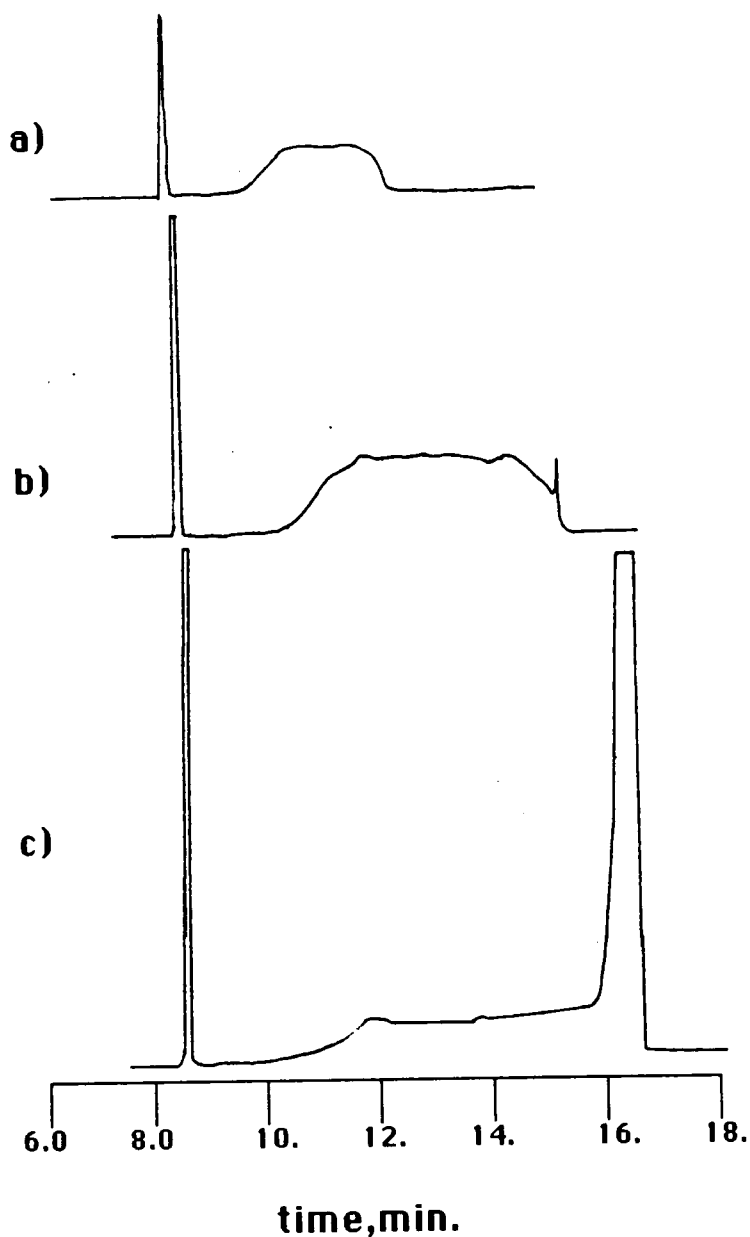


FIGURE 9 Avidin-Biotin Interactions in a Doped Mobile Phase with Avidin as the Limiting Reagent. The complex is formed in a a) 3.0×10^{-8} M, b) 1.5×10^{-8} M, and c) 7.5×10^{-8} M streptavidin doped-mobile phase. The injected biotin concentration in each electropherogram is 4.2×10^{-5} M. Methylamine is the internal standard in each case (retention time approximately 8.4 minutes).

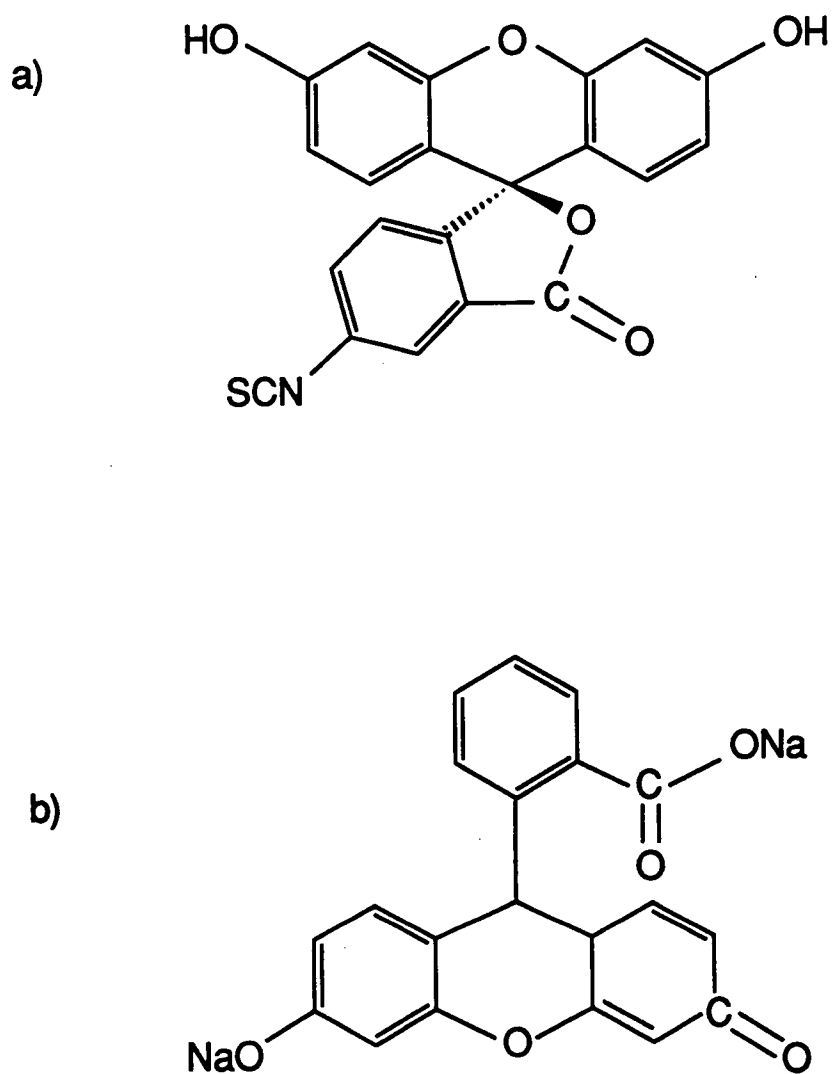
in excess. The efficiency of the "complex" peak decreases as the ratio of biotin to avidin increases (see Figure 9). This loss in efficiency is most likely a result of a mixture of complexes being formed, having different exchange rates and velocities. Thus, as the number of moles of biotin increases, the available binding sites of avidin become limited and a competition between the bound and free biotin exists. Figures 9a, 9b, and 9c show approximately a five, ten and twenty-fold biotin excess. Excess biotin in Figures 9b and 9c (retention time 15.1 and 16.1 minutes, respectively) elutes from the column without ever encountering avidin. In addition, the "complex," having a faster migration time than excess biotin, seems to broaden. This is presumably the result of a mixture of complexes being formed which have different exchange rates and velocities. The broadening can also result from adsorption of dopant to the capillary walls. Avidin in Figure 9c is probably completely consumed and, therefore, equilibrium is not reestablished. Because the K_a value is large, no free biotin species exist, making it nearly impossible to alter the velocity of the complex in a controllable manner as discussed in Chapter I, Section C.3.b. In order to resolve free biotin from complexed biotin, the concentration of biotin would have to be smaller than the limits of detection. Manipulation of pH and buffer ionic strength to reduce the K_a was not attempted because the avidin and the complex are stable in extreme experimental environments [38]. Other types of biotin may exhibit a different affinity toward avidin [37], which should be investigated in this system as a means for measuring K_a values.

Retention time reproducibility also needs to be addressed. In this preliminary experiment the retention time of the internal standard changes with electropherogram. This shift in retention time is caused by the following: a) injection of biotin before capillary equilibration was complete, and b) adsorption of streptavidin to the capillary wall. Capillary equilibration is essential when the doping method is non-continuous (see a more detailed discussion in Section C of this Chapter). If the injection is made before the dopant has established equilibrium with the capillary walls, electroosmotic flow changes can occur, as suggested by the change in retention time of the internal standard between each electropherogram. Adsorption of streptavidin can be removed by washing the capillary after each electrophoretic run. Unfortunately, when electrophoresis resumes more streptavidin will adhere to the walls, and, if the old protein had not been completely eliminated, alteration in the electroosmotic flow will result. Consequently, reproducible retention times are a problem, which should be further investigated in order for this technique to be of analytical merit. BioRad and Supelco [42,43] have developed surface modified capillary columns which virtually eliminate electroosmotic flow and minimize protein adsorption. Their columns are polymer-coated (BioRad) or carbon-based (Supelco) fused silica capillaries, which have been shown to produce reproducible results at various pH's. In the case of the BioRad column, 500 continuous runs can be performed in one column. These columns would assure reliable results and should be tried in future experiments.

B. ANTI-FITC-FITC SYSTEM

The avidin-biotin preliminary studies showed that an analyte's mobility could be altered in a doped mobile phase, but, due to the large K_a , manipulation of parameters did not provide the desired control of retention or the capability to determine K_a values. Common antibody-antigen systems have K_a values between 10^8 to 10^{12} M⁻¹ [44]. The K_a of the anti-FITC-FITC system was anticipated to be in the range of common immunological systems, therefore, it was chosen as an alternate ID-CZE test system. Furthermore, detection and efficiency were not expected to be problematic because labeling was not required.

FITC is a small molecule (m.wt. 389), having the structure shown in Figure 10a. FITC, because its molecular weight is less than 1000, must be conjugated to a large protein like bovine serum albumin (BSA) in order to elicit an immune response *in vivo*. This conjugation is to the NCS part of the FITC molecule, shown in the figure. Therefore, the antibody is directed against the ringed structure [45]. The anti-FITC used in this work was a monoclonal antibody derived from a hybridoma, produced by the fusion of mouse myeloma cells (tumor cells that grow indefinitely) and splenocytes (antibody producing cells) from an immunized mouse. This hybridoma is injected into the mouse and serum (ascites fluid) is collected from the mouse which contains the monoclonal anti-FITC IgG [45]. Ascites fluid also contains other serum proteins and some mouse antibodies that contaminate the ascites fluid. This monoclonal antibody was not affinity purified, therefore, all

**FIGURE 10**

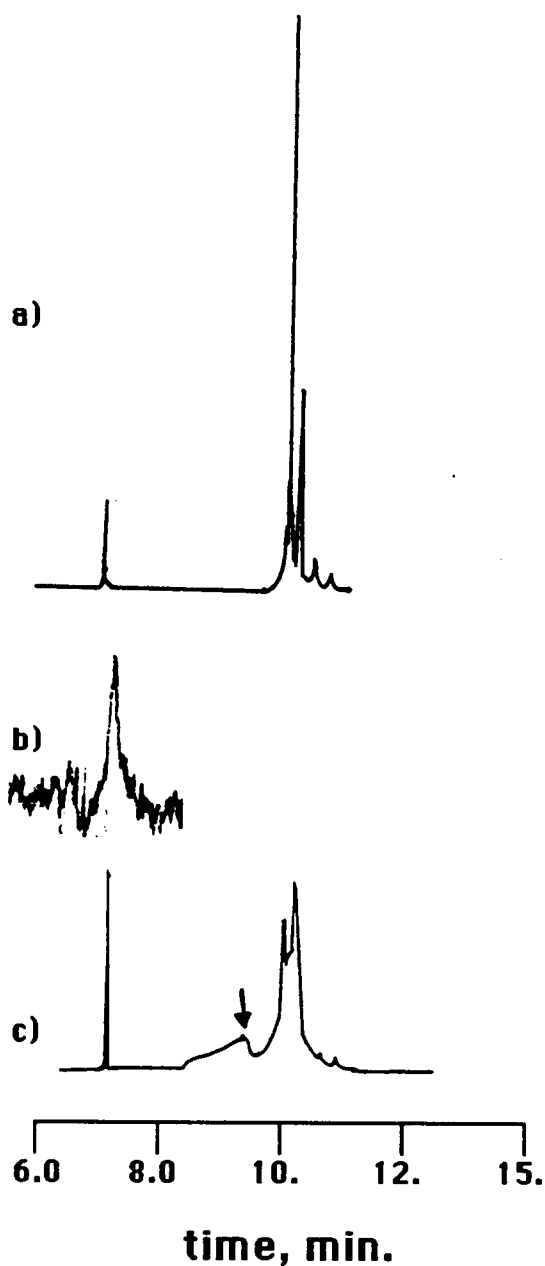
Structures of Fluorescein Isothiocyanate (FITC) and Sodium Fluorescein (NaFl).
a) FITC and b) NaFl

proteins contained in the ascites fluid were present upon injection into the ID-CZE experiment.

FITC, a hapten, has only one immunogenic site. Monoclonal anti-FITC interacts with only one FITC epitope. This hapten-anti-hapten interaction, described in Chapter I, Section B, only has two types of equations. Equations 8a and 8b describe this interaction. Hence, the stoichiometry for optimal complex formation should be two moles of FITC to one mole of anti-FITC.

Before doped mobile phases were used, preformed complexes were injected into an undoped mobile phase. These electropherograms are shown in Figures 11 and 12. FITC (concentration equal to 1.0×10^{-8} M) in Figure 11 has two large peaks, having a retention time of 10.2 and 10.6 minutes, respectively, and two smaller peaks of retention time 11.0 and 11.4 minutes. The two larger peaks are the FITC peaks, and the two smaller peaks are presumed to be degradation products containing, for the 11.4 minute species, sodium fluorescein. Figure 11b is an electropherogram of polyclonal FITC-labeled anti-mouse IgG at a concentration of 1 mg/mL, used to estimate the retention time of the unlabeled monoclonal anti-FITC (retention time 7.4 minutes). The FITC has a lower net mobility than the polyclonal FITC-labeled anti-mouse IgG.

Figure 11c is the electropherogram of the preformed anti-FITC-FITC complex at a one FITC to two anti-FITC molar ratio. A very broad and non-Gaussian band, having a retention time between FITC and the polyclonal FITC-labeled anti-mouse IgG, was observed. Band broadening

**FIGURE 11**

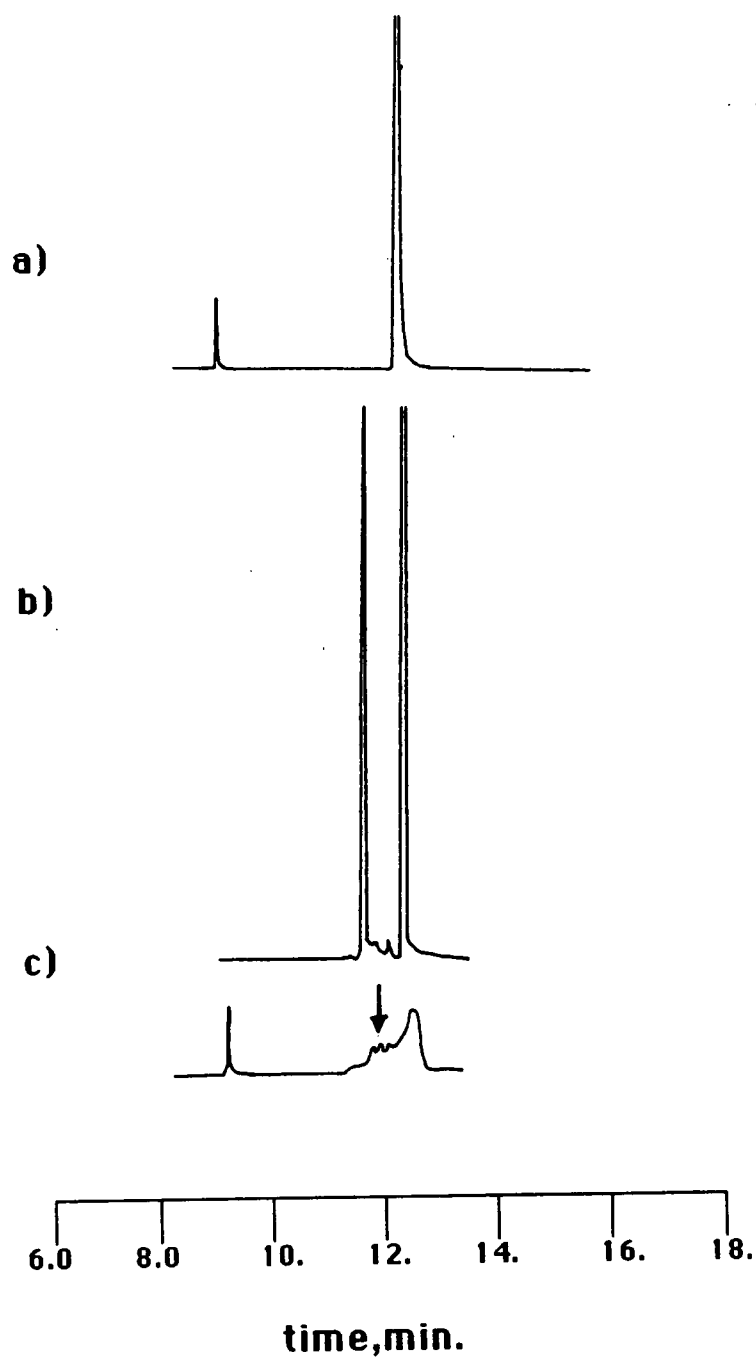
Anti-FITC-FITC Interaction in an Undoped Mobile Phase. a) FITC, 1.0×10^{-8} M; b) polyclonal mouse anti-IgG, 1 mg/mL; c) "preformed" FITC-anti-FITC complex, approximately one mole FITC to two moles anti-FITC (denoted with an arrow). Internal standard in a) and c) is methylamine (retention time approximately 7.4 minutes).

may be the result of two things. First, preformed complex injected into the mobile phase can fall apart, causing band dispersion. Second, other serum proteins in the system, with mobilities similar to that of the complex, may contribute to band dispersion. These problems may be reduced by formation of complex within the column and the selectivity of the ID-CZE system. Excess FITC evident indicates that the K_a for this system is lower than for the avidin-biotin system. Furthermore, the complex (at this stoichiometry) is resolved from excess FITC and measurement of K_a values, using the ID-CZE method discussed in Chapter I, Section C. b., would be possible. The results depicted in Figure 11 demonstrate that the capabilities of ID-CZE may be evaluated with this immunological system. Unfortunately, FITC is a fluorochrome that is commonly used to label proteins for detection in laser based systems. Therefore, the complex peak observed may also be the result of labeling of the FITC monoclonal antibody and other proteins in the ascites fluid.

Sodium Fluorescein, NaFl (approximate m.wt. 360), another fluorochrome, is not known to label proteins. Its structure (shown in Figure 11b) is similar to FITC, and, thus, the monoclonal antibody against FITC was expected to show some cross reactivity toward it (the monoclonal antibody would recognize NaFl). Hence, NaFl was used in subsequent experiments. Two questions were addressed: a) would the NaFl-monoclonal-FITC-antibody complex have a retention time equal to that of the FITC-monoclonal-FITC-antibody complex, or at least a retention time different than the free NaFl, and, b) could NaFl be used to study the possibility of altering an analyte's retention time in the

presence of an interferant (because it was thought originally that FITC and NaFl had identical retention times).

Figure 12 shows the results of an experiment designed to answer these questions. NaFl has a greater retention time than FITC (Figure 12b). Thus, NaFl is not an interferant. The NaFl-anti-FITC complex has a retention time different from the FITC-anti-FITC complex (approximately 11.9 and 9.0 minutes, respectively). In addition, the complex can be distinguished from the free NaFl. Unfortunately, the resolution of free NaFl from complex is quite low (probably on the order of 0.8, which is lower than the presumed FITC-anti-FITC complex). Recall (Chapter I, Section C. a and b) that resolution is a function of the dynamics of various processes in the separation (such as selectivity, $\mu_2 - \mu_1$, and efficiency, N) and the equilibrium properties of the system (defined by V^*_{AG}) [refer to Equations 4,12, and 13]. In order to increase the resolution between free NaFl and the NaFl-anti-FITC complex, N and V^*_{AG} should have been optimized. Increasing N by pH manipulation, for instance, might have degraded the separation and selective processes. V^*_{AG} , which is dependent on the K_a value and the distribution ratio, was also a parameter not easily manipulated. Because of the small difference in migration velocities of NaFl and the complex and the general lack of purity of the reagents, further experiments with the fluorescein system were abandoned.

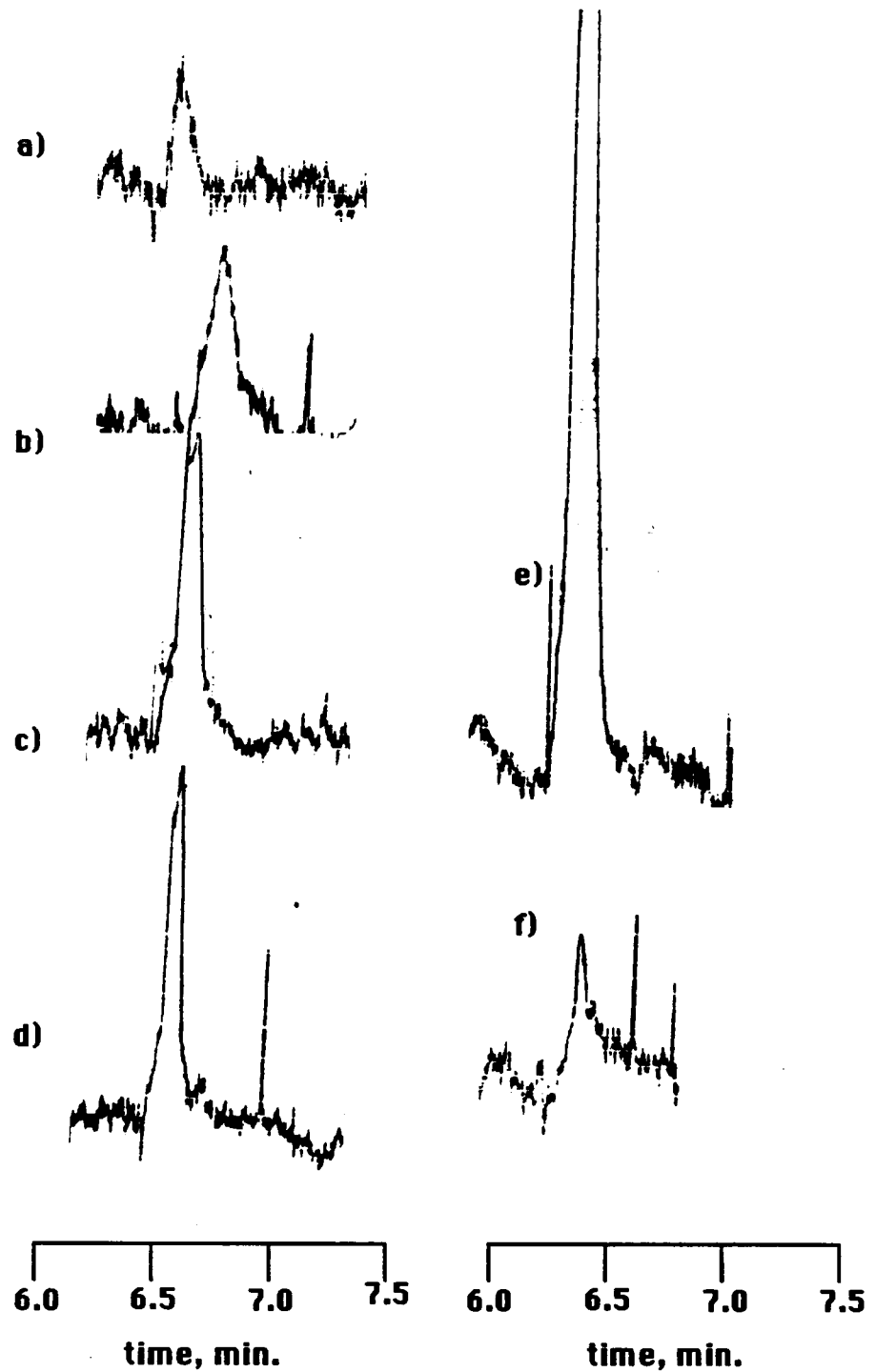
**FIGURE 12**

Anti-FITC-NaFl Interaction in an Undoped Mobile Phase. a) NaFl, 6×10^{-8} M; b) NaFl and FITC; c) "preformed" NaFl-anti-FITC complex, one mole NaFl to two moles anti-FITC (denoted with an arrow). Internal standard in a) and c) is methylamine (retention time approximately 9.1 minutes).

C. *ANTI-IgG-IgG SYSTEM*

The anti-IgG-IgG system was chosen as a immunologic prototype for ID-CZE systems. Experiments were performed to investigate several problematic aspects of ID-CZE systems. These problems include: reproducibility (due to the adsorption of doped protein to the capillary walls) and detection. Other problems that surfaced in the course of this investigation were mobility similarities (between free analyte and analyte affinity reagent complexes) and reactant stoichiometry considerations. These investigations will be discussed in this section as well as the immunoaffinity experiments that were attempted.

IgG [approximate m.wt. 160,000] is a large protein molecule that was produced, in this case, in rabbits (standard immunological procedures) [19,20]. The anti-IgG used in these experiments was produced in goat by injection of rabbit IgG into the goat and collection of serum. Anti-IgG also has an approximate m.wt. of 160,000. The structure of an IgG molecule is shown in Figure 1 (Chapter 1, Section B). The pI of IgG molecules varies with species and isolation preparation and is generally in the range of 6.0 to 9.0 [46]. Figure 13 is an electropherogram of different IgG and anti-IgG species injected into a CZE apparatus. The migration time of all specie IgG molecules is approximately 6.5 ± 0.3 and the migration time of two select (non-labeled) anti-IgG species is also in this range. Thus, resolution of rabbit IgG from anti-IgG was not expected in a mixture of IgG's. Increasing resolution involved either increasing the separation efficiency or altering the mobility of rabbit IgG or rabbit anti-IgG by

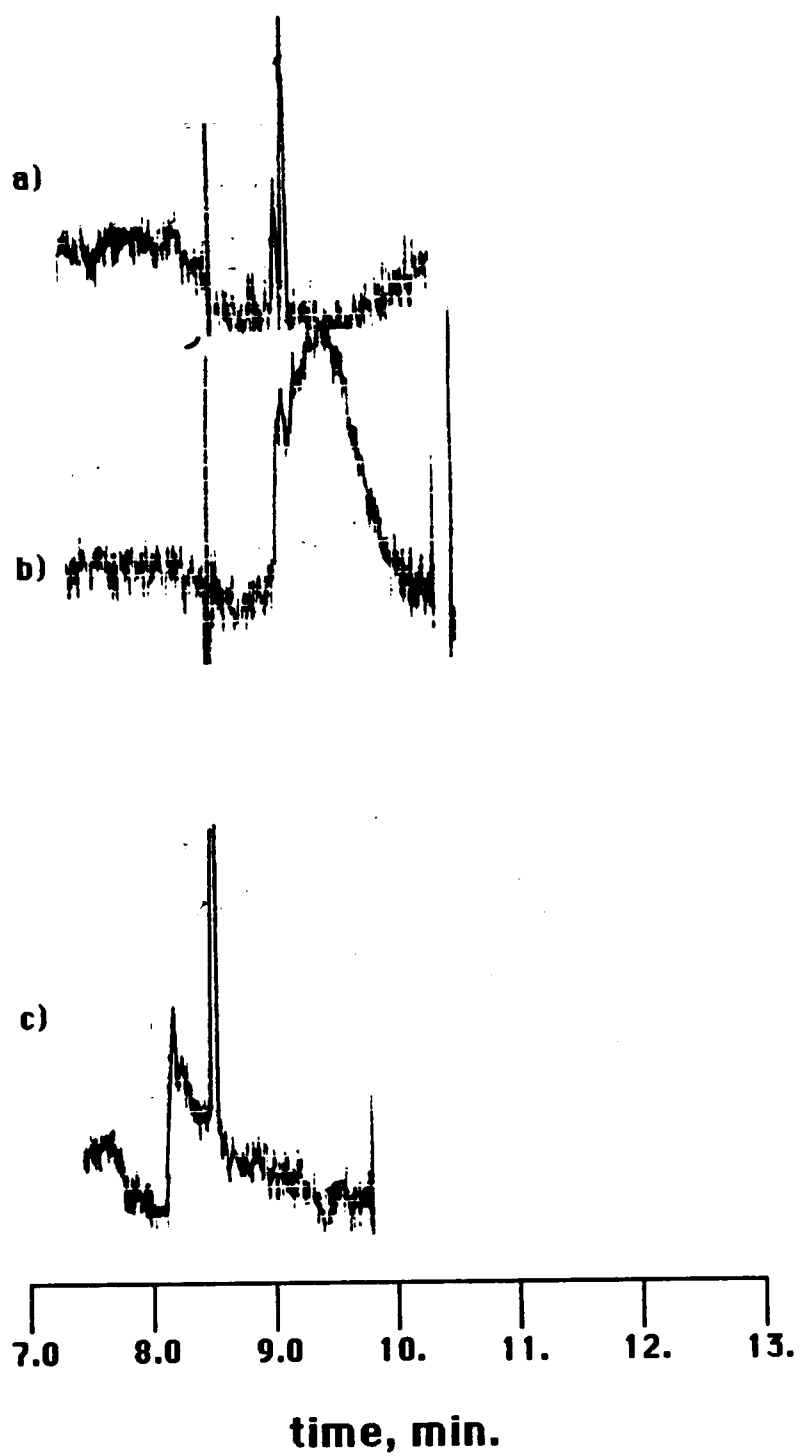
**FIGURE 13**

Mobility Similarities among IgG and Anti-IgG Species in an undoped mobile phase. a) human IgG (1 mg/mL), b) rabbit IgG Fab' (1mg/mL), c) mouse IgG (2 mg/mL), d) rabbit IgG (2 mg/mL), e) human anti-IgG (2 mg/mL), f) mouse anti-IgG (1 mg/mL).

structural modification (labeling or conjugating a charged species to the protein).

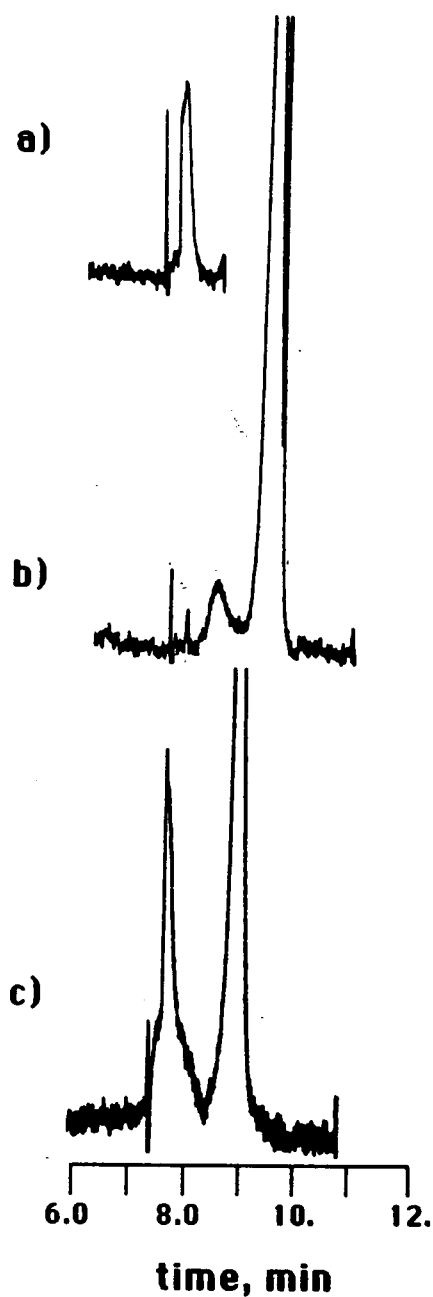
Improvements in CZE efficiency were discussed previously (Chapter I, Section A.2). Protein adsorption to the capillary walls can be reduced by pH manipulation or by coating the capillary walls with a reagent that reduces protein adsorption. Some band broadening is apparent in the proteins displayed in Figure 13, even at a pH of 9.0. Therefore, use of coated capillaries to reduce protein adsorption, and, thus, increasing the resolution of IgG species, was tried. Figure 14 is an electropherogram of rabbit anti-IgG and rabbit IgG, which were injected separately into a Sepulco coated capillary. Although better efficiency results for the rabbit anti-IgG, band broadening is increased for the rabbit IgG as compared to Figure 13. Resolution of rabbit anti-IgG and rabbit IgG was not anticipated. Hence, for this experiment, this type of capillary did not improve the overall efficiency and resolution of components. Labeling rabbit anti-IgG with biotin was investigated in order to improve resolution between rabbit IgG and rabbit anti-IgG. Biotin-labeled rabbit anti-IgG has a different retention time than the unlabeled species (see figure 15) because the label imparts a different charge to mass ratio on the IgG molecule. Two peaks are present for the biotin-labeled rabbit anti-IgG (refer to figure 15). The first peak is probably an impurity present in the preparation.

"Preformed" complexes at different antigen (rabbit IgG) to antibody (rabbit anti-IgG) ratios were injected into the capillary in

**FIGURE 14**

Behavior of IgG and Anti-IgG in a Coated Capillary.
a) rabbit anti-IgG, b) rabbit IgG, c) internal
standard (methylamine).

order to predict the relative migration time of an IgG-anti-IgG complex. Figure 15 depicts the results of this experiment with the antibody as a limiting reagent. Absorbance detection was utilized for these experiments, and, thus, complex detection limits were decreased. In Figure 15c, free labeled anti-IgG and free rabbit IgG are present at 9.0 and 7.8 minutes, respectively. The free rabbit IgG peak is broadened at the base line, indicating that complex may be present but undetectable in the background. Undetectable complex could be the result of four conditions: a) complex may be falling apart when injected as a "preformed" species, b) complex may have a retention time similar to either IgG or anti-IgG, c) the detection limits of the system may limit visualization of complex, and d) stoichiometry of reactants may not be optimal. In a doped mobile phase, the first condition would subside (complex would remain associated) unless the K_a was small. If the second condition was true, resolution of complex from free species would be impossible even in a doped mobile phase. Detection of proteins, as discussed previously (Chapter I, Section A), is a compromise between good efficiency (little band broadening) and good visualization. In these experiments, efficiency was more important. Reactant stoichiometry was important in the avidin-biotin system. For instance, when biotin was the limiting reagent the mobility of the complex was similar to the mobility of the dopant (avidin). Hence, stoichiometry probably plays an important part in complex formation in this system. When doping the mobile phase with antibody these conditions were considered.

**FIGURE 15**

IgG-Anti-IgG Interaction in an Undoped Mobile Phase.
a) rabbit IgG (16 mg/mL), b) rabbit anti-IgG (2 mg/mL), c) rabbit IgG-anti-IgG "preformed" complex (8 mg IgG to 1 mg anti-IgG).

The mobile phase was doped with two different concentrations of antibody (1×10^{-8} M and 1×10^{-9} M). The first experiments involved injection of rabbit IgG at different concentrations to see if a complex could be formed and if the mobility of the analyte (rabbit IgG) could be altered. These experiments are shown in Figure 16. No complex is seen. Injection of "preformed" complex into these doped mobile phases, to force the equilibrium to the right (complex formation), was tried. Again no complex was seen (data not shown). Therefore, it was concluded that either the detection limits of the system were not adequate or that the complex had the same retention time as one of the free species and could not be identified. Furthermore, the mobility of the rabbit IgG in the 10^{-9} M (lower) doped mobile phase is slightly faster than when it is in the 10^{-8} M (higher) doped mobile phase. In addition, band broadening is reduced at the lower dopant concentration. The mobility and band broadening differences observed between the 10^{-8} M and 10^{-9} M mobile phases is probably the result of less protein in the 10^{-8} M mobile phase. Hence, the electroosmotic flow is not changed and no adsorption effects are observed.

Reproducibility is another problem encountered in the ID-CZE system. Reproducibility was problematic in the avidin-biotin system. Recall that the retention time of the internal standard (methylamine) varied with time, which was due to irreproducible adsorption of doped protein to the capillary wall. Two injection schemes were investigated for improving the reproducibility of the system. The first injection scheme, termed non-continuous, involved injection of analyte after the

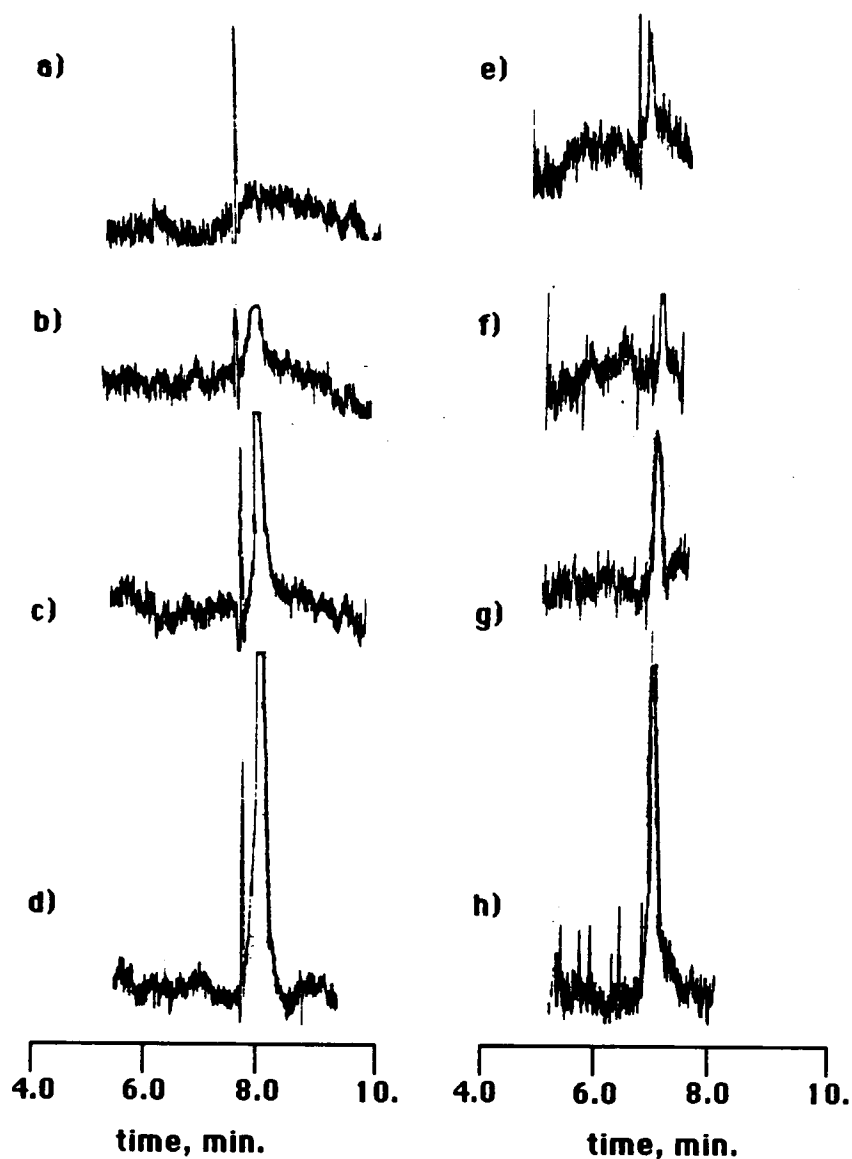


FIGURE 16

IgG-Anti-IgG Interaction in a Doped Mobile Phase.

a)-d) 10^{-8} M and e)-h) 10^{-9} M biotin-labeled rabbit anti-IgG. Rabbit IgG injected concentrations are as follows: a) and e) 0.25 mg/mL, b) and f) 0.5 mg/mL, c) and g) 1 mg/mL, and d) and h) 2 mg/mL.

column had been rinsed with buffer and then reequilibrated with new dopant. This type of injection was employed in the avidin-biotin system. The second scheme, continuous injection, involved equilibration of the capillary at the start of the experiment with dopant, sequential injection of analyte, and regeneration of column after experiments with dopant. This injection method was used in this system and it is the preferred ID-CZE scheme because the capillary can be regenerated with minute amounts of dopant. Unfortunately, large protein molecules, e.g., IgG, adsorb to the capillary walls. This problem is exacerbated with high voltage, which degrades proteins. Columns, which may be more practical in a continuous injection ID-CZE system may be polymer-coated columns [42] or sugar coated columns [47]. These columns should be investigated in future experiments.

CHAPTER IV

CONCLUSIONS

The objective of this study was to test the feasibility of developing ID-CZE as an immunological technique. Alteration of an analyte's mobility in a doped mobile phase was the primary investigation that was considered. Of the three systems investigated, only the avidin-biotin system showed that an analyte's mobility could be altered. Unfortunately, the K_a for this system was enormous and alteration of the analyte's mobility within a given range by manipulation of experimental parameters could not be accomplished. The study of the other two systems, FITC-anti-FITC and IgG-anti-IgG, did not prove to be successful. The preformed complex formed in the FITC-anti-FITC experiments probably was a FITC labeling product and not the result of an affinity interaction. The complex resolution from free species in the alternative FITC system, NaFl-anti-FITC, was lower than in the FITC system and, therefore, inadequate. Hence, doping the mobile phase was not accomplished. The complex in the IgG system, preformed or doped, was not visualized. This result was most likely due to either complex and free reactant mobility similarities or decreased detection limits.

Experimental problems existed in these preliminary experiments. These problems include: reproducibility (adsorption of protein to the capillary walls), detection, and resolution. Adsorption of protein to the capillary walls can present problems in ID-CZE by changing the

electroosmotic flow, which reduces the efficiency. Electroosmotic flow changes were seen in the avidin-biotin system, i.e., the retention time of the marker changed with time. Tailing of proteins (antibodies in the IgG system) was also observed, which probably altered IgG's actual retention time. These problems could be eliminated by using coated columns or a polymer-based mobile phase, which reduce charged sites on the capillary walls.

Detection of proteins in CZE is an obstacle. Labeling with a fluorescent compound can decrease efficiency by producing a mixture of labeled compounds, and, thus, degrade the separation. Hence absorbance detection is the preferred detection scheme. But, for ID-CZE, as potentially exhibited in the IgG system, complexes may not be visualized using absorbance detection. Therefore, alternate CZE protein detection schemes should be investigated before ID-CZE can be of any immunological merit.

Resolution is sometimes inherent to a system. In the avidin-biotin system resolution of free species from complex in the doped mobile phases could not be accomplished due to the large K_a . Mobility similarities were probably the cause of decreased resolution in the IgG system. In the FITC system, decreased complex efficiency affected resolution. Resolution of analyte from interferences is also an important factor in determining ID-CZE separation performance. Hence, optimization of resolution should be a primary concern when investigating other ID-CZE systems.

The preliminary systems investigated were pure. Most biological systems are composed of a complex mixture of proteins. In order for ID-CZE to be of any merit, separation of this complex mixture and selection (and quantitation) of the protein of interest would have to be accomplished. This separation is not an easy project, considering the problems with CZE, discussed in Chapter I, and the problems with ID-CZE encountered in this research. But, developing ID-CZE for this purpose would be invaluable to biotechnologists.

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VITA

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She is currently employed as a Research Associate at the University of Michigan (Ann Arbor), under the direction of Dr. Chris J. Dickinson (Department of Pediatric Gastroenterology).