Mathematical Treatment of Electrophoretically Mediated Microanalysis

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A new concept in reaction-based chemical analysis is introduced and theoretically described. By utilization of the variability in electrophoretic mobilities among charged species, spatially distinct zones of chemical reagents can be electrophoretically merged under the influence of an applied electric field. *Electrophoretically mediated microanalysis* (EMMA) exploits this phenomenon as a basis for chemical analysis utilizing capillary electrophoretic systems. EMMA is described in terms of the four stages required for reaction-based analysis: (1) analyte and analytical reagent metering; (2) initiation of reaction; (3) control of reaction conditions and product formation; (4) detection of species whose production or depletion is indicative of the concentration or quantity of the analyte of interest. The method is illustrated by the enzymatic oxidation of ethanol to acetaldehyde by alcohol dehydrogenase with the concurrent reduction of NAD^+ to NADH monitored at 340 nm. Experimental results for both substrate and enzyme determinations are shown to agree with the presented theory.

INTRODUCTION

Analytical determinations are frequently based upon direct instrumental measurements of analytes. When interfering substances preclude direct analysis, a separation method or a chemical reaction is often added to increase detection specificity. Combinations of separation methods and specific chemical reactions provide maximal specificity. This paper will focus on the use of specific chemical reactions in conjunction with electrophoresis for analytical determinations.

Electrophoresis is the migration of charged species under the influence of an electric field.[1] The electrophoretic mobility μ_{ep} of an ionic species is described by the equation

$$\mu_{ep} = \frac{Ze}{6\pi\eta a} \quad (1)$$

where Z is the effective net charge of the species, e is the electrical charge, η is the solution viscosity, and a is the hydrodynamic radius of the species. The electrophoretic velocity with which the species migrates through a solution under the influence of an applied electric field is the product of the electrophoretic mobility and the electric field strength E

$$v_{ep} = \mu_{ep}E \quad (2)$$

When capillary electrophoresis (CE) is performed in fused silica capillaries, negative charges at the capillary wall cause the formation of an ionic double layer which electrophoretically migrates toward the negative electrode under the influence of an electric field. Bulk liquid in the capillary is pulled along by a phenomenon known as electroosmosis.[2] The resulting migration velocity v_m of any substance in a CE system is the sum of both the electrophoretic v_{ep} and electroosmotic v_{eo} transport velocities

$$v_m = v_{ep} + v_{eo} = (\mu_{ep} + \mu_{eo})E \quad (3)$$

where μ_{eo} is the specific transport mobility due to electroosmotic flow.

Because the electroosmotic component is equal for all substances, differences in transport velocity in a CE medium must be imparted by variations in electrophoretic mobility among charged substances. Cations in a fused silica capillary migrate with a positive electrophoretic velocity in the same direction as the electroosmotically induced flow. In contrast, anions migrate against the bulk flow with a negative electrophoretic velocity. Differences in electrophoretic mobility among charged species have been widely used in electrophoresis as a separation technique.[3,4]

It will be shown in this paper that exploitation of differential transport velocities is equally useful as a mixing technique. Spatially distinct zones of chemical reagents of different electrophoretic mobility can be made to interpenetrate under the influence of an applied electric field as shown in Figure 1. Based upon this observation, electrophoretic methods will be used to initiate chemical reactions by mixing analyte and analytical reagents, control reagent contact time, separate the reactants and detectable product, and transport the detectable product to a detector. This technique is termed *electrophoretically mediated microanalysis* (EMMA). Preliminary studies by Bao and Regnier[5] reported the assay of glucose-6-phosphate dehydrogenase in a CE system via its enzymatic reaction with glucose 6-phosphate and nicotinamide adenine dinucleotide phosphate (NADP) to produce 6-phosphogluconate and reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH exhibits a unique absorbance at 340 nm, thereby serving as an indicator of the extent of the reaction and, consequently, the quantity of glucose-6-phosphate dehydrogenase injected. The purpose of the work presented here is to identify by theory and experimentation those variables that mediate such chemical analyses in a CE system.

MATERIALS AND METHODS

Instrumentation. All experiments were performed using a CE system built in-house. Polyimide-coated, fused silica capillaries (Polymicro Technologies, Phoenix, AZ) of 75 μm inner diameter and 360 μm outer diameter were utilized. The capillaries were of 40 cm total length with separation lengths (distance from injection to detection position) of 25 and 15 cm for the ethanol and ADH determinations, respectively. Electric fields were applied with a Spellman (Plainview, NY) Model FHR 30P 60/EI

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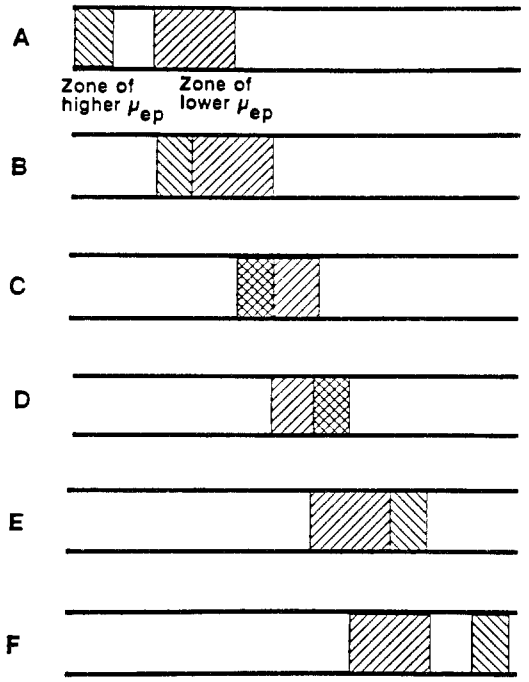

Figure 1. An illustration of electrophoretic mixing of two zones. Spatially distinct zones (A), beginning engagement (B), fully interpenetrated (C), beginning disengagement (D), fully disengaged (E), and continuing to traverse the capillary (F). The zone of higher electrophoretic mobility (μ_{ep}) is half the width of the zone of lower μ_{ep} and migrates with twice the transport velocity.

power supply. Detection was achieved using an ISCO (Lincoln, NE) CV^4 CE variable-wavelength absorbance detector. NADH, proteins, and mesityl oxide were monitored at 340, 200, and 254 nm, respectively. Data were collected either by a Linear (Reno, NV) 1200 strip-chart recorder or an i486 personal computer interfaced with a PC-LPM-16 I/O board and NI-DAQ DOS software (National Instruments Corp., Austin, TX).

Reagents. Yeast alcohol dehydrogenase (YADH), nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), glycine buffer solution (0.5 M, pH 9.0), tris[hydroxymethyl]aminomethane, and tris[hydroxymethyl]aminomethane hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Absolute ethanol and mesityl oxide were purchased from Midwest Solvents Co. of Illinois (Pekin, IL) and Aldrich Chemical Co. (Milwaukee, WI), respectively. For the determination of ethanol, 50 mM glycine CE running buffer was prepared by diluting the glycine buffer solution with degassed, double-distilled, deionized water. A 50 mM Trizma CE running buffer solution for the determination of ADH was made by dissolving tris[hydroxymethyl]aminomethane and tris[hydroxymethyl]aminomethane hydrochloride in degassed, double-distilled, deionized water. The CE running buffers were adjusted to pH 9.0 with either 1.0 M HCl or 1.0 M NaOH. The analytical reagent solutions were prepared by dissolving the appropriate analytical reagents (8 units/mL YADH and 10 mM NAD^+ for determinations of ethanol depicted in Figures 2 and 3; 75 units/mL YADH and 10 mM NAD^+ for the ethanol determination depicted in Figure 4; 50 mM ethanol and 10 mM NAD^+ for the determination of ADH shown in Figure 5) in the appropriate running buffer solutions.

Electrophoresis Procedures. The capillaries were treated with 1 M NaOH for 10 min and then rinsed with buffer solution for 10 min prior to use. The capillary and buffer reservoirs were filled by vacuum with the appropriate analytical reagent/running buffer solution. A plug of analyte solution was then hydrodynamically injected by manually placing the anodic end of the capillary into the sample vial and raising for a fixed height and time (typically 5–10 cm for 2–5 s). The assay was effected by applying an electric field strength of 125 V/cm and monitoring the absorbance electropherogram of NADH at 340 nm. All assays were performed either under the influence of a constant potential or with an intermittent time period at zero potential. Electrophoretic mobilities were determined by injecting the species of interest along with a neutral marker (mesityl oxide) and monitoring the migration times of the two species. Electrophoretic mobility was calculated as

$$\mu_{ep} = \frac{(t_{MO} - t_A)l}{t_A t_{MO} E} \quad (4)$$

where t_{MO} and t_A are the migration times of the analyte and mesityl oxide, respectively, and l is the separation length of the capillary.

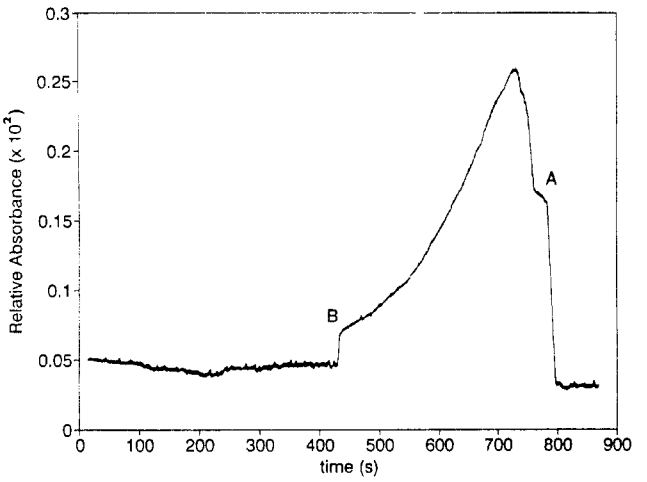

Figure 2. An electropherogram of an EMMA determination of a 0.5 g/L sample of ethanol. A shows the formation of NADH at the reagent interfaces prior to the application of potential while B indicates truncation of the NADH concentration profile as unreacted ethanol migrates past the detection window. Experimental conditions stated in text.

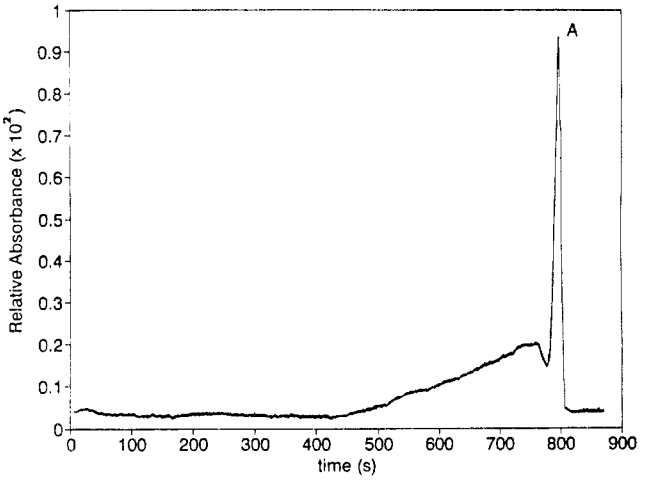

Figure 3. An electropherogram of an EMMA determination of a 0.5 g/L sample of ethanol. A indicates the accumulation of NADH at the reagent interfaces as the injected plug of ethanol is allowed to incubate for 120 s prior to the application of electric field. Experimental conditions are stated in the text.

RESULTS AND DISCUSSION

The reaction-based determination of an analyte requires four operations: (1) *analyte and analytical reagent metering*; (2) *initiation of reaction*; (3) *control of reaction conditions and product formation*; (4) *detection of species whose production or depletion is indicative of the concentration or quantity of the analyte of interest.* This paper demonstrates that CE systems, as employed by EMMA, are capable of performing each of these tasks.

The chemical system employed in a reaction-based determination typically involves the reaction of an analyte A with one or more analytical reagents R to produce or deplete a

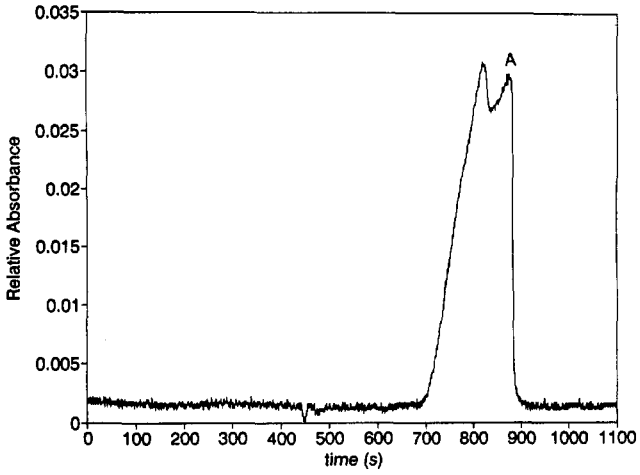

Figure 4. An EMMA determination of ethanol utilizing elevated concentrations of analytical reagents (75 units/mL YADH; 10 mM NAD^+) relative to those of Figure 2 (8 units/mL YADH; 10 mM NAD^+). No truncation of the NADH profile is observed. A indicates NADH production at the reagent interfaces prior to the application of the electric field. Experimental conditions stated in text.

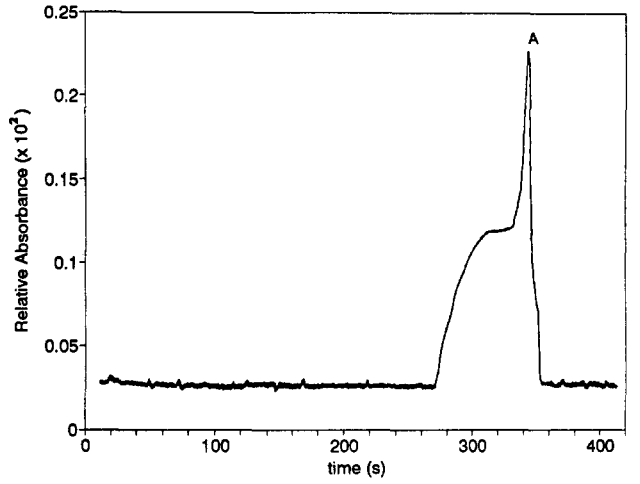

Figure 5. An EMMA determination of 5 units/mL sample of YADH. A indicates NADH accumulation at the reagent interfaces prior to the application of the electric field. Experimental conditions are stated in the text.

detectable species D which is stoichiometrically indicative of the quantity or concentration of the analyte present in the sample. This reaction may be depicted simplistically as

$$aA \xrightarrow{R} dD$$

The chemical system chosen for this study is the enzymatic reaction of alcohol dehydrogenase (ADH; alcohol:NAD oxidoreductase, EC 1.1.1.1), which reversibly catalyzes the oxidation of ethanol to acetaldehyde:

$$C_2H_5OH + NAD^+ \overset{ADH}{\leftrightarrow} CH_3CHO + NADH + H^+$$

The concurrent reduction of coenzyme NAD^+ to NADH can be directly monitored by the increase in absorbance at 340 nm as a measure of the extent of reaction.

Two modes of analysis are possible utilizing this enzymatic system: (1) the determination of ethanol in which ethanol serves as the analyte, ADH and NAD^+ as the analytical reagents, and NADH as the detectable species; (2) the determination of ADH in which ADH serves as the analyte, ethanol and NAD^+ as the analytical reagents, and NADH as the detectable species. Figures 2, 3, and 4 illustrate experimental EMMA determinations of ethanol while Figure 5 shows an EMMA ADH determination. The methodologies chosen here for the analysis of both ethanol and ADH require a forward reaction. The equilibrium, which lies far to the left at neutral pH, can be forced to the right by buffering at pH 9 and by trapping the acetaldehyde with an agent, such as hydrazine, thereby inhibiting the reverse reaction.[6] The following sections describe the EMMA procedures in terms of the stated four required steps in a chemical analysis.

1. Analyte and Analytical Reagent Metering. The EMMA methodology requires that the analyte and analytical reagents be sequentially introduced into the capillary. There are numerous potential metering modes varying both by their method of introduction (*e.g.* hydrodynamic, electrokinetic, and vacuum methods) and the size (*i.e.* plug width) of the reagent regions. The initial spatial positioning of the analyte and analytical reagent zones in the capillary is determined by the sign and magnitude of the electrophoretic mobilities of the various species involved so that the appropriate reagents approach and engage each other under the influence of an applied electric field. In the methodologies chosen for the determinations of both ethanol and ADH, the capillary is filled by vacuum with the analytical reagents contained in the CE running buffer. The analytical reagent solution is also maintained in the cathodic and anodic buffer reservoirs. The sample containing the analyte is then injected into the anodic end of the capillary via a hydrodynamic injection. In the analysis of ethanol, since the neutral ethanol has a greater transport velocity than either the negatively-charged ADH or NAD^+, the analyte must be positioned "behind" the analytical reagent zone contained in the filled capillary and cathodic buffer reservoir so that interpenetration of the zones occurs upon application of an electric field. In the determination of ADH, the ADH has a lower transport velocity than either the ethanol or NAD^+. Consequently, the analyte must be positioned "in front" of the analytical reagents contained in the anodic buffer reservoir so that the reagent zones merge upon the application of an electric field.

As described, both the ethanol and ADH determinations employ what may be termed the "reagent filled capillary" approach in which the capillary and buffer reservoirs are initially filled with reagents. Since the analytical reagent solution is maintained as a continuous stream within the CE system, upon electrophoretic mixing the analyte remains engaged with the analytical reagent zone throughout its traversal of the capillary. This strategy permits an analyte to encounter a maximal volume of analytical reagents and allows for the logistically simplest method of analytical reagent metering. Prior to each assay, the capillary is simply refilled with the analytical reagent solution. Conversely, one or more zones of analytical reagent(s) of finite width may be introduced into the capillary. The analyte encounters analytical reagent only during the time period in which the two regions are merged. The width of reagent zones introduced depends upon the desired reagent contact time as described in the next section. This approach allows for minimal consumption of analytical reagents.

Analytes are typically introduced in EMMA as a zone of finite width employing traditional CE injection methods, including hydrodynamic,[7,8] electrokinetic,[3,7,9] and microinjection techniques.[10,11] As determined by the Poiseuille equation,[11] approximately 2 nL of sample was injected for the determinations illustrated in Figures 2, 3, and 5. The

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(11) Wallingford, R. A.; Ewing, A. G. *Anal. Chem.* **1988**, *60*, 1972.
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analysis depicted in Figure 4 utilized approximately a 10-nL analyte injection volume. Depending upon the sensitivity of a particular assay and the physical dimensions of the CE system employed (*i.e.* capillary diameter), typical injection volumes in EMMA can range from 0.1 to 20 nL.

The small dimensions of the CE systems utilized are a primary advantage of the EMMA methodology. The ultramicroinjection volumes allow for the determination of microsamples, such as those encountered in the analysis of interstitial fluids or the intracellular fluids of a single cell. Furthermore, EMMA consumes small volumes of analytical reagents. The consumption of analytical reagent per assay depends upon the width of the analytical reagent plug used. However, the limiting case of maximal consumption of analytical reagent solution, in which the analytical reagents are maintained in the running buffer, is equal to the volumetric flow rate of the bulk buffer solution V_f

$$V_f = \pi r^2 \mu_{eo} E \tag{5}$$

where r is the radius of the capillary. Depending upon the electroosmotic flow and the radius of the capillary employed, typical volumetric flow rates are on the order of a few microliters per hour.

Due to their electrophoretic mobility, charged reagent species are selectively depleted from buffer reservoirs. Consequently, reagent concentrations can change significantly over time for species with large positive or negative electrophoretic mobilities if relatively small buffer reservoirs are employed. Based upon the experimental capillary diameter of 75 μm and an electric field strength of 125 V/cm, the ethanol and ADH determinations depicted in Figures 2 and 5, respectively, consume the analytical reagent/running buffer solutions at rates of approximately 9.5 and 12 μL/h, respectively, as calculated by eq 5. A single ethanol assay as depicted in Figure 2 requires 13 min, thereby consuming approximately 2.1 μL of analytical reagent/running buffer solution, 16 mmol of NAD^+, and 0.01 unit of ADH. The 6-min ADH determination shown in Figure 5 consumes approximately 1.2 μL of analytical reagent/running buffer solution, 60 nmol of ethanol, and 9 nmol of NAD^+ per assay. The minimal reagent requirements of EMMA diminish the frequent concerns over cost and disposal of analytical reagents in chemical analysis.

2. Initiation of Reaction. Chemical reactions are generally initiated in traditional methods by blending solutions containing the analyte and the analytical reagents in an active process involving turbulent mixing. Mixing bulk solutions by turbulent methods inherently dilutes the reagents, disperses the reactants and products (as in flow injection analysis), and diminishes the sensitivity of detection by diluting the detectable species.[13,14] The mixing of the analyte and analytical reagents is accomplished in EMMA by exploiting the variability in transport velocity among the chemical species in the chosen electrophoretic medium. In the free zonal CE medium chosen for this study, each of the reagent species has a different electrophoretic mobility due to their variable charge densities as dictated by eq 1.

Following the metering of the reagents into the capillary in appropriate positions, electrophoretic mixing is initiated by the application of an electrical field. The reagent zones migrate at a differential rate dependent upon the deviation in electrophoretic mobility between the two components of interest $\Delta\mu_{ep}$ and the applied electric field strength

$$v_{diff} = \Delta\mu_{ep} E \tag{6}$$

Ignoring the effects of diffusional broadening of the reagent zones, the time required for two spatially separated reagent bands to initiate contact $t_{contact}$ (Figure 1B) can be estimated as

$$t_{contact} = d/\Delta\mu_{ep} E \tag{7}$$

where d is the distance between the leading edge (toward direction defined as positive mobility) of the zone of greater transport velocity and trailing edge (away from direction defined as positive mobility) of the zone of lesser transport velocity. If the reagent zones are metered adjacently (*i.e.* $d = 0$), interpenetration of the zones occurs immediately upon the application of the electric field. The interpenetration of the zones continues as the potential is maintained, and the substance of greater transport velocity migrates through that of lower transport velocity.

Equation 7 can be used to estimate the time required for the two zones to fully interpenetrate (*i.e.* the narrower zone is completely merged within the wider zone; Figure 1C) if d is defined as the shorter distance of that between the two leading edges or the two trailing edges of the respective zones. In the frequently encountered situation in which a relatively narrow reagent plug of width w is injected adjacent to a broad (*i.e.* capillary-filling) reagent zone, the time required to fully electrophoretically mix the two regions t_{mix} can be estimated as

$$t_{mix} = w/\Delta\mu_{ep} E \tag{8}$$

The complete immersion of the narrower zone within the wider zone continues until that time at which disengagement of the regions begins. The time at which this full interpenetration of the narrower zone within the wider zone ends (Figure 1D) can be estimated from eq 7 if d is defined as the greater distance of that between the two leading edges or the two trailing edges of the respective zones. If a reagent is maintained in an appropriate running buffer reservoir, as in the ethanol and ADH determinations described in this paper, its zonal width may be viewed as infinite, and the reagent zones will remain engaged throughout the remainder of the experiment.

Ignoring the effects of diffusional broadening and reaction-induced depletion of the reagent zones, the time interval during which total merging of the narrower zone within the wider zone is experienced Δt_{merge} can be estimated as

$$\Delta t_{merge} = \frac{\Delta w}{\Delta\mu_{ep} E} \tag{9}$$

where Δw is the difference in widths of the two zones. Zonal engagement ceases as the two zones completely pass each other.

The time at which the two zones fully disengage (Figure 1E) can be estimated from eq 7 when d is defined as the distance from the trailing edge of the zone of greater transport velocity to the leading edge of the zone of lesser transport velocity. While eqs 7, 8, and 9 neglect contributions of diffusion and reaction-induced depletion of reagents to zonal width, each illustrates the critical roles of differential electrophoretic mobility, the magnitude and duration of the electric field strength, and the widths and positioning of the respective reagent zones in the zonal engagement and disengagement processes.

In the determination of ethanol, neutral ethanol ($\mu_{ep} = 0$ cm^2/V s) is transported with the electroosmotic flow toward the negative electrode. ADH ($\mu_{ep} = -1.6 \times 10^{-4}$ $cm^2/V{\cdot}s$) and NAD^+ ($\mu_{ep} = -1.2 \times 10^{-4}$ $cm^2/V{\cdot}s$) are each negatively charged at pH 9 and electrophorese against the electroosmotic flow, thereby allowing each to interpenetrate the ethanol zone under the influence of an applied electric field. Since electroosmotic flow is of greater magnitude than either reagent's electro-

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phoretic mobility, ADH and NAD^+ each travel toward the negative electrode and the detection window. At an experimental electric field strength of 125 V/cm, it may be calculated from eq 8 that a 0.5 mm wide injected plug of ethanol, such as that estimated by the Poiseuille equation for the determination illustrated in Figure 2, is fully interpenetrated by adjacent ADH and NAD^+ zones in approximately 2.5 and 3.3 s, respectively, based upon the experimental differential values of electrophoretic mobility for the reagent species ($\Delta\mu_{ep,ethanol-ADH} = 1.6 \times 10^{-4}$ cm^2/V·s; $\Delta\mu_{ep,ethanol-NAD} = 1.2 \times 10^{-4}$ cm^2/V·s). In the determination of ADH, such as that depicted in Figure 5, an adjacent zone of ethanol fully merges with a 0.5 mm wide plug of ADH in only 2.5 s at an experimental electric field strength of 125 V/cm. In contrast, an adjacent zone of NAD^+ requires 10 s to fully interpenetrate the ADH zone due to the relatively similar values of electrophoretic mobility for ADH and NAD^+ ($\Delta\mu_{ep,ADH-NAD} = 4 \times 10^{-5}$ cm^2/V·s).

If the analyte and analytical reagents are metered into the capillary adjacent to each other, mixing will occur immediately upon the application of the potential, as previously noted. However, diffusional broadening of the reagent zones can allow interpenetration and concurrent reaction at the interfaces between the reagents to occur prior to the application of the electric field. This phenomenon is shown as the product formations designated by A in the determinations of ethanol in Figures 2 and 4 and ADH in Figure 5. This NADH accumulation occurs as the adjacent analyte and analytical reagent zones diffusionally interpenetrate and react during the approximately 5–10-s period that the analyte is being injected, and the electric field is being applied. This effect is confirmed by the large NADH accumulation indicated by peak A in Figure 3 in which the reaction is allowed to occur at the reagent interfaces for 120 s prior to the application of the electric field. This phenomenon can be eliminated by injecting "spacer" plugs between the reagent zones. These spacers contain only buffer and must be sufficiently broad to prevent diffusional interpenetration of the reagent zones prior to the application of the electric field.

There are numerous advantages to the electrophoretic mixing utilized in EMMA relative to the traditional methods of mixing of bulk solutions. As species electrophorese essentially independently of the bulk solution, electrophoretic mixing merges two or more zones of varying electrophoretic mobility without a concurrent change in volume and, therefore, dilution of the zones. In an EMMA system for which Joule heating does not cause substantial band spreading, lateral diffusion is often the only significant factor causing dilution[3,15] of the reagent zones other than reaction-induced depletion. When the electrophoretic mobilities of two reagents vary substantially, full interpenetration can be achieved in several milliseconds by the application of high electric field strengths. Furthermore, theoretical and experimental design considerations are much simpler when turbulent mixing does not have to be built into the analytical apparatus. A typical CE system is capable of performing numerous EMMA analyses of various modes.

3. Control of Reaction Conditions and Product Formation. The production of detectable species in an EMMA system is dictated by the kinetics of the chosen chemical system, the concentrations of the overlapping reagent zones, the duration of the zonal engagement, and the electrophoretic properties of the species involved. The concentration profile of the detectable species can be estimated based upon these factors. The following discussion illustrates the effects of kinetics upon the production of detectable species in the determinations of ethanol and ADH described in this study.

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The mechanism and kinetic parameters of YADH are well characterized.[16,17] At pH between 8 and 9, YADH exhibits a catalysis mechanism described as an ordered bi-bi model.[16] The kinetic constants of YADH at 25 °C and pH 8.9 have been experimentally determined by Dickenson and Dickinson.[17] To simplify the kinetic considerations, the reaction may be viewed as irreversible. In the determination of ethanol or ADH, the product NADH ($\mu_{ep} = -2.3 \times 10^{-4}$ cm^2/V·s), which inherently differs in electrophoretic mobility from either analyte, is electrophoresed away from the vicinity of the reaction. This separation inhibits the reverse reaction. The use of positively-charged hydrazine to complex the other product, acetaldehyde, further inhibits the reverse reaction both by trapping the acetaldehyde and by imparting a positive electrophoretic mobility upon neutral acetaldehyde. Acetaldehyde is consequently electrophoresed from the vicinity of the neutral ethanol under the influence of an applied electric field.

When the reaction is irreversible, the kinetics simplify to the initial rate expression

$$\frac{[ADH]}{v} = \phi_0 + \frac{\phi_{NAD}}{[NAD]} + \frac{\phi_{EtOH}}{[EtOH]} + \frac{\phi_{NAD-EtOH}}{[NAD][EtOH]} \quad (10)$$

where v is the velocity of the reaction and ϕ_0, ϕ_{NAD}, ϕ_{EtOH}, and $\phi_{NAD-EtOH}$ are kinetic constants.[17] The kinetic considerations can be further simplified if the experimental concentration of NAD^+ is chosen to be sufficiently high so that the YADH encounters enzyme-saturating concentrations of NAD^+, and insignificant depletion of NAD^+ occurs during the analyte zone's traversal of the NAD^+ region. Under these conditions, eq 10 simplifies to a pseudo-Michaelis–Menten model:

$$v = \frac{V_{max}[EtOH]}{K_{M,EtOH} + [EtOH]} \quad (11)$$

where V_{max} is the maximal rate of the reaction (equal to $[ADH]/\phi_0$) and $K_{M,EtOH}$ is the Michaelis–Menten constant for ethanol (equal to ϕ_{EtOH}/ϕ_0). Equation 11 reveals that at high ethanol concentrations ($[EtOH] \gg K_{M,EtOH}$), the ADH is saturated with substrate, and the velocity of the reaction approaches V_{max}. The reaction rate will remain relatively constant until the substrate is depleted or product accumulation produces inhibition. At low ethanol concentration ($[EtOH] \ll K_{M,EtOH}$), the reaction rate is directly proportional to the substrate concentration. The rate of product of detectable NADH is clearly dependent upon the concentration of the overlapping reagent zones.

In the determination of ethanol, as depicted in Figures 2, 3, and 4, the reaction rate and, therefore, production of NADH at high substrate concentrations is relatively constant and independent of ethanol concentration. Therefore, purely enzyme-saturating concentrations of substrate are of little analytical value in the determination of ethanol. However, as the ethanol zone is depleted, so that enzyme-saturating conditions no longer exist, the rate of the reaction and, therefore, production of NADH decrease until they are directly proportional to the substrate concentration. Consequently, the plug of ethanol is depleted as it passes through the zones containing ADH and enzyme-saturating concentrations of NAD^+, and the rate of the reaction lessens as illustrated by Figure 6A. This figure depicts the time dependence of the reaction velocity due to ethanol depletion as predicted by eq 10 utilizing the kinetic constants of Dickenson and Dickinson[17] and a constant enzyme-saturating concentration of NAD^+.

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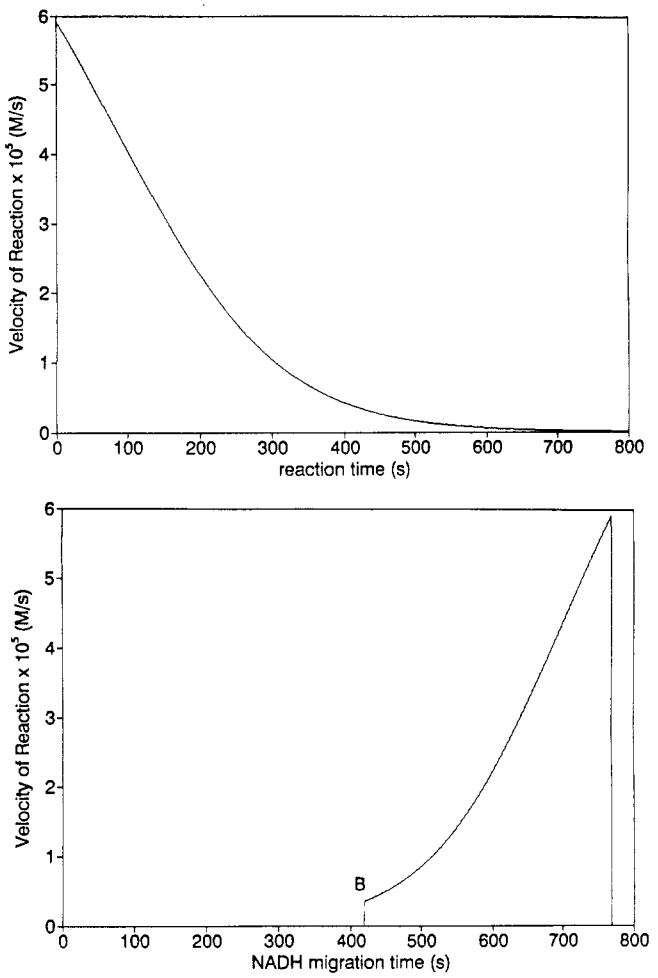

Figure 6. (A) Reaction velocity for the enzymatic oxidation of 0.5 g/L ethanol as a function of time as determined by eq 10. Kinetic constants are those of Dickenson and Dickinson[16] for YADH at pH 8.9 and 25 °C (ϕ_0 = 0.0019 s^{-1}; ϕ_{NAD} = 0.28 μM s; $\phi_{ethanol}$ ~ 23 μM s; $\phi_{NAD-ethanol}$ = 19700 μM^2 s). YADH and NAD^+ concentrations are those of experimental determination depicted in Figure 2 (8 units/mL and 10 mM, respectively). (B) Theoretical electropherogram for an EMMA determination of 0.5 g/L ethanol obtained by converting the reaction times of Figure 6A to migration times by eq 17. Instrumental parameters are those of experimental determination depicted in Figure 2 (E = 125 V/cm; l = 25 cm). Electrophoretic properties are stated in the text. B designates expected truncation of NADH concentration profile due to incomplete depletion of the ethanol zone as it migrates past the detection position.

The kinetics are very different for the determination of ADH as depicted in Figure 5. As the enzyme plug passes through the ethanol and NAD^+ regions, ADH is not depleted. If enzyme-saturating concentrations of both ethanol and NAD^+ are maintained, the rate of the reaction is relatively constant and directly proportional to the concentration of ADH:

$$v = V_{max} = [ADH]/\phi_0 \quad (12)$$

Furthermore, the velocity of the reaction is directly proportional to the turnover number (defined as the reciprocal of ϕ_0) of the enzyme. However, if sufficient depletion of one or more substrates occurs, so that enzyme-saturating conditions do not prevail, the velocity of the reaction is dependent upon the concentrations of substrate as dictated by eq 10 and is no longer directly proportional to the concentration of enzyme.

The reaction phase may be performed in a constant or zero potential mode. In the constant potential mode, as utilized in the determinations illustrated in this paper, two or more reagent zones, which have been previously electrophoretically mixed, continually electrophorese through each other under the influence of an applied electric field as the reaction progresses. A zone of analyte can be made to engage many times its own volume in analytical reagents by allowing a small zone of the analyte to migrate through a larger plug of the analytical reagent. Maximal analytical reagent is contacted when the analyte is metered adjacent to a continuous stream of analytical reagents (*i.e.* capillary and buffer reservoirs filled with analytical reagent solution as in this study) so that the analyte zone encounters analytical reagent throughout its traversal from injection until migrating past the detection position. In this mode, R_{vol} (defined as the ratio of analytical reagent volume encountered to analyte volume) depends upon the difference in electrophoretic mobility between the analyte and analytical reagent species $\Delta\mu_{ep,A-R}$, capillary distance from injection to detection, electrophoretic mobility of the analyte $\mu_{ep,A}$, electroosmotic flow, and the width of the injected analyte zone

$$R_{vol} = \frac{\Delta\mu_{ep,A-R} l}{(\mu_{ep,A} + \mu_{eo})w} \quad (13)$$

In the determination of ethanol depicted in Figure 2, eq 13 estimates that a 0.5-mm plug of analyte encounters approximately 170 times its volume in ADH and 120 times its volume in NAD^+. In the ADH assay depicted in Figure 5, a 0.5-mm zone of analyte interpenetrates about 110 and 30 times its own volume in ethanol and NAD^+, respectively, prior to passing by the detection position. Reaction under the influence of an applied electric field allows fresh analytical reagents to be transported to the analyte throughout the reaction without the concurrent dilution experienced in the mixing of bulk solutions.

Reactions in the zero potential mode are quite different. At zero potential, two or more reagent zones, which have been electrophoretically mixed by the previous application of an electric field, are allowed to incubate in the absence of an electric field in order to allow the detectable effects of the reaction (*e.g.* production or depletion of detectable species) to accumulate for a fixed period of time as previously described.[5] Following the zero potential time period, the potential is again applied to transport the detectable species to the detector.

If the analyte and detectable species differ in electrophoretic mobility, and the reaction proceeds under the influence of an applied electric field, the detectable species is continually transported away from the analyte region throughout the duration of the reaction. This separation occurs at a rate $v_{disengagement}$ which is dependent upon the difference in electrophoretic mobility between the analyte and the detectable species $\Delta\mu_{ep,A-D}$ and the applied electric field strength

$$v_{disengagement} = \Delta\mu_{ep,A-D} E \quad (14)$$

Consequently, the concentration profile of the detectable species is broadened relative to the zone width of the analyte. Minimum band broadening and maximum sensitivity are obtained in the constant potential mode when the two species differ little in electrophoretic mobility and at low electric field strengths. Under these conditions, the detectable species is allowed to accumulate in the vicinity of the reaction.[5] Optimum sensitivity is obtained in the zero potential mode because the detectable species is not separated from the vicinity of the analyte. This effect allows maximum accumulation of product. As no electrophoretic separation occurs in the absence of an applied electric field, the difference in electrophoretic mobility of the analyte and detectable species is not important in the zero potential mode. Sensitivity in this case is limited by diffusional band broadening of the accumulating detectable species, production inhibition, and depletion of the analytical reagents. The zero potential mode

is of particular value in those determinations for which maximum sensitivity is desired, such as the analysis of dilute analytes and in those cases for which the kinetics are slow (*i.e.* low turnover number enzymes).

4. Detection. Detection in EMMA is generally performed by electrophoretically transporting the detectable species to the detection system. The rate of transport is dependent upon the detectable species' electrophoretic mobility and the electroosmotic flow. In both the determination of ethanol and ADH, NADH has a lower transport velocity than either analyte. Therefore, the first NADH formed is the last to be detected. The first NADH which can be detected is that which last forms before the analyte zone (ADH or unreacted ethanol) migrates past the detection position. Therefore, there is a detection "time window" t_{det} during which NADH can be observed as defined by the apparent mobilities of the analyte and the detected product

$$\frac{l}{(\mu_{ep,A} + \mu_{eo})E} \leq t_{det} \leq \frac{l}{(\mu_{ep,D} + \mu_{eo})E} \tag{15}$$

The detection time window for the ethanol assay described in Figure 2 corresponds to NADH detection times of 420–770 s based upon the experimental values of electrophoretic mobility and electroosmotic flow. In contrast, the detection window for the ADH determination in Figure 5 spans from approximately 270 to 350 s. In an EMMA assay for which the detectable species has a greater transport velocity than the analyte, the first detectable species formed is the first to be detected, and the last detectable species which can be detected is that which forms as the analyte zone passes by the detection position. In this case, the detection window is defined as

$$\frac{l}{(\mu_{ep,D} + \mu_{eo})E} \leq t_{det} \leq \frac{l}{(\mu_{ep,A} + \mu_{eo})E} \tag{16}$$

Since the quantity of detectable species produced or depleted at a given time is dependent upon the rate of the reaction, and the detectable species is electrophoresed away from the vicinity of the reacting analyte at a rate indicated by eq 14, the resulting peak in the electropherogram provides a profile of the velocity of the reaction as the analyte traverses the capillary. When the assay is performed at constant applied potential, the total time required for the observed detectable species to migrate to the detector t_{mig} is the sum of the time t_A prior to the reaction during which the analyte moves with its electrophoretic mobility $\mu_{ep,A}$ a certain distance d from the point of injection and the time t_D required following the reaction for the resulting detectable species to traverse the remaining distance $(l - d)$ to the detector position with its unique electrophoretic mobility $\mu_{ep,D}$

$$t_{mig} = t_A + t_D = \frac{l + (t_A \Delta\mu_{ep,D-A} E)}{(\mu_{ep,D} + \mu_{eo})E} \tag{17}$$

where $\Delta\mu_{ep,D-A}$ is the difference in electrophoretic mobility between the detectable species and the analyte. The correlation between t_A and t_{mig} allows for the estimation of the time at which a given observed detectable species was formed in the assay

$$t_A = \frac{[t_{mig}(\mu_{ep,D} + \mu_{eo})E] - l}{\Delta\mu_{ep,D-A}E} \tag{18}$$

where t_A includes the time required to electrophoretically engage the analytical reagent zones and the reaction time following mixing. If electrophoretic mixing occurs rapidly, t_A approximates the time of reaction as dictated by the kinetics of the chemical system. Therefore, it is often possible to estimate the resultant electropherogram depicting the concentration profile of the detectable species based upon knowledge of the electrophoretic properties and kinetics of the system. Utilizing the same kinetic parameters as in Figure 6A and the experimental values of electrophoretic mobility and electroosmotic flow and the instrumental parameters of Figure 2, Figure 6B illustrates a theoretical NADH profile obtained by converting the reaction times of Figure 6A to electrophoretic migration times (*i.e.* NADH detection times) as dictated by eq 17. There is a definite similarity in appearance between the theoretical and experimental concentration profiles of Figures 2 and 6B, respectively, thereby verifying the stated kinetic and electrophoretic effects upon peak shape. The experimental NADH peak of Figure 2 is convoluted by other factors, including the interfacial accumulation described previously, diffusion of the reagent and product zones, the width of the injected analyte zone, and uneven depletion of the analyte zone due to the time required for electrophoretic mixing, which are not considered in the simple model depicted in Figure 6B.

Clinical substrate assays are often based on an end-point method in which the reaction is allowed to essentially reach completion prior to taking a spectrophotometric reading. An EMMA end-point determination of ethanol requires all of the substrate to react prior to passing the detection window. As a result, the area of the NADH peak is directly proportional to the quantity of ethanol injected. The total reaction time t_{rxn} available to fully deplete the substrate is equal to the time required for the ethanol to migrate from the injection point to the detection window

$$t_{rxn} = \frac{l}{(\mu_{ep,A} + \mu_{eo})E} \tag{19}$$

When the reaction is not completed prior to passing the detection window, the peak is abruptly truncated as NADH produced beyond this point is not observed by the detector. This phenomenon is illustrated in Figure 2 as the truncation of the NADH profile at position B as the approximately 420-s migration time of the ethanol zone through the analytical reagents' zone is insufficient to fully react the 1 ng (2 nL, as estimated by the Poiseuille equation, of 0.5 g/L sample) of ethanol injected. The theoretical NADH concentration profile of Figure 6B predicts this truncation based upon the expected detection window determined by eq 15. This truncation effect places the upper limit on the linear range of the technique as the area contained within the peak is no longer indicative of the quantity of substrate injected. The linear range can be extended either by increasing the available reaction time or by increasing the rate of the reaction. The available reaction time may be increased by operating at lower electric field strengths or by increasing the effective plug width of analytical reagents encountered by the analyte zone. In the reagent filled capillary mode, the latter effect can be accomplished by increasing the separation length of the capillary. However, elevating the available reaction time by decreasing the electric field strength or increasing the separation length concurrently lengthens the analysis time as dictated by the upper limits of eqs 15 and 16. The linear range may be extended without adversely affecting the analysis time by increasing the rate of the reaction by elevating the concentrations of analytical reagents. Although the turnover number of an individual enzyme is not altered, raising the concentration of the ADH proportionally increases the value of V_{max} by raising the number of enzyme molecules capable of operating at a given turnover number. This effect is shown in the ethanol determination of Figure 4 in which the concentration of ADH is increased from approximately 8 to 75 units/mL relative to the assay depicted in Figure 2. A 5-ng (10 nL, as estimated by the Poiseuille equation, of 0.5

g/L) sample of ethanol does not experience truncation as does the 1-ng sample of Figure 2. The NADH profile returns to the baseline at a migration time of 690 s, corresponding to a required reaction time of approximately 180 s as estimated by eq 18.

In the determination of an enzyme performed at constant potential and enzyme-saturating concentrations of substrates, such as that depicted for ADH in Figure 5, the rate of production and separation of detectable species is relatively constant as indicated by eqs 12 and 14, respectively. Therefore, the resulting concentration profile of detectable species is a plateau as previously described.[5] The width of the plateau is dependent upon the relative electrophoretic mobilities of the analyte and detectable species and the applied electric field strength as dictated by eq 15 or 16. The height of the plateau and, therefore, the sensitivity of the technique can be approximated by

$$h = \frac{\epsilon b k_3 [\text{enzyme}] w}{\Delta\mu_{\text{ep,A-D}} E} \tag{20}$$

where h is the height of the plateau in absorbance units, ϵ is the molar absorptivity of the detectable species, b is the cell path length, k_3 is the turnover number of the enzyme, and w is the injected plug width of the enzyme. Consequently, the height of the plateau is directly proportional to the quantity of enzyme injected, and the sensitivity of the method is directly proportional to the turnover number of the enzyme and inversely proportional to the rate of separation of the detectable species from the analyte.

In the zero potential mode, the detectable species accumulated in the absence of an electric field appears as a peak superimposed upon a plateau. The plateau results from the applied potential intervals which are required to electrophoretically mix the reagents prior to the zero potential period and to transport the detectable species to the detector position following the zero potential interval. The applied potential migration time of the zero potential peak can be estimated by eq 17 if t_A is defined as the time prior to the zero potential interval during which constant potential is maintained.

The lower limits of detection (LLD) observed using EMMA vary widely depending upon the chemical nature of the analysis and the detection system. In the determination of substrates, if sufficiently high concentrations of analytical reagents are utilized so that the reaction is nearly instantaneous and insignificant band broadening occurs due to the kinetic effects previously described, the LLD for the assay corresponds closely to that observed for a normal injection of the detectable species. For the EMMA determination of ethanol, we have reported a LLD of 1×10^{-4} M corresponding to the detection of approximately 3×10^{-13} mol of ethanol by UV absorption.[18] EMMA determinations of enzymes offer much lower LLDs than those encountered with substrates due to the amplifying nature of the enzymatic reaction. Wu and Regnier,[19] utilizing the zero potential mode and gel-filled capillaries to minimize diffusional band broadening, have reported the detection of 7.6×10^{-12} M (5×10^{-20} mol) alkaline phosphatase by the VIS absorption of the *p*-nitrophenol reaction product. The LLD can be further lowered by the use of more sensitive detection methods. Detecting reaction product 4-methoxy-β-naphthylamine by time-resolved laser-induced fluorescence, Miller *et al.*[20] have reported the determination of 6×10^{-13} M (7×10^{-22} mol) leucine aminopeptidase by the zero potential mode in free solution.

CONCLUSIONS

EMMA offers numerous advantages to traditional chemical methods of analysis. The use of electrophoretic mixing allows reagent zones to be merged without the concurrent dilution experienced in bulk methods, does not require turbulence and the resulting band spreading, and allows an analyte to encounter many times its own volume in analytical reagents. The small dimensions of the CE systems employed in EMMA allow analyses to be performed on ultramicrosamples with minimal consumption of analytical reagents. While this paper has focused on the use of electrophoresis as a means for mixing and transport in chemical analysis, EMMA will become most powerful when this capability is combined with the intrinsic separative capacity of electrophoresis. Present day chemical analyses often require that the detectable species possess a unique detection characteristic such that the reaction may be monitored free of interferences from the other chemical species involved in the reaction. This necessity can often force the experimenter to couple the analytical reaction to one or more secondary reactions in order to produce a substance with unique detection characteristics. The separative capability of EMMA can allow a species produced in the analytical reaction with similar detection characteristics but unique electrophoretic properties to serve as the monitored species without the need to couple it to a secondary reaction. Furthermore, the separative capability of CE can allow simultaneous analyses to be performed as either analytes or monitored species which differ in electrophoretic mobility can be separated prior to or following the analytical reactions.

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