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Laboratory Experiment

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Understanding Electrophoresis through the Investigation of Size, Shape, and Charge of pH Indicators

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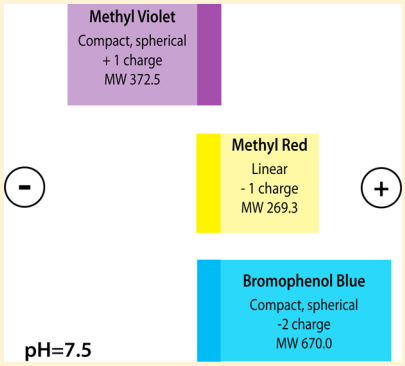
Supporting Information

ABSTRACT: A laboratory experiment was designed for upper-level students in a Chemical Analysis course to illustrate the theoretical and practical applications of 0.8% agarose gel electrophoresis and to reinforce an understanding of weak acids/bases using easy-to-visualize pH indicators. The careful choice of indicators included acid and base types with amphiprotic intermediates, species that become either singly or doubly deprotonated, and molecules with molecular weights that span from 269 to 670 g/mol. Students measure the rate of migration of the pH indicators relative to the rate of migration of a red dye standard. The relative rates are interpreted with respect to the indicators' fractions of deprotonation (and resulting molecule charges), molecular weights, and shapes. This procedure occurs under acidic (pH 4.0) and basic (pH 7.5) conditions with lower and higher applied voltages to understand the impact of changing protonation/deprotonation. This experiment has been successfully completed by over 200 analytical chemistry students with consistent results. Overall, this procedure teaches students to evaluate the controls on molecule movement in an electrical field critically, while gaining insight into weak acid/base equilibria.



KEYWORDS: *Upper-Division Undergraduates, Electrophoresis, Analytical Chemistry, Acids/Bases, Laboratory Instruction*

INTRODUCTION

Electrophoresis is a key method of analysis in many biochemical studies. Gel electrophoresis separates species by mass, charge, and shape by applying an electric voltage to a gel in a buffered solution. Samples are placed in wells inside the gel and travel through the gel toward either the positive or negative electrode located on opposite sides of the electrophoresis chamber. Smaller and highly charged species travel the farthest over a given time due to their respective charges by overcoming the frictional forces of the gel. Samples are then compared to a known standard, a solution of species with known masses and constant charges, to reduce inherent variations related to gel and buffer composition.

JUSTIFICATION

This experiment provides an understanding of the fundamentals of electrophoresis without delving into the biochemical applications. There is a plethora of specific types of electrophoresis, each with its own unique characteristics, migration media, and techniques for visualization. However, all electrophoreses operate on the same fundamental principle: charge migration under the influence of an electric field. Instead of requiring expensive chemicals or necessary staining and UV light to observe DNA fragments,[1−4] this investigation uses common pH indicators as a relatively straightforward technique to learn the fundamentals of electrophoresis in an undergraduate analytical chemistry course without the emphasis on biochemical molecules. It is not unique to use pH indicators to investigate the theory behind electrophoresis,[5−8] as acid/base indicators are themselves weak acids or bases whose colors and net charges depend on pH. However, this procedure is novel in that it compares the behavior of acid/base indicators across variable buffer media and voltages by requiring students to connect migration distances with specific properties such as molecular shape, weight, and charge. The goals of this experiment are to expose students to electrophoresis techniques and reinforce an understanding of weak acids/bases through the variable migration of acid/base indicators. In addition, students develop the analytical techniques of preparing buffers and agarose gels.

BACKGROUND

If a molecule with a negative charge is placed between a positive and negative plate, the ion will migrate toward the positive plate. The migration velocity, u_{ep} (cm/s), of the ion will not only depend on the electric field potential (E, V/cm) it experiences, but also on a variety of other factors all combined into a term called the electrophoretic mobility μ_{ep} (cm^2 V^{-1} s^{-1}) (eq 1).

$$u_{ep} = \mu_{ep} E \tag{1}$$

Factors that influence the magnitude of the electrophoretic mobility include a direct relationship to the net average charge on the analyte (Coulomb's Law: higher charge, more force) and an inverse relationship to frictional forces that impede the ion's progress. The frictional forces experienced by an ion are

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J. Chem. Educ. XXXX, XXX, XXX−XXX

Table 1. Properties of the Standard and Indicators Used in the Experiment[9−13]

Properties of Standard and Indicators					Fraction of Dissociation	
Indicator	Molar Mass (g/mol)	pK[a]	Indicator Type	Shape	pH 4.0	pH 7.5
FD&C Red 3	879.9		N/A	Spherical	-	-
FD&C Red 40	496.4	-	N/A	Linear	-	-
Bromothymol Blue	624.4	7.3[b]	Acid	Spherical	0.00	0.64
Phenol Red	354.4	7.9	Acid	Spherical	0.00	0.30
Bromophenol Blue	670.0	3.9	Acid	Spherical	0.55	1.00
Cresol Red	382.4	1.0, 7.7	Acid	Spherical	0.00	0.41
Methyl Orange	305.4	3.8[c]	Acid	Linear	0.60	1.00
Methyl Red	269.3	5.0	Acid	Linear	0.09	1.00
Methyl Violet 2B	372.5	1.3	Base	Spherical	1.00[d]	1.00[d]

[a]For the acid-type indicators, the pK value is the pK_a. For the base-type indicator, the pK is the pK_b (see Supporting Information for Students for additional information). [b]Although not listed, both bromothymol blue and phenol red presumably have a second pK_a that is similar to the first pK_a for cresol red, accounting for all three molecules being amphoprotic at pH 4.0. Bromphenol blue also has a second pK_a similar to cresol red. [c]Methyl orange and methyl red are acid-type indicators under the pH conditions present in this laboratory experiment, where the zwitterion behaves as an acid. At lower pH levels, where the zwitterion is completely protonated, methyl orange and methyl red may be considered base-type indicators and would be expected to migrate toward the negative terminal. [d]Fraction of association.

affected by components such as ion size (molecular weight), ion shape, and the nature of the medium through which the ion is traveling. The frictional forces can be controlled to some extent by choosing the appropriate media. For example, separation by size exclusion can be accomplished by using a medium with a gradient in pore sizes. In the case of large biomolecules, such as proteins that can fold into a variety of conformations, it is not only the mass of the species but also its shape that impacts the observed frictional forces. Since many biomolecules, especially proteins, possess acid/base properties, the average charge of the ions is often controlled by pH buffers. If, under a given set of conditions, the rate of migration between different species is sufficiently different, the species will separate over a given period of time.

The electrophoretic mobility constant, however, has its limits. It can only be used to compare ions that are subjected to the same conditions. For example, any change in the medium in which the ion is traveling would result in a different electrophoretic mobility constant. When gel electrophoresis is carried out in molecular biology, a standard is typically used to compare and measure protein samples that corrects for subtle changes in gel and buffer composition. Known molecules with a range of masses are used as standards, with each molecule having an identical charge-to-mass ratio. Samples are compared to the standards and the masses can be determined. For the purpose of this experiment, any substance can be used as a standard for gel electrophoresis, as long as it is visible and its charge is constant and known.

Both Type 1 (acid-type, HIn/In^-) and Type 2 (base-type, In/InH^+) indicators are used in this experiment to provide opposite migration directions in an electric field. Most applications of gel electrophoresis, such as analyzing DNA, involve only negatively charged molecules; however, using Type 2 indicators helps show that migration of positively charged molecules is possible. The exact nature of the acid/base reactions and the charge state of the indicator will be dependent upon the structure of the indicator molecule, as well as the pH.

EXPERIMENTAL AND LEARNING OBJECTIVES

This experiment is appropriate for an upper-level analytical chemistry course and provides a general understanding of the fundamentals of electrophoresis without additional hazards associated with stains or UV light. Specific learning objectives are to understand relationships between pH, charge, molecular weight, and electrophoretic mobility, as well as the impact of electric field potential. Students also gain a better understanding of protonation/deprotonation of weak acids/bases through the comparison of migration distances under different pH conditions.

EXPERIMENTAL DETAILS

Students work in groups of two or three to complete the lab within a 4 h time period. An agarose gel is used as the migration medium, which has microscopic pores that act as a molecular sieve. Four experiments are completed to investigate the role of pH and voltage on the migration of acid/base indicators. Indicators are chosen to have a range in molecular weight, shape, and charge to provide insight into frictional forces. One buffer solution is prepared from a concentrated stock buffer with a final pH of approximately 7.5, while the second buffer solution is prepared using acetic acid and sodium acetate to pH 4.0 with a final acetic acid concentration of 0.1 M. Four 0.8% agarose gels are made, with two gels from each prepared buffer solution. The electrophoresis chamber is filled with the buffer solution that correlates with the gel prepared at the same pH. Detailed procedures are available in the Supporting Information.

Each indicator (Table 1) is mixed with a small amount of glycerin to increase the density of the sample so that the indicator remains localized within the sample well and migrates through the gel instead of diffusing into the overlying buffer solution. A standard solution of red and blue food coloring is used to show color separation and the red color (FD&C red 3 and 40) is used to normalize for mild differences in gel composition and voltage variations.

Gels of each buffer are run at low (∼50 V) and high (∼80 V) voltages, and the time of migration is recorded. Rates of migration are calculated from the distance traveled per unit time. The relative rate of migration for each indicator is calculated as the ratio to the rate of migration for the standard (red food coloring), which optimizes comparisons among gels.

HAZARDS

The gel electrophoresis should be run in a fume hood due to the evolution of hydrogen gas from the chamber. Otherwise,

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J. Chem. Educ. XXXX, XXX, XXX−XXX

agarose gels are not toxic. In preparing the dyes for use, it should be noted that they are all hazardous when ingested or inhaled and are irritants on the skin or in the eyes. To minimize the hazard to students, dyes are used in very small quantities from dropper bottles. Appropriate precautions include lab coats, goggles, and gloves. The power supply used in this experiment produces a voltage that is high enough to cause severe electrical shock if handled improperly. The electrophoresis chamber or its contents should not be handled unless the power supply is turned off.

RESULTS AND DISCUSSION

Results presented here are representative of the results obtained over the past decade by over 200 students (Figure 1) in a

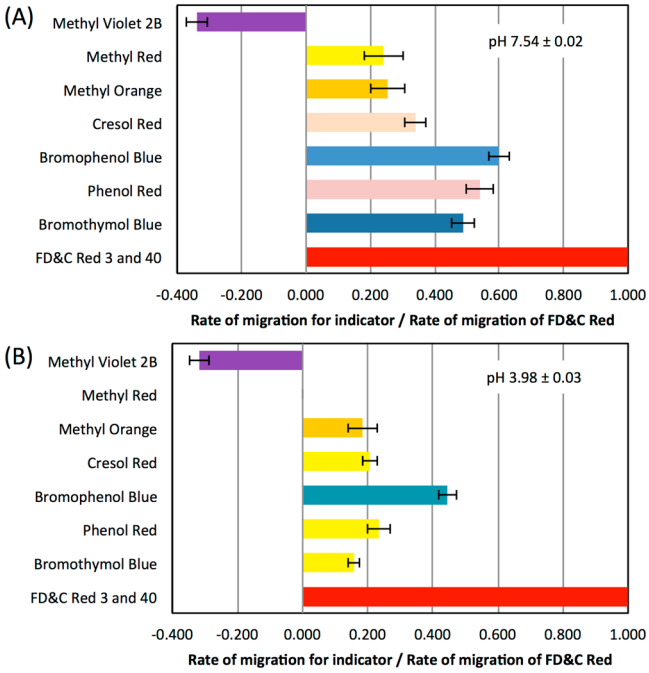

Figure 1. Average ratios of the indicator rate of migration to the rate of migration of the standard (FD&C red) at 80 ± 10 V with standard deviations, indicator colors, and positions on gel of indicators for pH (A) 7.54 ± 0.02 (n = 5) and (B) 3.98 ± 0.03 (n = 4). A change in color for an indicator between A and B means that the indicator color is different under these two pH conditions. Note that methyl red had no migration under the more acidic conditions.

Chemical Analysis course. All of the indicators had faster absolute rates of migration with the higher voltage than with the lower voltage because the greater difference in electric potential between the electrodes resulted in a greater attraction between the indicators and the electrodes. When plotted relative to the rate of migration of red, the lower and higher voltages had ratios that were the same at the 95% confidence level. Whether the indicator was Type 1 (acid-type) or Type 2 (base-type) dictated the direction of migration. Methyl violet 2B was the only indicator that migrated toward the negative electrode because it is a Type 2 indicator and had a positive charge under both pH conditions.

Some of the indicators were a different color when the buffer was changed (methyl red, cresol red, phenol red, and bromothymol blue, Figure 1). Comparisons between the two pH buffers showed the effect of changes in protonation. There was no statistical difference at the 98% confidence level between the relative rates of migration for methyl violet 2B in the two buffers, because it was nearly completely protonated under both pH conditions (Table 1). The remaining indicators had faster relative rates of migration in the pH 7.5 buffer than in the pH 4.0 buffer because of the greater extent of deprotonation and their higher charges. Bromothymol blue, phenol red, and cresol red are amphiprotic and have a −1 charge at pH 4.0, which accounted for their migration toward the positive electrode, whereas methyl red was the only indicator almost completely in the neutral form at pH 4.0 so it did not appreciably migrate. When we focus on indicators with similar shape and molecular weight, it is possible to see the effect of charge on the relative rate of migration under constant pH conditions. For example at pH 4.0, comparisons between methyl orange versus methyl red or bromothymol blue versus bromophenol blue emphasize the greater relative rates of migration for molecules that have higher average charges.

The shape and size of molecules can also directly affect the relative migration rates of indicators. Compact, spherical molecules migrate with a faster rate than linear molecules, which have greater frictional interactions with the gel. Comparisons of the relative migration rates for methyl orange and methyl red versus methyl violet 2B at pH 7.5 clearly exhibit the greater frictional interactions that linear molecules experience and the correspondingly slower relative migration rates (Figure 1A). Focusing on indicators with similar shapes and charge but variable molecular weights can illustrate how smaller molecules will have greater relative migration rates. For example, relative migration rates for bromothymol blue versus bromophenol blue or cresol red versus phenol red show that larger molecules will have slower relative migration rates when shape and charge are similar. However, shape or size does not always dictate the extent of migration. Although bromothymol blue and bromophenol blue are the largest molecules, their −2 charges resulted in relatively faster migration rates. Further questions that students explored can be found in the Supporting Information, as well as generic answers provided by the instructor.

SUMMARY AND CONCLUSION

This laboratory experiment provided a basic method for understanding the processes involved in gel electrophoresis. Students were able to comprehend the process of electrophoresis and how it was affected by voltage and pH, as seen through student reports and responses to postexperiment questions (included in the Supporting Information for Students). Students also gained insight into weak acid/base equilibria and explored the relationship between mass, shape, and charge with respect to changes in pH. The pH range used in this experiment was appropriate for this suite of indicators; however, students potentially could explore additional acid/base behavior by expanding the selection of buffer solutions to accommodate other pH values and/or using other indicators. Although the species being examined were quite simple, the same concepts can be applied to proteins, amino acids, and other biochemical molecules.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/ed500223d.

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J. Chem. Educ. XXXX, XXX, XXX−XXX

Student experimental procedure includes appropriate background on acid/base indicators and postexperiment questions (PDF, DOC)
Information for Instructors includes detailed procedures, CAS numbers, modifications for a shorter lab period, options for a more guided inquiry-based approach, and answers to questions provided to students (PDF, DOC)

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Notes

The authors declare no competing financial interest.

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J. Chem. Educ. XXXX, XXX, XXX−XXX