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The production, analysis and application of a self-generating mobile phase pH gradient in thin-layer chromatography

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The Production, Analysis and Application of a Self- Generating Mobile Phase pH Gradient in Thin Layer Chromatography

by

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A dissertation submitted to the Graduate Faculty in Chemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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18 January 1995
Date

David C. Lorke
Chair of Examining Committee

Jan. 19, 1995
Date

Executive Officer

Supervisory Committee

The City University of New York

Abstract

THE PRODUCTION, ANALYSIS AND APPLICATION OF A SELF - GENERATING MOBILE PHASE pH GRADIENT IN THIN LAYER CHROMATOGRAPHY

by

Alexander R. Altman

Advisor: Dr. David C. Locke

Several thin layer chromatographic systems are presented in which a mobile phase gradient is generated during development. Potentiometric, spectrophotometric, photographic and gravimetric analysis of the plates during and after development provide evidence for the mechanism by which the gradient is formed. The effect of the gradient on the separation of metal ions is investigated. The gradient is employed in the separation of poly - L - glutamic acid fractions on the basis of their molecular weights.

Acknowledgements

I would like to thank my Mother, Mrs. Renee Altman, for her constant and loving support throughout the years.

I would also like to acknowledge the assistance provided by the Queens College Chemistry Department staff especially my advisor Prof. David Locke, the secretaries Alice Brickman and Jeanne Deutsch and the CLT's Joe Badalamenti and Bob Wurman.

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Introduction

Shortly after the introduction of thin layer chromatography as an analytical technique, chemists began to realize that isocratic development was not sufficient to separate all of the components in every mixture. Various gradient techniques have been devised. Niederwieser and Honegger (1) discussed techniques by which adsorbent gradients can be prepared. These include various plate pretreatments as well as the preparation of continuously varying adsorbent layers. Niederwieser (2) described several ways in which elution gradients can be produced in TLC. These include polyzonal TLC in which a solvent composed of components of different polarity unmixes as development occurs, mechanical alteration of the developing solvent during development, vapor impregnation where the adsorbent is exposed to the vapors of a volatile solvent during development, and flux gradient TLC where some of the mobile phase is removed during development. Niederwieser also classified the gradients produced. Gradients that are perpendicular to the direction of development are termed "orthogonal". Those gradients in which the gradient direction is in the same direction as the development, in which the analyte is moving into a region of greater mobility, are called "parallel". Those in which the gradient direction is in the opposite direction as the development, in which the analyte is moving into a region of lesser mobility are called "antiparallel". The merits of antiparallel vs. parallel gradients are further discussed by

Geiss et al. (3).

In 1949 and 1950 Golumbic and Orchin (4,5) showed that the partition coefficients of weak acids and bases are dependent on the pH of the media they are in. They formulated the equation:

$$\log k' = pH + \log k - pK_a$$

where k' is the observed partition coefficient and k is the partition coefficient of the unionized substance. This relationship can be utilized in the gradient separation of closely eluting substances with slightly differing values of pK_a.

Several researchers have exploited pH gradients in thin layer chromatographic separations of weak acids and bases. In 1964, Stahl (6,7) introduced a technique for preparing acid - base gradients in silica gel layers. A thin layer spreader was modified by placing a diagonal divider across the trough. An acidic silica gel slurry was placed in one half and a basic slurry in the other. When plates were coated with this spreader a gradient from pH = 0 to 10 was prepared. These plates were most often used in an orthogonal direction. Bands of analyte were applied parallel to the direction of the gradient and the plates were developed perpendicular to the gradient. The developed analytes appeared as curves similar to titration curves. The acid - base properties of the compounds could be inferred from the shapes of these curves. These orthogonal pH gradients were used by Stahl

and Dumont (8) to separate monobasic and polybasic acids, ampholytes, organic bases, alkaloids and pH indicators; by Stahl and Muller for the separation of fluorescent dyes (9) and benzodiazepins (10); and by Quirin (11) for the separation of phosphatides.

Mobile phase pH gradients were used as early as 1949 by Mitchell et al. (12) for the separation of enzymes on a chromatopile. These researchers placed the mixture to be separated on top of a pile of 350 - 450 filter paper disks and allowed a developing solvent to pass through the pile. They then separated the disks and assayed each for enzyme activity. Improved separation was noted when a mechanically prepared gradient of pH, organic solvent or salt concentration was used. A mechanically prepared developing solvent pH gradient was used by Wieland and Determann (13) in the separation of adenine-nucleotides on thin layers of DEAE-Sephadex.

Self-generated elution gradients are much simpler to use. Self-generating mobile phase polarity gradients have been employed in the separations of derivatized amino acids (14), lipids (1), and polymers (15). Self-generated pH gradients were first mentioned as an anomaly.

In 1956 Peterson and Sober (16) introduced the use of derivatized celluloses as ion-exchange chromatographic adsorbents. Their work described the preparation of diethylaminoethyl- (DEAE), epichlorotriethanolamine- (ECTOLA), carboxymethyl- (CM), and phospho- (P) celluloses and their uses in the column separations of

proteins. In 1968 Cozzi, Desideri & Lepri (17) first noted the appearance of a self-generated mobile phase pH gradient when thin layers of CM cellulose in the Na^+ form were developed with solutions of nitric acid. The gradients measured were small, 0.25 pH unit/11.4 cm development. In a later work (18) the same authors investigated the formation of a self-generated pH gradient on thin layers of alginic acid. Solutions of sodium perchlorate produced gradients ranging from 1.1 pH unit/10 cm for 1.0 M to 0.3 pH unit/10 cm for 0.1 M with the lower pH values reported at the solvent front. They explained these results as being due to ion exchange followed by a "transfer of hydrogen ions towards the solvent front." Acetate buffer solutions were also used as developing solvents for alginic acid layers. When the concentration of sodium acetate was varied and the pH of the solution was kept at a constant 2.70 by the addition of HNO_3, a gradient of 0.6 pH unit/9 cm was measured in the layer with the lower pH being measured at the solvent front. The curves were similar but the 0.1 M solution produced the highest curve and the 0.4 M the lowest. This gradient was attributed to ion-exchange. When the sodium acetate concentration was held at 0.20 M and the pH varied by the addition of HNO_3 only a slight pH gradient, (0.3 pH unit/9 cm), was observed for an initial solvent pH of 1.40. The pH increased towards the solvent front. This gradient was attributed to "absorption". Those solutions of lower initial pH 1.00 and 1.22

produced no gradient. Similar experiments were carried out using carboxymethyl cellulose. The layers were developed with equimolar sodium acetate/acetic acid buffer solutions. When the acid form of CM cellulose was used the pH remained constant for the first 7 cm of development and showed a drop off of 0.2 pH units at the solvent front (9 cm) when 1.0 M and 0.5 M solutions were used. When a 0.1 M solution was used the pH measured quickly dropped after 5 cm and remained constantly acidic to the solvent front. When the sodium form was used no pH gradient was produced with the 1.0 M and 0.5 M solutions but the 0.1 M solution showed a larger gradient, almost 2 pH units/9 cm, with a pH of 6.5 at the solvent front. They also observed the behavior of metal ions chromatographed on CM cellulose in the sodium form using acetate buffers. Although no other experiments besides pH and metal ion R_f were mentioned, Cozzi, Desideri and Lepri asserted that the gradients were "due to: [1] An ion-exchange process; [2] A process of absorption of acid by the substrate." These authors published no further experiments on the possible mechanisms of formation of these gradients or on their exploitation in chromatographic separations. However the same authors and Coas (19) explained anomalies in their separations of amino acids on alginic acid layers by stating that "[t]he discrepancy between the theoretical and experimental values. . . is probably due to a pH gradient along the plate." Lepri, Desideri, Landini and Tanturli (20) explained anomalies in their separation of phenols on layers of

Dowex 50 X4 by saying that "[s]uch disagreement between theoretical and experimental values may be correlated with the different pH on the layer with respect to that of the eluent. . . . we have measured the pH on the layer and found pH values between 9.2 and 9.5 for sodium hydrogen carbonate and between 10.3 and 9.8 for sodium carbonate." Similarly Lepri, Desideri and Coas (21) explained anomalies in their separations of aminophosphonic acids on Dowex 50 X4 by saying that "[t]he discrepancy between the theoretical and experimental values is probably due to a pH gradient along the layer."

The Masters research of the current writer (22) concerned the purposeful production of a self-generating pH gradient in thin layer chromatography. The development of CM cellulose plates with a pH 7 phosphate buffer did not produce a continuous variation in pH as determined by spraying the developed plate with a universal pH indicator solution. Instead a discrete change in pH was observed at a height proportional to the strength of the buffer. A continuous gradient of 1.6 pH units/10 cm was measured using a pH meter equipped with a flat surface combination electrode on C-18 silica gel thin layers developed with a triethylamine/triethylammonium chloride buffer in ethanol-water. The more acidic measurement was made toward the solvent front. No experiments were performed to account for the mechanism by which the gradient was formed. It was believed to have been due to the progressive neutralization of the basic mobile

phase by the naturally acidic stationary phase. Considering that triethylamine has a higher volatility than its chloride salt, a flux gradient may have been responsible. The polarity of triethylamine is lower than its salt so polyzonal development may have also been occurring. The gradient produced showed little effect on the separations of amino acids, small peptides, nitroanilines or nitrophenols. It was able to separate samples of polyvinylalcohol on the basis of their molecular weights.

The current Doctoral research demonstrates several reproducible thin layer chromatographic systems in which a self-generated mobile phase pH gradient is produced. Several techniques for measuring the pH of thin layer plates were developed. Experiments were performed on both developed and developing plates using gravimetric, potentiometric, spectroscopic and photographic methods to determine the manner and mechanism by which the gradient is formed. The effect of the gradient on the separation of metal ions was investigated. The gradient was employed in the fractionation of samples of poly-L-glutamic acid.

TECHNIQUES FOR MEASURING GRADIENTS

In any experiment involving gradient chromatographic techniques, the most fundamental question must be: Has a gradient been formed and if so what is its magnitude and form? Several experimental techniques have been used for making these measurements.

Cozzi, Desideri and Lepri (18) measured the pH of their alginic acid and carboxymethyl cellulose thin layer plates using two techniques. "(1) Direct pH measurement on the surface of the layer using a special combined electrode (Polymetron).
(2) Measurement of the pH of the suspensions obtained by dispersing equal portions of the layer (bands) in 5 mL portions of distilled water after elution. A glass electrode was used for the measurements, which were carried out after the suspensions had been agitated for 2 minutes"

During the Master's research by the current researcher, (22) the pH of a C-18 Silica gel thin layer chromatographic plate was measured using a flat surface combination pH electrode (Fisher Scientific). Since the mobile phase used in those experiments was volatile at room temperature, measurements were carried out while

the plate was still in the developing tank. The electrode, held by a Bunsen clamp, was lowered into the tank perpendicular to the plate. At the end of the Bunsen clamp was a pointer indicating distance on a meter stick, thus indicating the vertical distance along the plate at which the pH measurement was being made.

In the same research, pH changes in carboxymethyl cellulose plates developed with non-volatile mobile phases were measured by spraying the plates with a universal indicator solution, (Fisher Scientific), and comparing the colors with a standard chart provided by the manufacturer.

In the current research, several techniques for measuring the pH of a thin layer were employed. Most commonly, a Corning Model 115 pH meter equipped with a Flat Membrane pH electrode (MI-404) and a Micro-Reference Electrode with Flexible Barrel (MI-402) from Microelectrodes, Inc. (Londonderry, NH) (Figure 1) was used. After development, the plates were removed from the chamber and the portion in contact with the developing solvent blotted. The plate was then placed on a slanted surface, and a specially prepared template (Figure 2) (fabricated by Mr. Ed Kuhner of the Physics Department of Queens College), was placed on top of the plate, with the etch mark placed at the top of the portion of the plate that was submerged in developing solvent. This

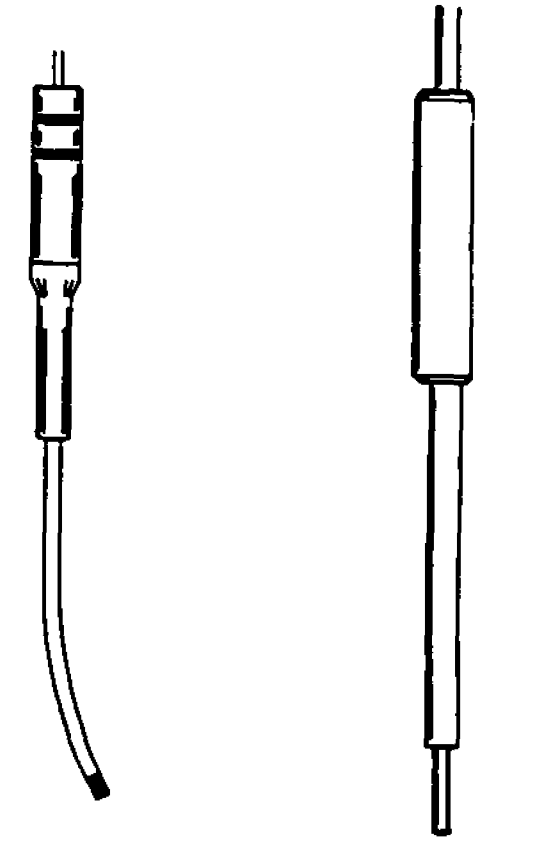


Figure 1 Microelectrodes. Illustrations from operations manual from Microelectrodes, Inc. (Londonderry, NH)

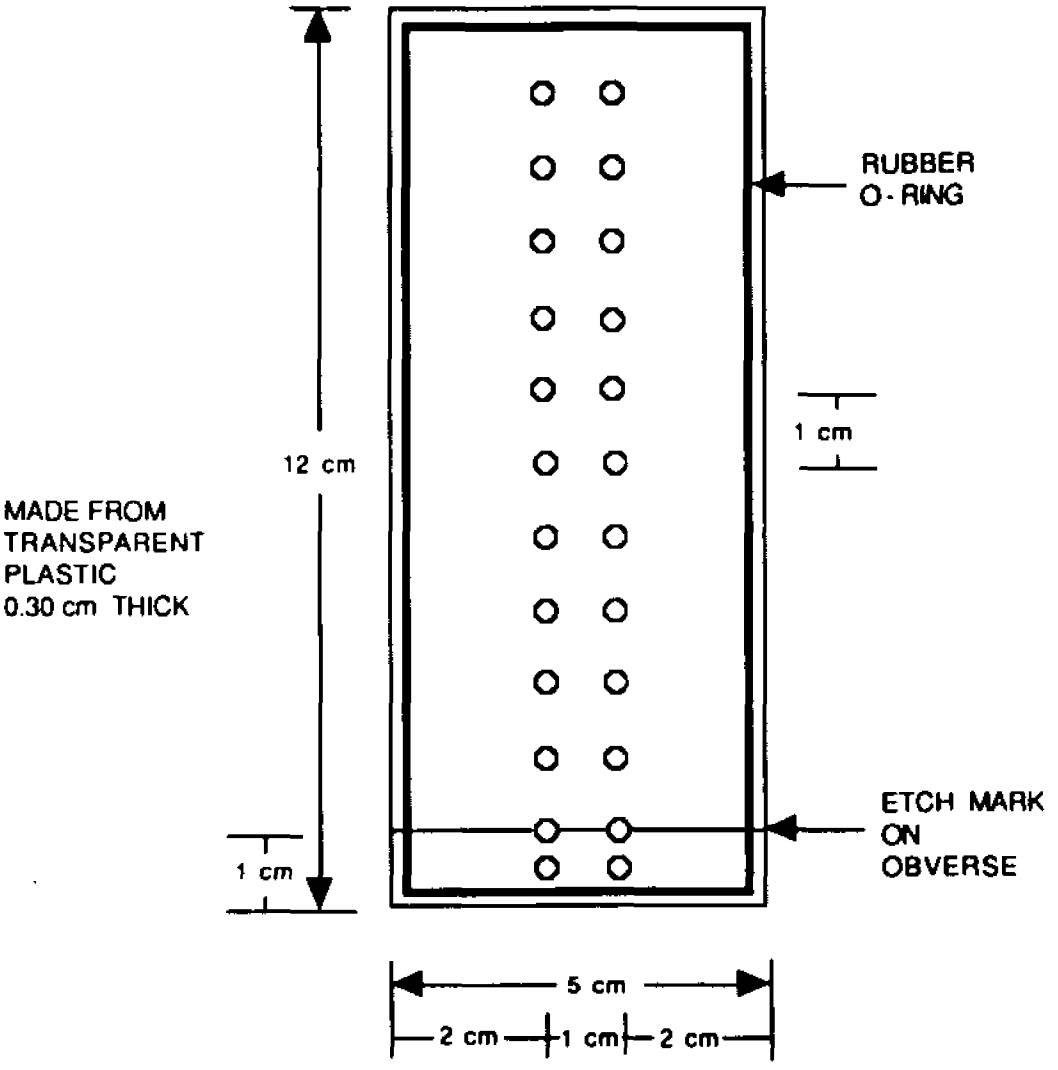


Figure 2. Template fabricated for the measurement of the pH of thin layer chromatographic plates

template served two functions. The rubber o-ring seal helped to minimize possible after-development pH changes due to evaporation, and the precisely drilled holes insured accurate and reproducible measurement of distance. The pH and reference microelectrodes were then placed in adjacent holes and held against the thin layer, and the pH at that distance was measured. Stable pH measurements were usually obtained within 10 sec.

Measurements on a flat surface correlated well with those in solution. The electrodes were calibrated against a pH 7.00 phosphate buffer and a pH 4.00 phthalate buffer. Samples of these buffers were then applied to pieces of filter paper and measured using the flat surface electrodes. These measurements were 7.00 and 4.00, respectively.

The same template was also used to measure the pH at one particular distance while the plate was being developed. Small holes were bored in rubber gas chromatography septa, (1 cm dia., cut to 0.2 cm in thickness) which were affixed using cyanoacrylate glue (Krazy Glue, Borden Inc., Columbus, OH) to both sides of the template over the holes at 1 cm above the start line. These allowed the reference and pH electrodes to be held firmly in place. A special cell was created, (Figure 3) to hold the thin layer plate by affixing 10 cm X 2.5 cm glass plates to a larger 5cm X 20 cm plate using cyanoacrylate

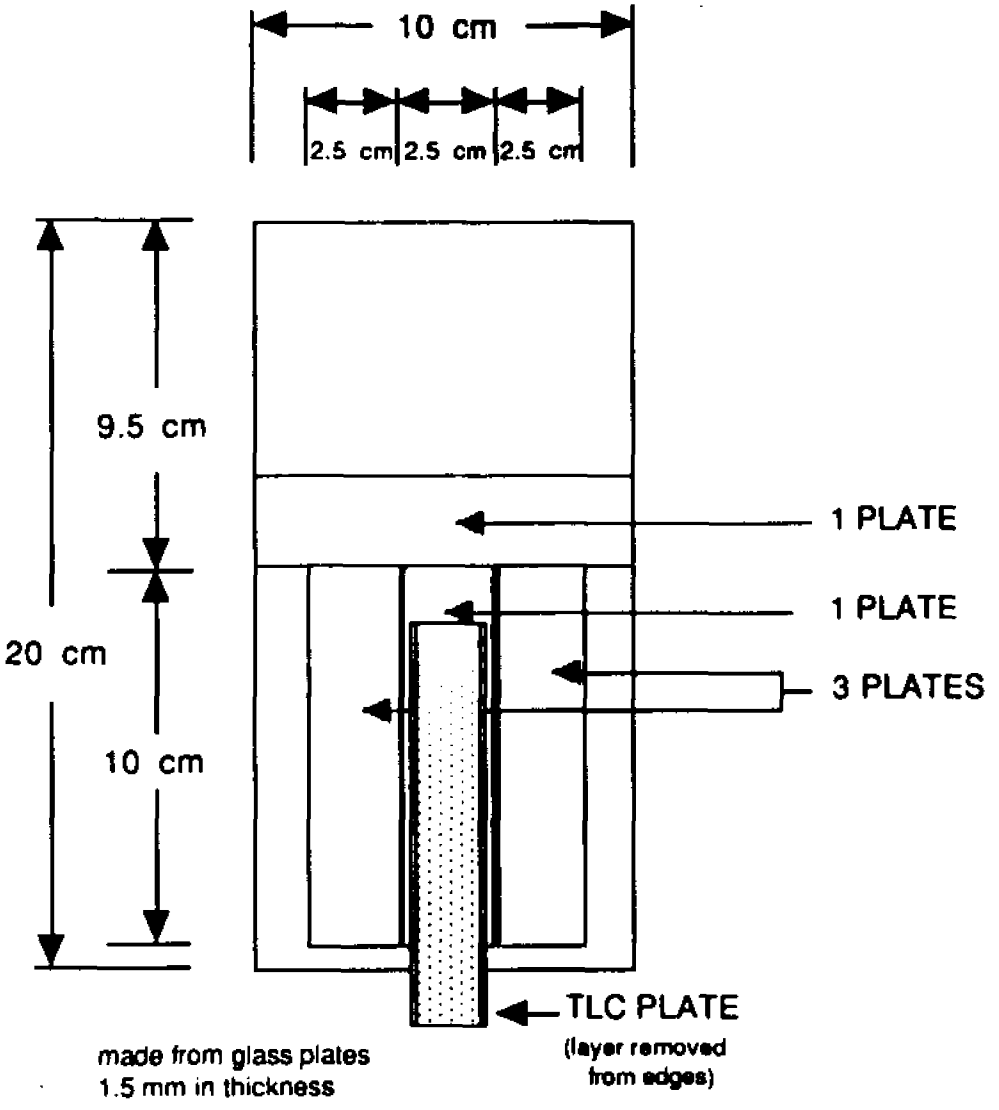


Figure 3. Bottom of a Special Cell Fabricated for the Continuous pH Measurement of a Thin Layer Chromatographic PLate

glue. The thin layer plate was then attached to the special cell using transparent tape. Approximately 1 cm of the thin layer plate was allowed to protrude from the cell. The cell was covered by the template which was equipped with flat surface pH and reference electrodes. The whole apparatus was then held vertically on top of a petri dish into which the thin layer plate was allowed to protrude. Developing solvent was then added to the petri dish to begin development. The signal from the pH meter was fed through a level shifting amplifier (designed and fabricated by Mr. Bob Bunche of the Physics Department of Queens College) . This signal was then sent to a Shimadzu R-111 strip chart recorder providing a read out of pH vs. time This plot was then correlated to pH vs. height of solvent front.

The pH of a thin layer was also determined during development colorimetrically. Several pH indicators had been determined to have little or no mobility on the thin layers studied. Vertical bands 0.3 cm in width were incised in the thin layer plates. A thin layer plate was placed on a glass plate (5 X 20 cm) and covered with two other glass plates to mask all but one vertical band. The resulting "sandwich" was held together by binder clips and placed upright in a hood. An approximately 0.1% (w/v) ethanolic or methanolic solution of a given indicator was applied using a 250 mL TLC sprayer (PYREX No. 5000) to cover the exposed band, and allowed to air dry. The masking plates were removed and the next band was similarly treated

with a different indicator. Up to 6 different indicators could be applied to a 10 cm X 5 cm plate. A special cell was fabricated from glass plates and cyanoacrylate glue (Figure 4) Approximately 0.3 cm of the stationary phase was removed from the vertical edges of the thin layer plate to which the indicators had been applied . The thin layer plate was then affixed by transparent tape to the bottom half of the cell, leaving approximately 1 cm protruding from the bottom of the cell. The top half was then placed atop the bottom, and the two held together using binder clips. The back of the cell was covered with a layer of filter paper to act as a light diffuser. The cell was held vertically atop a petri dish, and was back lit with light from a GE photoflood bulb. Developing solvent was placed in the petri dish to begin development. Photographs were taken at 1 cm intervals of development, using a Seagull DF-300 35mm SLR Camera, equipped with Rolev close-up lenses and a Mizco tripod.

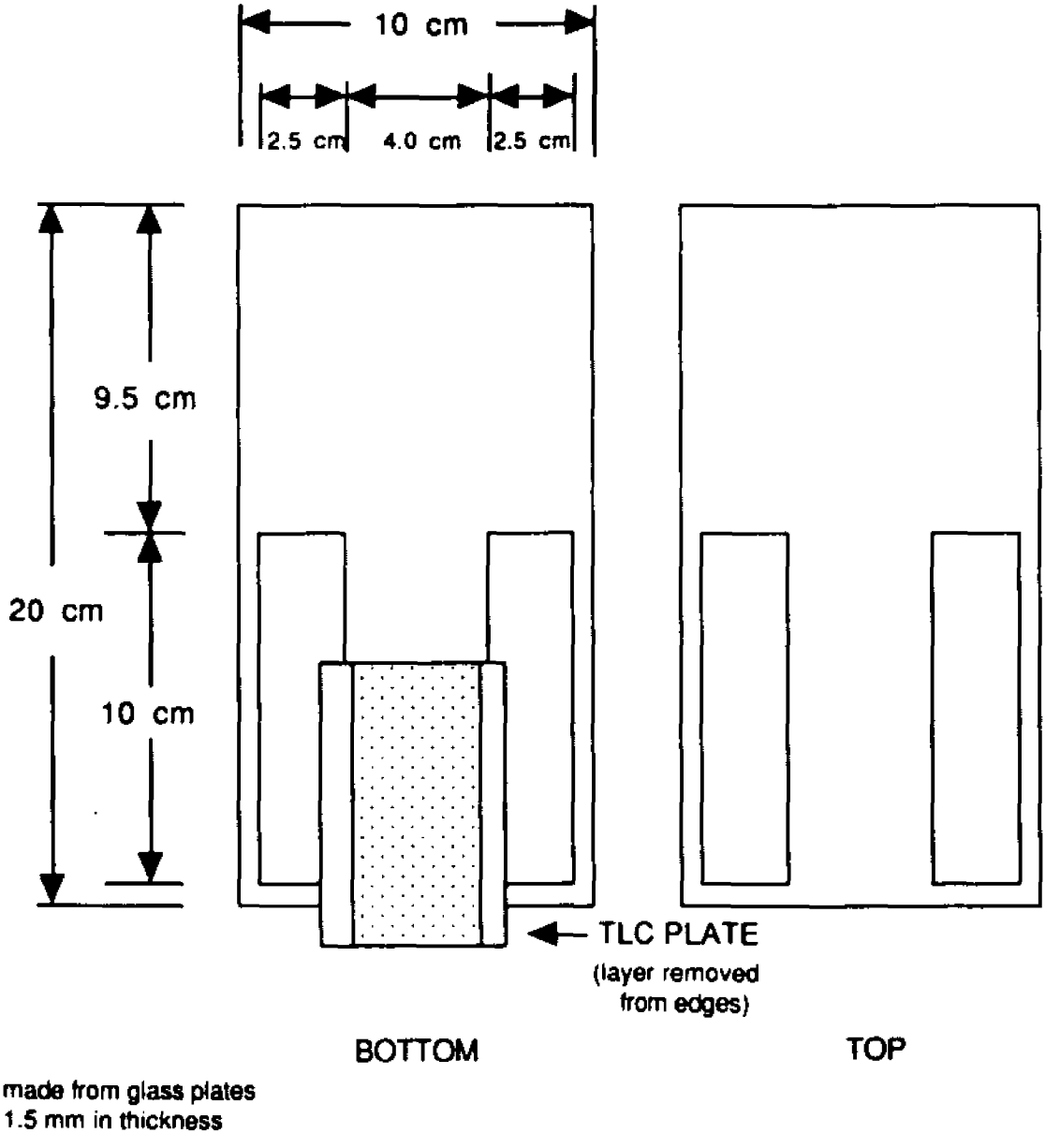
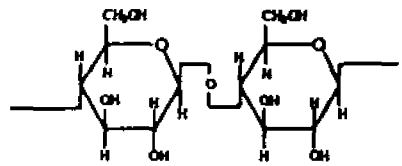


Figure 4. Special Glass Cell Fabricated for the Photography of a Developing Thin Layer Chromatographic Plate

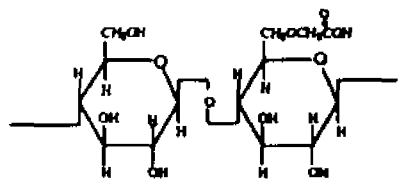
Choice of Stationary Phase.

Although C-18 Silica was used to produce a self-generating mobile phase gradient in previous work (22) , it was felt that the acid-base properties of derivatized silicas were neither well defined nor consistent. Those stationary phases intended for use in ion exchange thin layer chromatography usually possess well defined acidic or basic moieties . The concentrations of these moieties are usually well controlled. The stationary phases most often employed are the derivatized celluloses. For the current work, carboxymethyl and diethylaminoethyl celluloses were used (Figure 5) . Polyester sheets pre-coated with 100 μm layers of these celluloses are commercially available from Whatman and are known as Cellulose 300 CM [4410-223] and Cellulose 300 DEAE [4410-224], respectively. Similar DEAE Cellulose plates, known as Polygram CEL 300 DEAE are produced by Machery - Nagel.

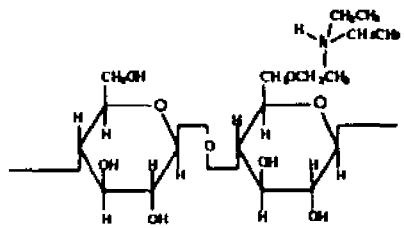
The Whatman CM cellulose plates as received were in the protonated form. This was apparent as a Cellulose 300 CM plate developed with distilled water and measured using the template and pH electrodes technique showed an average pH of 5.4. Development with solvents of increasing ionic strength showed lower pH values. But in all cases, development with aqueous NaCl solutions did not produce a pH gradient (Figure 6).



Cellulose



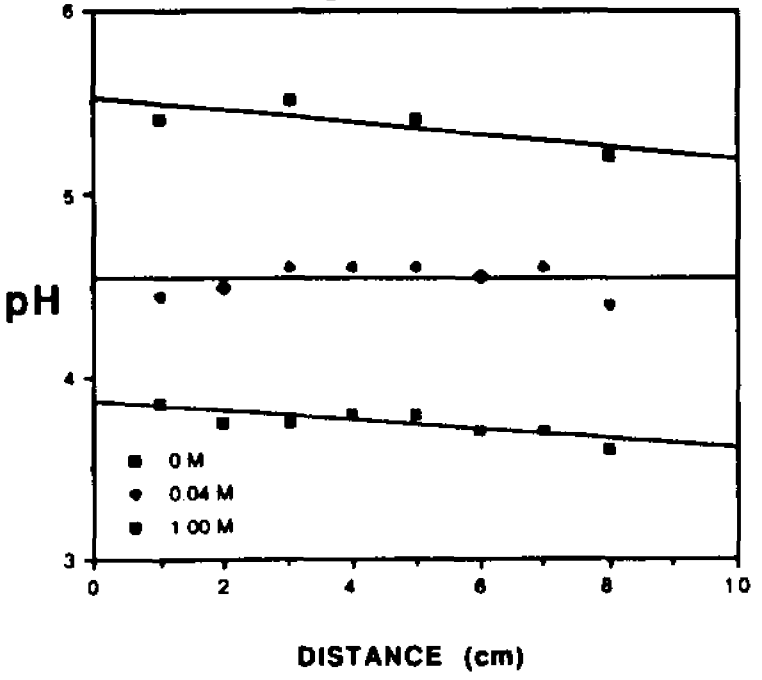
Carboxymethyl Cellulose



Diethylaminoethyl Cellulose

Figure 5. Cellulose and its Derivatives

Figure 6. Curves of pH vs. Distance for CM Cellulose plates developed with solutions of varying NaCl concentrations



In order to determine the sodium ion concentration necessary for the maximal exchange of protons, a titration was performed. A 0.157g sample of CM cellulose was removed from the Whatman pre-coated plates and was suspended in 20 mL of distilled water. Aliquots of 3.0 M NaCl were added via a buret and the pH was measured using a Corning 115 pH meter equipped with a glass combination electrode. The results showed that increasing the sodium ion concentration led to a decrease in pH. But above approximately 1.00 M increasing the sodium concentration yielded no further decrease in pH. The resulting curve is shown in Figure 7. Following this the suspended resin was then titrated with 0.0208 M NaOH. This titration revealed that the concentration of carboxymethyl groups on the cellulose was approximately 0.09 meq/g and that the apparent pKa of the titratable proton was approximately 4.8. This is very close to the tabulated pKa of acetic acid, 4.74. The resulting curve is shown in Figure 8.

The Machery - Nagel DEAE Cellulose plates were also received in the protonated form, as the average pH of a plate developed with distilled water was 4.75.

Figure 7. Titration of a suspension of CM cellulose from Whatman TLC plates by 3.0M NaCl

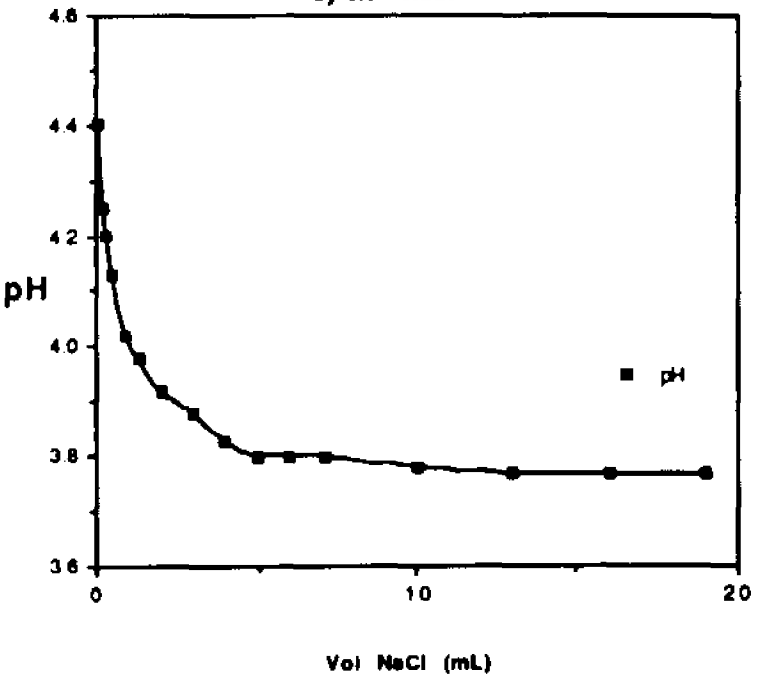
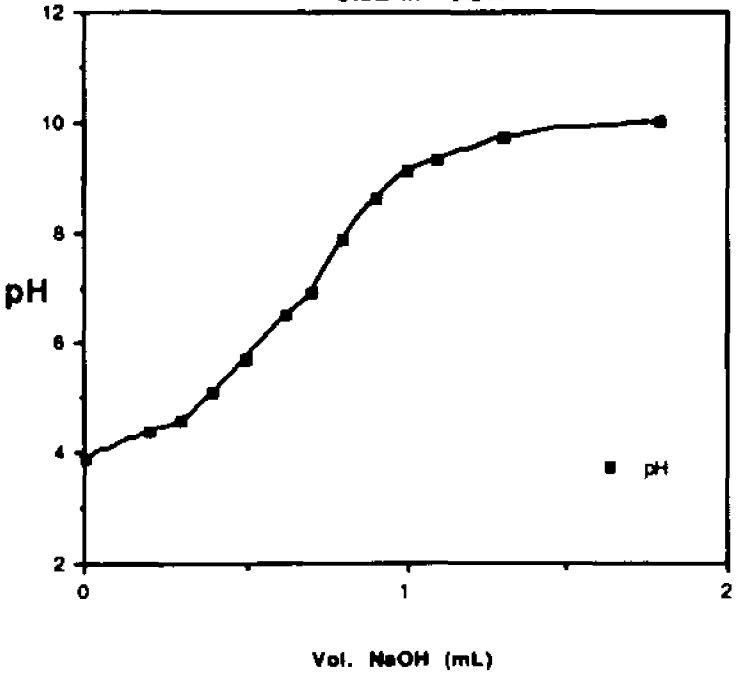


Figure 8. Titration of CM Cellulose suspended in1.5 M NaCl by 0.02 M NaOH



Developing Solvents

Throughout this work a distinction is made between the terms "developing solvent" and "mobile phase". The term developing solvent is used to denote the liquid into which a thin layer chromatographic plate is placed. The term mobile phase is used to denote the liquid which passes through the stationary phase. It is often assumed that these two are synonymous and in many chromatographic systems the two are identical. The aim in this work is to produce and study chromatographic systems in which they are not.

Since one of the stated goals is to produce a mobile phase pH gradient, a developing solvent capable of undergoing gradual changes in pH is required. Given that the commercially available ion exchange thin layer plates are supplied in the protonated form, one must use a basic developing solvent if any pH altering reaction is to take place between the mobile and stationary phases. The use of strong base solutions, (e.g. NaOH) would involve a complete neutralization reaction which would produce a non-gradient basic pH throughout the length of the plate. It was felt that solutions of the conjugate bases of weak acids, themselves basic, would be good candidates for use as developing solvents for the production of gradients. Several criteria were used in the selection of these salts. These compounds must have appreciable aqueous solubility in both

their acidic and basic forms, as we want them to travel with the mobile phase and not precipitate as they traverse the plate. Good solubility is also necessary since we need to be able to maintain a fairly large cation concentration, approximately 0.5M or greater, to facilitate the release of protons from the stationary phase. Also, the pK_b of the conjugate base must be sufficiently low to insure a meaningful reaction with the stationary phase. Many salts were evaluated for their ability to produce a gradient. They are listed in Table 1.

TABLE 1 Selected properties of some weak acids and bases

ACID	pKa[L]	MOLAR SOLUBILITY of ACID IN WATER	SALT USED	MOLAR SOLUBILITY of SALT IN WATER
acetic	4.76	∞[L]	Na	5.67[L]
formic	3.75	∞[L]	Na	14.3[C]
propionic	4.87	∞[L]	Na	
hydrofluoric	3.18	∞[C]	Na	1.00[C]
carbonic	6.38	.033 [C]	Na	0.82 (0)[C]
	10.25	-	Na_2	0.67 (0)[C]
o-phthalic	2.95	.0033[(25)L]	K	0.50 (25)[C]
	5.41	-	K_2	3.09[S]
tetraboric	4 9		Na_2	.053 (0)[C]

BASE	pKb[L]	MOLAR SOLUBILITY of BASE IN WATER	SALT USED	MOLAR SOLUBILITY of SALT IN WATER
ammonia	4.76	52.8[L]	Cl	5.55[(0)C]
triethylamine	3.28	∞[L]	Cl	10.9 (28)[L]

All solubilities measured at 20°C, and pK's measured at 25°C unless specified

L = Lange's Handbook of Chemistry (23)

C = CRC Handbook of Chemistry and Physics (24)

S = Solubilities, Inorganic & Metal-Organic Compounds (25)

Experiments in Self Generating Mobile Phase pH Gradient Production

The flexible, precoated, derivatized cellulose thin layer chromatographic sheets were used as received without activation. The 20 cm X 20 cm sheets were cut with a pair of scissors into smaller plates measuring 5 cm X 10 cm. A mechanical pencil was used to scratch away a thin, horizontal line of stationary phase at a desired distance from the bottom of the plate, usually 8.0 or 9.0 cm. This prevented overdevelopment of the plate.

Approximately 30 mL of the developing solvent was placed in a 400 or 1000 mL beaker and the plate was lowered along the walls of the beaker and placed into the developing solvent. The beaker was then covered with a watch glass and the plate was allowed to develop to the scratch mark. The plate was immediately removed and the pH along the length of development was measured using the template and microelectrodes technique. Aqueous mobile phases were able to develop 8.0 cm in these cellulosic stationary phases in usually under 30 minutes.

Developing solvents were prepared from solutes used as received which were dissolved in deionized water. The results of

these gradient forming experiments are shown graphically as plots of the pH measured on the surface of the plate vs. the distance of development at which that pH measurement was made, in Figures 9-20.

As seen in Figure 9, a repeatable pH gradient may be obtained by developing DEAE cellulose plates with a developing solvent of 1.0 M sodium acetate. A nearly linear gradient was obtained with the pH near the solvent front measuring on average 5.5 while the pH measured at the point 1 cm above the level of the developing solvent was on average 7.5. The results of developing CM cellulose plates with 1.0 M solutions of sodium formate, sodium acetate and sodium propanoate are shown in Figures 10 through 12. The average curves for all three are shown in Figure 13. Here, the first 6 cm of development show a modest decrease in pH of approximately 1 pH unit, while the last 2 cm of development show a more steep decrease in pH, approximately 2 pH units. Note also that the curve for 1.0M sodium formate is significantly lower that those of 1.0M sodium acetate or 1.0M sodium propanoate which are rather close together.

The effect of varying the concentration of sodium acetate solutions used to develop of DEAE cellulose plates is shown in Figure 14. The decrease from 1.0M sodium acetate to 0.5M produces only a small difference in the gradient produced. The curves are generally

Figure 9. Curves of pH vs. Distance for Three DEAE Cellulose Plates Developed with 1.0 M sodium acetate

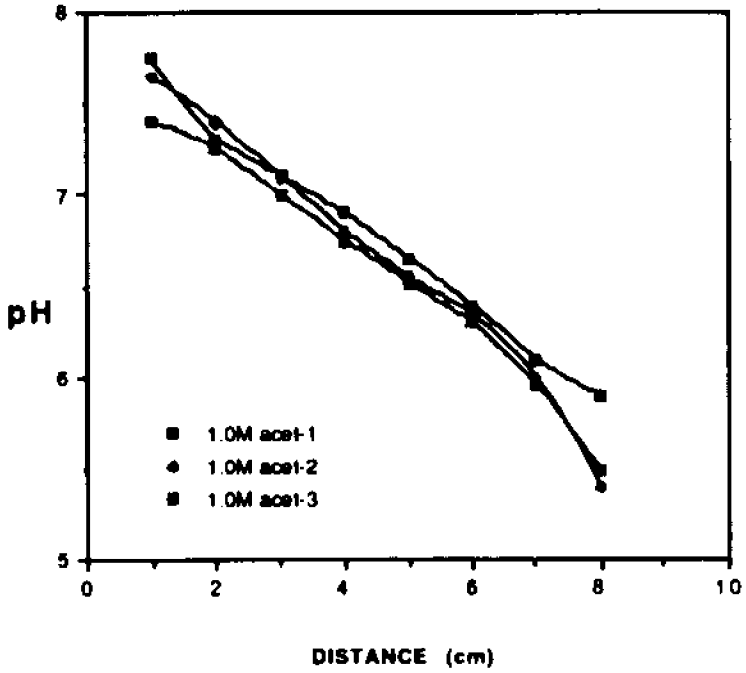


Figure 10. Curves of pH vs. Distance for Three CM Cellulose Plates Developed with 1.0M sodium formate.

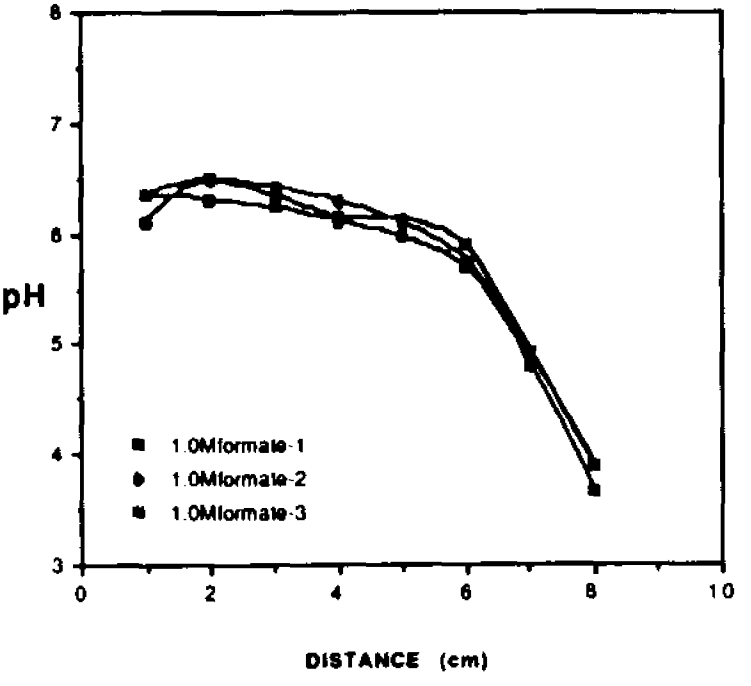


Figure 11. Curves of pH vs. Distance for Three CM Cellulose Plates Developed with 1.0 M sodium acetate

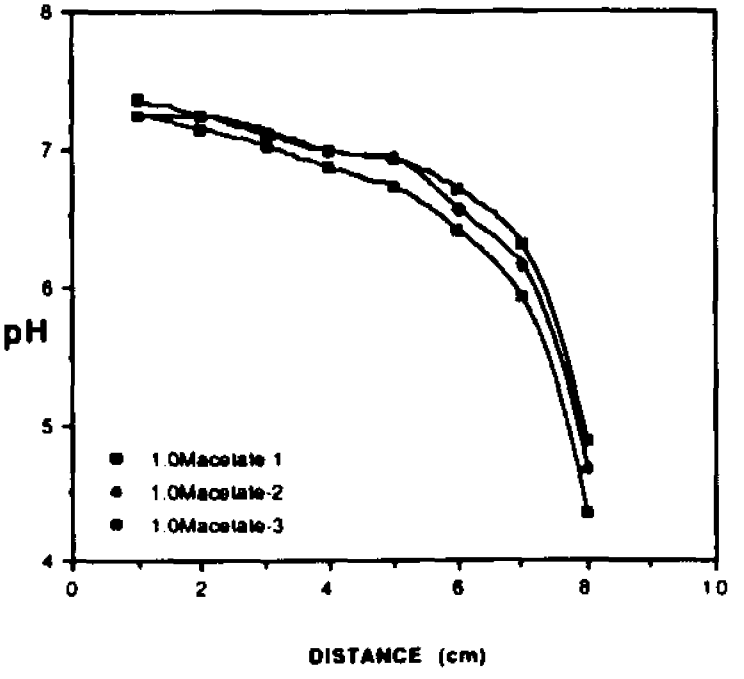


Figure 12. Curves of pH vs. Distance for Three CM Cellulose Plates Developed with 1.0 M sodium propanoate.

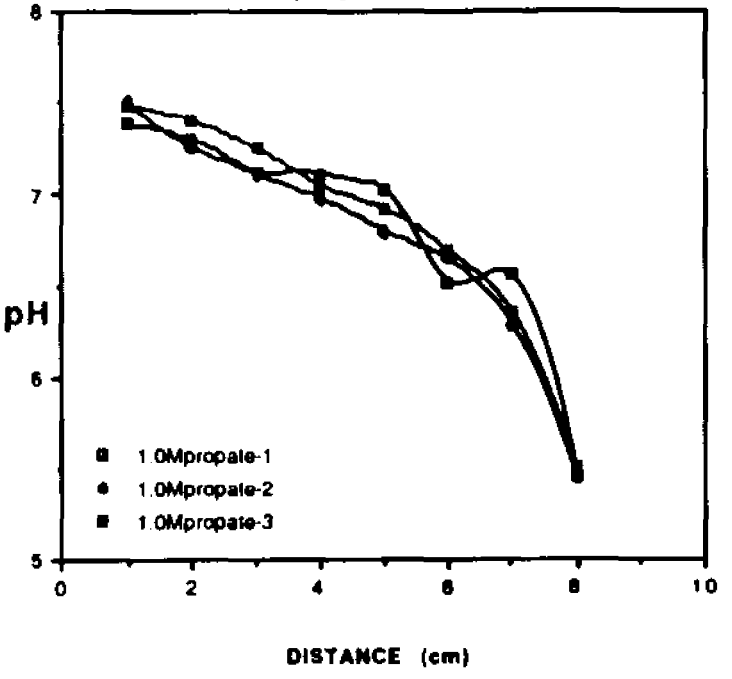
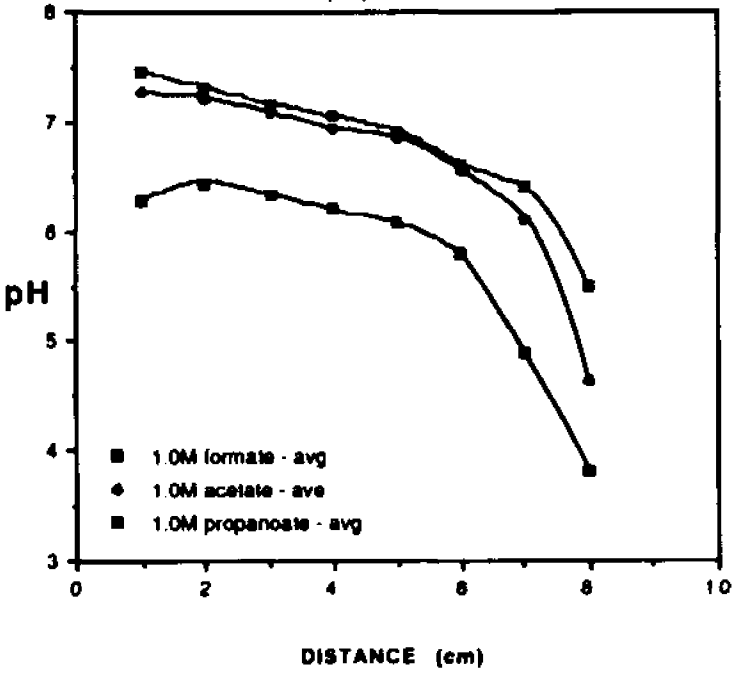


Figure 13. Curves of Average pH vs. Distance for CM Cellulase Plates Developed with 1.0M sodium formate, acteate and propanoate



of the same form, but the curve for 0.5M is slightly lower. The curve obtained using 0.3M is again lower, but also shallower until 7 cm of development, after which a sharper drop off in pH is seen in the last cm of development. When a 0.03 M solution was used, a rapid drop in pH was observed in the lower 4 cm of development and an almost unchanged pH from 4 - 7 cm with an increase in pH toward the solvent front.

The effect of changing the initial pH of the 1.0 M sodium acetate solution used as a developing solvent for DEAE cellulose in shown in Figure 15. These sodium acetate solutions were prepared by adjusting the pH of the solutions with concentrated hydrochloric acid. This maintained a constant sodium ion concentration and a practically constant acetic acid formality. When the initial pH was 7.0, the pH measured in the lower 4 cm of the developed plate showed a constant pH of 6.95 with a decrease in the upper 4 cm down to pH 5.30. When the initial pH was 6.0, the pH remained at a constant 6.15-6.25 for the lower 6 cm of development with a fall to 5.85 only in the last two cm of development.

Aqueous solutions of sodium salts of other weak acids were used as developing solvents. Figure 16 shows the result of using a 0.5 M sodium fluoride solution. The curve measured shows two continuous portions separated by a discontinuous change in pH between 5 and 6 cm.

Figure 14. Curves of Distance vs. pH for DEAE Cellulose Developed with Solutions of sodium acetate of Varying Molarites

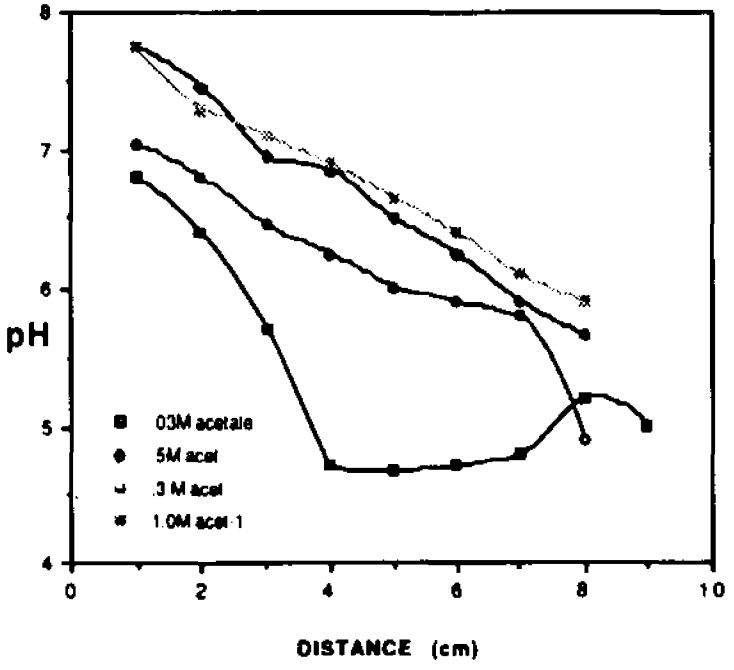


Figure 15. Curves of pH vs. Distance for DEAE Cellulose Developed with Solutions of 1.0 M sodium acetate of Varying Initial pH Values.

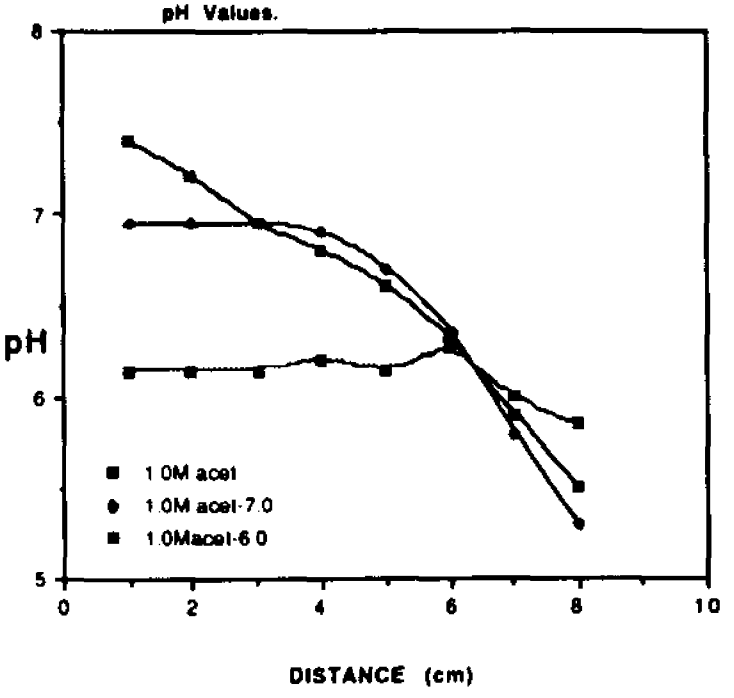
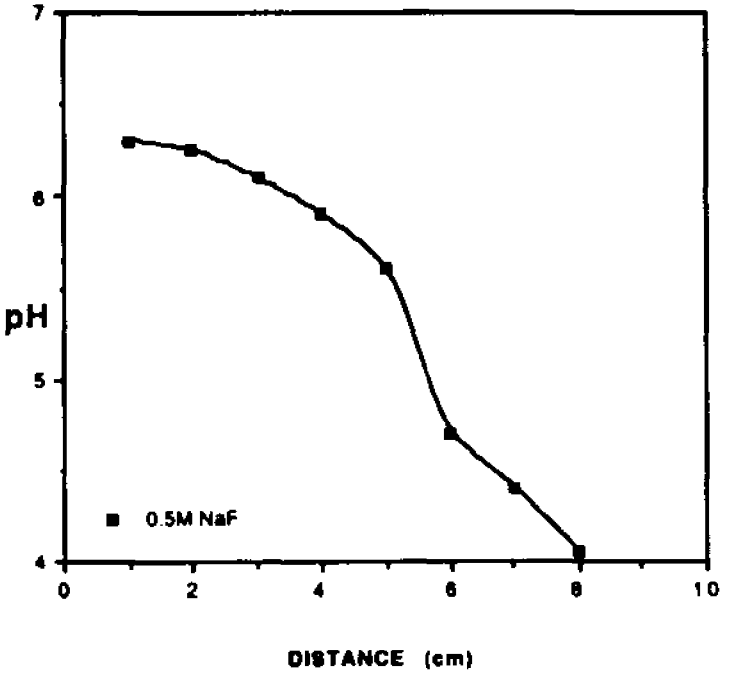


Figure 16. Curve of pH vs. Distance for CM Cellulose Developed with 0.5 M sodium fluoride.



Sodium carbonate and bicarbonate solutions were also used. Figure 17 shows a nearly constant pH for all but the last cm of development and a significantly lower pH value near the solvent front when 0.5 M sodium carbonate was used as a developing solvent for DEAE cellulose . A similar effect is noted in Figure 18. Here the 0.5 M sodium carbonate also showed a constant pH until the region near the solvent front. The more dilute 0.05 M sodium carbonate solution showed a constant pH for the lower 5 cm followed by a discontinuity between 5 and 6 cm and a decrease in the upper 2 cm. The 0.5 M sodium bicarbonate solution showed, but lower similar curve to that of the 0.5 M sodium carbonate solution.

The use of solutions of sodium tetraborate to develop DEAE cellulose in shown in Figure 19. Using a 0.10 M sodium tetraborate solution, a nearly constant pH was measured for the lower 6 cm with a sharp decrease in the upper 2 cm down to pH 5.0. When using the more dilute 0.01M sodium tetraborate, the same sharp decrease was observed , but it occurred in the lower 2 cm, with the upper 6 cm remaining near pH 5. A similar effect is seen in Figure 20 where a 0.01M sodium tetraborate solution was used to develop CM cellulose. Here the sharp decrease in pH was observed after 2 cm of development, and the region between 3 and 7 cm showed a nearly constant pH 5.3, followed by another decrease near the solvent front.

While these experiments show that with a properly chosen

Figure 17. Curve of pH vs. Distance for DEAE Cellulose Developed with 0.5 M sodium carbonate

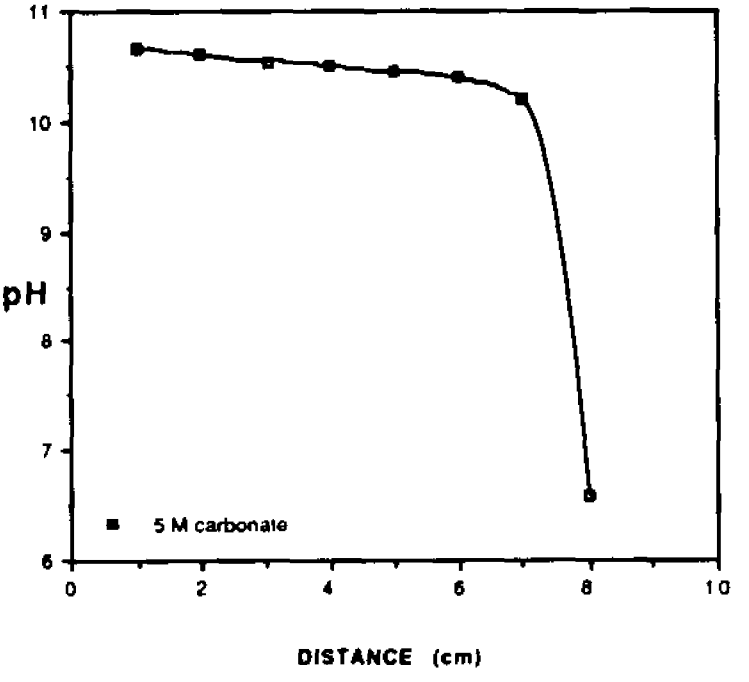


Figure 18. Curves of pH vs. Distance for CM Cellulose Developed with Solutions of sodium carbonate and sodium bicarbonate.

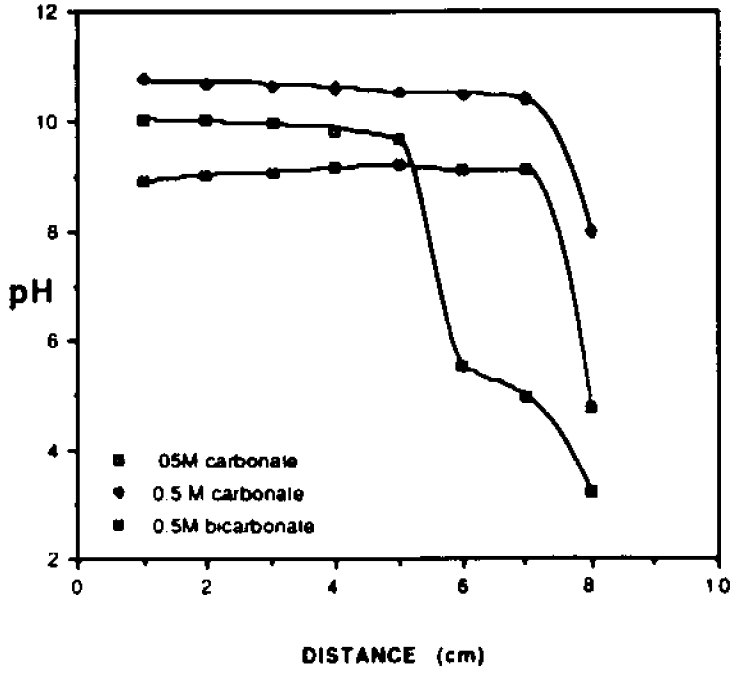


Figure 19. Curves of pH vs. Distance for DEAE Cellulose Developed with Solutions of sodium tetraborate.

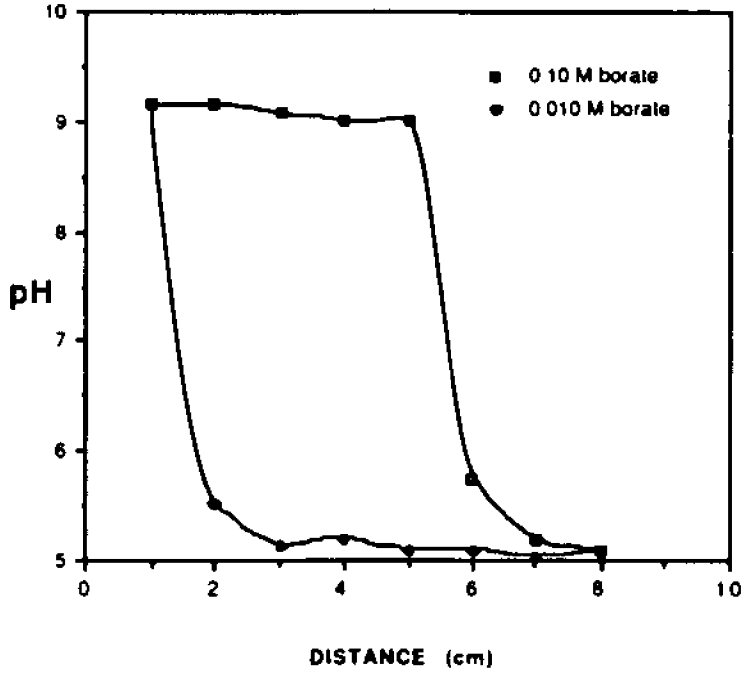
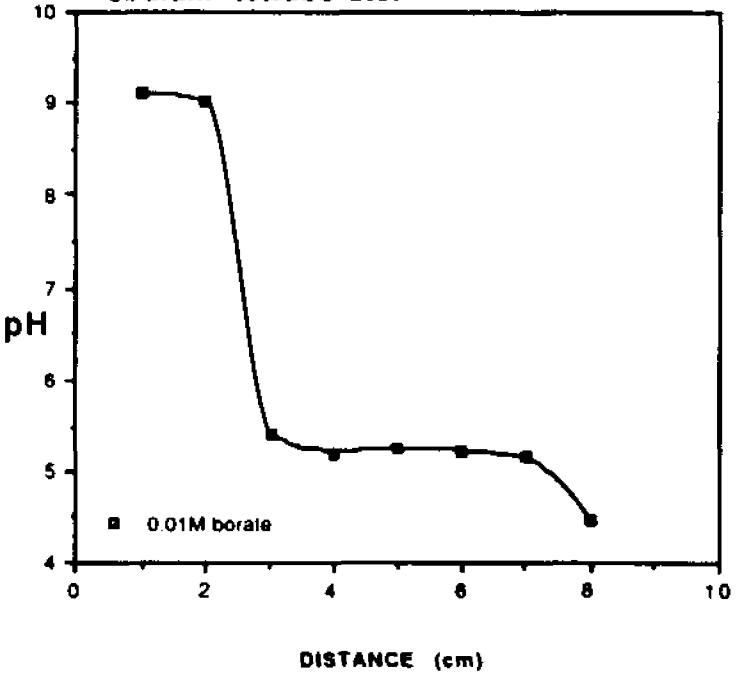


Figure 20. Curve of pH vs. Distance for CM Cellulose Developed with 0.01 M sodium tetraborate.



developing solvent, the pH measured at the bottom of a developed derivatized cellulose thin layer plate will not be the same as that measured near the solvent front, they also raise several questions. Namely: What is the mechanism by which these gradients are produced? Why are some curves continuous and some show discontinuities? Why do some mobile phases produce a nearly constant pH for the lower portion of the plate with a steep decline near the solvent front? How do pH measurements made on a developed plate correlate to the pH environment for an analyte? , i.e. What pH does an analyte "feel"? Further experiments designed to answer these questions are described in the following section.

Experiments toward elucidating the mechanism of self-generating pH gradient formation on carboxymethyl cellulose.

Carboxymethyl cellulose was chosen for the mechanistic experiments primarily because its mechanical stability is greater than that of DEAE cellulose. The DEAE cellulose layers used tended to be rather friable, writing on them with pencil was difficult and removing specific areas of the layer was almost impossible. The DEAE layers also tended to erode easily when touched with the pH microelectrodes.

One could postulate several possible mechanisms by which a basic mobile phase could create a self-generating pH gradient when passing through a protonated ion exchange resin. Four of them are given here.

a) Migration-Titration: A gradient could be formed as a basic mobile phase migrates through the layer and is gradually titrated by the protonated carboxymethyl cellulose. One could picture the stationary phase layer as being divided into n horizontal bands. As the basic mobile phase enters the first band, it encounters some protons and a limited amount of mobile phase cation is exchanged for H^+ and the pH diminishes. In the next band, the same block of mobile phase now

reacts with more protons and its pH diminishes still further until it reaches the n^{th} band where it is most acidic. This mechanism would also explain why the gradient is flatter near the bottom of the plate. After the bottom band of stationary phase has been transited by several bands of mobile phase the number of protons available for exchange has been diminished so the pH will change only slightly. If this mechanism were correct an analyte remaining near the bottom of the plate would always experience a basic mobile phase. If that analyte were to move with the mobile phase the environment surrounding it would become progressively more acidic.

b) Mobile Phase Separation: As early as 1958, Pickering (26) noted that when developing filter paper with an aqueous mobile phase containing NaOH or HNO_3 that two fronts were observed. The developed papers were moistened with universal indicator and it was observed that the upper aqueous front was followed by a front containing either OH^- or H^+. The R_f value of this second front increased with the solute concentration.

The failure of one component of the developing solvent to migrate with the mobile phase could cause a change in the pH measured on a thin layer plate. One would expect to find a discontinuity in the pH vs. distance curve representing the second

front similar to the curves shown in Figures 19, 20 and 21. As in Figure 19, one would expect the height of the discontinuity to increase with increasing solute concentration. One would also expect to be able to observe a discrete change in solute concentration at the same distance as the discrete change in pH.

c) selective retention: If a developing solvent contains more than two components it is possible that one or more of the components will be selectively retained by the stationary phase. This will cause a change in the composition of the mobile phase during development. This was the mechanism employed by Armstrong et al. (15) who separated polymers using a self-generating mobile phase polarity gradient. The pH of a buffered mobile phase is determined by the ratio of the acidic and basic forms of the buffer. If one of the two forms were to be selectively retained by the stationary phase one would predict that a pH gradient would be formed. This selective retention could be due either to ion exchange or to adsorption. This mechanism need not be considered in determining the how a pH gradient forms on CM cellulose since a gradient was observed when the mobile phase was composed of only one form of a weak acid, i.e. acetate. (The concentration of acetic acid in a 1.0 M sodium acetate solution is calculated as 2×10^{-5} M)

d) ion exchange - neutralization: As a band of mobile phase moves through an undeveloped band of the protonated ion exchange resin, the cations in the mobile phase exchange for the protons in the resin. The released protons cause the pH of the mobile phase to drop but the effect is buffered by the presence of the anion which is basic. As more bands of mobile phase pass through that same band of stationary phase, there are increasingly fewer protons available for exchange, and the pH of the mobile phase passing through that band becomes more basic and begins to approach that of the developing solvent. If this mechanism were correct, one would predict that the pH measured at the solvent front would be acidic. An analyte spotted near the bottom of the plate would first experience an acidic environment which gradually became more basic. One would expect to find that the concentration of the anion was nearly constant along the length of a developed plate, but that there should be fewer bound cations at the solvent front than there are near the bottom of a developed plate.

The experimental results that follow provide evidence which will permit the determination of the major mechanism involved.

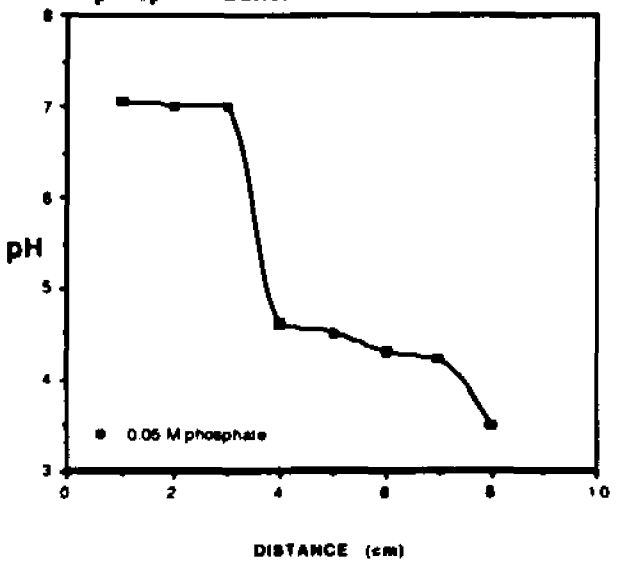
1. Qualitative Determinations of Mobile Phase constituents:

Qualitative experiments showed that the mobile phase

separation mechanism could not be the cause of the results shown in Figure 21. In this experiment a CM cellulose plate developed with a 0.05 M commercially prepared pH 7.00 phosphate buffer (Fisher Scientific) showed a discrete pH change between 3 and 4 cm of development. However when the developed dried plate was sprayed with a Dittmer - Lester phosphate detecting spray (27) the presence of a purple color indicated the presence of phosphate ion up to 7.5 cm of development. The total height of development was 8.5 cm. The change observed in pH is probably not due to a failure of the phosphate ion to migrate with the mobile phase. This is not the case in Figure 20. CM cellulose plates developed with a 0.01 M solution of $Na_2B_4O_7$ were sprayed with a turmeric indicator solution. The solution was prepared by boiling 1g of turmeric (Ehler's) in 40 mL of ethanol. This was filtered through paper and the filtrate acidified with 1 mL of glacial acetic acid and diluted to 50 mL with ethanol. This stock solution was diluted 1:10 with ethanol before use. For use on dried plates the solution was further diluted 1:1 with water. The turmeric indicator spray which is sensitive for borates gave a purple-brown color only in the region of the plate directly in contact with the developing solution and slightly above. This seems to indicate that for the case of sodium tetraborate which has a low aqueous solubility that the mobile phase separation mechanism could explain the discrete change in pH occurring in Figure 20.

Further quantitative experiments, described later, were

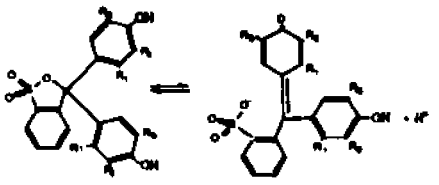
Figure 21. Curve of pH vs. Distance for CM Cellulose Developed with a 0.05 M pH 7.00 phosphate Buffer



performed on CM cellulose plates developed with phthalate solutions. These showed that the mobile phase separation mechanism did not play a role in pH change there. It is likely that this phenomenon is only significant when solutes with poor aqueous solubility are used for making developing solutions.

2. Use of pH Indicator Compounds:

Commercially available pH indicators were extensively used in the current research. Not only do they have well defined acid-base properties, but they require no post-chromatographic visualization. Several indicators were examined to determine their chromatographic behavior on CM cellulose plates developed with 1.0 M sodium acetate. The results of these experiments as well as data pertaining to these indicators are given in Table 2. Those indicators which change color in the range expected for the pH gradient and which show little or no movement in the system studied were used for the photographic determination of the process of the pH gradient. Those which are expected to be involved in an acid-base reaction in the range expected for the pH gradient and which show an R_f greater than 0.3 were considered for use in "R_f-on-the-fly" experiments. The indicators chosen for these experiments are all weak acids as can be seen in Figure 22. This will insure that there is no ion-exchange mechanism involved in the movement of the analytes as CM cellulose is a cation exchange resin. In these



INDICATOR	R_1	R_2	R_3
[illegible]	H	Br	Br
[illegible]	CH_3	Br	Br
[illegible]	H	Cl	H
[illegible]	H	Br	CH_3
[illegible]	CH_3	Br	$-CH(CH_3)_2$
[illegible]	H	Br	H
[illegible]	Br	H	H
[illegible]	H	CH_3	H

Figure 22 Structures of Indicators Used in "R on the Fly" Experiments

Table 2. Selected Data for pH Indicators

Indicator	Acid color	Basic color	Transition range(24)	Rf on CMC*
alizarin	yel	red	5.6 - 7.2	0.10
alizarin red S	yel	red	4.6 - 6.0	0.10 streak
bromcresol green	yel	blue	3.8 - 5.4	0.31
bromcresol purple	yel	purple	5.2 - 6.8	0.51
bromphenol blue	yel	blue	3.0 - 4.6	0.31
bromphenol red	yel	red	5.2 - 6.8	0.68
bromthymol blue	yel	blue	6.0 - 7.6	0.04
chlorophenol red	yel	red	5.2 - 6.8	0.64
congo red	blue	red	3.0 - 5.0	0.00
cresol red	yel	red	7.0 - 8.8	0.38
eriochrome black T	red	blue	5.0 - 6.5	0.00
ethyl orange	red	yel	3.4 - 4.8	0.07
fluorescein	col	grn fl.	4.0 - 4.5	0.37
methyl orange	red	yel	3.2 - 4.4	streak
methyl red	red	yel	4.8 - 6.0	0.34
phenol red	yel	red	6.6 - 8.0	0.68
phenolphthalein	col	red	8.2 - 10.0	0.02

* 1.0M sodium acetate used as a developing solvent

experiments the indicator is spotted on a CM cellulose plate which is placed in the cell fabricated for photographic work (Figure 4) . The cell is clamped vertically over a petri dish into which the developing solvent is placed and a ruler is clipped to the front of the cell. This permits the measurement of the height of both the solvent front and the analyte spot so that the R_f of the analyte can be measured several times while the plate is being developed.

These measurements were made using both gradient-producing (1.0 M sodium acetate) and non-gradient-producing (1.0 M sodium chloride) developing solvents. This will help to ascertain the mechanism by which a self-generated mobile phase pH gradient is formed . Plots of Rf values measured vs. the height of the solvent front when the measurements were made are given in Figures 23-28.

The pKa values of these indicators were determined spectrophotometrically. A dilute solution of an indicator was prepared in 0.1M acetic acid + 0.1M phosphoric acid. Acetate and phosphate help resist changes in pH in the region of interest. The pH of the solution was measured and the visible spectrum of an aliquot from the solution was determined using a Hewlett - Packard 8425A Diode Array Spectrophotometer controlled by the HP 89531A operating system running on a dtK 286 IBM- compatible computer. The pH was incrementally increased by the addition of drops of a saturated NaOH solution and spectra were obtained at

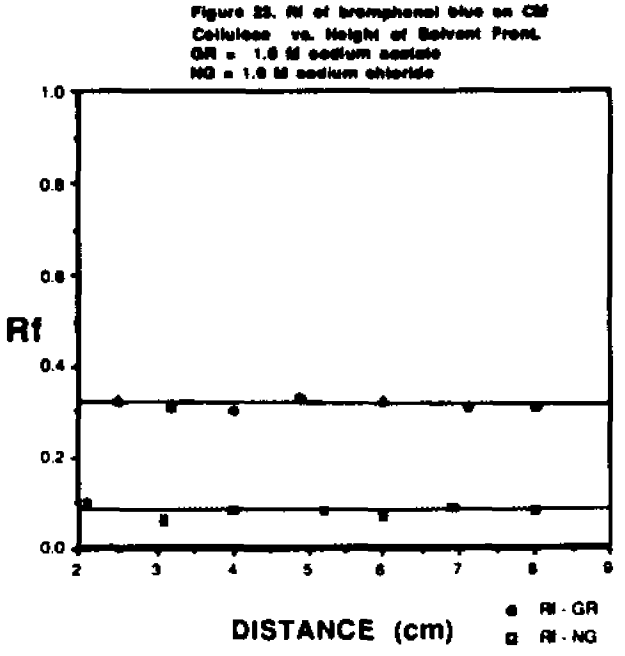
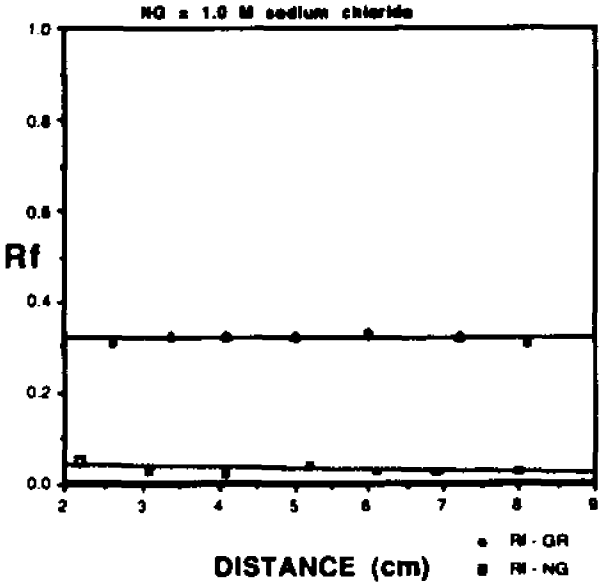

1.0
0.8
0.6
Rf
0.4
0.2
0.0
2
3
4
5
6
7
8
9
DISTANCE (cm)
Rf - GR
Rf - NG

Figure 24. Rf of bromcresol green on CM Cellulose vs. Height of Solvent Front.
GR = 1.0 M sodium acetate
NG = 1.0 M sodium chloride



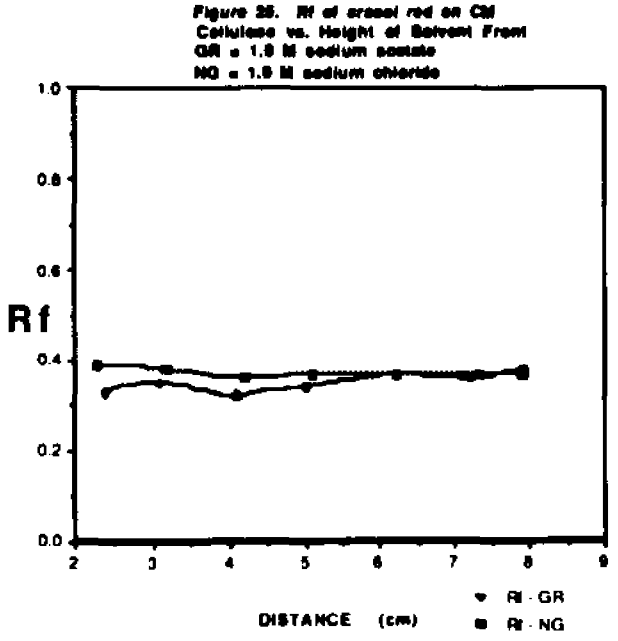


Figure 25. Rf of cresol red on CM
Cellulose vs. Height of Solvent Front
GR = 1.0 M sodium acetate
NG = 1.0 M sodium chloride
1.0
0.8
0.6
Rf
0.4
0.2
0.0
2
3
4
5
6
7
8
9
DISTANCE (cm)
Rf - GR
Rf - NG

Figure 28. Rf of phenol red on CM Cellulose vs. Height of Solvent Front.
GR = 1.0 M sodium acetate
NG = 1.0 M sodium chloride

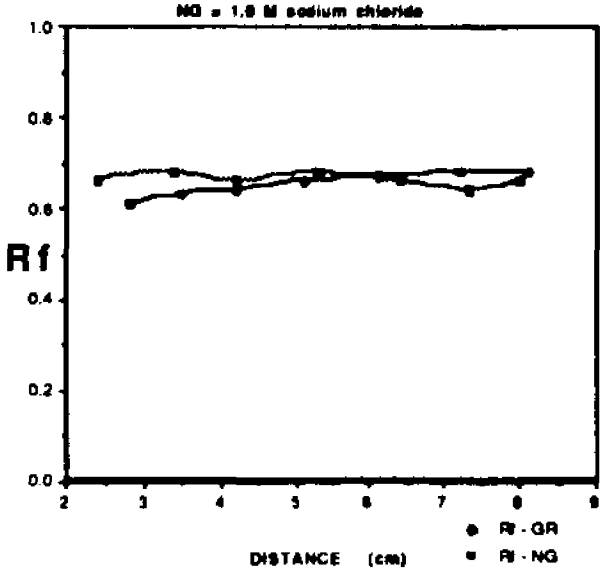


Figure 27. Rf of bromphenol red on Cbl Cellulose vs. Height of Solvent Front.
GR = 1.0 M sodium acetate
NG = 1.0 M sodium chloride

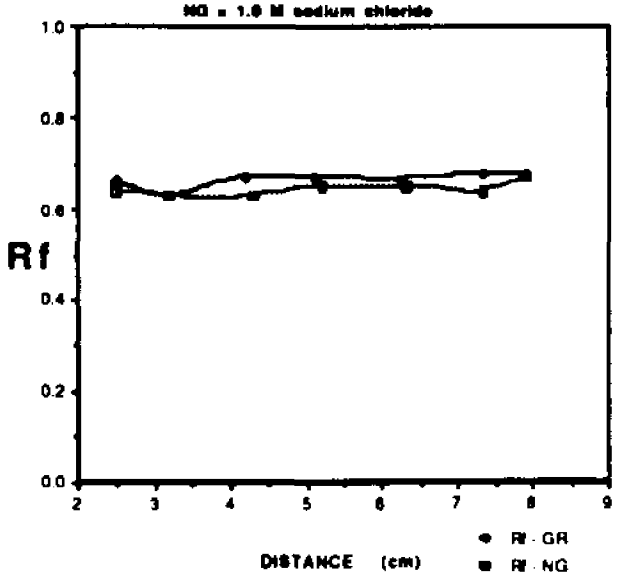
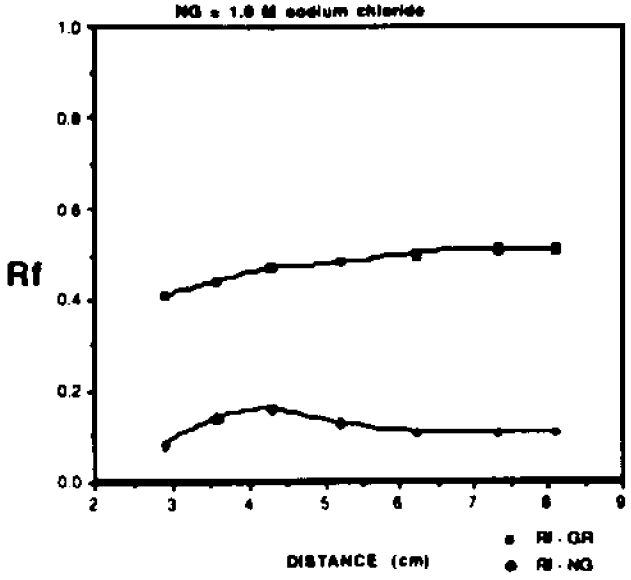


Figure 28. Rf of bromocresol purple on CM Cellulose vs. Height of Solvent Front.
GR = 1.0 M sodium acetate
NG = 1.0 M sodium chloride



approximately 0.5 pH unit intervals. The spectra generated for one indicator, chlorophenol red, are shown in Figure 29.

The pH and pKa of an indicator are related by the equation:

$$pH = pKa + \log ([In^-]/[Hin])$$

If the wavelengths of maximum absorbance are sufficiently different for the two forms of the indicator then the fraction of indicator molecules in the unprotonated form

$$f (In^-) = (A_i - A_0)/(A_{max} - A_0)$$

where A_i is the absorbance of a solution at the the wavelength where the unprotonated form absorbs maximally, A_{max} is the absorbance at the same wavelength when all of the molecules present are in the unprotonated form and A_0 is the absorbance when all of the molecules are in the protonated form. The fraction of molecules existing in the protonated form is simply

$$f(HIn) = 1- \{(A_i - A_0)/(A_{max} - A_0)\}$$

Then

$$[In^-] /[HIn] = \{(A_i - A_0)/(A_{max} - A_0)\} / 1- \{(A_i - A_0)/(A_{max} - A_0)\}$$

Figure 29. Visible Spectra of chlorophenol red Obtained at Various pH Values.

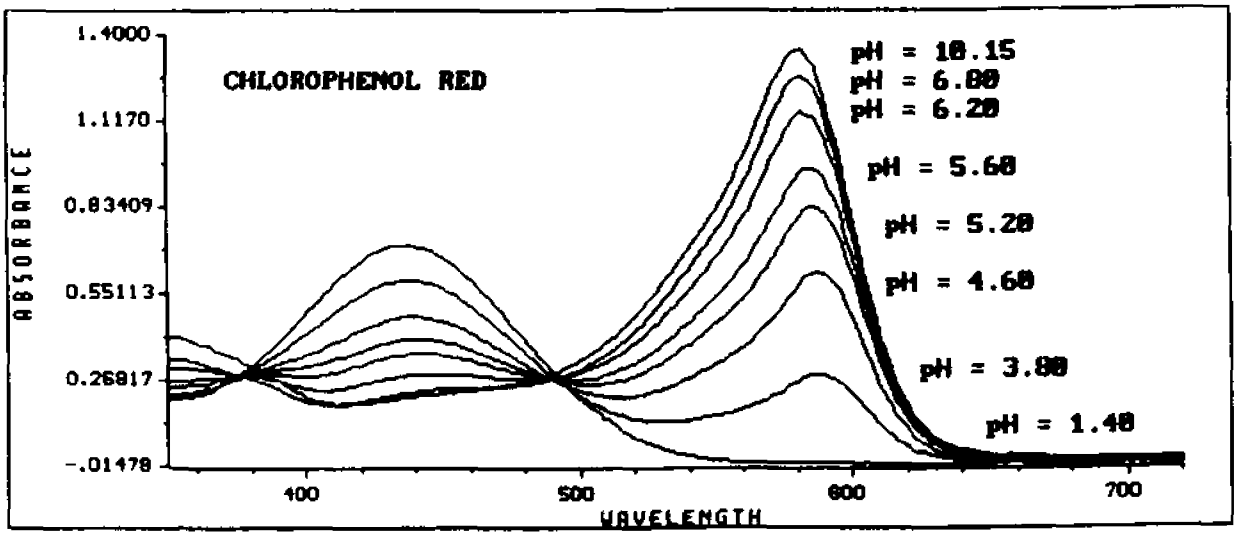
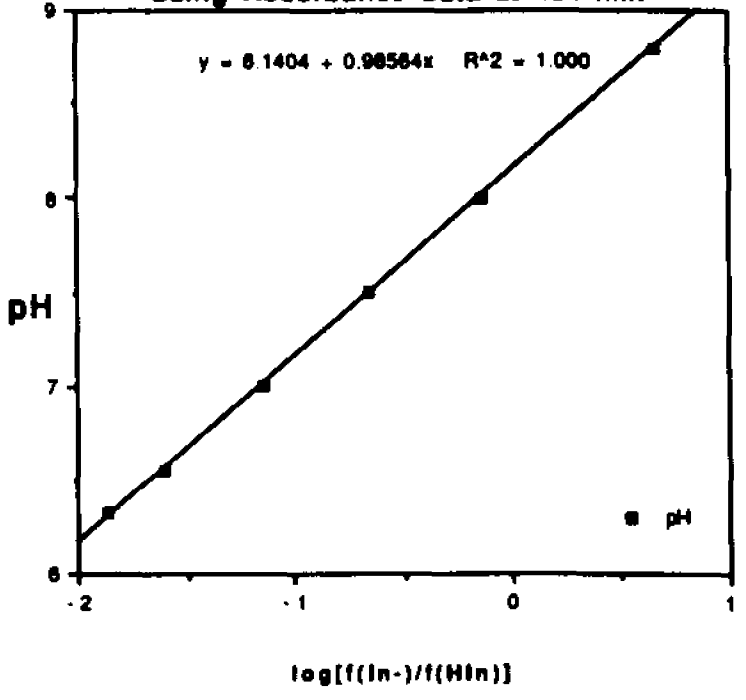


Figure 30. Spectrophotometric Determination of the pKa of cresol red Using Absorbance Data at 434 nm.



and a plot of pH versus $\{(A_i - A_0)/(A_{max} - A_0)\} / 1 - \{(A_i - A_0)/(A_{max} - A_0)\}$
yields a straight line whose y-intercept is the pKa. A representative graph for cresol red is shown in Figure 30. The pKa values that were determined spectrophotometrically are listed in Table 3.

The relationship between the R_f and pH in thin layer chromatography for acids is given by Geiss (28) as:

$$R_f' = \frac{1}{1+K(A_s/A_m)[1+10^{pKa-pH}]}$$

where A_s/A_m is the phase ratio and K the distribution coefficient.

If we assume a hypothetical case where the product $K(A_s/A_m) = 1$, a series of curves can be generated for different values of pKa showing the change in R_f predicted by the above equation when the pH of the mobile phase is changed from 3.75, (which was the measured constant pH when CM cellulose plates were developed with 1.0 M NaCl), to a new pH, pH'. These curves are given in Figure 31.

Figure 31. Predicted Change in R_f when pH of Mobile Phase is Changed from 3.75 to pH'

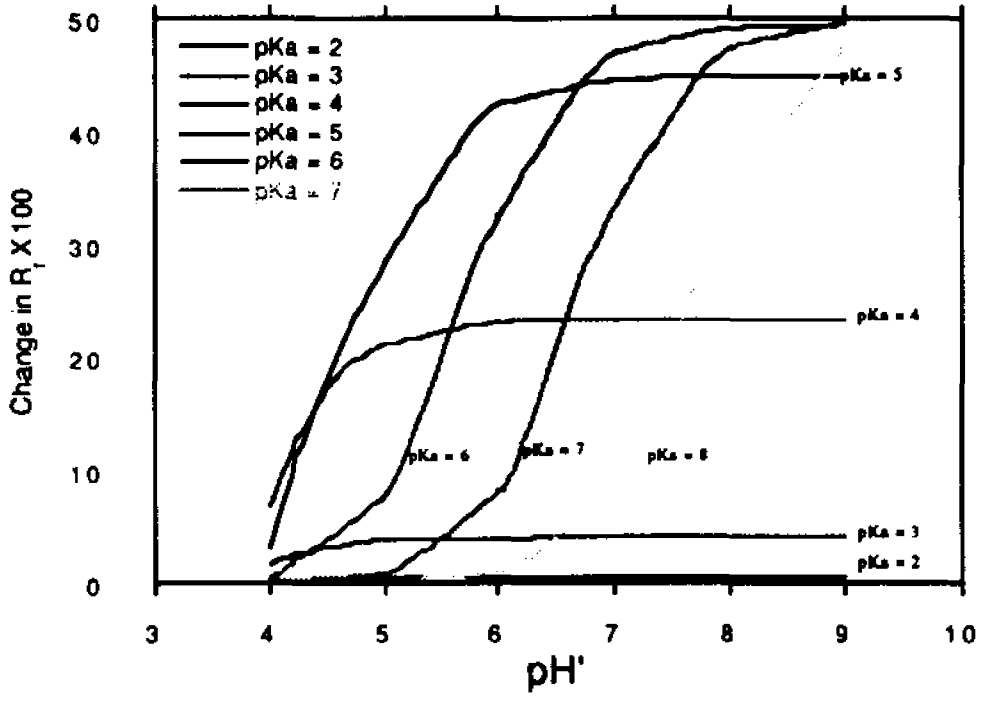


Table 3. Spectrophotometrically determined pKa values for some pH indicators

Indicator	Visual Transition Range	pKa
bromcresol green	3.8 - 5.4	4.65
bromcresol purple	5.2 - 6.8	6.07
bromphenol blue	3.0 - 4.6	3.99
bromphenol red	5.2 - 6.8	7.75
chlorophenol red	5.2 - 6.8	4.75
cresol red	7.0 - 8.8	8.14
phenol red	6.6 - 8.0	7.80

From these curves one could make several qualitative predictions about the chromatographic behavior of weak acids when the pH of the mobile phase used to develop them is changed. Then from a consideration of the actual behavior of indicators developed with gradient and non-gradient development one could determine the actual pH conditions present during gradient development.

The curves generated for pKa = 4 and pKa = 5 indicate that an increase in R_f would be predicted anytime the pH of the mobile phase used to develop these weak acids was increased above 3.75. The graphs of R_f vs. distance of development for bromphenol blue (pKa = 3.99) and bromcresol green (pKa = 4.56) (Figures 23 and 24) demonstrate an increase in R_f when the developing solvent is changed from 1.0M sodium chloride (non-gradient) to 1.0 M sodium acetate (gradient). One can conclude from this only that the pH of the mobile phase passing through the analyte spots is greater than 3.75.

From a consideration of the theoretical change in R_f vs. pH' curve one could predict that a weak acid with a pKa of 8 would experience a large change in R_f if the mobile phase pH were greater than 6, but little change if the pH were below 6. The graphs of R_f vs. distance for cresol red (pKa = 8.14), phenol red (pKa = 7.80) and bromphenol red (pKa = 7.75) (Figures 25, 26 and 27) indicate little or no change in R_f between gradient and non-gradient development. From this one can conclude that the mobile phase pH experienced by these analytes is below 6.

For a weak acid with a pKa of 6 one would again predict an increase in R_f if the mobile phase pH was greater than 3.75. From a consideration of the graph of R_f vs, distance for bromcresol purple (pKa = 6.07) (Figure 28) one can conclude that the mobile phase pH must be greater than 3.75 . One can also observe that for gradient development that the R_f is increasing with distance of development. From this one can determine that the pH experienced by the analyte is increasing as the plate develops.

The fact that the mobile phase pH is seen to be acidic, between 3.75 and 6, and the fact that the analytes experience an increasing pH support the ion exchange - neutralization mechanism.

Indeed the pH of the mobile phase passing through the analyte spots can be ascertained from the colors of the indicators themselves. In Figure 32, four indicators are shown spotted on a CM cellulose plate which has been placed in the cell for photography and clamped above a petri dish. The plate was then developed with 1.0 M sodium acetate. The plate is shown before development, as the mobile phase passes through the spots and as the mobile phase has just passed the spots. The indicators used were (from left to right on the undeveloped plate) ethyl orange, bromcresol green, methyl red and bromthymol blue. The colors on the undeveloped plate are consistent with a pH of approximately 4 since the methyl red is red and the bromcresol green is yellow and the ethyl orange is orange, (approximately half way between red (pH = 3.4) and yellow (pH =

4.8)). As the mobile phase passes through the spots it can be seen that the pH increases, as is evident by the ethyl orange turning yellow and the bromcresol green turning blue, but the bromthymol blue remains yellow indicating a pH less than 7.6 and the methyl red becomes orange and not yellow indicating a pH closer to 5.4, (approximately half way between red (pH = 4.8) and yellow (pH = 6.0)). This confirms the acidic pH of the mobile phase when CM cellulose is developed with 1.0 M sodium acetate.

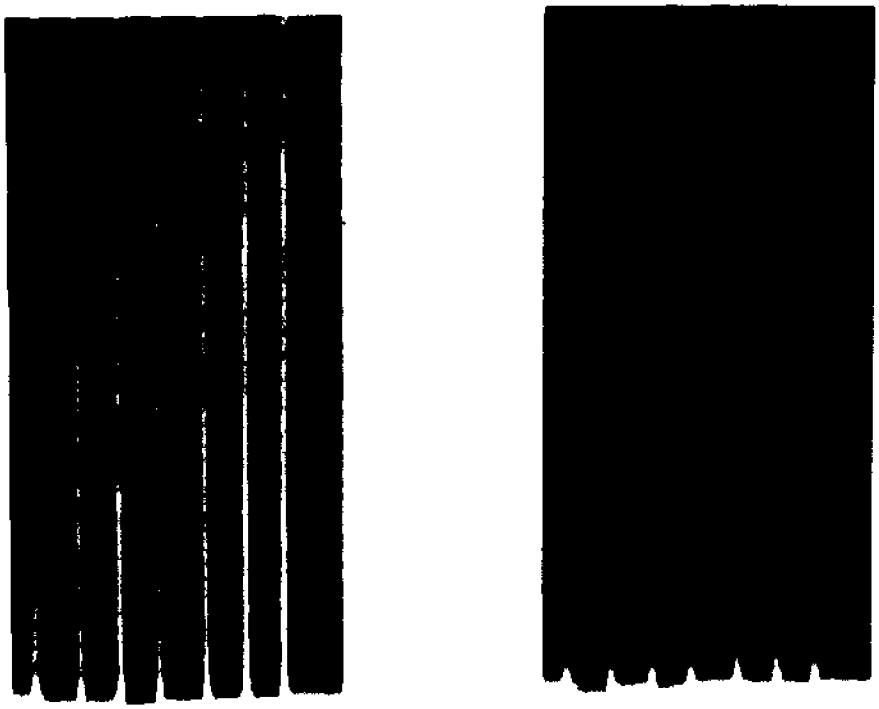
While this technique is useful for determining the pH of the mobile phase at one point in the development we would like to know the pH throughout the entire development of the plate. To this end several indicators that exhibit low R_f when developed with 1.0 M sodium acetate and exhibit color changes in the pH range expected were applied in vertical bands using the technique described earlier. This plate was clamped vertically in the cell fabricated for photography and held over a petri dish and developed with 1.0 M sodium acetate. Photographs were taken of the developing plate at each centimeter of development. These are shown in Figure 33. The indicators used and the pH ranges over which they change colors are given in Table 4. It can be seen by comparing the treated bands with the untreated periphery that with the exception of alizarin red S the presence of the indicator did not impede the movement of the mobile phase. It should also be noted that although methyl red and bromcresol green did exhibit some movement with the mobile phase

Figure 32. Photographs of Indicators Spotted on CM Cellulose Undergoing Development with 1.0 M sodium acetate.

Before Development. | As Solvent Front Passes Spots. | After Solvent Front Has Passed

(left to right) ethyl orange, bromcresol green, methyl red and bromthymol blue.

Figure 33. CM Cellulose Plates with Indicator Bands Undergoing Development with 1.0 M sodium acetate.



Before Development | 1 cm

(from left to right) alizarin red S, bromcresol green, eriochrome black T, methyl red, alizarin and bromthymol blue

Figure 32 (continued)

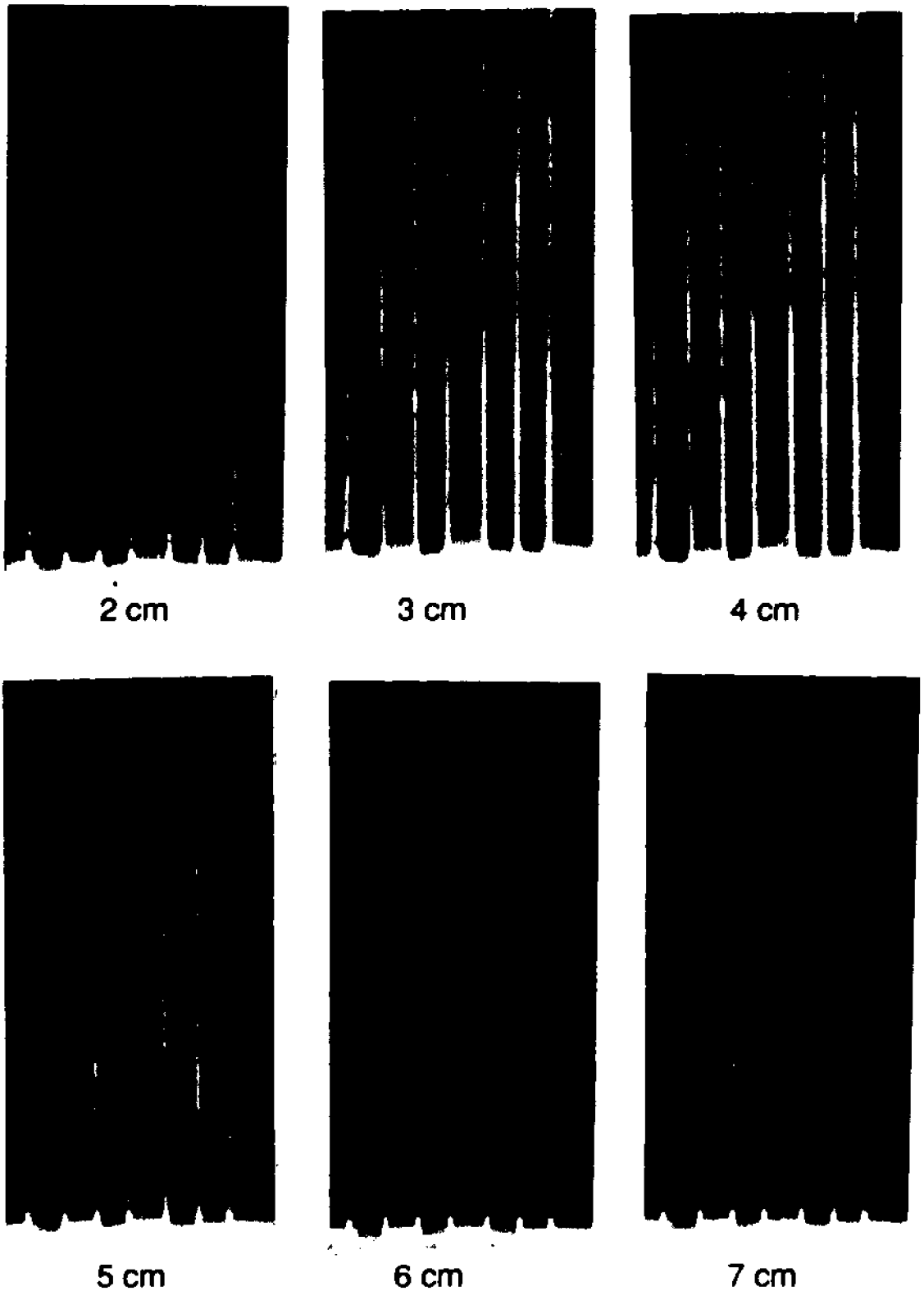


Table 4. Data for Indicators Used in Photographic Work.

Indicator	acid	base	pH range
Used as spots for analysis of passing solvent front			
ethyl orange	red	yel	3.4 - 4.8
bromcresol green	yel	blue	3.8 - 5.4
methyl red	red	yel	4.8 - 6.0
bromthymol blue	yel	blue	6.0 - 7.6
Used for whole plate vertical bands			
alizarin red S	yel	red	4.6 - 6.0
bromcresol green	yel	blue	3.8 - 5.4
eriochrome black T	red	blue	5.0 - 6.5
methyl red	red	yel	4.8 - 6.0
alizarin	yel	red	5.6 - 7.2
bromthymol blue	yel	blue	6.0 - 7.6

however one can still see the color changes of these indicators. It can be seen that bromthymol blue always remained yellow with just a faint tinge of green toward the bottom of the fully developed plate indicating that the pH was always below 7.6. For all of the other indicators, the region near the solvent front indicated the acidic color and the lower portion the basic color. Note that although all of the indicators change color in different pH regions, the distance on the plate at which a color change is seen is not greatly different for the different indicators. This indicates that the pH changes greatly near the solvent front. This is consistent with the steeper slope observed between 6 and 8 cm of development in the graph of pH vs. distance for CM cellulose developed with 1.0 M sodium acetate (Figure 11). Note also that the pattern of color change is fairly constant from 1 cm of development through full development. That is to say that the relative height (R_f) at which the color change occurs is constant.

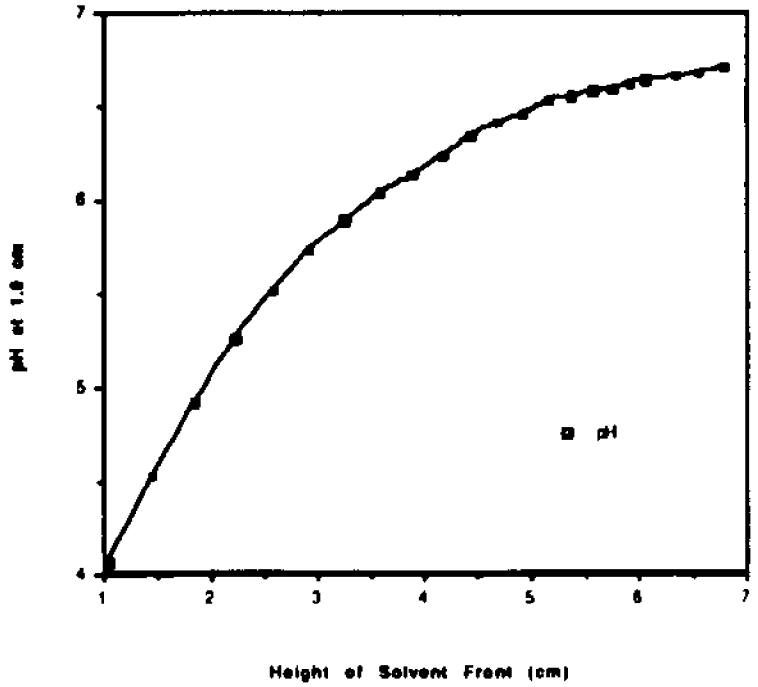
These observations provide evidence as to whether the Migration-Titration or the Ion Exchange - Neutralization mechanism is operating. If the Migration-Titration mechanism were operating, one would expect to find that in the initial centimeters of development that the indicators would show their basic colors at the solvent front and that as the plate developed that the colors at the solvent front would change to the acidic colors. If the Mobile Phase Separation mechanism were operating one would expect to find a discrete

distance where all indicators exhibited a change in color . But in actuality each indicator changes color in a continuous fashion and each at a slightly different distance. The observations made in the photographic documentation of the indicator color changes that occur during gradient development all support the ion exchange - neutralization mechanism. Namely the facts that the solvent front is observed to be consistently acidic and that if one focuses on one distance, e.g. 2 cm from the start line, that that point will be seen to be at first acidic and then gradually become basic.

3.Continuous pH measurement during development:

While pH indicators may give us a qualitative feel for the pH changes occurring during development, an indicator can only provide gross information about pH changes over a small range. Using a technique previously described it was possible to measure the pH of one point on a developing layer as the mobile phase passed through that point. Figure 34 shows the pH measured at 1.0 cm above the point of contact of a CM cellulose plate and developing solvent as a function of the height of the solvent front when that measurement was made. In this case the developing solvent used was 1.0 M sodium formate. Similar curves were obtained for 1.0M sodium acetate and 1.0 M sodium propionate. It can be seen in this case that the pH measured when the solvent front has just passed the point of measurement is acidic (4.0). The pH quickly increases before

Figure 34. Constant pH measurement at 1.0 cm on a CM Cellulose Plate Being Developed with 1. 0 M sodium formate.



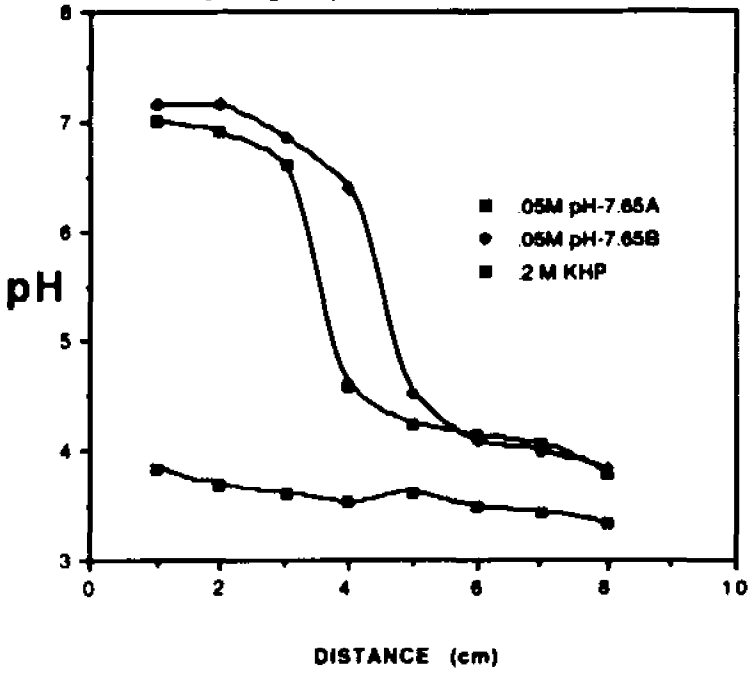
leveling off at 6.7 after 6.8 cm of development. When a CM cellulose plate was developed with 1.0 M sodium chloride and measurements made in the same fashion an unchanging acidic pH was measured. These observations support the ion exchange - neutralization mechanism. As the solvent front first moves through a previously undeveloped point, the resin has the maximum number of protons available for exchange for the mobile phase cation, (here Na^+), so the pH will be low. As a new band of mobile phase passes that point fewer protons are now available for exchange and the smaller quantity of protons can be more easily neutralized by the mobile phase anion, (here formate). After many bands of mobile phase have passed through that point the reservoir of protons has been depleted and the pH of the plate approaches the pH of the developing solvent. If the migration-titration mechanism were operating one would expect to measure a basic pH as the solvent front passed through the point of measurement. The pH value would then gradually decrease as the basic component of the mobile phase were neutralized. This is the opposite of what was observed. The observations made in the continuous pH measurements can not be used as evidence for or against the mobile phase separation mechanism as this separation could have occurred at a point above the point where measurements were made.

4. Total species profiles:

While all of the previously mentioned measurements are consistent with the ion exchange - neutralization mechanism they all involve the measurement of only one component in the mechanism, namely the H^+ ion. As an additional piece of evidence we would like to know how the concentrations of all of the components of the mobile phase change during development. A mobile phase was chosen whose components are amenable to measurements. A phthalate mobile phase was chosen since phthalate is commercially available in a pure form as potassium acid phthalate and the phthalate ion also strongly absorbs UV light facilitating its measurement. Experiments using potassium acid phthalate as a mobile phase for CM cellulose failed to produce a gradient. This is not surprising as pKa_1 for o-phthalic acid is 2.95. However a solution of the dipotassium salt did produce a pH gradient, $pKa_2 = 5.41$, as shown in Figure 35. The mobile phase of dipotassium phthalate was produced by titrating the desired mass of potassium hydrogen phthalate dissolved in 60 mL of deionized water with 1.5 M KOH until a pH of 7.65. The resulting solution was then diluted to 100.0 mL with deionized water.

Two 5 cm X 10 cm CM cellulose plates were developed with 0.05 M dipotassium phthalate (pH = 7.65) . The gradient produced was measured using the template and electrodes technique and the plates were then dried in a vacuum oven overnight. Vertical lines

Figure 35. Curves of pH vs. Distance for CM Cellulose Developed with phthalate and hydrogen phthalate Solutions.



were incised in the layer 1.8 cm from each edge and horizontal lines were incised at the top of the region of immersion of the plate, 0.5 cm above that, and at 1 cm intervals above that. Each of the resulting segments of developed stationary phase was scraped into tared test tubes using a single edge razor blade. The mass of each sample was approximately 5 mg. This provided 2 parallel sets of samples from each plate. The samples in one set were suspended in 5.00 mL of deionized distilled water which extracted only the free potassium ions. The samples from the other set were suspended in 5.00 mL of 0.10 M HCl which extracted all of the potassium ions, the H^+ ions displacing K^+ from the resin. This also insured that all of the phthalate extracted would be in the protonated form. This is necessary since the protonated and deprotonated forms maximally absorb UV light of different wavelengths. The phthalate concentration of the resin was measured by comparing the absorbance at 230 nm of the acidic extracts to standard KHP solutions made in 0.1M HCl . Concentrations were reported as moles phthalate/g dried resin. Measurements were made using the Hewlett - Packard 8425A Diode Array Spectrophotometer described earlier. The potassium concentrations of both the acidic and water extracts were determined using a Perkin-Elmer 3030B Atomic Absorption Spectrophotometer equipped with a hollow cathode lamp. An air/acetylene flame was used and measurements were made at 766.5

nm. Potassium concentrations were reported as moles K^+/g dried resin. The potassium concentration measured in the water extracts was called "free K^+" and that from the acidic extract the "total K^+". The amount of "bound K^+", that K^+ attached to the ion-exchange resin, was determined by subtraction. Figure 36 shows the concentrations of K^+, free and bound, and total phthalate ion as a function of distance to the center of the band of resin sampled. It can be seen that the concentration of phthalate ion is essentially constant throughout the entire length of the plate. This demonstrates that the pH gradient is not being produced by the mobile phase separation mechanism. If it were one would expect to find a sharp drop off in the concentration of phthalate ion somewhere along the plate. The concentration of potassium ion, both free and total, decreases with distance. This indicates that potassium ions are being retained by the resin, most likely by an ion exchange mechanism. The ratio of free to bound K^+ ions as a function of development distance is given in Figure 37. Near the solvent front, 8 cm, the ratio is small. This result is in accord with the model in which the mobile phase at the solvent front encounters a resin with the maximum number of protons available for exchange. This presents the greatest possibility for ion exchange and consequently the greatest fraction of K^+ ions will be bound to the resin. Blocks of resin closer to the start line have been

Figure 36. Concentrations of Mobile Phase Components vs. Distance in a CM Cellulose Plate Developed with 0.05 M phthalate

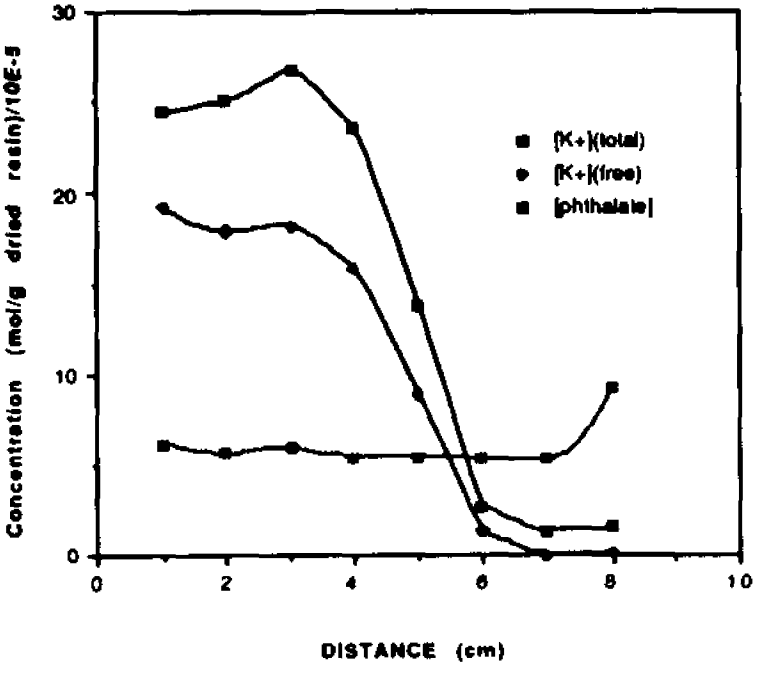
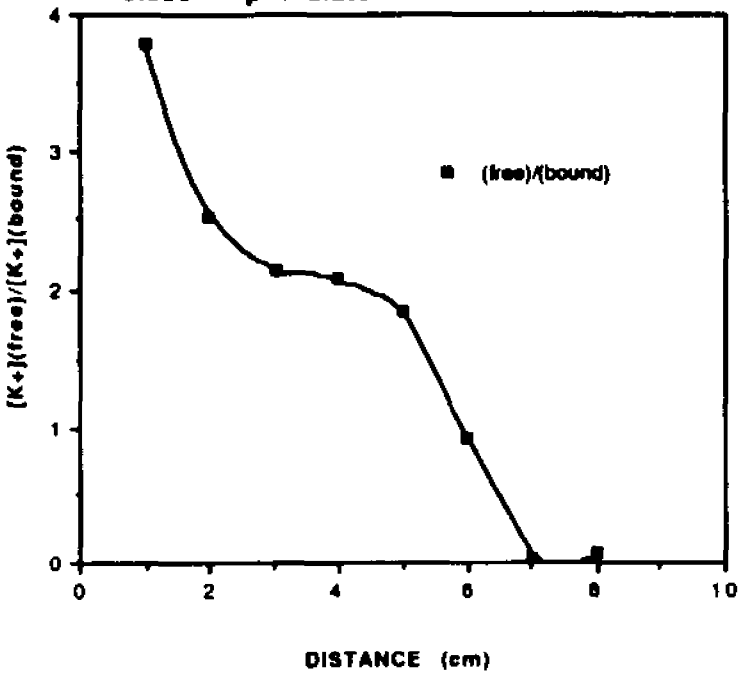


Figure 37. Ratio of [K+](free)/[K+] (bound) for a CM Cellulose Plate Developed with 0.050 M phthalate.



transversed by more bands of mobile phase and have already undergone substantial ion exchange. The diminished number of protons available for ion exchange make it less likely that a K^+ ion will be able to undergo ion exchange and hence more likely that a K^+ ion will be free. This is in fact observed in Figure 37 where the ratio of free to bound K^+ is highest at the bottom of the developed plate and lowest at the solvent front.

Measurements made of the mobile phase components in a developed plate rule out the possibility that the pH gradient produced by the phthalate system was formed by the mobile phase separation mechanism.

Of the two remaining mechanisms these measurements seem to support more the ion exchange - neutralization mechanism. If the migration - titration mechanism were the major mechanism one would expect to find that the ratio of free to bound K^+ ions followed the trend observed as the only way for the resin to the release protons affecting the neutralization would be by ion exchange. In speaking of ion exchange resins Day and Underwood state that:

"...in contrast with ordinary electrolytes, the anion is permanently attached to the polymer matrix; it cannot migrate throughout the aqueous phase within the resin pores, nor can it escape to the external solution. The fixation of the anion in turn restricts the mobility of the cation, H^+. Electrical neutrality is maintained within the resin, and H^+ will not leave the resin phase unless it is replaced by some

other cation, which is the ion exchange process." (29)

Although the ratio of free to bound K^+ ions would not be constant there is no reason to expect that the total concentration of K^+ ions would not be constant. The variation of total K^+ ion concentration with distance shown in Figure36 favors the ion exchange - neutralization mechanism.

The difference between the migration - titration and the ion exchange - neutralization mechanisms is quantitative; that is to say that in both mechanisms the same chemical processes are involved in the production of the gradient but that in each mechanism a different process is dominant. The migration - titration mechanism assumes that the concentration of exchangeable protons in the resin is small with respect to the concentration of basic component in the mobile phase and/or that the ion exchange equilibrium is not favorable for the release of protons. A band of mobile phase would need to pass through several bands of stationary phase in order for its basic component to have been titrated effectively. In other words the pH of the mobile phase is determined by the presence of the basic component which is gradually titrated by the few protons released by ion exchange. The ion exchange - neutralization mechanism assumes that the concentration of exchangeable protons large with

respect to the concentration of basic component in the mobile phase and that the ion exchange equilibrium is favorable for the release of protons. The pH of the mobile phase is therefore determined by the released protons the effect of which is neutralized by the presence of a basic component in the mobile phase.

5. A test of the mechanism:

If the ion exchange - neutralization mechanism is correct, as all previous evidence seems to indicate, we could alter the gradient produced by changing the amounts of cation and basic substance present. It is not necessary that both come from the same compound. In these experiments the developing solvent used was an aqueous solution of sodium chloride and ammonia. One would predict that no gradient would be formed when a solution containing only one of the components was used as a developing solvent. This was previously shown to be true when a sodium chloride solution was used. A solution containing only ammonia has a very low cation concentration and should produce few protons by ion exchange, consequently the pH change should be small. This is demonstrated in Figure 38. It was shown that the maximum release of protons could be obtained by using a 1.0 M NaCl solution as a developing solvent. If a series of solutions of varying concentrations of NH_3 in 1.0M NaCl were used, varying gradients should be generated. Three such curves are shown in Figure 39. The magnitude of pH change is the same in all

Figure 38. Curves of pH vs. Distance for CM Cellulose Plates Developed with 0.18 M ammonia and with 1.0M NaCl.

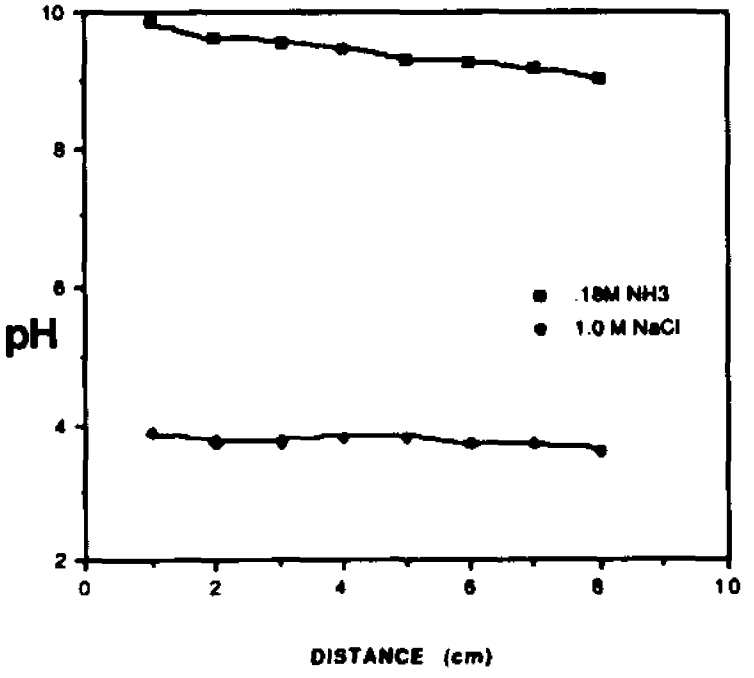
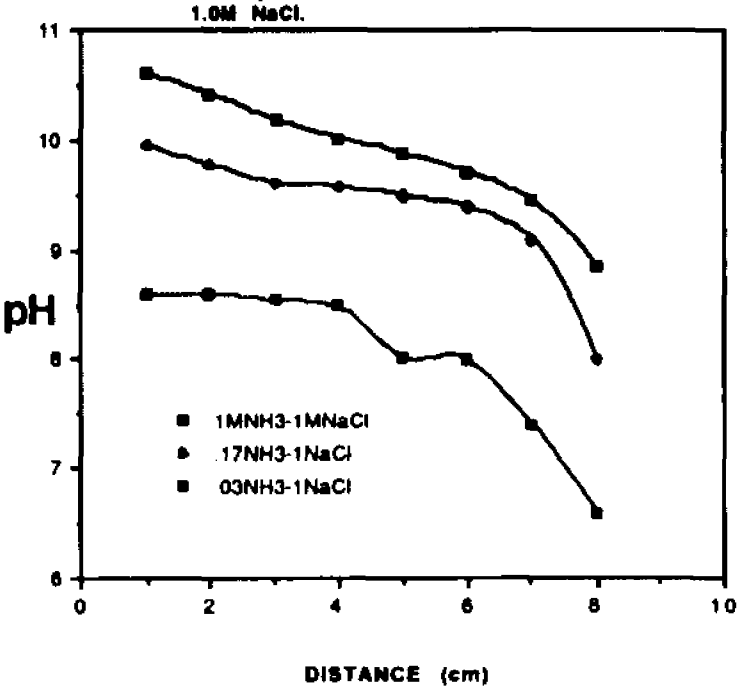


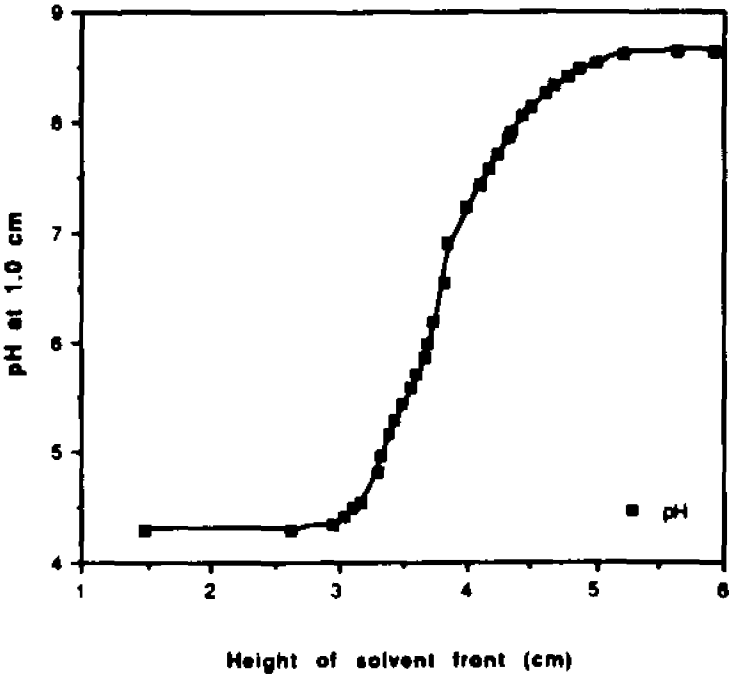
Figure 39. Curves of pH vs. Distance for CM Cellulose Plates Developed with Varying Concentrations of ammonia in 1.0M NaCl.



curves but as expected the larger the ammonia concentration the higher the measured pH. It is also useful to note that the larger the ammonia concentration the more linear the curve appears. The curve for 1.0 M NH_3 + 1.0 M NaCl is nearly linear with a deviation from linearity only at 8 cm. The curve for .18 M NH_3 + 1.0 M NaCl also exhibits a linear region but the deviation from linearity occurs earlier and is steeper. This trend is continued in the curve for .03 M NH_3 + 1.0 M NaCl. This effect could be due to the phenomenon mentioned by Pickering cited earlier. There may be in effect two solvent fronts, a Na^+ ion front and an NH_3 front. When the concentration of NH_3 is large (1.0 M) the R_f for the NH_3 front is close to 1, that is the two fronts merge. As the concentration of NH_3 decreases the R_f similarly decreases and the NH_3 front trails the Na^+ front. If this is so there is little NH_3 available to buffer the protons released by exchange of Na^+ ions from the resin at the leading Na^+ front. This explains the steep drop off in pH near the observed solvent front. This may also explain the curve shape noted in other graphs most dramatically Figure 14. Here similarly the curves are linear for the 1.0M and 0.5M acetate solutions but a marked region of non-linearity is noted for the acetate solutions of lower concentration. This explanation is supported by the constant pH measurement made at 1.0 cm above the start line on a CM cellulose plate developed with 0.03 M NH_3 in 1.0M NaCl, Figure

40. The pH measured as the observed solvent front passes through the point of measurement is acidic. The pH increases only very slightly until the observed solvent front is 3 cm above the developing solvent and only then does the pH begin to rise. The pH then increases in sigmoidal form as the passing bands of ammonia neutralize the released protons until a nearly constant pH is measured as the observed solvent front passes 5.5 cm of development. This same phenomenon was not observed in Figure 34. Here the developing solvent had a higher concentration of the basic component , (1 M formate ion), and so there was effectively only one solvent front, i.e. sodium and formate traveled together. The pH was therefore observed to increase as soon as the solvent front passed the point of measurement. However the complete sigmoidal curve was not observed only the upper portion. This can be explained by a consideration of the pKa of the conjugate acids of the basic components. The pKa of formic acid is 3.75. When sodium formate encounters the stationary phase with its released protons at pH 4 it is experiencing a pH already above the pKa of formic acid. All that is observed is the above pKa neutralization of protons by formate and hence only the upper portion of the sigmoidal curve. This is in contrast with ammonia. The pKa of ammonium ion is 9.24. When the ammonia encounters the stationary phase with its released protons at pH 4 it is experiencing a pH considerably below the pKa of ammonium ion consequently the entire sigmoidal curve is observed

Figure 40. Constant pH Measurement at 1.0 cm on a CMC Plate Being Developed with 0.03 M ammonia in 1.0 M NaCl.



as the ammonia neutralizes the released protons. The final pH observed is 8.6 which is the pH predicted for a 0.03 M NH_3 solution itself.

Applications of a self-generating pH gradient on carboxymethyl cellulose.

1. Metal ions.

Traditionally carboxymethyl cellulose thin layer chromatographic plates have been used for the separation of metal ions. Cozzi, Desideri and Lepri (18) published data for the separation of 19 metal ions on carboxymethyl cellulose layers using sodium acetate - acetic acid buffers of varying strengths. Their method did not separate well Zn^{2+} from Ni^{2+} or Mn^{2+} from Ca^{2+}. Their results are presented in Table 5 as Rf1-Rf3. It was felt that a pH gradient might be helpful in resolving these ions. The effect of pH on the chromatographic behavior of metal ions is twofold. First the metal ion attached to the ion exchange resin can be exchanged for a proton from the mobile phase and displaced from the stationary phase.

$$nR^{-}M^{n+} + n\,H^{+} \rightarrow nR^{-}H^{+} + M^{n+}$$

Another factor affecting the mobility of metal ions is their ability to form insoluble hydroxides. Above a critical pH each metal ion will form an insoluble hydroxide which will remain precipitated on the stationary phase and not move into the mobile phase. The potential for a metal ion to precipitate out as an hydroxide can be estimated from its K_{sp}. The pH above which a 1.0M solution of a given ion is expected to form an hydroxide precipitate is given in Table 5. From a

consideration of both of these factors it can be seen that an acidic mobile phase will increase the mobility of metal ions on carboxymethyl cellulose.

These experiments were carried out before the mechanism of pH gradient formation was established. The working hypothesis at that time was that the migration-titration mechanism was producing the gradient. If that were true then a metal ion analyte spotted on a CM cellulose plate would first experience a basic pH and would either be converted to its insoluble hydroxide or would find few protons available for exchange in the mobile phase and would in either case exhibit little mobility. As the mobile phase pH began to decrease the metal ion would begin to move. As the critical pH values for hydroxide precipitation are different for Zn^{2+} and Ni^{2+} (5.8 and 6.4) as well as for Mn^{2+} and Ca^{2+} (7.4 and 11.4) it was felt that a pH gradient would provide what Niederweiser (2) terms an anti-parallel gradient which would improve resolution. If in fact the ion exchange - neutralization mechanism were in effect the gradient would ruin the separation. The analyte metal ion would experience a more acidic environment at the top of the spot that at the bottom and consequently the top would accelerate and the spot become elongated. The poor chromatographic performance of metal ions in the self generated pH gradient observed provides further evidence for the ion exchange - neutralization mechanism. It should be noted

that the non-gradient producing developing solution used as a control did provide better separation that the literature values.

Standard solutions were prepared by dissolving the appropriate masses of $ZnCl_2$, $NiCl_2$, $CaCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$ and $MgSO_4 \cdot 7H_2O$ to produce solutions with metal ion concentrations of 20 g/L. The addition of 5 drops of 6 M HCl was needed for the dissolution of $ZnCl_2$. All solutions except Ca^{2+} were diluted to 2 g/L before use. Spots were detected by spraying the developed plates with a 1% solution of 8 -quinolinol in ethanol. After the evaporation of the ethanol the plates were viewed under long wave UV light. Ca^{2+}, Mg^{2+}, and Zn^{2+} form fluorescent complexes with 8 - quinolinol while the Mn^{2+}, Ni^{2+}, and Cu^{2+} complexes appear as dark spots under UV light.

As predicted, metal ions exhibited very low R_f values on CM cellulose when developed with a 0.03M NH_3 developing solution. Not only is the pH high, but the cation concentration is very low. The analytes appeared as a streaks from the start line. When a gradient- producing developing solvent of 0.03M NH_3 in 1.0M NaCl was used mobility did increase but the spots produced were elongated and were often streaks. It is interesting to note that the higher the critical hydroxide pH value for a given ion, the higher was

its R_f value. In fact those ions whose critical hydroxide pH was unlikely to be reached in the pH gradient, Ca^{2+} (11.4) and Mg^{2+} (8.4), experienced little streaking and gave smaller spots. The other ions whose critical hydroxide pH was within the range of pH values produced by the pH gradient produced elongated spots and often streaks.

The ions which showed poor development also form chloro complexes. The log of the formation constants for the tetrachloro complexes are: $CuCl_4^{-2} = 5.62$, $NiCl_4^{-2} = 1.20$, $ZnCl_4^{-2} = 0.88$ (30). The poor development could be due to the partial formation of a soluble complex anion leaving some uncomplexed metal cation behind. In order to insure that the presence of chloride ion was not responsible for the poor separation a developing solvent of 0.03 M NH_3 in 1.0 M $NaClO_4$ was used. This also produced a pH gradient but perchlorate ion is a poorer ligand than chloride ion. This resulted in no improvement in separation.

The developing solvent intended to serve as a control, 1.0 M $NaClO_4$, provided better separation than the gradient- producing solvent. Smaller spots were observed for Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, although Cu^{2+} and Ni^{2+} still showed streaking. The separation was better that that reported in the literature. Zn^{2+}, Mn^{2+}, Ca^{2+} and Ni^{2+} were clearly separated with the perchlorate

developing solvent. These ions all showed similar R_f values when separated with an acetate buffer (18). The R_f values obtained in all of these experiments are given in Table 5.

While the self-generating mobile phase pH gradient produced in the current experiments did not improve the separation of metal ions the poorer separation serves as an additional proof of the ion exchange - neutralization mechanism. Gradients such as those produced here where the analyte first experiences an acidic mobile phase which gradually becomes more basic would be more useful in the separation of analytes that experience a greater mobility in a basic medium and decreased mobility in an acidic medium i.e. weak acids like the polyglutamic acid samples presented in the next section. If a series of such analytes were exposed to such a gradient then those analytes least affected by the acidic mobile phase would begin to move first leaving behind those more affected by the acidic medium. As the pH of the mobile phase passing the analyte increased more and more analytes would begin to move. Spot sizes would also be minimized. The top of the spot would experience a less favorable mobile phase pH and would be braked while the bottom of the spot would experience a more favorable mobile phase pH and would be accelerated. This would result in a more compact spot.

Table 5. R_f Values of Some Metal Ions on CM Cellulose

METAL ION	CRITICAL pH *	Rf X 100						
		Rf1	Rf2	Rf3	Rf4	Rf5	Rf6	Rf7
Ca^{2+}	11.4	57	35	4	0-34	70-78	69-78	83
Cu^{2+}	4.6	55	28	0	0-3	1-13	0-10	7-21
Mg^{2+}	8.4	78	53	9	0-5	77-85	73-84	85
Mn^{2+}	7.4	58	38	8	0-3	17-70	26-53	73
Ni^{2+}	6.4	61	38	4	0-3	47-81	13-48	65
Zn^{2+}	5.8	62	35	4	0-2	9-28	6-21	56

Rf1 = 1N sodium acetate - 1 N acetic acid (18)
Rf2 = 0.5N sodium acetate - 0.5 N acetic acid (18)
Rf3 = 0.1N sodium acetate - 0.1 N acetic acid (18)
Rf4 = 0.03M ammonia
Rf5 = 0.03M ammonia in 1.0 M NaCl
Rf6 = 0.03M ammonia in 1.0 M $NaClO_4$
Rf7 = 1.0 M $NaClO_4$

* Estimated pH = 14 - $\log(K_{sp})^{1/2}$ above which a 1.0 M solution of the ion will form an hydroxide precipitate.

2. The molecular weight characterization of poly - L - glutamic acid.

According to the model of Tanford (31) the square of the end-to-end distance for a flexible polymer (h_0^2) is given by:

$$h_0^2 = \beta^2\sigma$$

where β is the effective bond length between segments

σ is the number of monomer elements in a polymer chain. This equation is based on a model which treats the segments as independent units and assumes that any segment may occupy any space. This is not true as some space is occupied by other polymer segments. To compensate for this the Flory-Fox empirical factor α was introduced so that the square of the actual end-to-end distance h^2 is:

$$h^2 = \alpha^2\beta^2\sigma$$

This α factor also accounts for attractive forces. In a polymer solution there may be a stronger attraction between the polymer segments and the solvent molecules than there is between the polymer segments themselves. This solvent is termed a good solvent. Here a polymer segment is more likely to be adjacent to a solvent molecule than to another segment so the total excluded volume is larger than the physical volume occupied by the polymer segments themselves. In such a case α is greater than one. If the segment-solvent interactions are not stronger than the segment-

segment interactions a polymer segment is more likely to be adjacent to another and the solvent is termed a poor solvent. In such a case α is less than one and the polymer will not be soluble in such a solvent. In either case since the total physical excluded volume and the net sum of the attractive interactions both increase with the number of segments the value of α is also dependent on σ. The maximum possible variation is given as.

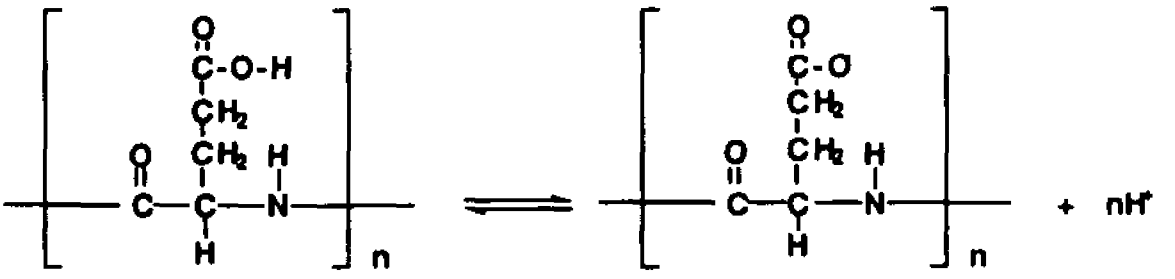
$$\alpha = (\text{constant})\ \sigma^{0.10}$$

If the segments possess charged moieties the situation is more complicated. If all of the charges are of the same type (e.g. all negative) then electrostatic repulsions make compact configurations highly unfavorable and the polyelectrolyte assumes an expanded configuration. If the moiety is a weakly dissociating electrolyte (e.g. -COOH) then a basic solvent which would promote ionization would cause the polymer to assume a more expanded configuration and would be a good solvent for that polymer while an acidic solvent which suppresses ionization would be not as good.

The solubility of a polymer decreases with increasing molecular weight. If a polydisperse sample of a polymer containing a weakly dissociating electrolyte moiety is insoluble in an acidic solvent ($\alpha < 1$) and it is exposed to a slightly more basic solvent a certain fraction of the groups on the polymer will ionize. This will result in a more expanded configuration for the polymer and an increase in α. The

value of α for the smaller chains may increase to a value greater than 1 at which point they will become soluble while the larger chains remain insoluble. There will be a critical pH for each chain length at which it becomes soluble. If a sample of such a polymer were spotted at the bottom of a thin layer chromatographic plate and exposed to an acidic mobile phase no analyte movement would occur. As the pH of the mobile phase increased the smaller chains would begin to move and as the pH increased eventually all of the chains would begin to move. In the developed plate one would expect to find the greatest R_f value for the shortest polymer chains.

The polymer chosen for this experiment, poly - L - glutamic acid, is shown in Figure 41 . Samples of the sodium salt of poly - L - glutamic acid of varying molecular weight distributions were obtained from SIGMA Chemical Co. , St. Loius MO. The samples were characterized by Sigma using viscosity measurements and size exclusion chromatography using low angle laser light scattering (SEC-LALLS) detection. Data provided by Sigma for the samples are given in Table 6. Samples were dissolved in deionized water at a concentration of 50 mg/mL. Analytes were detected by drying the developed plates in a 100° oven for five minutes followed by spray of 1% $CuSO_4$ in water (w/v) and repeated oven drying. Poly-L-glutamate appeared as light blue spots. When sodium formate was used as a developing solution the background appeared as a paler



poly -L- glutamic acid poly -L- glutamate

Figure 41. The Structures of poly - L -glutamic acid and poly - L - glutamate

Table 6. Data on poly - L - glutamic acid samples provided by Sigma Chemical

SIGMA LOT #	AVG. MW (by viscosity)	AVG. MW (by LALLS)	80% FRACTION (by SEC-LALLS)
62H-5514	14, 300	9,700	7,000 - 13,000
52H-5522	17, 500	15, 300	7,000 - 21,000
123H-5525	64,900	53,500	24,600 - 69,000
13H-5511	82,300	100,000	67,300 - 132,700

blue and analyte identification was facilitated by exposing the sprayed, dried plate to HCl gas (from the mouth of an open bottle of concentrated HCl solution) followed by a few minutes in a 100° oven. This caused the analyte to appear as blue-green but left the background unaffected. When the developing solution contained chloride ion the background was yellow owing to the formation of the $CuCl_4^{2-}$ complex. Since all of the samples are polydisperse and separation is predicted to be a function of molecular weight the analytes should appear as elongated spots covering a range of R_f values proportional to the range of molecular weights of the polymers.

The only moiety capable of dissociating in poly - L - glutamic acid is the -COOH group. Based on data obtained from titrations of ribonuclease, Tanford & Hauenstein (32) reported that the intrinsic pK_a for this group is 4.7.

As expected when the samples were chromatographed on caroboxymethyl cellulose plates using 0.03 M NH_3 in 1.0 M NaCl as a developing solvent all of the samples exhibited a high R_f. Even though this developing solvent produces a gradient on CM cellulose, the lowest pH measured for the solvent front in Figure 40 is 4.3. At this pH approximately 30% of the -COOH groups are predicted to be ionized. The pH then rises steeply to well over 8.0. This mobile phase was always a good solvent for all of the samples.

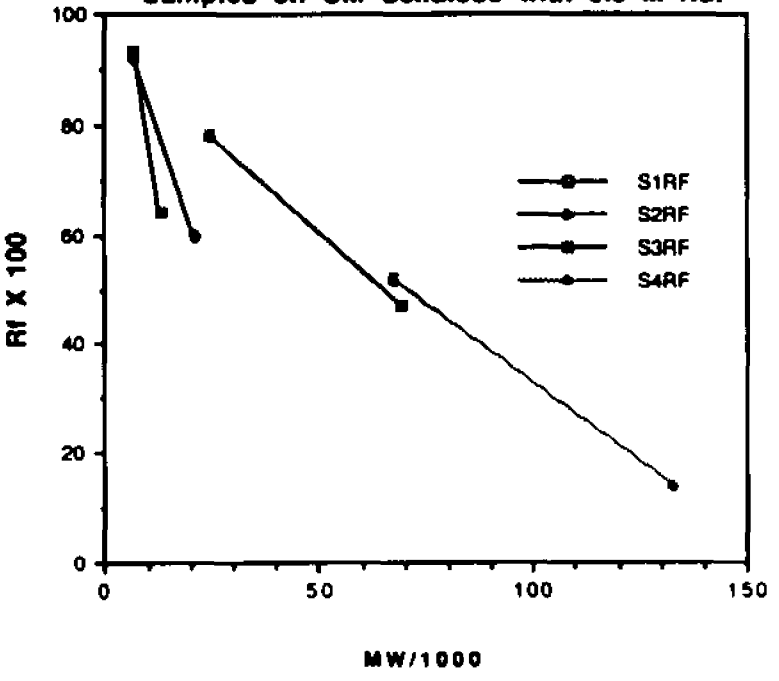
When 1.0 sodium formate was used there was better separation but even the highest molecular weight sample showed a rather large R_f (77-91). The better separation can be explained by the fact that the initial pH of the solvent was lower, 4.0, as seen in Figure 34. At this pH a smaller fraction, approximately 17%, of the -COOH groups are predicted to be ionized. The pH also increases more shallowly, reaching a maximum below 7.0.

A developing solvent of 0.5 M sodium fluoride was also used to separate the samples. Although 0.5M NaF produces a gradient similar to 1.0 M sodium formate on CM cellulose (Figures 13 and 16), the samples showed lower R_f values and better separation. This is possibly due to the lower ionic strength of the developing solvent. The R_f range observed for each sample is plotted against the molecular weight range into which 80% of the sample falls in Figure 43.

An unsuccessful attempt was made to assure that the elongated analyte spots observer were due to the polydispersity of the sample and not simply due to poor separation. If a sample were to be exposed to the same pH gradient in two dimensions an elongated spot produced by polydispersity would become a diagonal line while one due to poor separation would become a large oval. Since the formation of the gradient is due to the conversion of the protonated form of the resin to the sodium form, it is not possible to form the same pH gradient on the same plate twice. An experiment was

performed in which one sample of poly-L-glutamic acid was

Figure 43. Rf Ranges Observed for Polydisperse poly - L -glutamic acid Samples on CM Cellulose with 0.5 M NaF



chromatographed on CM cellulose using 0.5 M NaF as a developing solvent. The plate was then dried and cut vertically adjacent to the sample and grafted onto an unused plate and development was attempted with the same solvent in a perpendicular direction. The mobile phase failed to cross the gap neatly between the old plate and the new plate so the experiment was abandoned.

A non-gradient-producing acidic developing solvent of approximately equal ionic strength to the formate solution was also used to develop the poly - L - glutamic acid samples. A solution of 0.01 M H_2SO_4 in 0.5 M Na_2SO_4 produced some movement for the lighter samples and very little for the heavier samples. Even at approximately pH 2.0 where 0.20% of the -COOH groups are ionized, the smaller chains show some slight solubility. The R_f values produced in all of these experiments are given in Table 7. This method could serve as an inexpensive quality control screen for incoming raw polymeric materials. A semi- quantitative non-instrumental determination of the molecular weight distribution of a sample may be obtained quickly; the separations in these experiments required about 20 minutes. As always in thin layer chromatography the ability to analyze many samples simultaneously provides for greater throughput.

Table 7. R_f values for poly-L-glutamate fractions chromatographed on CM cellulose.

AVG. MOL. WT. (by viscosity)	Rf X 100			
	Rf1	Rf2	Rf3	Rf4
14, 300	88-100	94-100	64-92	14-44
17, 500	84-96	84-98	60-92	16-38
64, 900			42-78	11
82, 300	84-96	71-91	14-52	0

Rf1 = 0.03 M NH_3 in 1.0 M NaCl
Rf2 = 1.0M sodium formate
Rf3 = 0.5 M sodium fluoride
Rf4 = 0.01M H_2SO_4 in 0.5 M Na_2SO_4

Summary and Conclusion

Since many analytes are either weak acids or bases their chromatographic behavior on a thin layer plate can be greatly affected by the pH of the mobile phase to which they are exposed. Often one is called upon to separate molecules which are very similar and differ only in their acid base properties. These separations may be enhanced by use of a mobile phase pH gradient.

Several techniques were explored for the determination of pH gradients. The pH was measured at several points along a thin layer chromatographic plate by placing a specially made template over the plate and applying micro pH electrodes to the plate. This technique was also modified to permit the measurement of the pH on the plate as it was being developed. Indicator compounds were also used extensively. Qualitative measurements of the pH were obtained by observing the colors of various indicators which were either spotted on the bottom of a plate or which were applied in vertical bands along the plate.

In order to insure the presence of well defined and controlled acid base properties in the stationary phases, derivatized celluloses were used. Both carboxymethyl cellulose and diethylaminoethyl cellulose layers were commercially available precoated on flexible sheets. The developing solvents used were aqueous solutions of the sodium or potassium salts of weak acids. Several systems were

investigated in which a pH gradient was observed on the developed plates.

The mechanism by which the gradient forms on carboxymethyl cellulose was determined by means of several experiments. The use of spray reagents , "R_f-on-the-fly" experiments, observations of indicator colors, continuous pH measurements and total species profiles using atomic absorption and visible light spectroscopy all indicated that the most likely mechanism is the ion exchange - neutralization mechanism. In this mechanism cations from the mobile phase are exchanged for protons in the stationary phase. The effect of the released protons is neutralized by the weakly basic component in the mobile phase. The proposed mechanism was verified by the use of solutions of ammonia in 1.0 M sodium chloride.

The effect of the gradient on the separations of two classes of analytes was explored. The gradient did not improve the separations of metal ions. However the non-gradient control did provide better separations that were found in the literature. The gradient was able to bring about the separation of polydisperse samples of poly-L-glutamic acid on the basis of their molecular weights.

REFERENCES

(1) Niederwieser, A. ; Honegger, C. C. Advances in Chromatgraphy **1966** , 2, 123 - 167

(2) Niederwieser, A. Chromatographia **1969**, 2, 362 - 375

(3) Geiss, F. ; Sandroni, S.; Schlitt, H. J. Chromatogr. **1969**, 44, 290-299

(4) Golumbic, C.; Orchin, M.; Weller, S. J. Am. Chem Soc. **1949**, 71, 2624

(5) Golumbic, C.; Orchin, M.; J. Am. Chem. Soc. **1950**, 72, 4145

(6) Stahl, E. Chemie-Ing. Techn. **1964**, 36, 941

(7) Stahl, E.; Dumont, E. Talanta **1969**, 16, 657-669

(8) Stahl, E.; Dumont, E. J. Chromatogr. Sci. **1969**, 7, 517-525

(9) Stahl, E.; Muller, J. J. Liq. Chromatogr. **1980**, 3(6), 775-787

(10) Stahl, E.; Muller, J. J. Chromatogr. **1981**, 209, 484 - 483

(11) Quirin, K. W. J. Chromatogr. **1983**, 281, 179 - 197

(12) Mitchell, H.; Gordon, M.; Haskins, F. J. Biol. Chem **1949**, 180, 1071-1076

(13) Wieland, T.; Determann, H. Experientia **1962**, 18, 431 - 432

(14) Niederwieser, A.; Brenner, M. Experientia **1962** , 21, 105

(15) Armstrong, D. W. ; Bui, K. H. ; Boehm R. E. J. Liq. Chromatogr. **1983**, 6(1), 1 -22

(16) Peterson. E. L.; Sober, H. A. J. Am. Chem. Soc. **1956**, 78, 751

(17) Cozzi, D.; Desideri, P. G.; Lepri, L; Ciantelli, G. J. Chromatogr. **1968**, 35, 396-404

(18) Cozzi, D.; Desideri, P. G.; Lepri, L; J. Chromatogr. **1969**, 42, 532

(19) Cozzi, D.; Desideri, P. G.; Lepri, L; Coas, V. J. Chromatogr. **1969**, 40, 138 - 144

(20) Lepri, L; Desideri, P. G.;Landini, M.; Tanturli, G. J. Chromatogr. **1975**, 109, 365 - 376

(21) Lepri, L; Desideri, P. G.;Coas, V. J. Chromatogr. **1970**, 52, 421 - 428

(22) Altman. A. R. The Creatioon of a pH Gradient Along a Thin Layer Chromatographic Plate and its Applications. M. S. Thesis, St. John's University, 1989

(23) Dean, J.A., ed. Lange's Handbook of Chemistry, 11th ed.; McGraw-Hill Book Co.: New York, 1973

(24) Weast, R. C. ed. CRC Handbook of Chemistry and Physics, 64th ed.; CRC Press, Inc. : Boca Raton, FL, 1984

(25) Linke, W. F. ed. Solubilities, Inorganic and Metal-Organic Compounds, Vol II; ACS Press: Washington, D.C., 1965

(26) Pickering, W. F. J. Chromatogr. **1958**, 1, 276

(27) Dittmer; Lester J. Lipid Research **1964**, 5, 126

(28) Geiss, Friedrich Fundamentals of Thin- Layer Chromatography (Planar Chromatography); Huethig: Heidelberg - Basel - NY, 1987

(29) Day ,R.A.; Underwood, A. L Quantitative Analysis, fifth edition; Prentice - Hall, Englewood Cliffs, NJ 1986. 586-7

(30) L. G. Sillen compiler, Stability Constants of Metal - Ion Complexes, London, The Chemical Society, 1964 pp. 283, 285 & 289

(31) C. Tanford, Physical Chemistry of Macromolecules, 1961, John Wiley & Sons, New York, pp. 162-4, 201, 489-91

(32) Tanford, C. ; Hauenstein, J.D., J. Am. Chem. Soc., **1956**, 78, 5287 cited in Tanford op. cit. p.556