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Developments in the use of pH and electric field strength gradients for the electrophoretic analysis of biomolecules

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2016-07-18
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Conference proceedings generated by this work

1. M.P. Bellini and K.L. Manchester "Isotachophoretic focusing of proteins" in **Abstracts of the Eleventh Congress of the South African Biochemical Society**, p.54, 28 June - 1 July 1992, Sun City, South Africa

2. M.P. Bellini and K.L. Manchester "The Synthesis of a New Acrylamido buffer for Isoelectric Focusing in Immobilized pH Gradients" in **Abstracts of the First Congress of the International Council of Electrophoresis Societies (ICES-ELPHO)**, p.46, June 2-4, 1993, Sandefjord, Norway

3. M.P. Bellini and K.L. Manchester "The Synthesis of a New Acrylamido Buffer for Isoelectric Focusing in Immobilized pH Gradients" in **Abstracts of the Twelfth Congress of the South African Biochemical Society**, p.100, 24-26 January 1994, Stellenbosch, South Africa

Acknowledgements

I would like to thank the following people for making my work on this thesis possible:

My supervisor **Prof. K.L. Manchester** of the Department of Biochemistry, University of the Witwatersrand, for encouraging me to follow up my original ideas and develop as a biochemist.

Dr. R.M. McGrath of the Department of Biochemistry, University of the Witwatersrand, for his generous gift of several chemicals necessary for my work.

Prof. J.P. Michael (organic chemistry) and **Prof. E.W. Neuse** (polymer chemistry) of the Department of Chemistry, University of the Witwatersrand, for their valuable advice.

The Foundation for Research Development for financial support during part of my studies.

The University of the Witwatersrand Scholarships Office for a **Postgraduate Merit Scholarship** which gave me financial support during part of my studies as well as for a **Postgraduate Travel Grant** which allowed me to present my work at a conference in Norway in 1993.

The trustees of the Freda Lawenski Postgraduate Merit Award which contributed towards the costs of my studies during the course of a year.

Lastly and most importantly, **my family** for their constant love, encouragement and prayers.

Abstract

The electric field strength and pH gradients generated in isotachophoretic systems may be used for the separation of biomolecules. Poly(2-acrylamido-2-methyl-1-propanesulfonic acid) polymers of a uniform distribution of molecular mass were synthesized and used as spacers in isotachophoresis in order to generate linear electric field strength gradients in which biomolecules could be focused. These novel spacers are designed to operate within sieving media, and hence are useful for the separation of nucleic acids.

A novel weakly basic acrylamido buffer, *N*-[bis(2-hydroxyethyl)aminomethyl]acrylamide (pK_a = 5.6), suitable for the preparation of immobilized pH gradients, was synthesized by means of the Mannich reaction of acrylamide, formaldehyde and diethanolamine. It exerts a buffering effect in the considerable pH gap between the pK_a 4.6 and 6.2 Immobilines®, and was shown to make possible the use of ultranarrow immobilized pH gradients centred around pH 5.6. However, this acrylamido buffer is expected to undergo slow hydrolysis in aqueous solution, limiting its usefulness. Hence more stable alternatives were investigated.

A range of α-aminomethylated acrylic acids, buffering in the neutral and basic pH ranges, were synthesized by the Mannich reaction of malonic acid, formaldehyde and a secondary amine. These compounds are much more stable than the corresponding acrylamide derivatives. A pH 7.3-7.5 IPG prepared using α-(*N*-morpholinomethyl)acrylic acid (pK_a 7.6) as the immobilized buffer was able to resolve myoglobin contaminants differing by as little as 0.005 pH unit.

Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Science of the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

Signed:



Marco Paolo Bellini

24th day of December, 1996.

Developments in the Use of pH and Electric Field Strength Gradients for the Electrophoretic Analysis of Biomolecules

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Marco Paolo Bellini

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg 1996

List of abbreviations

AAEE	*N*-Acryoylaminoethoxyethanol
AAP	*N*-Acryoylaminopropanol
AMPD	2-Amino-2-methyl-1,3-propanediol
AMPS	2-Acrylamido-2-methyl-1-propanesulfonic acid
BAPEDA	*N,N'*-Bis(3-aminopropyl)ethylenediamine[1]
BCPP	*N,N'*-Bis(2-carboxyprop-2-enyl)piperazine[2]
BHEAMA	*N*-[Bis(2-hydroxyethyl)aminomethyl]acrylamide
bpe	DNA base pair equivalent
BSA	Bovine serum albumin
EACA	ε-Aminocaproic acid[3]
EDTA	Ethylenediaminetetraacetic acid
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
ITP	Isotachophoresis

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[1] IUPAC name: 1,10-diamino-4,7-diazadecane

[2] IUPAC name: 2-[4-(2-carboxyprop-2-enyl)-piperazinylmethyl]propenoic acid.

[3] IUPAC name: 6-Aminohexanoic acid

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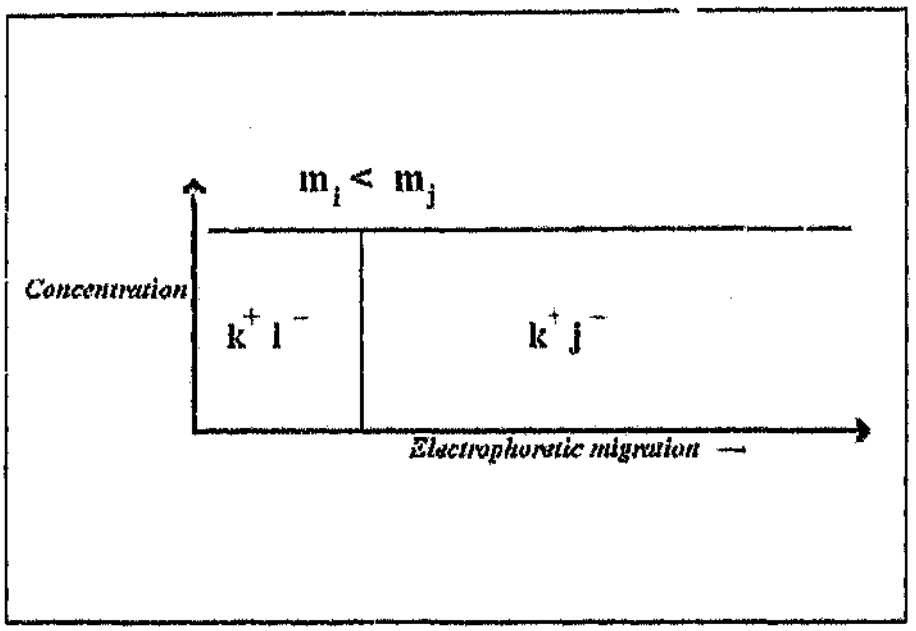


Figure 2 The initial condition in the separating column, with the concentrations of the adjacent salt solutions, k^+i^- and k^+j^-, arbitrarily set equal.

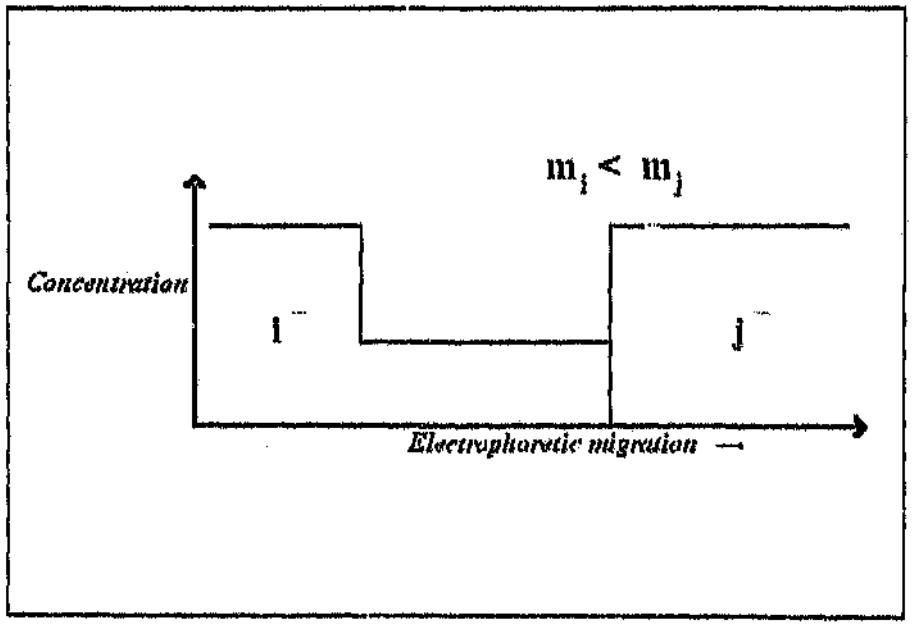


Figure 3 Attainment of the steady state. The concentration of i has been regulated according to the concentration of j.

direction. However, the j^- ions will soon leave the slower i^- ions behind, leading to the formation of an "ionic vacuum", ie. a region of lower molar conductivity Λ will appear. Since the current I at any part of the system is the same (as all sections of the conducting material are electrically in series), then the field strength E must rise due to Ohm's Law - $I \propto E\Lambda$. This causes an increase in the net velocity of the i^- ions, accelerating them until they catch up with the j^- ions. The concentrations of i^- and k^+ in the terminating electrolyte will adjust to the concentrations of j^- and k^+ in the leading electrolyte, in such a way that they will create a voltage gradient large enough to give the terminating zone the same speed as the leading zone.

Thus the concentrations of the i^- and k^+ ions in the terminating electrolyte (and hence the pH of the terminating electrolyte) are *regulated* by the ionic composition of the leading electrolyte. A stable moving boundary is formed between the j^- and i^- zones, with a *stepwise increase in field strength* and a *stepwise decrease in concentration* going from the j^- ions to the i^- ions. A *stepwise increase in pH* also occurs at the boundary of the j^- and i^- ions. This is due to the fact that the ionization of the common basic buffering counterion k^+ must decrease so as to maintain electroneutrality in view of the lower concentration of the i^- ions. This process is portrayed diagrammatically in Figures 2 and 3.

If a zone of a third ion, a^-, of an ionic mobility intermediate to those of the j^- and i^- ions, is introduced between the j^- and i^- zones, then the a^- ions will act as "terminating ions" with respect to the j^- ions, and as "leading ions" with respect to the i^- ions, as shown in Figures 4 and 5.

1.2 STEADY STATE MOVING BOUNDARIES

1.2.1 Historical background

The electrophoretic phenomenon, employed by Ornstein and Davis in 1964 for prefocusing of protein samples in discontinuous electrophoresis (the stacking effect), was discovered as early as 1897 by Kohlrausch, a German physical chemist. Kohlrausch observed that, on migration in an applied electric field, the boundary between a solution of ions of substance *j* of high mobility, and a solution of ions of substance *i* of low mobility and the same sign of charge, would be sharply maintained. Kohlrausch derived a "regulating function" defining the conditions required for the formation and maintenance of steady state moving boundaries.

1.2.2 The formation of steady state moving boundaries[1]

Imagine a system composed of two adjacent electrolytes K^+j^- (leading electrolyte) and K^+i^- (terminating electrolyte) where the ionic mobility of the *j* ions is higher than that of the *i* ions, and K^+ is the common basic buffering counterion. Once a potential difference is applied the *j* ions of high mobility and *i* ions of low mobility will start migrating in the same

[1] The theory of steady state moving boundaries was mainly developed by Longsworth (Longsworth 1959a, Hjelmeland and Chrambach, 1982). A detailed theoretical description of the equations describing the ionic composition of steady state moving boundaries is given in Appendix B.

A major advance in zone electrophoresis was the introduction of starch gel as an electrophoretic medium (Smithies, 1955) resulting in a spectacular gain in resolution - 20-25 serum components could be resolved as opposed to 6-7 by previous methods. Obviously a new principle was at work, and Smithies himself realised that it was molecular sieving, a principle that gained immense importance through the introduction of synthetic polyacrylamide gels by Raymond and Weintraub in 1959. Polyacrylamide gel is formed by the free radical-induced polymerization of acrylamide with the cross-linking agent methylene-*N,N'*-bisacrylamide. The pore size in polyacrylamide gels is dependent on the concentration of acrylamide used, for example, a 2.5% gel sieves molecules of a molecular mass of 10^5 to 10^6 daltons, a 7% gel sieves molecules of a molecular mass of 10^4 to 10^5 daltons, and a 30% gel sieves molecules of a molecular mass of 2×10^4 to 2×10^5 daltons.

The greatest factor limiting resolution in polyacrylamide gel electrophoresis is diffusion, which causes protein bands to spread with time during the electrophoretic run. To limit the loss of resolution, the protein samples loaded onto the gels had to be small and highly concentrated. This problem was overcome by the introduction of "disc electrophoresis" in 1964 by Ornstein and Davis, in which discontinuities in the gel buffers caused the formation of steady state moving boundaries by which the sample proteins became concentrated into extremely thin starting zones. High resolution was thus achieved in very brief runs, with the added advantage that dilute protein samples could be loaded. The theory behind the steady state moving boundaries employed in discontinuous electrophoresis is elaborated in the following section.

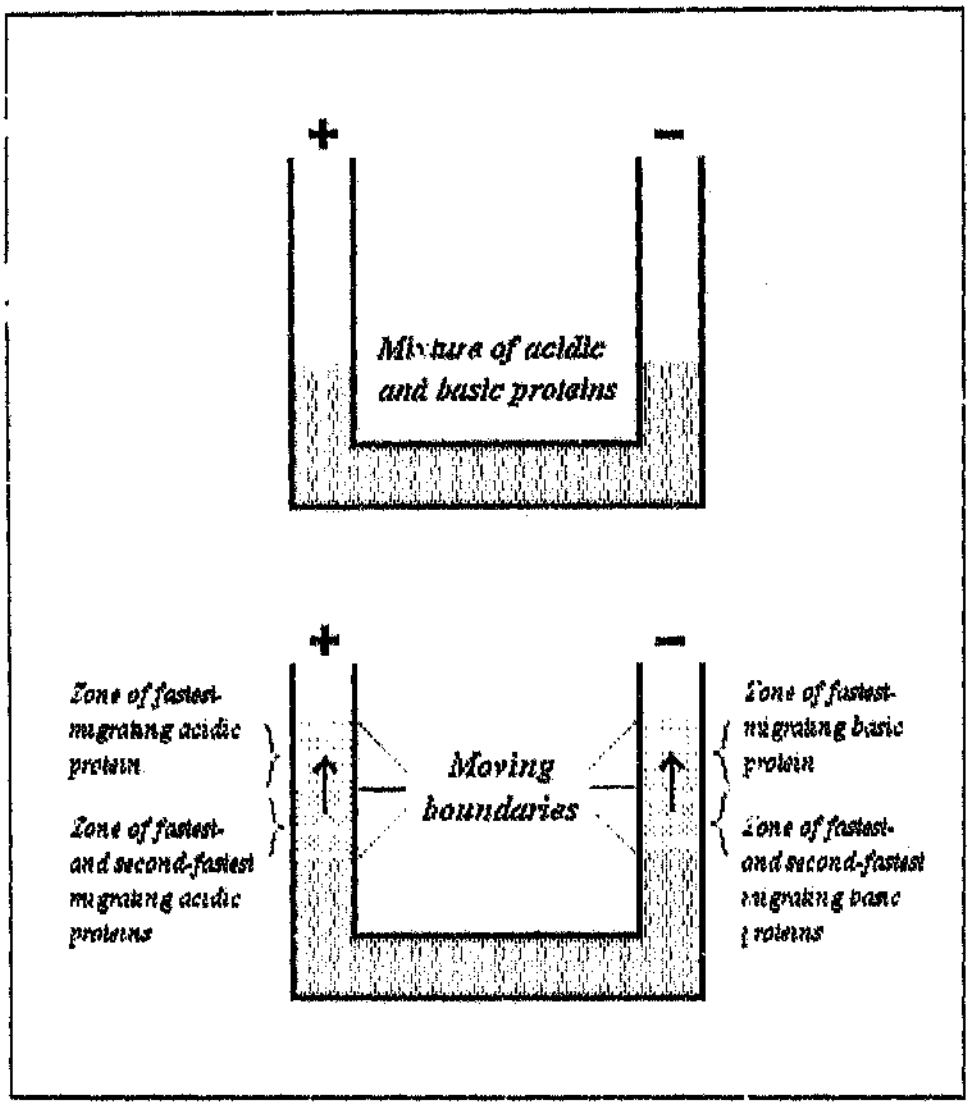


Figure 1 Moving boundary electrophoresis in a U tube.

Development of electrophoresis as an analytical tool in biochemistry

Electrophoretic analysis of protein mixtures in free solution started with Tiselius in 1937. In this technique, named moving boundary electrophoresis[1], the mixture of proteins was applied in a U-shaped cell filled with a buffer solution, at the end of which electrodes were immersed. Under the influence of the applied voltage, the proteins would migrate at different velocities towards the anode or cathode depending on their charge. Although the individual protein zones could never be completely separated from each other, the boundaries formed between the mixed protein zones could be detected using Schlieren optics, as shown in Figure 1. Using this technique, Tiselius was able to separate blood serum into about 5 or 6 fractions. He received the Nobel prize in Chemistry for this work in 1948.

During the 1950's, zone electrophoresis was introduced - so called because the sample constituents could be resolved into individual zones. Although this was true for small molecules such as amino acids, only a minimal increase in the resolution of proteins was obtained over moving boundary electrophoresis. In these early experiments in zone electrophoresis, supporting media such as cellulose paper or cellulose acetate strips were used. The use of supporting media solved the problem of convection currents caused by temperature fluctuations which are a problem in electrophoresis conducted in free solution.

[1] Reviewed in Longsworth (1959*a*,*b*).

$$\mathcal{F}' = fv \qquad (1\text{-}3)$$

where f is the *frictional coefficient*.

The two forces act in opposite directions, and the ions reach a terminal speed, the *drift speed*, when the accelerating force $\mathcal{F}$ is balanced by the viscous drag $\mathcal{F}'$. The net force is zero when:

$$v = \frac{QE}{f} \qquad (1\text{-}4)$$

The ratio of Q/f is a constant for any ion, and may be replaced with a quantity defined as the *ionic mobility*[1] m (in $cm^2 \cdot V^{-1} \cdot s^{-1}$):

$$v = mF \qquad (1\text{-}5)$$

The frictional coefficient is a function of the hydrodynamic size of a molecule. Increasing the molecular size increases the frictional coefficient. Shape also plays a role in large molecules like nucleic acids and proteins, of which the length-to-width ratios can vary widely. Therefore the ionic mobility of a molecule is a function of size, shape and charge.

[1] In the older literature the ionic mobility is often represented as μ or u.

Theory of electrophoresis[1]

When two electrodes a distance L (in cm) apart are at a potential difference V (in volts), the ions in the solution between them experience a uniform electric field of magnitude (in $V \cdot cm^{-1}$):

$$E = \frac{V}{L} \qquad (1\text{-}1)$$

In such a field an ion of charge Q experiences a force $\mathcal{F}$ of magnitude:

$$\mathcal{F} = QE \qquad (1\text{-}2)$$

A cation responds by accelerating towards the negative electrode (cathode) and an anion responds by accelerating towards the positive electrode (anode). However as the ion moves through the solvent it experiences a frictional retarding force $\mathcal{F}'$ that is proportional to its velocity, v (in $cm \cdot s^{-1}$):

$$\mathcal{F}' \propto v$$

This relationship may be rewritten as:

[1] A more in depth treatment of electrophoretic theory is given in Appendix A.

My interest was also stimulated in the use of pH gradients for the separation of proteins. Isoelectric focusing in immobilized pH gradients (IEF-IPG) caught my eye, due to the stability of the pH gradients generated by this technique, as well as the prospect of pH gradient engineering. Since the acrylamido buffers required for IEF-IPG are quite expensive, an opportunity presented itself for research into alternative, cheaper acrylic-based buffers.

1.1.2 Use of electrophoresis in biochemistry[1]

Electrophoresis is the most powerful and frequently used analytical technique in the study of charged macromolecules. It utilises differences in charge and molecular mass for resolution of proteins. Techniques such as denaturing gel electrophoresis, using detergents such as sodium dodecyl sulfate, and pore gradient gel electrophoresis depend solely on molecular mass differences to effect a separation. Isoelectric focusing utilizes solely differences in electric charge, namely, differences in pI. Of the two approaches, isoelectric focusing has generally the greater resolving power for the analysis of proteins.

[1] Some reviews of the development of electrophoresis as an analytical tool in biochemistry have been written by Rilbe (1983) and Vesterberg (1989, 1993).

Chapter 1 - Introduction

1.1 GENERAL BACKGROUND

1.1.1 Aim of project

The project as originally intended was to evaluate the resolving power of polyacrylamide gel electrophoresis (PAGE) in non-denaturing buffers for the analysis of lactate dehydrogenase (LDH) isozymes from rat and fruit bat tissues with a view to comparing their patterns and correlating these with metabolic activity. The failure of the LDH-5 isozyme to enter the gels during discontinuous electrophoresis provoked my interest in the physico-chemical mechanisms behind the stacking process. I soon became deeply interested in the concepts involved behind the electrophoretic focusing of proteins in voltage gradients. Eventually the focus of my Ph.D project reversed - from using electrophoresis to study protein patterns, I used protein patterns as a means of evaluating the resolving power of electrophoretic processes.

The stacking of proteins in discontinuous electrophoresis is caused by the generation of voltage gradients across a moving boundary between two ionic species (chloride and glycine anions in the Ornstein-Davis system). This physicochemical phenomenon is the underlying principle of action of isotachophoresis (ITP). Since ITP to date has not achieved a widespread use in biochemistry for the analysis of biomolecules, in spite of its potentially good resolving power, I recognised the opportunity for novel research in a little-known field.

TEMED	*N,N,N',N'*-Tetramethylethylenediamine
TEPA	Tetraethylenepentamine[1]
TETA	Triethylenetetramine[2]
Tris	Tris(hydroxymethyl)aminomethane[3]
UV	Ultraviolet
VS	Vinyl sulfonate

[1] IUPAC name: 1,12-diamino-3,6,9-triazadodecane

[2] IUPAC name: 1,8-diamino-3,6-diazaoctane

[3] IUPAC name: 2-Amino-2-(hydroxymethyl)-1,3-propanediol

LDH	Lactate dehydrogenase
MABH	Maleic acid benzylidene hydrazide
MAMH	Maleic acid monohydrazide
MCT	Monochlorotriazine
NAD	β-Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PEHA	Pentaethylenehexamine[1]
PIPES	1,4-Piperazinebis(2-ethanesulfonic acid)
polyAMPS	Poly(2-acrylamido-2-methyl-1-propanesulfonic acid)
PrIME	Preparative isoelectric membrane electrofocusing
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPADNS	2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalenedisulfonic acid
TBE	Tris-borate-EDTA

[1] IUPAC name: 1,14 diamino-3,6,9,12-tetraazatetradecane

1.3.2 The performance of carrier ampholyte spacers

The resolution of complex protein mixtures by ITP using carrier ampholyte spacers has often been very unsatisfactory. This problem is caused by the fact that some time is required for an isotachophoretic stack to reach the steady state (see Figure 8). In order to provide a sufficient spacing effect the carrier ampholytes must be used in large excess, and very often the isotachophoretic steady state is not reached by the end of the electrophoretic run, resulting in poor resolution of the sample proteins in polyacrylamide gel ITP (Chrambach *et al.*, 1972; Griffith *et al.*, 1973; Griffith and Catsimpoolas, 1974; Baumann and Chrambach, 1976). Nevertheless, excellent resolutions have been obtained by, for example, Acevedo (1989) who obtained over 30 bands upon subjecting human serum to TP in agarose gels. (The good resolution may have been due to the high voltages used - up to 1200 V.)

Another approach has been the use of telescope electrophoresis (TEP). In TEP an electric field strength gradient is still generated, albeit less steep than in a pure ITP system (Schafer-Nielsen and Svendsen, 1980,1981). However, TEP systems have the important advantage in that the steady state (ie. the formation of discrete, albeit mixed, spacer zones) is immediately achieved (see Figure 9), so that fractionation of the sample proteins in an electric field strength gradient takes place from the very start of electrophoresis, leading to improved resolution (Acevedo, 1989; Bringard and Charlionet, 1990).

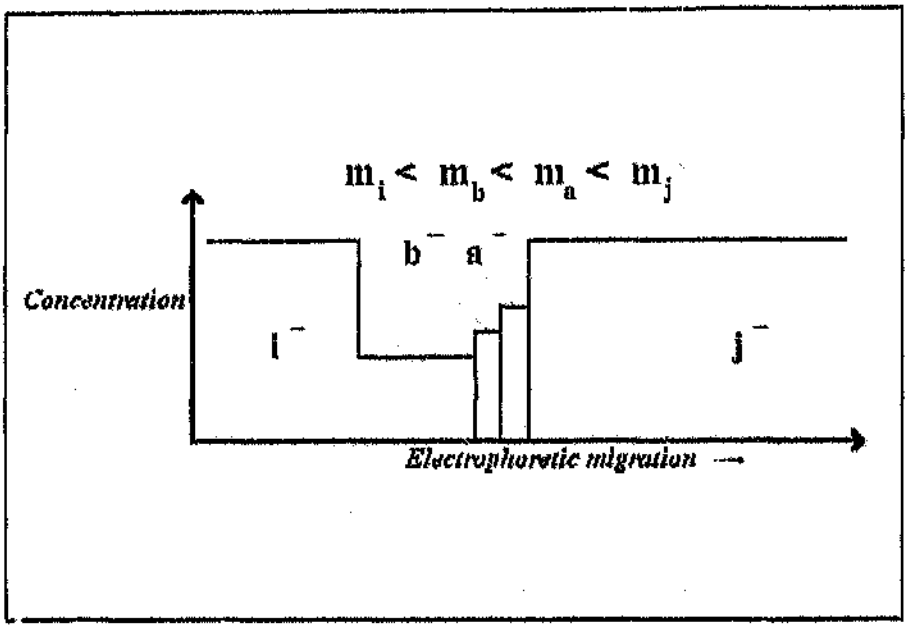


Figure 10 Steady state conditions for a sample containing two ions, *a*⁻ and *b*⁻. Although their zones have been completely separated, they cannot be visually distinguished.

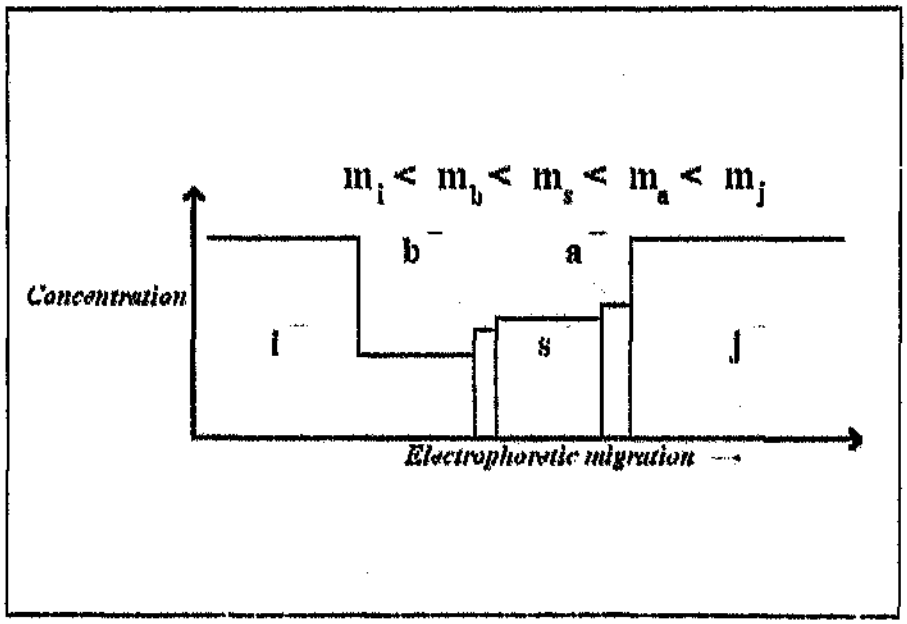


Figure 11 The effect of the addition of a spacer ion *s*⁻ of intermediate ionic mobility to *a*⁻ and *b*⁻.

the stepwise increase in field strength from one zone to the next. As this increase in field strength is generally accompanied by a slight increase in temperature, thermocouples can also be used to detect the boundaries from one zone to the next.

In order to distinguish protein zones by conventional stains, it is necessary for the protein zones to be physically separated from each other. This can be achieved in ITP by using "spacers", ie. charged molecules, such as amino acids, having ionic mobilities intermediate to those of the proteins of interest, as shown diagrammatically in Figures 10 and 11. By using large concentrations of spacers in proportion to the sample proteins, the spacer zone becomes long enough for the protein zones to be distinguished.

Highly heterogeneous mixtures of spacers of varying ionic mobility are required for the resolution of complex mixtures of proteins. The only chemicals meeting this requirement at present are the carrier ampholytes used for isoelectric focusing (Svendsen and Rose, 1970; Routs, 1973; Acevedo, 1989). The carrier ampholyte spacers migrate behind the leading electrolyte (eg. Cl^-) in order of decreasing acidity, so that a pH gradient, as well as an electric field strength gradient, is formed within the isotachophoretic stack.

For example, a sample ion lagging behind its own zone will enter a region of higher electric field strength which will cause the protein to accelerate back into its own zone (see equation (1-5)). Conversely, if a sample protein diffuses ahead of its own zone, it will enter a region of lower electric field strength which will cause it to slow down and be caught up by its own zone. The pH gradient existing across zone boundaries has the identical zone-sharpening effect.

Hence, it would be expected that the theoretical superiority of ITP over conventional forms of electrophoresis would lead to its widespread use for the analysis of proteins. However this has not been the case, for reasons discussed in the following section.

1.3 ISOTACHOPHORESIS[1]

1.3.1 The need for spacer ions in ITP

The use of ITP in agarose or polyacrylamide gels for the analysis of protein samples is complicated by the fact that the zones are contiguous, and cannot be distinguished by conventional protein stains. In capillary ITP (Everaerts *et al.*, 1973; Everaerts and Verheggen, 1983), generally used for the analysis of mixtures of small charged molecules, this is not a problem since the boundaries between the sample zones can be detected by micro-conductivity meters which measure

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[1] General reviews: Haglund (1970), Karol and Karol (1978). Theoretical aspects: Martin and Everaerts (1970); Everaerts and Routs (1971), Everaerts *et al.* (1973), Schafer-Nielsen *et al.* (1980). Preparative aspects: Svendsen and Rone (1970), Holloway and Battersby (1984).

Since, in the electrophoretic setup of Figure 9, each zone does not migrate as fast as its particular leading zone, the electric field strength gradient that develops will be shallower than that which develops in the ITP system of Figure 8. However this system does have an advantage in that the steady state is immediately attained upon commencement of electrophoresis, ie. the formation of discrete zones, albeit mixed. These zones will grow at different rates during electrophoresis in a fashion not unlike the individual tubes in a sliding telescope that is being extended; hence the term *telescope electrophoresis*[1] (TEP) has been coined by Schafer-Nielsen and Svendsen (1980,1981) to describe these modified isotachophoresis systems.

1.2.4 The theoretical superiority of isotachophoretic systems over zone electrophoresis

The greatest cause of poor resolution in zone electrophoresis is the progressive diffusion with time of the sample zones. In discontinuous electrophoresis the pre-concentration of the sample proteins into extremely thin starting zones diminishes, but does not solve, the deleterious effect of diffusion. However, in ITP and modified isotachophoretic systems such as TEP, the boundaries between zones do not diffuse with time due to the presence of the electric field strength and pH gradients that exist across zone boundaries.

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[1] The term *telescope electrophoresis*, although trivial, has been adopted by other workers in the field of electrophoresis to describe this particular modification of ITP. Hence this term will also be used within the text of this thesis wherever this technique has been employed.

Figure 9 shows a setup similar to the one described for the ITP system in Figure 8, except that in this case the sample ions a^- and b^- have been added to the leading electrolyte before the onset of electrophoresis. Thus the leading electrolyte is, in effect, composed of a mixed (j^- + a^- + b^-) zone, of a net ionic mobility equalling the average of the ionic mobilities of the constituent ions.

Upon commencement of electrophoresis in this system the a^- and b^- ions in the leading electrolyte will, by virtue of their lower net ionic mobility, be overtaken the faster leading ions j^- and thus be left behind the leading electrolyte boundary. In doing so, they form a permanent mixed (a^- + b^-) zone, of a net ionic mobility equal to the average of the ionic mobilities of the constituent a^- and b^- ions.

During the course of electrophoresis, more a^- and b^- ions join the mixed (a^- and b^-) zone, where they achieve a steady state net mobility similar to the other a^- and b^- ions in this zone. Thus, in this system, the migration velocity of the (a^- and b^-) zone must be lower than that of the leading electrolyte boundary, since otherwise they will accumulate to infinite concentration just behind the boundary. Likewise the velocity of the b^- zone must be lower than that of the (a^- and b^-) zone, ie. we have:

$$v_{(a^- + b^-)} < v_{(a^- + b^-)} < v_{j^-} \qquad (1\text{-}7)$$

In the ITP setup shown in Figure 8, the velocities of the individual zones are equal:

$$v_{j^-} = v_{a^-} = v_{b^-} = v_{j^-} \qquad (1\text{-}8)$$

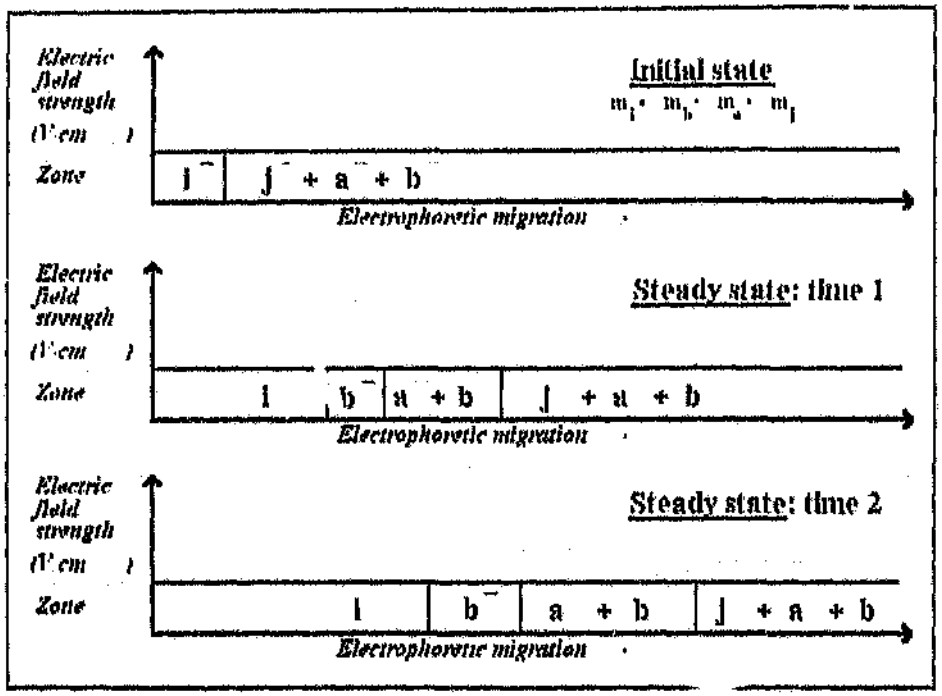


Figure 9 A modification of isotachophoresis in which the leading ion j^- is contaminated with two sample ions a^- and b^-, where a^- has a higher ionic mobility than b^-. Hence the leading electrolyte is composed of the mixed zone ($j^- + a^- + b^-$), of a net ionic mobility equalling the average of the ionic mobilities of the constituent ions. As the leading ion j^- migrates in the applied electric field, it leaves behind the slower moving a^- and b^- ions, resulting in the formation of a permanent mixed zone ($a^- + b^-$), where the net ionic mobility of the zone equals the average of the ionic mobilities of the constituent a^- and b^- ions. In a similar fashion the a^- ion leaves behind the slower moving b^- ion, resulting in the formation of a pure zone of b^- ions. These zones grow in a telescopic fashion during the course of electrophoresis - hence this modification of isotachophoresis has been named *telescope electrophoresis* by Schafer-Nielsen and Svendsen (1980,1981). Although the electric field strength gradient is shallower in telescope electrophoresis than in isotachophoresis, it has the advantage that the steady state is immediately attained, ie. the formation of discrete zones.

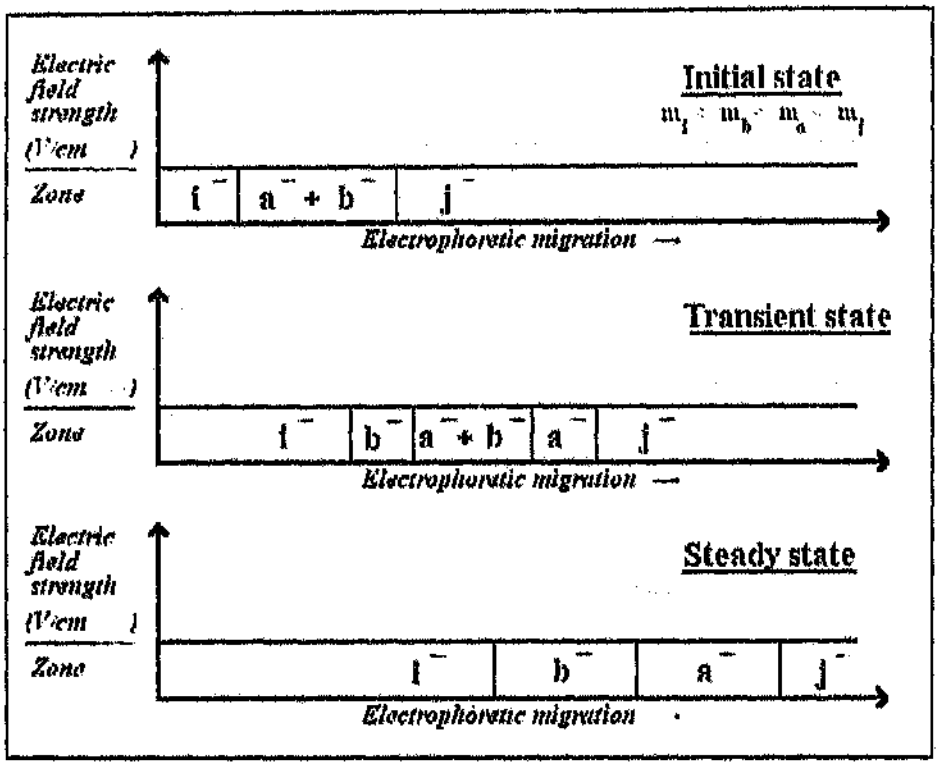


Figure 8 Isotachophoresis of two sample ions a^- and b^-, where a^- has a higher ionic mobility than b^-. As the leading ion j^- migrates in the applied electric field, a stepwise electric field gradient develops across the boundaries of adjacent zones so that all the zones migrate at an equal velocity. The $(a^- + b^-)$ mixed zone gradually becomes separated into two distinct zones. However, while it exists, the $(a^- + b^-)$ mixed zone behaves as a unique zone, of a net ionic mobility equalling the average of the ionic mobilities of the constituent a^- and b^- ions.

1.2.3 The formation of multiple steady state moving boundaries

The same principle works for multiple sample ion systems as for single sample ion systems - the ions form discrete zones in order of their ionic mobilities, with stepwise gradients of increasing pH and electric field strength, and decreasing concentration, from the leading to the terminating ions. Multiple ion discontinuous electrophoretic systems are generally referred to as isotachophoresis[1] (ITP) or displacement electrophoresis. Hence, in isotachophoretic steady state systems, the ionic composition of the leading zone determines the ionic compositions of all the following zones, according to the principles of the Kohlrausch regulating function.

However, a certain amount of time is required for an isotachophoretic system to reach the steady state, particularly if the sample ions differ only slightly in their ionic mobilities. Figure 8 shows the gradual formation of steady state moving boundaries during the isotachophoretic separation of two sample ions a^- and b^-, where a^- has a higher ionic mobility than b^-. The mixed zone of a^- and b^- gradually becomes separated into two distinct zones. However, while it exists, the $(a^- + b^-)$ mixed zone behaves as a unique zone, of a net ionic mobility equalling the average of the ionic mobilities of the constituent a^- and b^- ions. If the leading electrolyte were contaminated with a^- and b^- ions, then the $(a^- + b^-)$ mixed zone would exist permanently as portrayed in Figure 9.

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[1] From the Greek meaning "same speed", since all the zones migrate at an equal velocity.

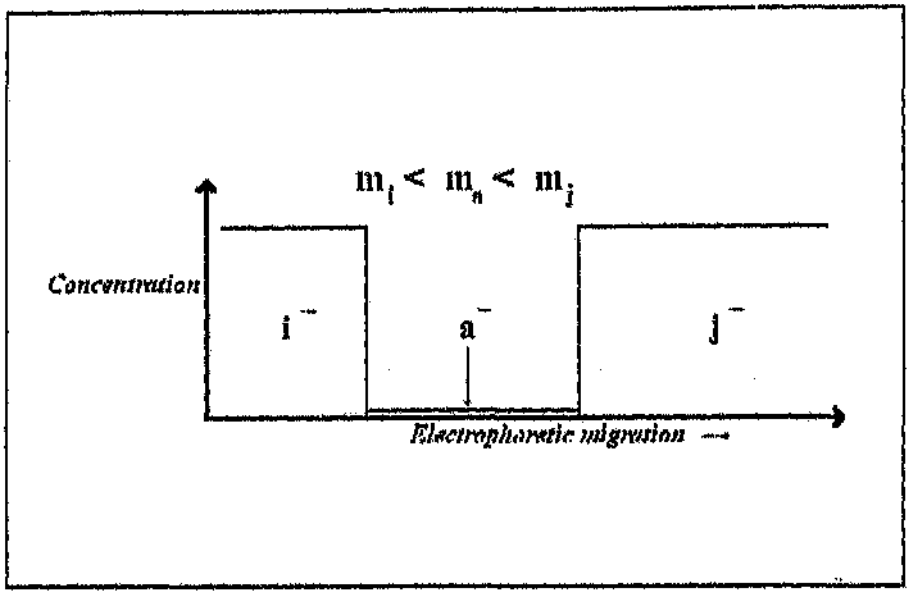


Figure 6 The starting conditions: a dilute zone of sample ion a^- has been introduced between the leading and terminating ions, j^- and i^-, respectively.

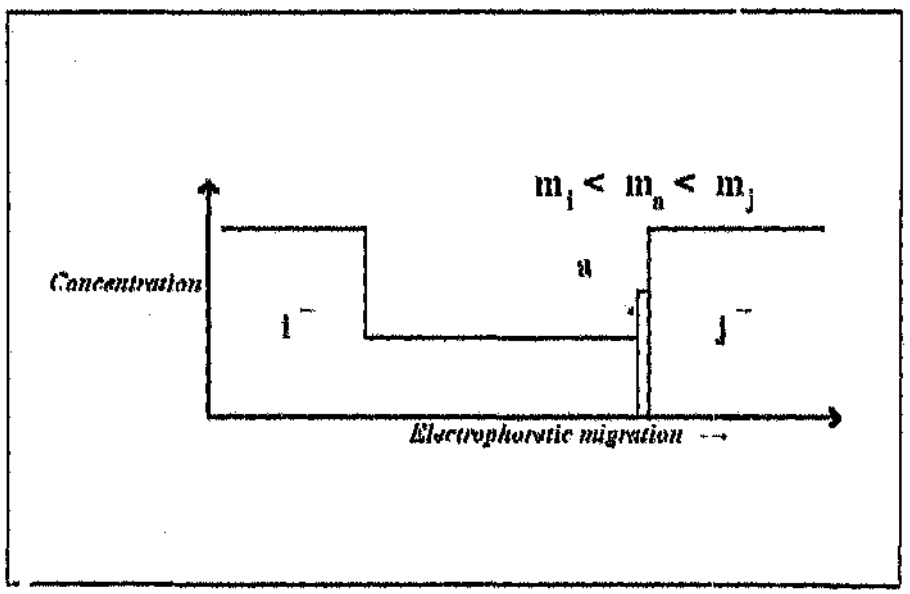


Figure 7 Sample stacking effect: the concentration of the sample ion, a, has been regulated according to the concentration of the leading ion j.

The preceding principles are expressed by the Kohlrausch regulating function[1],

$$C_i = C_j \cdot \frac{m_i}{m_j} \cdot \frac{m_k - m_j}{m_k - m_i} \qquad (1\text{-}6)$$

where C is the molar concentration, m is the ionic mobility, and j is the leading ion, i is the terminating ion, and k is the common buffering counterion. C_i is regulated by C_j, with the ratio of concentrations directly proportional to the ratio of the ionic mobilities of j and i.

Figure 5 shows that the concentration of the sample ions is automatically regulated by the Kohlrausch regulating function to that of the leading ions, irrespective of the original concentration of the sample ions. Thus, if the concentration of the leading ions is relatively high compared to the original concentration of sample ions, a remarkable concentration effect will result, as portrayed diagrammatically in Figures 6 and 7. This is the principle of the stacking effect in discontinuous electrophoresis (Ornstein, 1964). The concentration of the sample proteins into very thin starting zones increases the resolving power of this technique over that of conventional zone electrophoresis.

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[1] Derived as equation (C-15) in Appendix C. Appendix C develops the fundamental equations of steady state moving boundaries, and applies them to the calculation of the ionic parameters of the discontinuous electrophoresis systems of Ornstein and Davis (1964), and Tamura and Ui (1972).

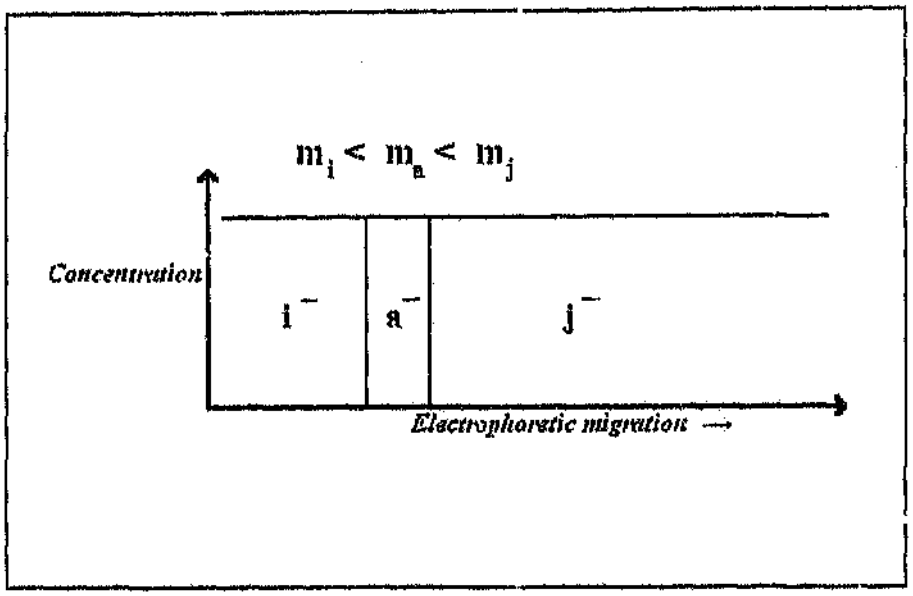


Figure 4 The starting conditions: a zone of intermediate ion a^- has been introduced between the leading and terminating ions, j^- and i^-, respectively.

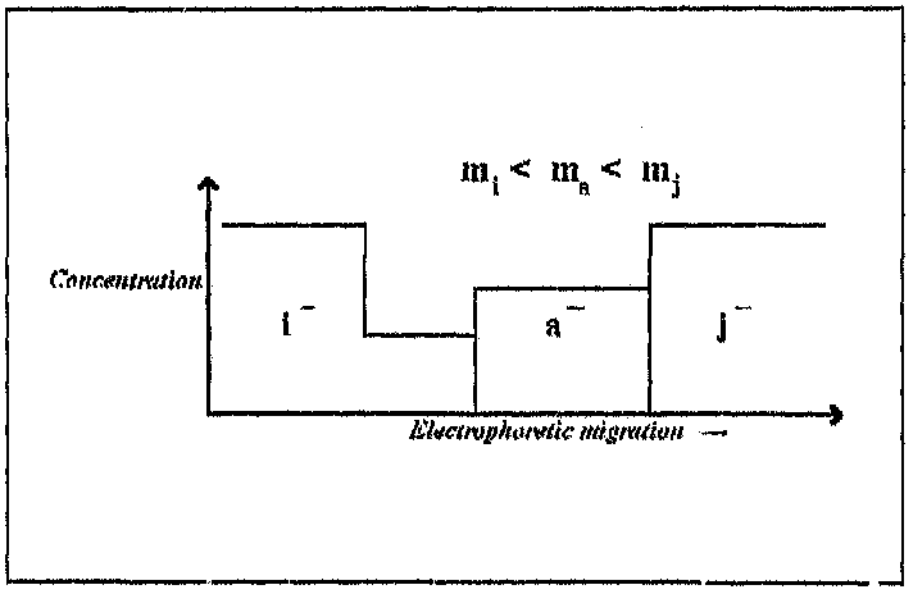


Figure 5 Steady state: the concentrations of a^- and the terminating ion i^- have been regulated according to the concentration of the leading ion j^-.

al., 1981; Nelson, 1984; Bundgaard, 1985), and although it is slow enough to allow satisfactory focusing, decomposition of *N*-(dialkylaminomethyl)acrylamide solutions during storage rules them out as valid additions to the 'Immobiline' range.

What will the next generation of 'Immobilines' look like?

It is unlikely that acrylamide or its derivatives will lose their place as the monomers of choice for the preparation of gel media for electrophoresis, due to their hydrophilicity and ease of polymerization. The main problem of acrylamides has always been their sensitivity towards alkaline hydrolysis (most electrophoretic separations take place at a pH of 8.5 or above), so that polyacrylamide gel matrices cannot be reused. Reusable polyacrylamide matrices would be highly desirable for techniques such DNA sequencing and PrIME.

Recently, a hydrophilic acrylamide derivative, *N*-acryloylaminopropanol (AAP), which is extremely resistant to alkaline hydrolysis, has been synthesized (Simó-Alfonso *et al.*, 1996a,b). The structure of AAP is shown below[1]:

$$CH_2=CH-CONH-CH_2-CH_2-CH_2-OH$$

[1] The chemistry behind the high resistance of *N*-acryloylaminopropanol to alkaline hydrolysis is discussed in Appendix II, Section II.4.

in p*K* values may be extrapolated to predict the p*K* values expected in those hypothetical basic Immobiline[1] buffers in which the amine groups are separated from the amide groups by only one carbon atom. The *N*-(morpholinomethyl)acrylamide compound should actually prove quite useful, since its expected p*K* value, 5.4, fills in the considerable gap between the p*K* 4.6 and 6.2 Immobilines®.

It so happens that these *N*-aminomethylated acrylamido buffers are easily synthesized in a one-pot Mannich-type reaction[1] involving acrylamide, formaldehyde and a secondary amine (Sébille, 1969; Bartoli *et al.*, 1975):

$$CH_2{=}CH\,CONH_2 + CH_2O + HNR'R'' \rightarrow CH_2{=}CH\,CONH\,CH_2\,NR'R'' + H_2O$$

acrylamide — formaldehyde — secondary amine — *N*-(dialkylaminomethyl)-acrylamide — water

N-Aminomethylated polyacrylamides have been in production for some time by chemical companies such as American Cyanamid, Dow Chemical Company and Hoechst. Their major uses are as polyelectrolyte flocculants in waste-water treatment, and as binders to increase paper strength (Schiller and Suen, 1956; Tramontini *et al.*, 1988). Unfortunately, the aminomethylation reaction is reversible (Bundgaard and Johansen, 1980, 1981; Loudon *et*

[1] A Mannich reaction involves the condensation of a molecule with an acidic hydrogen with an aldehyde (usually formaldehyde) and a secondary amine, resulting in an aminomethylated end product. Formaldehyde rapidly condenses with secondary amines to form highly reactive hydroxymethyl amines (or iminium ions in acid-catalyzed reactions); it is these species which react with the *N*-acidic compounds. The mechanism of the reverse reaction is explained in Section 3.4.1 of the Results and Discussion. Reviews of the Mannich reaction: Tramontini (1973) and Tramontini and Angiolini (1990).

of these electron withdrawing groups. This phenomenon can be seen in Table 2:

Table 2: Effect of electronic and steric factors on the pK values of the Immobiline range of acrylamido buffers

Number of carbon atoms separating amine from amide group	pK	Structure	Name
1	5.47	$CH_2{=}CH\ CONH\ CH_2\ N\langle\ \rangle O$	*N* (Morpholinomethyl) acrylamide*
2	6.2	$CH_2{=}CH\ CONH\ (CH_2)_2\ N\langle\ \rangle O$	*N* (2 Morpholinoethyl) acrylamide
3	7.0	$CH_2{=}CH\ CONH\ (CH_2)_3\ N\langle\ \rangle O$	*N* (3 Morpholinopropyl) acrylamide
1	7.77	$CH_2{=}CH\ CONH\ CH_2\ N(CH_3)_2$	*N* (Dimethylaminomethyl) acrylamide*
2	8.5	$CH_2{=}CH\ CONH\ (CH_2)_2\ N(CH_3)_2$	*N* (2 (Dimethylamino) ethyl)acrylamide
3	9.3	$CH_2{=}CH\ CONH\ (CH_2)_3\ N(CH_3)_2$	*N* (3 (Dimethylamino) propyl)acrylamide

* Hypothetical Immobiline acrylamido buffer.

If the number of carbon atoms separating the amine groups from the amide groups is decreased from three to two, there results a decrease of 0.8 units of the pK of the basic Immobiline, due to the electron withdrawing properties of the amide group. It may be also noticed that the pK of the morpholino derivatives are greatly lowered compared to the dimethylamine derivatives due to steric reasons, since, being in a ring structure, the protonated form of the nitrogen atom is then considerably strained and therefore less easily formed. These shifts

1.4.2 Can the commercial Immobiline chemicals be improved upon?

Parts of the pH scale not sufficiently buffered by Immobilines

There are gaps in the pH range that are not sufficiently buffered by the Immobiline range (see Table 1). This can be a problem when electrofocusing in narrow pH gradients, as the deficiency in buffering power must be compensated for by increasing the concentration of Immobilines. The resulting increase in charge density can lead to excessive swelling of the IPG gel during the washing and staining steps. Other acrylamido buffers have been synthesized which provide buffering power in the gaps between the p*K* 6.2 and 7.0 Immobilines (Chiari *et al.*, 1990*a*, 1991*a*,*b*), and between the p*K* 7.0 and 8.5 Immobilines (Chiari *et al.*, 1990*a*,*b*). However, no monoprotic acrylamido buffer has yet been synthesized that buffers in the gap between the p*K* 4.6 and 6.2 Immobilines, which is a rather important range for proteins (Chiari and Righetti, 1992).

Design of acrylamide derivatives which buffer in the pH 4.6 to 6.2 range

The basicity of an amine group is decreased by the presence of electron withdrawing groups, such as hydroxyl and amide groups, in its vicinity. Protonation of the amine with the resulting gain of a positive charge is hence rendered more difficult due to the already slightly positive polarization of the amine caused by the vicinity

Four additional acrylamido derivatives must be utilized for the production of pH 2.5 11 IPG gels (Chiari and Righetti, 1992): 2-acrylamido 2-methyl-1-propanesulfonic acid, a strong acid; acrylamidoglycolic acid, an acidic buffer with a p*K* of 3.1 (Righetti *et al.*, 1988c); *N*-[3-(diethylamino)propyl]acrylamide, a basic buffer with a p*K* of 10.3 (Gelfi *et al.*, 1987; Bossi *et al.*, 1994*b*); and 3-acrylamidopropyl(trimethyl)ammonium hydroxide, a strong base. Although they are not included in the Immobiline® buffer range, they can be purchased individually from other chemical suppliers such as Fluka.

This technology has recently been developed by Prof. Pier-Giorgio Righetti of the University of Milan, in collaboration with Ciba-Geigy, to allow the large-scale purification of valuable proteins. This technology, known as PrIME[1] (Preparative Isoelectric Membrane Electrofocusing), was only made available to the biotechnology industry in 1993, and is being marketed by Hoefer, a Californian company specialising in separation science. The chemistry of the Immobiline® compounds and of the physical chemistry of IPG's in general is described in more detail in Appendix D.

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[1] References on PrIME: Faupel *et al.*, 1987; Righetti *et al.*, 1987; Wenisch *et al.*, 1987; Righetti *et al.*, 1988b; Wenisch *et al.*, 1992; Righetti *et al.*, 1993; Chiari *et al.*, 1994c; Daniels and Landers (1996).

difference in pI of only 0.001 units (Righetti et al., 1989a; Righetti, 1990).

Since the buffering groups are covalently bonded to the acrylamide matrix and hence immobilized, the resulting pH gradient is indefinitely stable, unlike the gradients generated by carrier ampholytes. The IEF step in 2D electrophoresis is now nearly always performed with immobilized pH gradients (IPG's) due to its high degree of reproducibility (Righetti et al., 1983; Görg et al., 1988; Righetti, 1990; Sinha et al., 1992; Görg, 1993; Rabilloud et al., 1994).

Table 1: Immobiline range of acrylamido buffers

pK	Structure	Name
3.6	CH_2=CH CONH CH_2 COOH	*N* acryloylglycine
4.4	CH_2=CH CONH $(CH_2)_2$ COOH	3 acrylamidopropionic acid (discontinued)
4.6	CH_2=CH CONH $(CH_2)_3$ COOH	4 acrylamidobutyric acid
6.2	CH_2=CH CONH $(CH_2)_2$ N⟨ ⟩O	*N* (2 morpholinoethyl) acrylamide
7.0	CH_2=CH CONH $(CH_2)_3$ N⟨ ⟩O	*N* (3 morpholinopropyl) acrylamide
8.5	CH_2=CH CONH $(CH_2)_2$ N$(CH_3)_2$	*N* [2 (dimethylamino) ethyl]acrylamide
9.3	CH_2=CH CONH $(CH_2)_3$ N$(CH_3)_2$	*N* [3 (dimethylamino) propyl]acrylamide

1.4 ISOELECTRIC FOCUSING IN IMMOBILIZED pH GRADIENTS

1.4.1 Background to immobilized pH gradient technology

In 1982, a Swedish biochemical company, LKB (now part of the Pharmacia Biotechnology AB group), introduced a novel technique by which proteins could be electrofocused in a pH gradient of unprecedented stability and reproducibility (Bjellqvist *et al.*, 1982; Righetti *et al.*, 1988a; Righetti, 1990). Their ingenious concept involved the synthesis of acrylamide derivatives which bear acidic and basic buffering groups of varying p*K* values (see Table 1). Copolymerization of these acrylamide derivatives (commercially known as Immobilines®) with acrylamide and methylene-*N,N'*-bisacrylamide results in gels suitable for electrophoresis bearing covalently bonded buffering groups. The ratios of the acidic and basic acrylamido buffers, calculated using the Henderson-Hasselbalch equation, can be varied so that the resulting gel (a type of mixed-bed ion exchanger) buffers in any region of the pH range. The concentrations and ratios of the acrylamido buffers can be varied gradually down the length of the gel by using a gradient maker to pour the gel, giving pH gradients suitable for isoelectric focusing (IEF).

Computer programs can be used to calculate the acrylamido buffers required, as well as their concentrations, to generate any pH gradient in the range 2-12 (Righetti, 1990; Celentano *et al.*, 1991; Tonani and Righetti, 1991; Righetti and Tonani, 1991). Even pH gradients extending over only one pH unit or less can be prepared, allowing resolution of proteins having a

It is envisaged that problems may be experienced with the use of polyAMPS and related polyanions as spacers for the isotachophoretic separation of ampholytic biomolecules, due to ionic interactions. Therefore it seems that polyanionic spacers will be restricted to the separation of negatively-charged biomolecules such as DNA. For the separation of ampholytic biomolecules such as proteins, the synthesis of monoanionic polymeric spacers would be logical. However, since ITP has never, to my knowledge, been applied to the separation of nucleic acids, I restricted myself to the synthesis of polyAMPS spacers. Apart from its novelty, this work may have practical applications in areas such as DNA sequencing.

1.3.5 From isotachophoresis to isoelectric focusing

Although ITP has been used in the past for the separation on a charge basis of ampholytic biomolecules such as proteins, isoelectric focusing (IEF) is normally the method of choice. My interest in IEF was stimulated during the course of my research in the field of ITP, since pH gradients are generated in isotachophoretic stacks, especially if carrier ampholytes are used as spacers. My interest was particularly drawn to the recently-introduced technique of IEF in immobilized pH gradients (IEF-IPG). IEF-IPG has many advantages over the traditional carrier ampholyte-generated pH gradients - the pH gradients are permanently stable, and can be engineered to encompass any pH range between pH 2.5 and 11. However the chemicals required for this technique are expensive, and I saw that there was scope for the synthesis of cheaper alternatives.

independent of pH, their net charges must stay constant over the pH range 3 to 10, ie. they must be strong acids or bases. Since ionic mobility is a factor of charge and molecular mass, it follows that the ionic mobility of the spacers must then depend solely on differences in molecular mass.

However, this introduces the possibility that the ionic mobilities of the spacers may be affected by the degree of molecular sieving exerted by the gel media. It has been customary in the past to perform ITP in non-restrictive gel media with separation mainly on the basis of net charge - limiting ITP to the separation of non-denatured proteins. However there seems to be no logical reason why separation on the basis of molecular sieving should not be allowed to occur. In fact, for macro-molecules possessing a constant charge/mass ratio but differing in molecular mass, such as nucleic acids and SDS-denatured polypeptides, molecular sieving is required for separation.

Hence, it is likely that molecular sieving of the spacers would be unavoidable if ITP were attempted in restrictive gel media. Therefore it seems logical to design spacers in which ionic mobility differences would occur on the basis of molecular sieving itself. The simplest way to do this is to synthesize charged polymers of varying lengths, by the controlled polymerization of an acidic vinyl derivative. For this purpose heterogeneous mixtures of poly(2-acrylamido-2-methyl-1-propane-1-sulfonic acid) (polyAMPS) of various molecular mass ranges have been synthesized. The higher molecular mass polyAMPS molecules are suitable spacers for performing ITP in low-percentage acrylamide gels; the lower molecular mass polyAMPS spacers being optimal for separations in high-percentage polyacrylamide gels.

However, carrier ampholytes have a number of deficiencies which affect their suitability as spacers:

(i) *Their ionic mobility is pH-dependent.* The ionic mobility of carrier ampholytes increases as the pH is raised since their net negative charge increases. This means that the pH at which ITP is conducted cannot be changed without altering the net ionic mobilities of the carrier ampholyte spacers. For example, if the pH of the isotachophoretic separation is raised, in order to keep the ionic mobilities of the spacers constant, the use of more basic carrier ampholytes is necessitated. This makes ITP more time consuming, and less reproducible.

(ii) *They possess a buffering power of their own.* The formation of a wide pH gradient within an isotachophoretic stack is undesirable since it introduces another variable: the net charge of the sample proteins is altered, and hence their ionic mobilities.

It seems that no attempt has been made as yet to synthesize spacers for ITP that are free of these deficiencies. The characteristics of ideal spacers for ITP may be summarised as follows.

(i) *The spacers must be composed of a highly heterogeneous population of molecules of varying ionic mobility* - in order to allow the resolution of complex samples.

(ii) *The spacers must not possess a buffering power of their own* - in order that the pH is strictly determined by the buffering counterion.

(iii) *The ionic mobility of the spacers must be independent of the pH* - in order that the pH of the isotachophoretic separation may be altered for the purposes of optimization of resolution without necessitating changing the spacer species.

The last condition is the hardest to meet strictly. In order for the ionic mobilities of the spacers to be

"cascade stacking", ie. conventional ITP using a mixture of 10 amino acids/zwitterionic buffers with pK_a values[1] ranging from 6.4 to 11.3, with Tris as the buffering counterion. The pH gradient within the resulting isotachophoretic stack spanned the range 5.5 to 8.5. Although BSA, phycoerythrin, ferritin and haemoglobin were all separated from each other, the resolution was generally poor.

Acevedo (1991), using as spacers a mixture of 23 amino acids and zwitterionic buffers with pK_a values ranging from 6.1 to 10.8, could only obtain about 15 bands from human plasma samples in polyacrylamide gel ITP, compared with at least 30 using carrier ampholytes as spacers (Acevedo, 1989). Similar experiments performed on a preparative scale have shown amino acid spacers to be useful only for the separation of protein mixtures into their subgroups (Kopwillem *et al.*, 1976; Bier *et al.*, 1977; Johansson *et al.*, 1987; Acevedo, 1993). Hence it appears that a highly heterogeneous mixture of spacers of varying ionic mobility is necessary for the resolution of complex protein mixtures.

1.3.4 Problems occurring in the use of carrier ampholytes as spacers for ITP

At the present time carrier ampholytes have been the only source of heterogeneous spacers for use in ITP.

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[1] The acidic dissociation constant pK_a is used to describe the ionisation of both acidic and basic protolytic species, for reasons explained in Section B.5.1 of Appendix B. For purposes of simplicity the term pK will be used from henceforth within the text of this thesis - it being taken for granted to stand for the pK_a value of the protolytic species.

1.3.3 The attempted use of discrete mixtures of spacer buffers for the generation of moving electric field strength/pH gradients

It is very doubtful whether the zones of individual carrier ampholyte species of closely-spaced pI values are ever completely resolved in practice. Thus the electric field strength and pH gradients generated by carrier ampholyte spacers are linear rather than stepwise. Hence it is correct to view ITP as a technique in which sample ions are focused in *moving* electric field strength/pH gradients[1] as opposed to *stationary* pH gradients as generated in IEF.

A question that can be asked is whether it is possible to generate linear pH/electric field strength gradients with a limited number of spacers. Carrier ampholytes are very expensive, and it would be advantageous if other potential spacers such as amino acids could be used, especially in preparative applications.

Attempts have been made by Chrambach and coworkers (Nguyen *et al.*, 1977, Nguyen and Chrambach, 1978) to generate linear pH/electric field strength gradients within an isotachophoretic stack using what they called

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[1] If a single buffering counterion is used, there will not be a large difference in pH across the isotachophoretic stack, and the resolution of the proteins, on the basis of their net ionic mobilities, will be largely accomplished by means of the electric field strength gradient (Bringard and Charlionet, 1990). If a mixture of buffering counterions is used so that there is an even buffering power over a few pH units, there will be a large difference in pH across the isotachophoretic stack, and the resolution of the proteins will be largely accomplished by the pH gradient, on the basis of their pI values (Acevedo 1989, '991, 1994).

2% agarose gels (Boehringer Mannheim Agarose MP), and 3.5%, 8%, 12%, and 15%[1] polyacrylamide gels, in TBE buffer (62.5 mM Tris, 52 mM boric acid and 0.6 mM Na_2-EDTA, pH 8.7). The electrode strips were composed of filter paper strips soaked in 16x TBE buffer, and applied to the extremities of the gel, upon which were laid the platinum electrodes. The polyAMPS samples were composed of 30 mM solutions of polyAMPS polymers in TBE buffer, containing bromophenol blue and xylene cyanol FF as migration markers. Electrophoresis was conducted at 200 V at room temperature, and terminated when the bromophenol blue was 1 cm away from the filter paper electrode strip. The polyAMPS polymers were stained by immersing the gels in a 0.5% solution of methylene blue, a cationic dye which has proved suitable for the staining of other polyanions in the past such as RNA (Peacock and Dingman, 1967).

The lengths of the pAMPS polymers could be expressed in terms of the number of base pairs of those DNA molecules of equal ionic mobility; the standard size ranges of DNA separated by the above gels obtained from the 1996 FMC BioProducts Catalogue. It was noted that in all the gels, the polyAMPS fractions exhibited streaking patterns; moreover precipitation of polyAMPS polymers occurred in the polyacrylamide gels near the sample wells. This was taken be caused by entanglement of the longer polyAMPS polymers with the gel fibres.

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[1] The 3.5% polyacrylamide gel contained 4% methylene-*N,N'*-bisacrylamide crosslinker, and the 8%T to 15%T polyacrylamide gels contained 2.5% crosslinker.

The polymerization mixtures were composed of 5 ml aliquots in glass test-tubes of 3.0 M (62.1%) AMPS, 300 mM (1%) hydrogen peroxide, 10 μM (0.0003%) $FeSO_4$ and 10 mM (0.2%) ascorbic acid, containing increasing concentrations of thioglycolic acid from zero mM (control) to 500 mM. Ascorbic acid was added last to start the reaction. The reaction mixtures were allowed to polymerize at room temperature for 2 hr. (The polymerization reaction was exothermic and the test-tubes became quite hot to the touch.) The more fluid polyAMPS solutions were diluted to 1.5 M with distilled water and tested for suitability as spacers for TEP.

2.2.2 Characterization of polyAMPS polymers

Preparation of gels

Flatbed polyacrylamide gels (0.5-1 mm thick) were prepared by pouring the acrylamide solutions into molding cassettes, one side of which was a glass plate treated with a 0.5% solution of 3-(trimethoxysilyl)propyl methacrylate in acetone, and the other side a glass plate treated with neat chlorotrimethylsilane. The gels were run in a horizontal format, with samples loaded directly in shallow wells on the gel surface.

Estimation of the molecular masses of polyAMPS polymers by gel electrophoresis

The molecular masses of the polyAMPS polymers were not directly measured but instead expressed in terms of "DNA base pair equivalents" (bpe values). The polyAMPS polymers were subjected to zone electrophoresis in 1% and

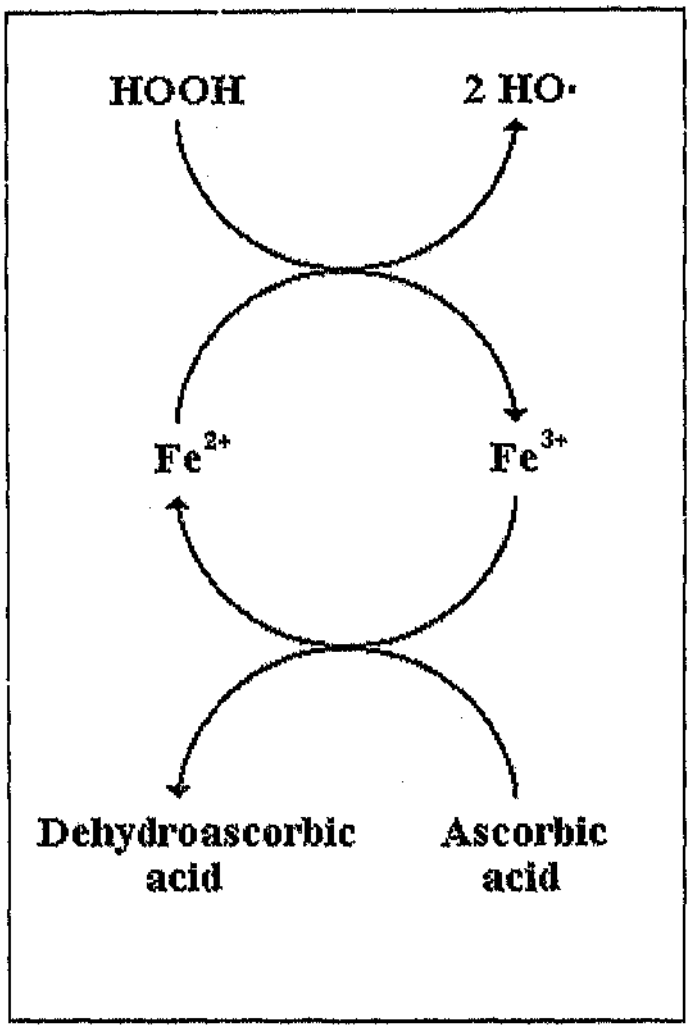


Figure 12 Mechanism of free radical production by Fenton's reagent.

2.2 ISOTACHOPHORESIS

2.2.1 Synthesis of polyAMPS polymers of a uniform distribution of molecular mass

General approach

Poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (polyAMPS) polymers of a uniform distribution of molecular mass were synthesized by the addition of chain transfer agents such as thioglycolic acid (mercaptoacetic acid) to the polymerization reaction mixture. PolyAMPS was chosen instead of polyacrylic acid since polyAMPS is 100% ionized over pH 3 and hence the net ionic mobility of the polyAMPS molecules is dependent solely upon the pore size of the polyacrylamide gels, and not upon the buffer pH. It was noted that polymerization of a 60% solution of AMPS resulted in the formation of a firm gel. Hence the decrease in average chain length of polyAMPS caused by the addition of increasing concentrations of thioglycolic acid could be qualitatively measured by the decrease in viscosity of the polyAMPS solution.

Polymerization of AMPS

A free radical initiation system was required that could operate under strongly acidic conditions. Hence the hydrogen peroxide-$FeSO_4$-ascorbic acid system known as Fenton's reagent (Uri, 1952; Blackshear, 1984) was chosen (Figure 12).

concentration of *p*-nitrophenol with gel length by densitometric measurement at 405 nm.

2.1.2 PAGE of LDH isozymes using the alkaline discontinuous buffer system of Tamura and Ui

Composition of gels

Polyacrylamide gels (0.5 mm thick) were prepared using the discontinuous buffer system of Tamura and Ui (1972):

Stacking gel: 3.75%T,4%C polyacrylamide
60 mM HCl - 66 mM AMPD buffer, pH 7.8
Resolving gel: 7.5%T,4%C polyacrylamide
60 mM HCl - 361 mM AMPD buffer, pH 9.5
Electrode buffer: 77 mM glycine - 13 mM Tris, pH 8.8

Preparation of tissue samples and electrophoresis

The same procedures for the preparation of the rat and fruit bat tissue samples, electrophoresis, and specific staining of the LDH isozymes were performed as for the Ornstein-Davis gels (Section 2.1.1).

1 isozyme. The homogenates were then transferred to Eppendorf tubes, and centrifuged for 10 minutes. The supernatants (20 µl) were loaded directly into the sample wells. The gels were run at 500 V in a cold room (10°C) until the xylene cyanol FF band was 1 cm from the end of the gel.

LDH staining

After the electrophoretic run, the LDH bands were stained[1] using a slight modification of the nitroblue tetrazolium method of Romero-Saravia *et al.* (1988). The gels were immersed in a 0.5 M Tris buffer solution, pH 8.5, containing 100 mM L-(+)lactate, 1 mM NAD, 0.1 mM phenazine methosulfate and 0.1 mM nitroblue tetrazolium. The phenazine methosulfate was added last as it is not stable in solution. The staining solution was then incubated at 37°C in the dark for 30 minutes, followed by washing in distilled water until the yellow background had disappeared.

Linear 4-20% polyacrylamide gradient gels

PAGE was repeated using the same buffer system and procedure in linear 4-20% polyacrylamide gradient gels. The gradient gels (0.5 mm thick) were prepared using conventional two-chamber gradient makers. The linearity of the gradient was verified by incorporating *p*-nitrophenol in the 20% acrylamide solution to a final concentration of 0.01%, and then checking the change in

[1] For the interest of the reader, comprehensive compilations of specific stains for enzymes are given in Heeb and Gabriel (1984) and Gabriel and Gerarden (1992).

2.1 NON-DENATURING DISCONTINUOUS ELECTROPHORESIS OF LDH ISOZYMES

2.1.1. PAGE of LDH isozymes using the discontinuous buffer system of Ornstein and Davis

Composition of gels

Polyacrylamide gels (0.5 mm thick) were prepared using the discontinuous buffer system of Ornstein and Davis (Davis ,1964; Blackshear, 1984):

Stacking gel: 3.75%T,4%C polyacrylamide[1]
60 mM HCl - 62.5 mM Tris buffer, pH 6.8
Resolving gel: 5%T,4%C polyacrylamide
60 mM HCl - 375 mM Tris buffer, pH 8.8
Electrode buffer: 192 mM glycine - 25 mM Tris, pH 8.3

Preparation of tissue samples and electrophoresis

The rat and fruit bat tissue samples (100-200 mg) were homogenized in 4 volumes of stacking gel buffer containing 20% glycerol, and a little bromophenol blue and xylene cyanol FF as migration markers. Xylene cyanol FF was found to migrate slightly faster than the rat LDH-

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[1] %T = Total concentration of acrylamide and methylene-*N,N'*-bisacrylamide measured in grams per 100 ml solution.

%C = Proportion of crosslinker (methylene-*N,N'*-bisacrylamide) measured in grams crosslinker per 100 grams of total monomer species.

Chapter 2 - Materials and methods

Spectra

Ultraviolet (UV) Spectra

All measurements of UV absorbance values were performed using a Shimadzu UV-160A spectrophotometer.

Nuclear magnetic resonance (NMR) spectra

The ^{1}H NMR and ^{13}C (proton-decoupled) NMR spectra were obtained at 200 MHz and 50 MHz respectively, using a Bruker AC200 instrument. D_2O was the solvent for all determinations. (Hence groups such as -COOH, $-NH_2$ and -OH did not give ^{1}H NMR signals due to rapid exchange of the protons with deuterium from the solvent.) The residual HDO signal (δ 4.7) was used as the internal standard for ^{1}H spectra, and sodium 3-(trimethylsilyl)-1-propanesulfonate was used as the internal standard for ^{13}C spectra.

Electrophoresis

Power packs

An LKB 3371D power pack was used for normal electrophoretic applications up to 1200 V. An LKB 2297 Macrodrive 5 power pack was used for isoelectric focusing in immobilized pH gradients up to 5000 V.

Electrophoresis units

Discontinuous electrophoresis was performed in the vertical format on home-made electrophoresis units. Isoelectric focusing and isotachophoresis were conducted in the horizontal format on a BioRad Model 1405 electrophoresis cell. Cooling was effected by connecting the electrophoresis cell to a refrigerated water bath.

polypeptides of any molecular mass from contaminating carrier ampholytes *via* a kind of affinity chromatography. I have attempted the synthesis of carrier ampholytes bearing hydrazide groups, which can be specifically and quantitatively removed from focused proteins by passing a mixture of the focused protein with the contaminating carrier ampholytes through a chromatographic matrix bearing aldehyde groups. Aldehyde groups can be easily introduced onto any polysaccharide chromatographic matrix by oxidation with periodic acid (Dyer,1956).

Unfortunately this work has been fraught with technical problems which I was not able to resolve within the time-frame of the Ph.D. However this concept, which promises to be a concrete advance in IPG technology, is certainly worthy of further effort.

from focused proteins.[1] For this reason the FDA has never permitted the preparative purification of pharmaceutical proteins using isoelectric focusing in carrier ampholyte-generated pH gradients.

Unfortunately, many pharmaceutically-important proteins cannot be purified by the use of IPG's due to insolubility problems caused by the low ionic strength. In analytical IPG's carrier ampholytes must often be added to overcome problems with protein precipitation[2] and poor conductivity (see Appendix D). Hence, there is a pressing need for carrier ampholytes that can be quantitatively removed from proteins in some manner.

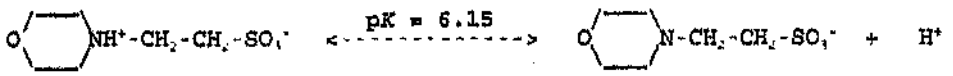
Carrier ampholytes bearing special functional groups which interact quantitatively with a derivatized chromatographic matrix seem to be a more promising approach. This would allow facile purification of

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[1] Since carrier ampholytes have a M_r below 1000, it is possible to separate them from proteins *via* techniques such as gel filtration and electrodialysis, provided that the buffer used is of a high ionic strength (0.1 - 0.5 M) in order to disrupt weak complexes which might form between carrier ampholytes and proteins by electrostatic interactions. However, carrier ampholytes bound by *hydrophobic interactions* (in particular the basic carrier ampholytes) would not be removed. The problem would be exacerbated for small polypeptides (M_r < 1500) which include many important biomolecules such as growth factors, hormones (such as oxytocin), endorphins, neuropeptides and enzyme inhibitors. This is because carrier ampholytes and small polypeptides have quite similar physicochemical properties, both in terms of net charge and mass, making them almost impossible to separate from each other.

[2] The addition of carrier ampholytes improves the solubility of proteins at their isoelectric points. Moreover carrier ampholytes shield the basic Immobilines from hydrophobic interactions with hydrophobic proteins (such as membrane-bound receptor proteins) which can lead to smearing or precipitation (Rabilloud *et al.*, 1987; Fawcett and Chrambach, 1988; Fawcett *et al.*, 1988).

the amine group, eg. 2-(*N*-morpholino)ethanesulfonic acid (MES) has a pK_a of 6.15:



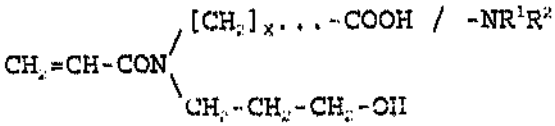
zwitterionic **acidic**

I have synthesized a series of α-aminomethylated acrylic acid derivatives, by means of a Mannich-type reaction, involving malonic acid, formaldehyde and a secondary amine (Pelletier and Franz, 1952; Krawczyk, 1995). These zwitterionic acrylic acid buffers can be successfully copolymerized with acrylamide to form IPG gels. Since they buffer in the pH range 7-10, they may be useful as hydrophilic alternatives to the basic Immobilines®, which in their free base forms are quite hydrophobic - leading sometimes to precipitation or smearing of sample proteins *via* hydrophobic interactions.

1.4.4 Can IPG technology be improved upon in other aspects?

When immobilized pH gradient technology was first introduced one of the main advantages claimed for the technique was that proteins could be purified without the problem of the subsequent removal of contaminating carrier ampholytes (Bjellqvist *et al.*, 1982). This is of especial importance for preparative purposes, since there is no technique presently available that guarantees the quantitative removal of contaminating carrier ampholytes

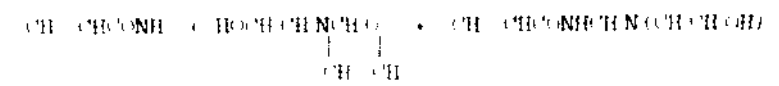
Hence it seems reasonable that the next generation of acrylamido buffers for IPG's will be derivatives of AAP:



No doubt scientists at Pharmacia Biotechnology AB are already actively involved in the synthesis of the above compounds. However, vinyl monomers other than acrylamides are certainly worthy of investigation as possible alternatives for the preparation of IPG's.

1.4.3 Development of buffers that are derivatives of vinyl monomers other than acrylamide

Hydrophilic acrylic ester derivatives have already been tried for the preparation of gels for electrophoresis (Zewert and Harrington, 1992) but have not been introduced commercially. This may be due to the fact that acrylic esters are even more sensitive to alkaline hydrolysis than acrylamides. Derivatives of acrylic acid would be very stable, but one would only be able to prepare zwitterionic buffers since a carboxylic acid group would always be present. Zwitterionic buffers (Good *et al.*, 1966; Good and Izawa, 1972; Ferguson *et al.*, 1980), which are some of the most commonly-used biological buffers, act in fact as weak acids, with pK_a values ranging from about 6 to 10, depending on the pK of



Reaction protocol

Paraformaldehyde (7.5 g, 0.25 mol of HCHO) and 26.3 g (0.25 mol) of diethanolamine were dissolved in 200 ml of chloroform and stirred at room temperature until the paraformaldehyde had dissolved. Then 17.8 g (0.25 mol) of acrylamide and 0.3 g of hydroquinone (polymerization inhibitor) were added and the resulting solution refluxed for 5 to 6 hours (chloroform boils at 50°C at the altitude of Johannesburg; raising the reaction temperature to 60° or 65°C by substituting the chloroform with methanol (Sébille, 1969) or carbon tetrachloride (Bartoli *et al.*, 1975), respectively, was found to result in considerable polymerization, irrespective of the quantity of inhibitor present).

After filtering through Whatman No. 1 paper, the chloroform was evaporated off *in vacuo* and the resulting viscous pale yellow oil dried over anhydrous K_2CO_3. Activated charcoal was added to bind the hydroquinone/benzoquinone present, followed by centrifugation to remove the activated charcoal. Attempts to purify the acrylamido buffer *via* partition chromatography on silica gel were unsuccessful. The concentration of BHEAMA in the crude oil was determined by titration (p*K* of BHEAMA determined to be approximately 5.6), and the oil diluted with *n*-propanol so as to give a 0.2 M solution of BHEAMA. This solution was used for the preparation of analytical IPG gels.

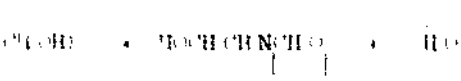
means of a heating mantle until the paraformaldehyde had dissolved. Then was added 0.25 mole of acrylamide (17.8 g) and 0.3-0.5 g of polymerization inhibitor. The reaction mixture was refluxed for 3 to 4 hours, followed by removal of the carbon tetrachloride by rotary evaporation to leave the crude acrylamido derivative as a viscous yellow oil.

At first hydroquinone was used as a polymerization inhibitor, but was later replaced with 4-methoxyphenol. This was necessitated by the fact that benzoquinone formed by the oxidation of hydroquinone by O_2 (in spite of the N_2 atmosphere) was rapidly decomposed under the alkaline conditions of the reaction (Bovey and Kolthoff, 1948) to intensely red-coloured degradation products.

Synthesis of *N*-[*N'*,*N'*-bis(2-hydroxyethyl)aminomethyl]-acrylamide

Overall reaction

The synthesis of *N*-[*N'*,*N'*-bis(2-hydroxyethyl)aminomethyl]acrylamide (BHEAMA) differs from that of other secondary amines in that the formaldehyde and diethanolamine react to form the cyclic carbinolamine ether *N*-hydroxyethyloxazolidine (Fernandez and Butler, 1963):



The *N*-hydroxyethyloxazolidine then reacts with acrylamide to form (BHEAMA):

gels were run at room temperature at constant voltage (8.5 W), with an initial voltage of 200 V, and a final voltage of 350 V. Electrophoresis was terminated when the bromophenol blue was 1 cm from the electrode wick. The DNA bands were stained as described in the previous section.

TEP of the DNA was performed at first using the polyAMPS-50 and -30 fractions as spacers. The polyAMPS-15 fraction was then tried in an attempt to get increased spacing between the DNA bands. However some distortion of the DNA bands occurred, most likely due to distortions in the electric field strength gradient caused by entanglement of the longer polyAMPS polymers. (*Cf.* Section 2.2.2.)

2.3 ISOELECTRIC FOCUSING IN IMMOBILIZED pH GRADIENTS

2.3.1 Preparation of immobilized pH gradients using novel acrylamido buffers synthesized in a Mannich reaction

Synthesis of novel acrylamido buffers

The protocol used was based on that of Bartoli *et al.* (1975). Basically, in a round-bottomed flask equipped with a reflux-condenser, a magnetic stirrer and a thermometer, and under a N_2 atmosphere, were added between 200 and 250 ml of carbon tetrachloride, to which was added 7.5 g of paraformaldehyde (equivalent to 0.25 mole of formaldehyde) and 0.25 mole of a secondary amine. The reaction mixture was stirred and gently warmed by

prior to the developing step in order to minimise the removal of formaldehyde by the Cannizzaro reaction:

$$2\ CH_2O \xrightarrow{OH^-} CH_3OH + COOH$$

(This is a precaution that is not mentioned in most silver staining protocols.)

5. Termination of development by placing the gels in fixing solution for 15 min.
6. Washing of the gels for 1 hr in distilled water. Leaving the gels in distilled water for a longer time was found to result in overswelling of the gels, since the polyAMPS polymers trapped within the polyacrylamide gel cause the gel to act as a cation exchanger.

TEP of DNA molecular mass markers using polyAMPS spacers

TEP was conducted in 5%T,4%C polyacrylamide gels. The gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers adjusted to pH 9.5 with ethanolamine. Both the anolyte and the catholyte (terminating electrolyte) were composed of 400 ml each of 100 mM EACA titrated to pH 9.5 with ethanolamine. The extremities of the gel were connected to the electrode buffer by filter paper strips soaked in electrode buffer.

Boehringer Mannheim molecular mass marker VI was used as sample DNA. 5 µl samples of a 40 µg/ml solution of DNA (= 200 ng DNA per well) in 15 mM of EACA-ethanolamine buffer, pH 9.5 were loaded in shallow wells on the polyacrylamide gel surface. The sample buffer contained bromophenol blue as a migration marker. The

DNA molecules, since they should exhibit similar electrophoretic behaviour.

PAGE of DNA molecular mass markers

PAGE of was conducted in 5%T,4%C polyacrylamide gels using TBE buffer (62.5 mM Tris, 52 mM boric acid and 0.6 mM Na_2-EDTA, pH 8.7). The electrode strips were composed of filter paper strips soaked in 16x TBE buffer, and applied to the extremities of the gel, upon which were laid the platinum electrodes. Boehringer Mannheim molecular mass marker VI was used as sample DNA. The mixture contains 15 fragments with the following number of base pairs (1 base pair = 660 daltons): 2176, 1766, 1230, 1033, 653, 517, 453, 394, 2x 298, 2x 234, 220, 2x 154.

Samples (5 µl) of a 40 µg/ml solution of DNA (= 200 ng DNA per well) in 15 mM of EACA-ethanolamine buffer, pH 9.5 were loaded in shallow wells on the polyacrylamide gel surface. The sample buffer contained bromophenol blue as a migration marker. The gels were run at 200 V at room temperature until the bromophenol blue was 1 cm away from the filter paper electrode strip.

The DNA bands were detected by a modification of the silver staining method of Sanguinetti *et al.* (1994):

1. Fixation of the gels for 15 min in 10% ethanol/0.5% acetic acid.
2. Impregnation of the gels for 15 min in a 0.2% solution of $AgNO_3$ in fixing solution.
3. Washing of the gels for 2 min in distilled water.
4. Development of the gels for 5-8 min in 3% NaOH and 0.1% formaldehyde. The formaldehyde was added just

to shallow wells on the gel surface. The gels were run at room temperature at constant voltage (8.5 W), with an initial voltage of 200 V, and a final voltage of 350 V. The ampholytic dyes were fixed by first placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol.

TEP of the 3B7T ampholytic dye fraction was performed at first using the polyAMPS-50 and -30 fractions as spacers. Slight smearing of the ampholytic dye bands occurred due to ionic interactions with the polyAMPS spacers. Fortunately these interactions could be inhibited by the inclusion of 8 M urea into the gel mixture. The polyAMPS-15 and -10 fractions were then tried in an attempt to get better spacing between the individual ampholytic dye species. The faster ampholytic dye species were well resolved, but severe distortion of the bands of the slower species was obtained. This problem was not alleviated by increasing the concentration of the urea to 9.5 M - indicating that the problem was not due to interactions between the ampholytic dye molecules and the polyAMPS spacers, but due to distortions in the electric field strength gradient caused by entanglement of the longer polyAMPS polymers (see Section 2.2.2).

2.2.4 TEP of DNA using polyAMPS spacers

TEP of DNA was attempted since DNA, like polyAMPS, is a polyanion, and should not undergo any ionic interactions with polyAMPS polymers. Moreover polyAMPS polymers, being similar in structure to DNA, should be ideal as spacers for the isotachophoretic separation of

interacted strongly with carrier ampholytes, resulting in smearing. Fortunately, these interactions could be inhibited by including 8 M urea and 30% dimethylformamide (DMF) in the gel solution. The IEF gels were composed of 5%T,4%C polyacrylamide, since DMF acts as a polymerization inhibitor. The gels contained 2% Pharmalytes, 10% sorbitol, 8 M urea and 30% DMF.

The ampholytic dye solutions were loaded directly on the IEF gel surface (up to 60 µl). The anode and cathode consisted of thin filter paper strips soaked in 0 5 M acetic acid or 1 M NaOH, respectively, upon which were laid the platinum electrodes. Focusing was performed at 5°C at an initial voltage of 200 V, with the voltage gradually increased to a final value of 1200 V. Focusing was continued at 1200 V for 16 hr, followed by 3500 V for a further 2 hr. The focused ampholytic dyes were fixed by placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol.

TEP of the 3B7T ampholytic dye fraction using polyAMPS spacers

The 3B7T ampholytic dye fraction was used for the iso-tachophoretic experiments since it was judged to have the best spread of isomeric subspecies. TEP was conducted in 5%T,4%C polyacrylamide gels. The gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers adjusted to pH 9.5 with ethanolamine. Both the anolyte and the catholyte (terminating electrolyte) were composed of 400 ml each of 100 mM EACA titrated to pH 9.5 with ethanolamine. The extremities of the gel were connected to the electrode buffer by filter paper strips soaked in electrode buffer. The 4T/B solution (20 µl) was applied

Analysis of the pI range of the ampholytic dyes by PAGE

A rough idea of the pI range of the individual ampholytic dye fractions was obtained by PAGE in a 4.5%T, 4%C polyacrylamide gel, with 15 mM HCl-ethanolamine, pH 9.5, as the gel buffer. Thin filter paper strips soaked in a 1.5 M solution of the same buffer were applied to the extremities of the gel, upon which the platinum electrodes were placed. 15 µl of the ampholytic dye fractions were loaded in narrow wells in the centre of the gel. The outermost wells contained bromophenol blue and xylene cyanol dissolved in gel buffer. Electrophoresis was conducted at 100 V at 5°C until the bromophenol blue had migrated 5 cm.

The ampholytic dyes were fixed by first placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol. The ampholytic dye molecules appeared as red zones against a deep yellow background. The function of the acetic acid in the first fixing step was to acidify the gel so that all the ampholytic dye molecules possessed a net positive charge, and so would form an insoluble complex with the picric acid. The fixation step also rendered the bromophenol blue dye undetectable, as it is coloured yellow in its acidic form.

IEF of the ampholytic dyes

IEF of the ampholytic dye fractions in a pH 8-10.5 gradient was performed in order to determine the number of the individual ampholytic dye species of each fraction. It was found that the ampholytic dye molecules

After reacting at room temperature for 24 hr, the reaction products were diluted by the addition of 20 ml of distilled water, and transferred to 500 ml conical flasks. The ampholytic dye product was precipitated by the addition of 100 ml each of 2-propanol, dichloromethane and tetrachloroethylene. The flasks were placed in the refrigerator overnight to ensure complete precipitation. The solvent was then removed by decantation. This precipitation step purified the ampholytic dye product from unreacted tetramine, and organic contaminants in the dye powder such as dodecylbenzene, a dedusting agent.

The precipitate was then washed with 150 ml of methanol to remove the ampholytic dye product from contaminating inorganic salts. The methanol solution was filtered through Whatman No. 1 filter paper, and the ampholytic dye product reprecipitated by the addition of 100 ml each of tetrachloroethylene, benzene and diisopropyl ether. The flasks were placed in the refrigerator overnight to ensure complete precipitation. The very fine precipitate could not be recovered *via* decantation, but had to be removed from the solvent by filtration through Whatman No. 1 filter paper. Most of the ampholytic dye product was removed from the filter paper by grinding the filter paper in 10 ml of distilled water. The pH of the ampholytic dye solutions ranged from 9.3 to 9.5, indicating that the average pI values of the ampholytic dyes lay in this range. The various ampholytic dye solutions were stored at 4°C.

prepared by dissolving 1 g of dye[1] in 5 ml of distilled water, followed by addition to 25 mmole aliquots of tetramine of varying composition in test-tubes. The solutions were mixed by inversion and allowed to react for 24 hr at room temperature. The composition of the various tetramine fractions is given in Table 3.

Table 3: **Composition of the tetramine fractions reacted with Remazol Red RB**

Composition of tetramine	Trivial name	BAPEDA	TETA
		25 mmole total	
100% BAPEDA	10B	4.4 g	none
90% BAPEDA/10% TETA	9B1T	3.9 g	0.4 g
80% BAPEDA/20% TETA	8B2T	3.5 g	0.7 g
70% BAPEDA/30% TETA	7B3T	3.1 g	1.1 g
60% BAPEDA/40% TETA	6B4T	2.6 g	1.5 g
50% BAPEDA/50% TETA	5B5T	2.2 g	1.8 g
40% BAPEDA/60% TETA	4B6T	1.7 g	2.2 g
30% BAPEDA/70% TETA	3B7T	1.3 g	2.6 g
20% BAPEDA/80% TETA	2B8T	0.9 g	2.9 g
10% BAPEDA/90% TETA	1B9T	0.4 g	3.3 g
100% TETA	10T	none	3.7 g

[1] Commercially produced reactive dyes always contain sodium chloride and phosphate buffer salts, as well as dedusting agents such as dodecylbenzene (Stead, 1987), so there is actually less than 1 mmole of Reactive Red RB present.

2.2.3 Interaction of ampholytic molecules with polyAMPS polymers

Synthesis of ampholytic dyes

Basic, ampholytic dyes were synthesized by reacting Remazol Red RB (Hoechst) with an excess of polyamines. Since the exact structure of Remazol Red RB is a trade secret, the molecular mass was arbitrarily taken to be 1000 daltons, assuming the dye to be of a related structure to other commercially available reactive dyes of known structure. It is, however, known from the literature (von der Eltz, 1982) that Remazol Red RB contains two reactive groups - one monochlorotriazine (MCT) group and one vinyl sulfonate (VS) group. Therefore a 25x molar excess of polyamine was used in order to ensure that the two reactive groups did not both react with one polyamine molecule.

Two tetramines were available - *N,N'*-bis(3-aminopropyl)ethylenediamine (BAPEDA) which was available as an isomerically pure species, and triethylenetetramine (TETA) which was available as a mixture of structural isomers. Hence the heterogeneity of the product could be varied by altering the ratios of BAPEDA and TETA. The use of BAPEDA alone was expected to yield four different ampholytic dye species of differing pI values.

Assuming the molecular mass of Remazol Red RB to be 1000 daltons, 1 mmol samples of Remazol Red RB were

TEP of dyes using polyAMPS spacers

The polyAMPS polymers from the 50 mM thioglycolic acid polymerization reaction (polyAMPS-50) were selected to test the ability of the polyAMPS polymers to generate an electric field strength gradient in a TEP system. Dyes were chosen as markers of resolving power. (Sample dyes in order of decreasing ionic mobility: SPADNS, orange G, bromophenol blue, azocarmine G and xylene cyanol FF.) The gel buffer was composed of a 10 mM solution of polyAMPS-50 spacer polymers adjusted to pH 9.5 with ethanolamine (p*K*=9.5). The anolyte was composed of 3 M ethanolamine titrated to pH 9.5 with boric acid. The catholyte was composed of 3 M EACA (p*K*=10.8) titrated to pH 9.5 with ethanolamine. Thin filter paper strips soaked in the anolyte and catholyte solutions respectively were applied to the extremities of the TEP gel, upon which the electrodes were laid. Solutions (15 µl) of the sample dyes (dissolved in 15 mM of terminating electrolyte) were applied to shallow wells on the gel surface. The gels (3.5%, 5% and 7% polyacrylamide) were run at 200 V at room temperature.

Zone electrophoresis of the same dyes in a 3.5% polyacrylamide gel was performed to compare resolution. The gel buffer was composed of 15 mM HCl titrated to pH 9.5 with ethanolamine. The electrode strips were soaked in a 1.5 M solution of the same buffer. Solutions (15 µl) of the sample dyes (dissolved in gel buffer) were applied to shallow wells on the gel surface. The gel was run at 200 V at 10°C.

The protocol for the preparation of the IPG gels is set out in Table 7:

Table 7: Protocol for the preparation of 4.5%T,4%C pH 7.3-7.5 IPG gels

Component	Acidic dense pH 7.3 (µl)	Basic light pH 7.5 (µl)
Distilled water	10000	10000
0.2 M α-(*N*-morpholino-methyl)acrylic acid in water	830	810
0.2 M Immobiline p*K* 9.3 in n-propanol	310	390
*Check pH**	7.25-7.35	7.45-7.55
30%T,4%C Acrylamide/methylere-*N,N'*-bisacrylamide	3000	3000
Glycerol	4000	-
Distilled water to	-> 20000	->20000
Cool in ice		
TEMED	10	10
40% Ammonium persulfate	20	20

* Since the pH values of the acidic and basic extremes of the IPG are so close, adjustment with Tris is unnecessary.

distilled water, followed by further washes (3 × 2 hr in distilled water). The gel was then air-dried to its original weight. Filter paper electrode strips (distilled water) were applied and prefocusing of the gel performed at a maximum power of 15 W until the salt fronts (seen as refractive lines or thin ridges) had migrated into the electrode strips. The same electrode strips were used for the rest of the electrofocusing procedure. The sample protein (about 10 mg/ml of equine myoglobin in 20% glycerol) was applied to shallow wells (75 µl in each well) on the surface of the gel. Focusing was performed at 5°C for 2 hr at an initial field strength of 1300 V, which was gradually raised to a final value of 4500 V. Focusing was performed at 4500 V for a further 2 hr. The gel was stained with Coomassie Blue G-250 in dilute perchloric acid according to Reisner (1984).

Preparation of a pH 7.3-7.5 IPG gel

α-(*N*-morpholinomethyl)acrylic acid was used as the weak acidic buffering component and the p*K* 9.3 Immobiline® as the strong basic titrant. The concentrations of the above chemicals required for the preparation of a pH 7.3-7.5 IPG was calculated using the DoctorpH program.

pH 7.3 solution:	8.6 mM α-(*N*-morpholinomethyl)acrylic acid. 3.1 mM Immobiline® 9.3.
pH 7.5 solution:	8.1 mM α-(*N*-morpholinomethyl)acrylic acid. 3.9 mM Immobiline® 9.3.

The protocol for the preparation of the IPG gels is set out in Table 6:

Table 6: Protocol for the preparation of 4.5%T,4%C pH 7.2-7.6 IPG gels

Component	Acidic dense pH 7.2 (µl)	Basic light pH 7.6 (µl)
Distilled water	10000	10000
0.2 M α-(*N*-morpholino-methyl)acrylic acid in water	1840	1290
0.2 M Immobiline p*K* 9.3 in *n*-propanol	570	710
Check pH	7.15-7.25	7.55-7.65
1 M Tris	150	-
Check pH	7.8	7.6
30%T,4%C Acrylamide/methylene-*N,N'*-bisacrylamide	3000	3000
Glycerol	4000	-
Distilled water to	20000	20000
Cool in ice		
TEMED	10	10
40% Ammonium persulfate	20	20

The IPG gel was poured with a two chamber gradient maker and left to polymerize at room temperature for 2 hr. The gel was soaked overnight in 200-300 ml of

distilled water, followed by further washes (3 x 2 hr in distilled water). The gel was then air-dried to its original weight. Filter paper electrode strips (distilled water) were applied and prefocusing of the gel performed at a maximum power of 15 W until the salt fronts (seen as refractive lines or thin ridges) had migrated into the electrode strips. Fresh electrode strips were then applied (anode - 10 mM glutamic acid, cathode - 10 mM histidine). The sample protein (about 10 mg/ml of equine myoglobin in distilled water) was applied to shallow wells (100 µl in each well) on the surface of the gel. Focusing was performed at 5°C for 2 hr at an initial field strength of 2000 V, which was gradually raised to a final value of 5000 V. The gel was stained with Coomassie Blue G-250 in dilute perchloric acid according to Reisner (1984).

Preparation of a pH 7.2-7.6 IPG gel

α-(*N*-morpholinomethyl)acrylic acid was used as the weak acidic buffering component and the p*K* 9.3 Immobiline® as the strong basic titrant. The concentrations of the above chemicals required for the preparation of a pH 7.2-7.6 IPG was calculated using the DoctorpH program.

pH 7.2 solution:	18.4 mM α-(*N*-morpholinomethyl)-acrylic acid. 5.7 mM Immobiline® 9.3.
pH 7.6 solution:	12.9 mM α-(*N*-morpholinomethyl)-acrylic acid. 7.1 mM Immobiline® 9.3.

The protocol for the preparation of the IPG gels is set out in Table 5:

Table 5: Protocol for the preparation of 4.5%T,4%C pH 6.9-7.9 IPG gels

Component	Acidic dense pH 6.9 (µl)	Basic light pH 7.9 (µl)
Mi[illegible]d water	10000	10000
0.2 M α-(*N*-morpholino-methyl) acrylic acid in water	1310	1160
0.2 M Immobiline pK 9.3 in n-propanol	240	840
Check pH	*6.85-6.95*	*7.85-7.95*
1 M Tris	200	-
Check pH	*8.0*	*8.0*
30%T,4%C Acrylamide/methylene-*N,N'*-bisacrylamide	3000	3000
Glycerol	4000	-
Distilled water to	20000	20000
Cool in ice		
TEMED	10	10
40% Ammonium persulfate	20	20

The IPG gel was poured with a two chamber gradient maker and left to polymerize at room temperature for 2 hr. The gels was soaked overnight in 200-300 ml of

Preparation of IPG gels using α-(*N*-morpholinomethyl)-acrylic acid[1]

Preparation of a pH 6.9-7.9 IPG gel

α-(*N*-morpholinomethyl)acrylic acid was used as the weak acidic buffering component and the p*K* 9.3 Immobiline[®] as the strong basic titrant. The concentrations of the above chemicals required for the preparation of a pH 6.9-7.9 IPG was calculated using the DoctorpH program[2].

pH 6.9 solution:	13.1 mM α-(*N*-morpholinomethyl)-acrylic acid. 2.4 mM Immobiline[®] 9.3.
pH 7.9 solution:	11.6 mM α-(*N*-morpholinomethyl)-acrylic acid. 8.5 mM Immobiline[®] 9.3.

[1] Analytical IEF-IPG is normally conducted in gels 0.5 mm thick. However the use of such thin gels often leads to the formation of air bubbles during the pouring of the immobilized pH gradients. Hence IPG gels of 2 mm thickness were used for this and all subsequent experiments in order ensure smooth and reproducible pouring. Obviously the increased thickness necessitated longer washing times of the IPG gels in distilled water - multiple washes over at least 24 hr were found necessary.

[2] The DoctorpH program was obtained from Hoefer. This program not only calculates the ratios of the acidic and basic buffers required, but also the concentrations required to maintain an even buffering power throughout the IPG gel. Linear, exponential and sigmoidal gradients can be calculated.

α-[N-methyl-N-(2-hydroxyethyl)aminomethyl]acrylic acid

After standing at room temperature for several weeks, the oil of α-[*N*-methyl-*N*-(2-hydroxyethyl)aminomethyl]acrylic acid was found to have solidified. The crude product was successfully used as seed crystals for a second synthesis. Small white crystals of α-[*N*-methyl-*N*-(2-hydroxyethyl)aminomethyl]acrylic acid were obtained upon crystallization from hot acetone/2-propanol. These crystals absorbed water from the atmosphere, but did not deliquesce like α-(*N*-morpholinomethyl)acrylic acid. Melting point: 105-106°C. Formula: $C_7H_{13}NO_4$ (159.19 Da). The pK_a value of a 10mM solution was determined to be 9.22 ± 0.08 at 22°C using the method of Albert and Serjeant (1984).

^{1}H NMR (D_2O, 200 MHz) δ 3.24 (brs, 3H, ND$^+$-CH_3), 3.7-4.1 (m, 6H, DO-CH_2CH_2-ND$^+$-CH_2-), 5.82 (s, 1H, =CH), 6.21 (s, 1H, =CH).

^{13}C NMR (D_2O, 50 MHz) δ 39.4 (ND$^+$-CH_3), 55.1 (DO-CH_2CH_2-ND$^+$), 57.1 (ND$^+$-CH_2-C=), 59.2 (DO-CH_2CH_2-ND$^+$), 130.5 (C=CH_2), 134.6 (C=CH_2), 172.3 (-COO$^-$).

None of the other α-aminomethylated acrylic acid derivatives were successfully crystallized.

Improved protocol for the synthesis of α-aminomethylated acrylic acids

The protocol used is essentially that of Krawczyk (1995). To a stirred mixture of malonic acid (10.4 g, 0.1 mol) and paraformaldehyde (6.6 g, 0.22 mol) in 150 ml of absolute ethanol was added 0.1 mol of the appropriate secondary amine. Upon heating a vigorous evolution of CO_2 commenced. The suspension was heated under reflux for 2-3 hr until no more CO_2 bubbles were evolved. The resulting solution was evaporated under reduced pressure to give an oily residue.

α-(N-morpholinomethyl)acrylic acid

Only the oil of α-(*N*-morpholinomethyl)acrylic acid readily crystallized on standing. The white, *deliquescent* solid of α-(*N*-morpholinomethyl)acrylic acid was recrystallized from hot acetone to give hygroscopic small white crystals. (Slow evaporation of the acetone solution yielded needle-shaped crystals.) Melting point: 105-106°C. Formula: $C_8H_{13}NO_3$ (171.20 Da). The pK_a value of a 10 mM solution was determined to be 7.59 ± 0.03 at 23°C using the method of Albert and Serjeant (1984).

^{1}H NMR (D$_2$O, 200 MHz) δ 3.39 (t, 4H, *J* = 7.1, C(CH$_2$C<u>H</u>$_2$)$_2$ND'), 3.99 (brs, 6H, O(C<u>H</u>$_2$CH$_2$)$_2$ND'-C<u>H</u>$_2$-), 5.90 (s, 1H, =CH), 6.30 (s, 1H, =CH).

^{13}C NMR (D$_2$O, 50 MHz) δ 53.9 (O(CH$_2$<u>C</u>H$_2$)$_2$ND'), 61.9 (ND'-<u>C</u>H$_2$-C=), 66.4 (O(<u>C</u>H$_2$CH$_2$)$_2$ND'), 133.7 (<u>C</u>=CH$_2$), 136.8 (C=<u>C</u>H$_2$), 175 (-COO$^-$).

Spectrophotometric monitoring of reaction

The progress of the reaction may also be measured by following the increase in UV absorption due to the carbon-carbon double bond of the α-aminomethylated acrylic acid product. This also provided some information on the kinetics of the reaction. Equal volumes of 1 M malonic acid, 2 M formaldehyde and 1 M morpholine were mixed and the resulting solution allowed to stand at room temperature. The progress of the reaction was followed by periodically measuring the change in the UV spectrum of the solution using a Shimadzu UV-160A spectrophotometer.

Synthesis of acrylic acid buffers: effect of water

Secondary amine (0.3 mol) was dissolved in 50 ml of distilled water (except for dimethylamine which was already in the form of a 26% (w/v) solution in water). Malonic acid (31.2 g, 0.3 mol) was added with cooling in ice-water. Formaldehyde solution (48.7 g of a 37% solution, 0.6 mol) was then slowly added with stirring and cooling in ice-water. The addition had to be slow since the reaction was very exothermic. A vigorous evolution of CO_2 ensued during the course of the reaction; this slowed down and was substantially over after 2-3 hr reaction at room temperature. Various secondary amine derivatives were synthesized and their p*K* values roughly determined by titration.

V until the salt fronts (seen as refractive lines or thin ridges) had migrated into the electrode strips, necessitating frequent replacing of the electrode strips with fresh strips. Bovine carbonic anhydrase and crude L-amino acid oxidase (*Crotalus adamanteus* venom) were applied to small rectangles of Whatman No. 1 paper near the anode. Focusing was performed at 5°C at 1500 V for 2 hr, then 2500 V overnight, followed by 4500 V for 2 hr. The gels were stained with Coomassie Blue G-250 in dilute perchloric acid according to Reisner (1984).

2.3.2 Preparation of immobilized pH gradients using acrylic acid buffers

First syntheses of acrylic acid buffers

Basic protocol

The protocol used was based on that of Pelletier and Franz (1952). Basically, malonic acid was dissolved in 37% formaldehyde solution (1:2 molar equivalents of malonic acid to formaldehyde). Then, with stirring and cooling in ice-water, one molar equivalent of morpholine was slowly added. The reaction is moderately exothermic, with simultaneous CO_2 release as quite a pronounced effervescence, which slowly decreased with time. The progress of the reaction was monitored by removing an aliquot and titrating it to determine the shift in p*K* values.

Table 4: Protocol for the preparation of 5%T,4%C pH 5.1-6.1 IPG gels

Component	Acidic dense pH 5.1 (µl)	Basic light pH 6.1 (µl)
Distilled water	3000	3000
0.2 M BHEAMA	1500	1500
0.2 M AMPS	1180	320
Distilled water to	-> 7500	-> 7500
pH adjusted to 8 with 1 M Tris		
30%T,4%C Acrylamide/methylene-*N,N'*-bisacrylamide	2500	2500
Glycerol	3000	-
Distilled water to	-> 15000	-> 15000
Cool in ice		
TEMED	6	6
40% Ammonium persulfate	15	15

The IPG gels were poured with a two-chamber gradient maker and left to polymerize at room temperature for 3 hr. They were washed in 100 mM ascorbic acid, pH 4.5, for 1 hour to reduce any potential amine oxides caused by persulfate oxidation of the tertiary amine of the basic acrylamido buffer. The gels were extensively washed in distilled water (8x30 min) and then air-dried to their original weights. Filter paper electrode strips were applied (anode - 10 mM glutamic acid, cathode - 10 mM histidine) and prefocusing of the gels performed at 500

Preparation of IPG gels using crude BHEAMA

The formulation of the gels was based on the protocols given in Righetti (1990) for the preparation of 1 pH unit-wide gradients, using 2-acrylamido-2-methylpropane-1-sulfonic acid (AMPS) as the strong acidic titrant, in ratios determined by means of the Henderson-Hasselbalch equation so as to give IPG's of 5.1 to 6.1. Unfortunately the IPG could not be accurately calculated (for example, with the help of the DoctorpH program, obtainable from Hoefer) since the acrylamido buffer used was not pure.

pH 5.1 solution: 20 mM BHEAMA, tertiary amine group 79% ionised at this pH ($pK \approx 5.6$), so 15.8 mM AMPS titrant required.

pH 6.1 solution: 20 mM BHEAMA, tertiary amine group 21% ionised at this pH, so 4.2 mM AMPS titrant required.

The protocol for the preparation of the IPG gels is set out in Table 4:

100 ml of distilled water to remove any contaminating hydrazine hydrate.

Finely crushed maleic anhydride (9.8 g, 0.1 mol) was added to the chloroform solution. An immediate precipitate of maleic acid benzylidene hydrazide (MABH) was obtained as a pale yellow solid, insoluble in almost all organic solvents in its free acid form. The MABH was vacuum-filtered, and washed with chloroform and with diethyl ether. The product obtained is identical to that obtained by Snyder et al. (1938). The ^{1}H and ^{13}C NMR spectra were not determined due to the insolubility of the compound in its free acid form.

Reaction kinetics of MABH with polyamines

A solution of MABH (0.1 mM) in methanol was allowed to react at room temperature with a slight excess of pentaethylenehexamine (PEHA). The rate of consumption of MABH was measured by following the decrease in absorbance at 310 nm.

Attempted synthesis of carrier ampholytes bearing hydrazide groups

Reaction of PEHA and MABH

PEHA (4.0 g, 0.017 mol) was dissolved in about 60 ml of methanol, to which was added 11.3 g MABH (0.05 mol), so as to give a 2:1 ratio of amine groups to carboxylic acid groups. The reaction flask was flushed with nitrogen and heated at 60°C (boiling point of methanol at the altitude of Johannesburg) for 22 hr. Aliquots of the

Attempted synthesis of carrier ampholytes bearing hydrazide groups

PEHA (7.0 g, 0.03 mol) was dissolved in about 15 ml of degassed distilled water, to which was added 11.7 g MAMH (0.09 mol). The volume was made up with degassed distilled water to 30 ml to give a 1 M solution of PEHA. The reaction flask was flushed with nitrogen and allowed to react at 70°C. Aliquots of the reaction mixture were taken at regular intervals, diluted in methanol, and their absorbance values read at 320 nm to determine the rate of consumption of MAMH. When the reaction was judged complete, the reaction mixture was cooled, transferred to a dark glass bottle, and stored at 4°C.

2.4.3 Attempted synthesis of hydrazide-containing carrier ampholytes by reacting maleic acid benzylidene hydrazide and PEHA

Synthesis of maleic acid benzylidene hydrazide

Benzylhydrazone was synthesized according to the method of Pross and Sternhall (1970). Benzaldehyde (10 g, 0.1 mol) is added very slowly to a vigorously-stirred solution of 20 g of hydrazine hydrate (0.4 mol). The clear hydrazine hydrate became cloudy-white during the addition due to the formation of benzyl hydrazone. Some benzaldehyde azine (a bright yellow solid) was also formed. After the addition of benzaldehyde was complete, the cloudy solution was carefully decanted from the contaminating benzaldehyde azine, and the benzyl hydrazone extracted with 3 x 100 ml of chloroform. The combined chloroform layers were then extracted with 3 x

filtered, and washed with 2 propanol and diethyl ether to give a white non hygroscopic powder. Melting point: 103-104°C[1]. Formula: HOOC-CH=CH-CONHNH$_2$ ($C_4H_6N_2O_2$, 130.11 Da).

^{1}H NMR (D_2O, 200 MHz) δ 5.91 (d, 1H, J = 12.1 Hz, DOOC-CH=), 6.43 (d, 1H, J = 12.1 Hz, =CH-CONDND$_2$).

^{13}C NMR (D_2O, 50 MHz) δ 122.7 (DOOC-<u>C</u>H=), 141.6 (=<u>C</u>H-CONDND$_2$), 168.4 (CONDND$_2$), 177.3 (COOD).

The ^{1}H and ^{13}C NMR spectra identify MAMH as an α,β-unsaturated carboxylic acid derivative, as can be observed by comparison with the NMR spectra of the isopropyl ester of *N,N*-diethylmaleamic acid (Matsumoto et al., 1991) ((H$_3$C)$_2$CH-OOC-CH=CH-CON(CH$_2$CH$_3$)$_2$):

^{1}H NMR (CDCl$_3$, 400 MHz) δ 5.95 (d, J = 12 Hz, ROOC-CH=), 6.54 (d, J = 12 Hz, =CH-CONR$_2$)

^{13}C NMR (CDCl$_3$, 15 MHz) δ 123.3 (ROOC-<u>C</u>=), 137.7 (=<u>C</u>-CONR$_2$), 164 (C-O), 166.0 (C=O)

<u>Reaction kinetics of MAMH with polyamines</u>

A solution of 0.5 mM MAMH and 4 mM tetraethylene-pentamine (TEPA) in methanol was allowed to react at room temperature, and the rate of consumption of MAMH measured by the decrease in absorbance at 320 nm.

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[1] The normal products of the reaction of maleic anhydride with hydrazine hydrate are 1,2-bis(3-carboxyacryloyl)hydrazine, a yellow solid with a melting point of 185-187°C (Feuer et *al.*, 1958) or 179-180°C (Liu et *al.*, 1974b), and 1,2-dihydro-3,6-pyridazinedione (maleic hydrazide), a white solid with a melting point of 296°C (Miller and White, 1956).

synthesized by reacting an α,β unsaturated acid such as acrylic acid, and a polyamine such as pertaethylene-hexamine (PEHA), using a 1:2 ratio of carboxylic acid groups to amine groups (Vesterberg, 1969). More recent protocols are merely slight modifications of the above reaction (Vinogradov, 1973; Righetti *et al.*, 1975; Binion and Rodkey, 1981). The synthesis of carrier ampholytes bearing hydrazide groups was attempted by reacting an α,β-unsaturated acid containing a hydrazide group (which may or may not be protected in some way) with PEHA, using a 1:2 ratio of carboxylic acid groups to amine groups.

2.4.2 Attempted synthesis of hydrazide-bearing carrier ampholytes by reacting maleic acid monohydrazide and PEHA

Synthesis of maleic acid monohydrazide

Carbon dioxide was bubbled for 30 min through a solution of 25.9 g hydrazine hydrate (0.5 mol) in 300 ml 2-propanol, with cooling and stirring, to form the carbazate salt of hydrazine. Finely-crushed maleic anhydride (49.0 g, 0.5 mol) was then gradually added with vigorous stirring and cooling in ice-water. A bright yellow solid (the carbazic acid derivative of maleic acid monohydrazide) formed, which was immediately vacuum-filtered. The yellow solid was redissolved in the *minimum amount* of warm distilled water (50°C). Carbon dioxide was vigorously evolved. Hot 2-propanol was added until a thick slurry of maleic acid monohydrazide (MAMH) crystals (rosette-like clusters of small transparent needles) was obtained. The yield of crystals was increased by allowing the solution to cool slowly to room temperature, followed by further cooling to 4°C. The MAMH crystals were vacuum

triazatetradec-1-ene). In other words, deamination had not occurred with the decarboxylation step as normally happens in the Mannich reaction of malonic acid. Extraction of the reaction mixture with cupferron (a Cu^{++}-chelating agent) in chloroform showed the proportion of free Cu^{++} in solution to be relatively small.

The reaction mixture was then neutralized with NaOH and loaded onto a Sephadex G-15 column (equilibrated in distilled water), in order to separate the linear tetramine derivative 14-amino-2-carboxy-4,8,11-triaza-tetradec-1-ene (if any) from the contaminating macro-cyclic 10-carboxy-1,4,8,12-tetraazacyclopentadecane (the macrocyclic compound would be easily separated as it would not be able to diffuse into the gel pores of Sephadex G-15 for steric reasons and hence would remain in the void volume). However only the blue macrocyclic 10-carboxy-1,4,8,12-tetraazacyclopentadecane compound was eluted from the column together with minor contaminants, none of which contained an acrylic acid group as determined by their UV spectra.

2.4 SYNTHESIS OF CARRIER AMPHOLYTES BEARING HYDRAZIDE GROUPS

2.4.1 General approach to the syntnesis of carrier ampholytes bearing hydrazide groups

A highly heterogeneous mixture of polyamino-polycarboxylic acids possessing good buffering capacity and conductivity at their isoelectric points[1] may be

[1] The necessary properties for a carrier ampholyte (Rilbe, 1976).

diamino-4,7-diazadecane) to 4.5 g (0.02 mol) of $CuCl_2.2H_2O$ in 10 ml of water. The $CuCl_2$ solution, which was a pale turquoise colour (A_{max} 807 nm), became a deep indigo colour (A_{max} 534 nm) upon addition of the tetramine[1]. Malonic acid (2.1 g, 0.02 mol) and formaldehyde (3.5 g of a 37% solution, just over 0.04 mol) were added and the reaction mixture left to react overnight at room temperature. The solution became an even darker indigo colour. (If a smaller volume of water, say 10 ml, was used as the reaction solvent, then a purple precipitate would form - indicating that the macrocyclic product is only moderately soluble in water).

No evolution of CO_2 was observed at all during the course of the reaction. However, upon acidification of the reaction mixture with HCl, vigorous evolution of CO_2 occurred. Obviously the cyclic condensation product of 1,10-diamino-4,7-diazadecane, formaldehyde and malonic acid had, upon acidification with HCl, been converted into 10,10-dicarboxy-1,4,8,12-tetraazacyclopentadecane, with spontaneous decomposition of one of the carboxy groups to CO_2.

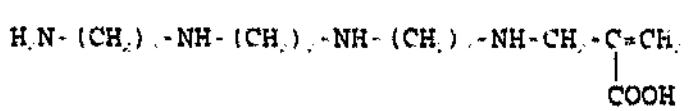
It was noted during the acidification step with HCl that very little decolorization of the reaction mixture occurred, indicating that most of the product was a Cu^{II} cyclic tetramine derivative instead of the expected Cu^{II} linear tetramine derivative (14-amino-7-carboxy-4,8,11-

[1] This is due to coordination of the Cu^{II} ion by the electron pairs of the four nitrogen atoms of the linear tetramine, with the formation of a macrocyclic structure. Addition of HCl will decolorize the solution, due to the disruption of the Cu^{II} tetramine complex. However, if the tetramine is cyclic, then the Cu^{II} ion will be too tightly coordinated for breakdown of the complex to occur. Hence this phenomenon can be used as a method to determine whether a linear tetramine has become cyclized or not.

the washing steps (due to the high charge density of the gel) resulting in damage to the surface of the acidic portion of the gel. Filter paper electrode strips soaked in distilled water were applied and prefocusing of the gel performed at a maximum power of 8.5 W until the salt fronts (seen as refractive lines or thin ridges) had migrated into the electrode strips. Fresh electrode strips were applied (anode - 10 mM glutamic acid, cathode - 10 mM arginine) and the sample proteins (about 10 mg/ml bovine carbonic anhydrase in 20% glycerol) loaded onto shallow wells on the surface of the gel (75 µl in each well). Focusing was performed at 5°C for 75 min at an initial voltage of 840 V which was gradually raised to a final voltage of 4700 V. The gel was stained with Coomassie Blue G-250 in dilute perchloric acid according to Reisner (1984).

Attempted synthesis of an α-tetraaminomethylated acrylic acid derivative

The attempted synthesis of 14-amino-2-carboxy-4,8,11-triazatetradec-1-ene:



was a modification of a protocol given by Lawrence and O'Leary (1987) for the synthesis of the macrocyclic tetramine 10,10-dicarboxylethyl-1,4,8,12-tetraazacyclopentadecane.

The macrocyclic copper-tetramine complex was prepared by adding 3.5 g (0.02 mol) of the tetramine *N,N'*-bis(3-aminopropyl)ethylenediamine (IUPAC name 1,10

Table 8: Protocol for the preparation of 6%T[a],4%C pH 4.9-8.2 IPG gels

Component	Acidic dense pH 4.9 (μl)	Basic light pH 8.2 (μl)
Distilled water	10000	10000
BCPP	150 mg[b]	150 mg[b]
0.2 M Immobiline p*K* 9.3 in *n*-propanol	1500	4000
Check pH	*4.9*	*8.2*
Acrylamide	1.2 g[d]	1.2 g[d]
Glycerol	4000	-
Distilled water to	-> 20000	->20000
Cool in ice		
TEMED	10	15[c]
40% Ammonium persulfate	20	30[c]

[a] Amount raised to allow satisfactory polymerization in view of the high concentration of *n*-propanol in the gel solution.
[b] To give a 20 mM concentration of BCPP.
[c] Final pH of solution following complete dissolution of BCPP, which generally took over an hour.
[d] To give a 6%T polyacrylamide gel.

The IPG gel was poured with a two chamber gradient maker and left to polymerize at room temperature for 3 hr. The gel was soaked overnight in 300-400 ml of distilled water, followed by further washes (3 x 2 hr) and then air-dried to its original weight. Excessive swelling of the acidic portion of the IPG occurred during

Preparation of pH 4.9-8.2 IPG gel using BCPP

Since BCPP is a bifunctional acrylic acid derivative, it can act as a crosslinker, and so it is not necessary to add methylene-*N,N'*-bisacrylamide. Since the buffering regions of the more weakly basic piperazine nitrogen and the carboxylic acid groups overlap (as seen in Figure 13), it is not possible to determine the p*K* values of these groups by titration. Hence it was necessary manually to titrate the "acidic dense" and "basic light" solutions (each containing 20 mM BCPP) to the desired pH values with the strongly basic p*K* 9.3 Immobiline titrant (0.2 M in *n*-propanol). This was a slow process as BCPP dissolves only very slowly in water.

The titration curve of BCPP (Figure 13) shows it to buffer linearly between pH 3 and 5, and between pH 8 and 10. A pH 4.9-8.2 IPG was prepared for the resolution of carbonic anhydrase isozymes However, since the titration curve rises steeply over pH 6, the pH 6-8 part of the IPG forms only a small proportion of the length of the IPG gel, so that in actual fact the carbonic anhydrase isozymes were resolved on a pH 5-7 IPG gel.

The protocol for the preparation of the pH 4.9-8.2 IPG gels is set out in Table 8:

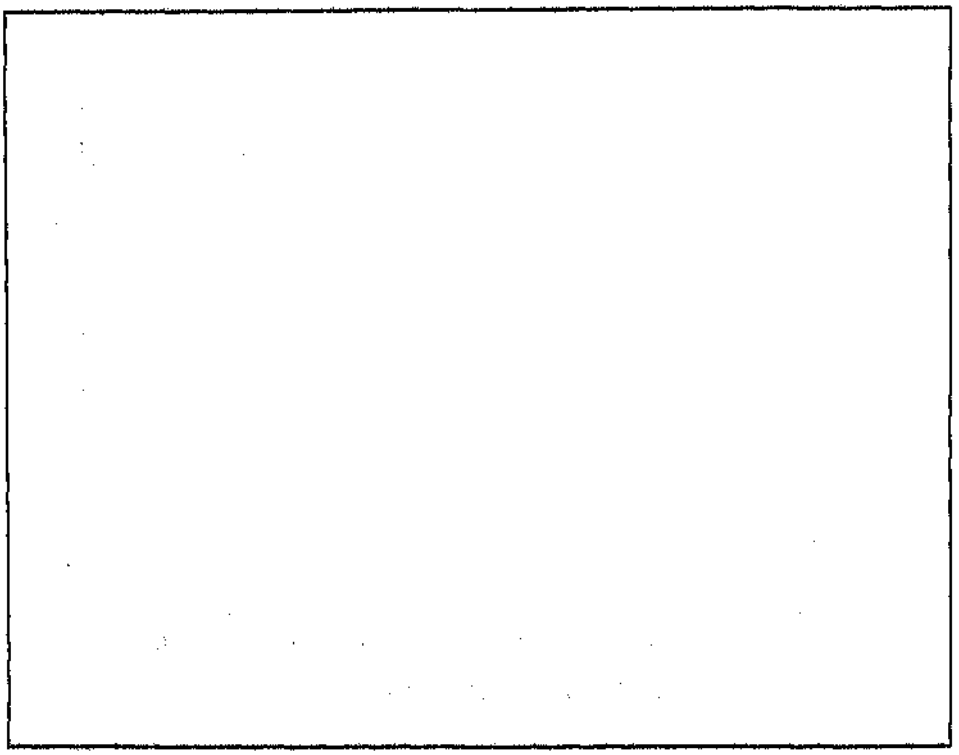


Figure 13 Titration curve of *N,N'*-bis(2-carboxyprop-2-enyl)piperazine (BCPP). BCPP·8H_2O (0.57 g = 2.5 mmole) was dissolved in 50 ml of 0.1 N NaOH, giving a 50 mM solution (pH 11.4). It was necessary to dissolve BCPP in an alkaline solution as BCPP is insoluble in its free acid form. The solution was titrated with 2.8 ml of 1 N HCl (2.8 mmole acid) at 25°C.

The white precipitate was then filtered and recrystallized from the minimum amount of hot water to yield crystals in the form of tiny rosette-like clusters of needles. The yield from the reaction itself was high, but a lot of product was lost in the recrystallization step. Drying a known mass of crystals at 80°C for 3 hr followed by reweighing determined the product to be in the form of an octahydrate. Melting point of anhydrous compound: 228-229°C with decomposition. Formula of anhydrous compound: $C_{12}H_{18}N_2O_4$ (254.29 Da).

^{1}H NMR (D_2O, 200 MHz) δ 3.45 (s, 8H, -ND'(CH_2CH_2)$_2$ND'-), 3.91 (s, 4H, 2 × ND'-CH_2-C=), 5.88 (s, 4H, 2 × =CH), 6.30 (s, 4H, 2 × =CH).

^{13}C NMR (D_2O, 50 MHz) δ 48.9 (-ND'(CH_2CH_2)$_2$ND'-), 58.7 (ND'-CH_2-C=), 131.5 (C=CH_2), 134.1 (C=CH_2), 172.2 (-COO).

A 50 mM solution of the sodium salt of *N,N'*-bis(2-carboxyprop-2-enyl)piperazine (BCPP) was prepared (pH 11.4) and titrated with 1 N HCl. The titration curve is shown in Figure 13.

The IPG gel was poured with a two-chamber gradient maker and left to polymerize at room temperature for 2 hr. The gel was soaked overnight in 200-300 ml of distilled water, followed by further washes (3 x 2 hr in distilled water). The gel was then air-dried to its original weight. Filter paper electrode strips (distilled water) were applied and prefocusing of the gel performed at a maximum power of 17 W until the salt fronts (seen as refractive lines or thin ridges) had migrated into the electrode strips. The same electrode strips were used for the rest of the electrofocusing procedure. The sample protein (about 10 mg/ml of equine myoglobin in 20% glycerol) was applied to shallow wells (75 µl in each well) on the surface of the gel. Focusing was performed at 5°C overnight at an initial field strength of 1000 V. The next day the electric field strength was gradually raised over the course of seven hours to a final value of 4900 V. Focusing was continued at 4900 V for a further 56 hr - this excessive length of time of focusing was found necessary as the proteins took a very long time to reach their isoelectric points due to the extreme shallowness of the pH gradient (0.02 pH unit/cm). The gel was stained with Coomassie Blue G-250 in dilute perchloric acid according to Reisner (1984).

Synthesis of *N,N'*-bis(2-carboxyprop-2-enyl)piperazine

Malonic acid (20.8 g, 0.2 mol) was dissolved in 50 ml of distilled water, and 19.4 g of piperazine hexahydrate (0.1 mol) was added with cooling in ice-water. Then 33 g of 37% formaldehyde solution (0.4 mol) was slowly added with stirring and cooling in ice-water. A very vigorous evolution of CO_2 then ensued, with the concurrent formation of a white precipitate. The reaction mixture was left to react overnight at room temperature.

the leading electrolyte. The calculation of the concentration of buffering counterion in the terminating electrolyte can become quite complicated, involving equations of mass balance and electroneutrality (Ornstein, 1964; Jovin, 1973*a*,*b*,*c*). It is probably this fact that deterred Tamura and Ui from designing their discontinuous buffer systems from first principles.

The fundamental physicochemical equations governing the stacking process

Electrophoresis systems involving the formation of the steady state moving boundaries, such as that between the leading ion (chloride) and the terminating ion (glycine) that causes the stacking effect in discontinuous electrophoresis, fall under the technique of isotachophoresis (ITP). Therefore it was necessary to study the equations governing the isotachophoretic steady state[1] in order to understand the stacking process. An in-depth discussion of the fundamental equations governing the formation and maintenance of steady state moving boundaries is presented in Appendix B[2]. In Appendix C I have applied these equations to the chloride-glycine system in discontinuous electrophoresis. These equations are set out below, for use with the Ornstein-Davis system. However by simply replacing Tris with AMPD the identical equations can be used for the Tamura-Ui system as well. It is assumed that the pH of the system is below 10, so that the concentration of OH^-

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[1] Theoretical treatments of the steady state boundaries existing in ITP may be found in Ornstein (1964), Everaerts and Routs (1971), Jovin (1973*a*) and Everaerts *et al.* (1974).

[2] This is preceded by a general introduction to theory of electrophoresis in Appendix A.

3.2.3 Investigation of the physicochemical mechanism of the stacking process

The need to design discontinuous electrophoretic systems from first principles

It was clear that the rat and fruit bat LDH-5 isozymes were still not stacking, even though they were certainly negatively charged due to increased pH of the discontinuous buffer system. It was at this point that I decided to begin a systematic study of the physicochemical mechanisms governing the stacking process in discontinuous electrophoresis, in an effort to solve this problem.

Up to this time I had used the discontinuous electrophoresis of Ornstein and Davis without really understanding how the stacking process worked - a deficiency that is probably widespread among users of the technique in the biological sciences. The key to the understanding of the stacking process lies in the Kohlrausch regulating function (Ornstein, 1964), which relates the concentration and electric field strength gradients across steady state moving boundaries to the difference in ionic mobility of the leading and terminating ions. These steady state moving boundaries have already been discussed in Section 1.2 of the Introduction.

However the concentration of the buffering counterion in the terminating electrolyte is also regulated in the steady state, so that the total ionic composition (and hence pH) of the terminating electrolyte is predetermined by the total ionic composition of

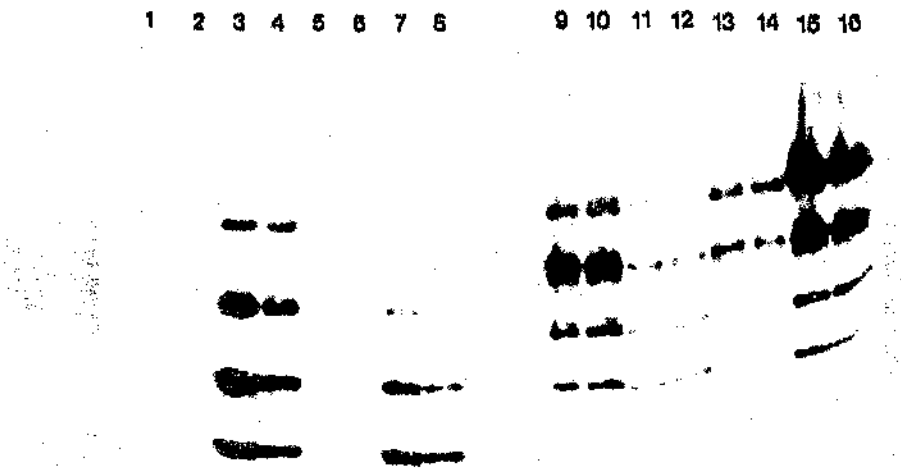


Figure 16 LDH isozymes separated on a 7.5% polyacrylamide gel, using the discontinuous buffer system of Tamura and Ui (1972). Lanes 1 and 2: rat skeletal muscle, lanes 3 and 4: rat heart, lanes 5 and 6: rat liver, lanes 7 and 8: rat kidney, lanes 9 and 10: fruit bat brain, lanes 11 and 12: fruit bat heart, lanes 13 and 14: fruit bat liver, lanes 15 and 16: fruit bat kidney. The tissue samples were homogenized in four volumes of stacking gel buffer containing 20% glycerol. Electrophoresis was conducted in a cold room (10°C) at 500 V, until the xylene cyanol FF dye marker was 1 cm from the bottom of the gel. The LDH isozyme bands were detected using a modification of the nitroblue tetrazolium stain of Romero Naravia *et al.* (1988).

sample proteins are left behind the chloride-glycine moving boundary as a thin starting zone in a region of constant pH and electric field strength, and now migrate as in ordinary zone electrophoresis.

The design of anionic discontinuous electrophoretic systems

1. *Choose a pH for the separating gel.* The pH of the separating gel rises by about half a pH unit from the original pH as the leading ions become replaced with terminating ions.

2. *Choose as the terminating ion a weak acid or amino acid with a pK up to one pH unit higher than that of the separating gel.* Thus at the actual running pH of the separating gel the terminating ions will have a net charge of at least 0.5, and their net ionic mobility will be higher than those of any of the sample proteins.

3. *Set the pH of the stacking gel 2 to 3 pH units less than the pK of the terminating ion.* In this way the terminating ions will have a net charge of 0.001 to 0.01, and so their net ionic mobility will be lower than those of any of the sample proteins.

4. *Choose as a buffer a base with a pK up to one pH unit less than the pH of the separating gel.* In this way the stacking gel also possesses a buffering capacity.

5. *Make the pH of the electrode buffer equal to the pK of the buffer.* The pH of the electrode buffer is not critical, so making it up this way minimizes the pH changes caused by the products of electrolysis.

Although the rules of Williams and Reisfeld appear very logical, the Tamura-Ui discontinuous buffer system did not succeed in resolving the LDH-5 isozyme (Figure 16).

The principles that Tamura and Ui used in designing their discontinuous buffer system

Tamura and Ui designed their discontinuous buffer system along the principles given by Williams and Reisfeld (1964) in their paper entitled "Disc electrophoresis in polyacrylamide gels: extension to new conditions of pH and buffer.". In order to set out their principles, I will give a brief résumé of their paper.

The basic mechanism of discontinuous electrophoresis

The stacking and separating gels contain a leading ion (chloride) of higher ionic mobility than any of the sample proteins. The electrode buffer contains a terminating, or trailing, ion (glycine) of lower net ionic mobility of any of the sample proteins. When a potential difference is applied to the system, the chloride ions run away from the glycine ions, leaving behind a zone of lower conductivity, and therefore increased electric field strength. In this electric field strength gradient the glycine ions are accelerated to keep up with the chloride ions. A steady state moving boundary is created which migrates down the gel, rapidly overtaking the sample proteins. Since the sample proteins possess ionic mobilities intermediate to the chloride and glycine ions, they are sandwiched between the chloride and glycine zones to form a very thin zone.

At the pH discontinuity between the stacking and separating gels, the degree of ionization of the glycine ions will be increased so that their net ionic mobility now becomes greater than any of the sample proteins, but still less of course than that of the chloride ions. The

3.2.2 The search for more alkaline discontinuous buffer systems

The discontinuous buffer system of Tamura and Ui

It occurred to me that the rat and fruit bat LDH-5 isozymes were not migrating into the gel because they were still positively charged at the stacking pH, and that a more basic discontinuous buffer system was necessary in order to detect the LDH-5 isozymes. A literature search revealed the existence of a basic non-denaturing discontinuous buffer system (Tamura and Ui, 1972) using 2-amino-2-methyl-1,3-propanediol (AMPD) as opposed to Tris as the buffer (p*K* 8.8 versus 8.1 respectively). The composition[1] of the Tamura-Ui buffer system is compared below with that of Ornstein and Davis:

Ornstein-Davis	Tamura-Ui
Stacking gel buffer	
60 mM HCl 62.5 mM Tris pH 6.8	60 mM HCl 66 mM AMPD pH 7.8
Resolving gel buffer	
60 mM HCl 370 mM Tris pH 8.8	60 mM HCl 161 mM AMPD pH 9.5
Electrode buffer	
192 mM glycine 25 mM Tris pH 8.3	77 mM glycine 14 mM AMPD pH 8.8

[1] In Tamura and Ui (1972) the concentration of AMPD in the stacking gel is stated to be 59 mM! Since the concentration of HCl is 60 mM, this would give the stacking gel buffer a pH of about 3 - obviously an error. Moreover in their paper the concentration of AMPD in the separating gel buffer is stated to be 262 mM, which would give the separating gel buffer a pH of 9.3 instead of 9.5. I have substituted the correct concentrations of AMPD required - 66 mM AMPD and 161 mM AMPD for the stacking and separating gel buffers respectively.

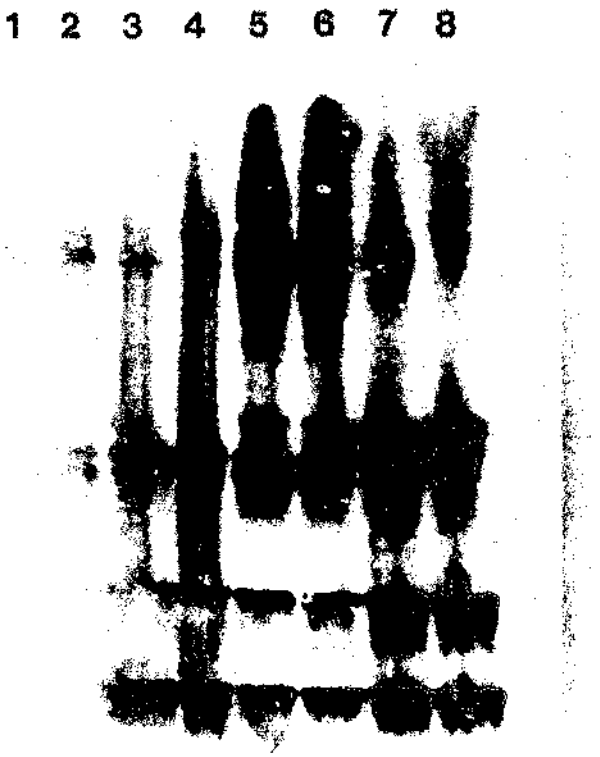


Figure 15 Fruit bat LDH isozymes resolved by a linear 4-20% polyacrylamide gradient gel, using the buffer system of Davis (1964) according to the formulation given in Blackshear (1984). Lanes 1 and 2: liver, lanes 3 and 4: heart, lanes 5 and 6: kidney, lanes 7 and 8: brain. The tissue samples were homogenized in four volumes of stacking gel buffer containing 20% glycerol. Electrophoresis was conducted in a cold room (10°C) at 500 V, until the xylene cyanol FF dye marker was 1 cm from the bottom of the gel. The LDH isozyme bands were detected using a modification of the nitroblue tetrazolium stain of Romero-Saravia *et al.* (1988).

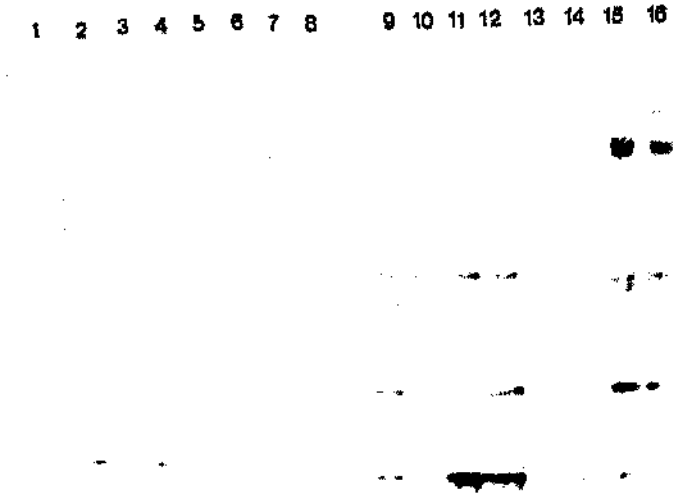


Figure 14 LDH isozymes resolved on a 5% polyacrylamide gel, using the buffer system of Davis (1964) according to the formulation given in Blackshear (1984). Lanes 1 and 2: rat skeletal muscle, lanes 3 and 4: rat heart, lanes 5 and 6: rat liver, lanes 7 and 8: rat kidney, lanes 9 and 10: fruit bat brain, lanes 11 and 12: fruit bat heart, lanes 13 and 14: fruit bat liver, lanes 15 and 16: fruit bat kidney. The tissue samples were homogenized in four volumes of stacking gel buffer containing 20% glycerol. Electrophoresis was conducted in a cold room (10°C) at 500 V, until the xylene cyanol FF dye marker was 1 cm from the bottom of the gel. The LDH isozyme bands were detected using a modification of the nitroblue tetrazolium stain of Romero-Saravia *et al.* (1988).

longer be homogeneous, and each LDH isozyme will exist as a number of allozymes, varying slightly in amino acid composition.

Since the B subunit is more acidic than the A subunit, LDH-1 migrates the fastest and LDH-5 the slowest in PAGE using alkaline buffers. The discontinuous buffer system of Davis (1964) is normally used. The tissues of interest are homogenized in four to nine volumes of stacking gel buffer containing 20% glycerol, centrifuged for 10 min, and loaded directly onto the gel. The LDH isozyme bands are detected by means of a specific stain employing tetrazolium salts (Romero-Saravia et al., 1988) (see p. 41).

Non-denaturing PAGE of LDH isozymes using the discontinuous buffer system of Ornstein and Davis

The LDH isozymes from various rat and fruit bat tissues were analyzed by non-denaturing PAGE using the discontinuous buffer system of Ornstein and Davis (Davis, 1964; Ornstein, 1964; Blackshear, 1984). Although a satisfactory resolution was obtained, the LDH-5 isozyme of both the rat and fruit bat failed to migrate into the gel (Figure 14).

Under the misconception that the failure to see LDH-5 was due to its very slow migration, I decided to run the LDH isozymes on gradient gels, so that I could prolong the time of electrophoresis without the risk of the LDH-1 isozymes migrating out of the bottom of the gel. Linear 4-20% polyacrylamide gradient gels were prepared for the separation of rat LDH isozymes. An improvement in the fruit bat LDH allozyme sub-banding patterns was achieved, however the LDH-5 isozymes still could not be seen (Figure 15).

3.2 THE DIRECTION OF MY RESEARCH PROJECT INTO THE FIELD OF ISOTACHOPHORESIS

During my B.Sc (Hons) year my project involved the purification of lactate dehydrogenase (LDH) from the fruit bat *Rousettus aegyptiacus* and the investigation of its kinetics. At the start of my postgraduate studies, I thought that it would be a good idea to clarify first a problem that I had experienced in the analysis of the fruit bat LDH isozymes by PAGE in discontinuous buffers, ie. the failure of the LDH-5 isozyme to migrate into the gel. Being forced to study the physicochemical mechanism behind discontinuous electrophoresis in an effort to solve this problem, I became fascinated by the ingenuity of the technique, so much so that I redirected my Ph.D project into the realm of electrophoresis.

3.2.1 Problems encountered in the analysis of LDH isozymes by PAGE using the discontinuous buffer system of Ornstein and Davis

The use of PAGE for the analysis of LDH isozymes

LDH is a tetramer, having a total molecular mass of 144 kDa. The subunits may be of two types, designated A and B. Hence there are five possible tetrameric arrangements - B_4, AB_3, A_2B_2, A_3B and A_4, which are named LDH-1 to LDH-5 respectively. Since the A and B subunits are coded for by different genes, the distribution of LDH isozymes present in a tissue is controlled by the ratio of A and B subunits produced, their combination into the various tetramers being random. If both alleles of a subunit gene are expressed, the subunit types may no

Chapter 3 - Results and discussion

3.1 GENERAL OVERVIEW: THE TRANSITION FROM DISCONTINUOUS ELECTROPHORESIS TO ISOTACHOPHORESIS AND ISOELECTRIC FOCUSING

At the start of my M.Sc. project, my task was to investigate whether the specialised metabolism of the fruit bat *Rousettus aegyptiacus* was reflected in changes in the activity of key enzymes involved in glycolysis, for example, lactate dehydrogenase (LDH). This involved the use of polyacrylamide gel electrophoresis (PAGE) in non-denaturing buffers for the analysis of LDH isozymes from rat and fruit bat tissues with a view to comparing their patterns and correlating these with metabolic activity. The problems that I experienced in the failure of the LDH-5 isozymes to enter the polyacrylamide gels stimulated my interest in the physical chemistry involved behind the stacking process in discontinuous electrophoresis. The stacking effect is caused by the generation of electric field strength gradients across steady state moving boundaries, by which the sample proteins become focused into thin layers. This improves resolution since the degree of band spreading caused by diffusion is minimized.

The above electrophoretic phenomenon is also the principle behind the technique of displacement electrophoresis, or isotachophoresis (ITP), in which multiple moving ionic boundaries are used to generate wide electric field strength gradients in which proteins can be focused. I found the physicochemical principles behind ITP to be extremely interesting, and my thoughts turned to the possibility of doing practical research in the field of ITP. ITP in polyacrylamide gels has been a

reaction mixture were taken at regular intervals, diluted in methanol, and their absorbance values read at 310 nm to determine the rate of consumption of MABH. When the reaction was judged complete, refluxing was stopped, and catalytic transfer hydrogenolysis of the benzyl hydrazone groups was attempted.

Attempted deprotection of the hydrazide groups by catalytic transfer hydrogenolysis

Simultaneous reduction of the benzyl hydrazone groups to *N'*-benzylhydrazides, and debenzylation of the *N'*-benzylhydrazides to hydrazides, was attempted by catalytic transfer hydrogenolysis using a palladium catalyst and the formate ion as the *H*-donor (Adger *et al.*, 1987; Ram and Spicer, 1987). Palladium on polyethylenimine beads (Pd-PEI) was used as the catalyst (Coleman and Royer, 1980). Pd-PEI (2 g) and formic acid (0.2 mol = 4 molar equivalents of benzyl hydrazone groups) were added to the reaction mixture, which was then stirred for 100 hr at room temperature. The Pd-PEI was then removed by filtration, and the methanol removed by rotary evaporation to yield a viscous oil. The oil was found to be insoluble in water, indicating that the debenzylation step was unsuccessful.

3.3.2 Synthesis of heterogeneous mixtures of polyAMPS as polymeric spacers for ITP

Synthesis of polyAMPS polymers of varying lengths

The principal stages of free radical-induced vinyl polymerization are:

1. *Chain initiation*

Peroxide/persulfate $R\text{-}O\text{-}O\text{-}R \rightarrow R\text{-}O\bullet$

$$R\text{-}O\bullet + CH_2{=}\underset{\displaystyle X}{CH} \rightarrow R\text{-}O\text{-}CH_2\text{-}\underset{\displaystyle X}{CH}\bullet$$

2. *Chain propagation*

$$R\text{-}O\text{-}CH_2\text{-}\underset{\displaystyle X}{CH}\bullet + CH_2{=}\underset{\displaystyle X}{CH} \rightarrow R\text{-}O\text{-}CH_2\text{-}\underset{\displaystyle X}{CH}\text{-}CH_2\text{-}\underset{\displaystyle X}{CH}\bullet$$

3. *Termination*

A. *Combination*

$$2 \times R\text{-}O\text{-}(CH_2\text{-}\underset{\displaystyle X}{CH})_n\bullet \rightarrow R\text{-}O\text{-}(CH_2\text{-}\underset{\displaystyle X}{CH})_n\text{-}(\underset{\displaystyle X}{CH_2\text{-}CH})_n\text{-}O\text{-}R$$

separation mainly on the basis of net charge - limiting ITP to the separation of non-denatured proteins. However there seems to be no logical reason why separation on the basis of molecular sieving should not be allowed to occur. In fact, molecular sieving is required for the separation of macromolecules possessing a constant charge/mass ratio but differing in molecular mass, such as nucleic acids and SDS-denatured polypeptides.

Since it is likely that molecular sieving of the spacers would be unavoidable if ITP were attempted in restrictive gel media, it seemed logical to design spacers in which ionic mobility differences would occur on the basis of molecular sieving. Thus it was reasoned that a heterogeneous mixture of anionic polymers of an even spread of molecular masses would have the desired characteristics. 2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPS) was chosen since its polymers are fully dissociated above pH 2[1]. Heterogeneous mixtures of polyAMPS of various molecular mass ranges were synthesized and evaluated for their suitability as spacers for isotachophoretic separations.

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[1] Poly(acrylic acid) is a much weaker acid than its monomer (pK_a 4.25) because of electronic interactions between the carboxylic acid groups, which are only separated by two carbon atoms. Hence a 0.01 M solution of poly(acrylic acid) in water will a have an apparent pK (pK') of 6.17 (Miller, 1964).

3.3 ISOTACHOPHORESIS - THE SYNTHESIS AND APPLICATION OF NOVEL POLYMERIC SPACERS

3.3.1 Need for alternative spacers to carrier ampholytes

As discussed in Section 1.3.4 in the Introduction, carrier ampholytes have a number of deficiencies which affect their suitability as spacers, most importantly being the fact that their ionic mobilities vary with pH. The pH at which the isotachophoretic separation is conducted must often be varied in order to optimise the resolution of the sample proteins. Thus the ionic mobilities of the spacer ions should be independent of pH in order to avoid continuous recalibrations being necessary. To quote from Bringard and Charlionet (1990): "*Concerning the nature of the spacers, we have shown that Ampholine 5-7 or Pharmalyte 6.7-7.7 are not ideal, because their electrophoretic mobility depends on pH. Highly diversified spacers of nonamphoteric nature and with adequate mobilities for the analysis of proteins would certainly be welcome.*"

This requirement means that the net charges of the spacer ions must stay constant over the pH range 3 to 10, ie. they must be strong acids (assuming that an anionic separation is occurring). Since ionic mobility is a factor of charge and molecular mass, it follows that the ionic mobility of the spacers must then depend solely on differences in molecular mass. However, this introduces the possibility that the ionic mobilities of the spacers may be affected by the degree of molecular sieving exerted by the gel media. It has been customary in the past to perform ITP in non-restrictive gel media with

enthusiasm for the technique, and I decided to redirect my research project into this field.

ITP has rarely been used for the separation of proteins in polyacrylamide or agarose gels (Acevedo 1989,1991). This is surprising since, in theory, isotachophoretic systems should have a higher resolving power over conventional electrophoretic systems, since the boundaries between zones do not diffuse with time due to the sharpening effect of the electric field strength and pH gradients across the zone boundaries.

Perhaps the main reason why ITP has not become more widespread in its use in biochemistry is that all the ion zones are contiguous. Hence protein zones cannot be distinguished by conventional stains unless they are physically separated from each other by means of spacer ions, ie. charged molecules, such as amino acids, having ionic mobilities intermediate to those of the proteins of interest. Highly heterogeneous mixtures of spacers of varying ionic mobility are required for the resolution of complex mixtures of proteins. The only chemicals meeting this requirement at present are the carrier ampholytes used for isoelectric focusing (Svendsen and Rose, 1970; Routs, 1973; Acevedo, 1989).

However, carrier ampholytes have a number of deficiencies which affect their suitability as spacers, in particular, the variation of their ionic mobilities with pH. This means that the pH at which ITP is conducted cannot be changed without having to change the carrier ampholyte spacers as well in order to keep the ionic mobilities of the spacers constant. This makes ITP more time consuming, and less reproducible. Thus there was scope for me to do novel research in this field.

is -9.1×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$ - probably much higher than that of the LDH-5 isozymes[1]. In fact it is surprising, considering the relatively high net ionic mobility of the glycine terminating ions, that any of the other LDH isozymes stacked at all.

It is clear that the Tamura-Ui buffer system will only stack proteins of a relatively high ionic mobility. It seems that the rules given by Williams and Reisfeld are inadequate for the design of new discontinuous buffer systems - it is necessary to calculate the necessary ionic parameters from first principles, using the isotachophoretic steady state equations.

3.2.5 From discontinuous electrophoresis to isotachophoresis

The isotachophoretic steady state equations derived in Appendices B and C allow the design of new discontinuous electrophoretic buffer systems. A more alkaline buffer system that successfully stacked LDH-5 would have allowed me to continue my research into the metabolism of the fruit bat *Rousettus aegyptiacus*. However, the understanding that I had acquired of the principles involved in isotachophoretic processes had enkindled my

---

[1] The ionic mobilities of the following proteins at pH 10.45 were determined by Baumann and Chrambach (1976):

Ovalbumin (pI 4.7)	12.5×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$
Bovine serum albumin (pI 4.9)	12.3×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$
Equine ferritin (pI 4.5)	8.3×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$
Human haemoglobin (pI 7.4)	7.5×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$

Considering that the rat and fruit bat LDH-5 isozymes have pI values of at least 8.9, it is likely that their net ionic mobilities would be low due their low net negative charges at pH 9.3 (stacking pH).

Stacking

Although the original composition of the terminating electrolyte (electrode buffer) is 77 mM glycine - 13 mM AMPD, pH 8.8, once electrophoresis is commenced the concentrations of glycine and AMPD in the terminating zone are adjusted by the Kohlrausch regulating function to 45 mM glycine - 52 mM AMPD, pH 9.3. Hence sample proteins of a pI of 9.3 or greater will not be stacked. Nor will proteins possessing a net ionic mobility of less than -9.1×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$ - the net mobility of glycine at pH 9.3.

Separation

The separating gel buffer is composed of 60 mM HCl - 361 mM AMPD, pH 9.5. Once the chloride-glycine boundary reaches the separating gel, the composition of the leading electrolyte becomes that of the separating gel buffer. The composition of the terminating electrolyte hence becomes readjusted by the Kohlrausch regulating function to 45 mM glycine - 346 mM AMPD, pH 9.9. The net mobility of glycine at the readjusted pH of 9.5 is -21.6×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$. The chloride-glycine moving boundary moves on ahead, while the sample proteins are now left in a homogeneous 45 mM glycine - 346 mM AMPD buffer, pH 9.9.

Discussion of the system with respect to LDH-5

It is now apparent why the rat and fruit bat LDH-5 isozymes were not stacked by the Tamura-Ui discontinuous buffer system. At the regulated pH in the terminating zone (pH 9.3), the net ionic mobility of the glycine ions

Discussion of the system with respect to LDH-5

It is clear that the pH of the stacking buffer was deliberately set at pH 6.8 by Ornstein in order that the regulated pH in the terminating zone (pH 8.9) would be nearly one pH unit below the p*K* value of glycine (p*K* = 9.8), thus ensuring that the net ionic mobility of the glycine molecules would be lower than that of any of the sample proteins. Likewise, the pH value of the separating buffer was deliberately set at pH 8.8, in order that at the regulated pH in the terminating zone (p*H* 9.5), the net ionic mobility of the glycine molecules would be higher than that of any of the sample proteins.

It seems that the rat and fruit bat LDH-5 isozymes must have pI values of at least 8.9, in order not to have been stacked by this system.

Calculation of the ionic composition of the leading and terminating electrolytes in the Tamura-Ui system

Starting conditions

In the Tamura-Ui discontinuous buffer system, chloride is the leading ion, glycine is the terminating ion, and AMPD is the common buffering counterion. The leading electrolyte (stacking gel buffer) is composed of 60 mM HCl - 66 mM AMPD, pH 7.8. The terminating electrolyte (electrode buffer) is composed of 77 mM glycine - 13 mM AMPD, pH 8.8.

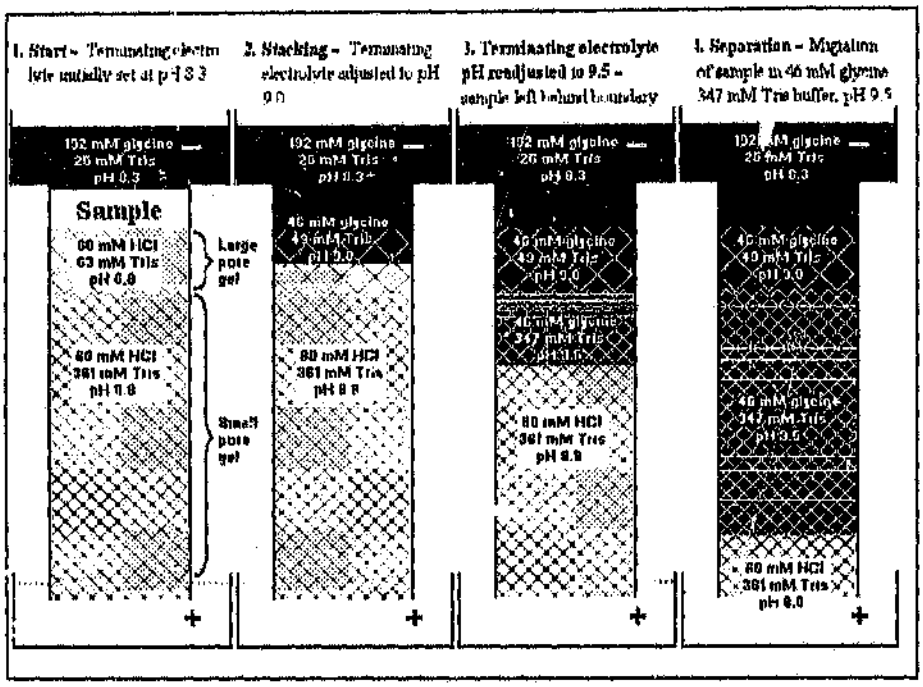


Figure 17 The mechanism of operation of the Ornstein-Davis discontinuous buffer system.

Starting conditions: The sample proteins are introduced between a leading electrolyte (stacking gel buffer) = 60 mM HCL - 63 mM Tris, pH 6.8, and a terminating electrolyte (electrode buffer), initially set at 192 mM glycine - 25 mM Tris, pH 8.3.

Stacking: The sample proteins are stacked between the leading electrolyte (stacking gel buffer) = 60 mM HCL - 63 mM Tris, pH 6.8, and the adjusted terminating electrolyte = 46 mM glycine - 49 mM Tris, pH 9.0.

Boundary passes sample proteins: When the chloride-glycinate boundary reaches the separating gel buffer, the composition of the leading electrolyte is changed to 60 mM HCl - 361 mM Tris, pH 8.8, and the terminating electrolyte is hence readjusted to 46 mM glycine - 347 mM Tris, pH 9.5. The sample proteins are left behind the chloride-glycinate boundary.

Separation of sample proteins: Normal zone electrophoresis of the sample proteins now occurs in 46 mM glycine - 347 mM Tris buffer, pH 9.5.

to 46 mM glycine - 49 mM Tris, pH 8.9 (Figure 17, step 2). Hence sample proteins of a pI of 8.9 or greater will not be stacked. Nor will proteins possessing a net ionic mobility[1] of less than -4.7×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$ - the net mobility of glycine at pH 8.9.

Separation

The separating gel buffer is composed of 60 mM HCl - 375 mM Tris, pH 8.8. Once the chloride-glycine boundary reaches the separating gel, the composition of the leading electrolyte becomes that of the separating gel buffer. The composition of the terminating electrolyte hence becomes readjusted by the Kohlrausch regulating function to 46 mM glycine - 361 mM Tris, pH 9.5 (Figure 17, step 3). The net mobility of glycine at the readjusted pH of 9.5 is -11.9×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$. Hence the glycine ions overtake the sample proteins, which are slowed down in the separating gel due to molecular sieving. The chloride-glycine moving boundary moves on ahead, while the sample proteins are now left in a homogeneous 46 mM glycine - 361 mM Tris buffer, pH 9.5 (Figure 17, step 4). Separation of the sample proteins now occurs on the basis of size and charge as in normal zone electrophoresis, except that the resolution is better because of the sample proteins have been concentrated into very narrow zones.

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[1] The ionic mobility of an ion is a signed quantity, given the same sign as the charge of the ion.

3.2.4 Use of the isotachophoretic steady state equations to calculate the change in ionic compositions during discontinuous electrophoresis

Calculation of the ionic composition of the leading and terminating electrolytes in the Ornstein-Davis system

Starting conditions

In the Ornstein-Davis discontinuous buffer system (Ornstein, 1964), chloride is the leading ion, glycine is the terminating ion, and Tris is the common buffering counterion. The leading electrolyte (stacking gel buffer) is composed of 60 mM HCl - 63 mM Tris, pH 6.8. The terminating electrolyte (electrode buffer) is composed of 192 mM glycine - 25 mM Tris, pH 8.3 (Figure 17, step 1).

Stacking

On starting of electrophoresis, the sample proteins become concentrated behind the chloride zone according to the Kohlrausch regulating function. By the time the chloride-sample proteins-glycine moving boundary reaches the separating gel, the sample proteins will have become concentrated into a very narrow zone.

Although the original composition of the terminating electrolyte (electrode buffer) is 192 mM glycine - 25 mM Tris, pH 8.3, once electrophoresis is commenced the concentrations of glycine and Tris in the terminating zone are adjusted by the Kohlrausch regulating function

Combined electroneutrality/equilibrium equations of glycine[1]

$$[gly]_{ionized} = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

where

$$a = (1 \quad 10^{(pK_{gly} - pK_{Tris})}) \tag{3-4}$$

$$b = [Tris^{Term}]_{total} + [gly]_{total}$$

$$c = [Tris^{Term}]_{total} \times [gly]_{total}$$

If one presets the concentration of chloride in, and the pH of, the stacking buffer (leading electrolyte), then the above equations can be used to calculate all the other ionic parameters of the leading and terminating electrolytes. The pH of the terminating electrolyte can be calculated by the Henderson-Hasselbalch equation:

$$pH^{Term} = pK_{gly} + \log_{10}\frac{[gly]_{ionized}}{[gly]_{total} - [gly]_{ionized}} \tag{3-5}$$

The net mobility of the glycine ions in the terminating electrolyte is found by:

$$\overline{m}_{gly} = \frac{[gly]_{ionized}}{[gly]_{total}} \times m_{gly} \tag{3-6}$$

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[1] Derived in Appendix C as equation (C-24).

ions is negligible. Hence, according to the electroneutrality principle:

$$[Cl]_{total} = [Tris^{Lead}]_{ionized}$$
$$[glv]_{ionized} = [Tris^{Term}]_{ionized} \qquad (3\text{-}1)$$

Chloride, being a strong acid, is fully ionized, so $[Cl]_{total} = [Cl]_{ionized}$. The fundamental steady state equations for the Ornstein-Davis discontinuous buffer system are:

Kohlrausch regulating function[1]

$$[glv]_{total} = [Cl]_{total} \cdot \frac{m_{glv}}{m_{Cl}} \cdot \frac{m_{Tris} - m_{Cl}}{m_{Tris} - m_{glv}} \qquad (3\text{-}2)$$

where m is the absolute ionic mobility of the ion.

Mass balance equation of buffering counterion[2]

$$[Tris^{Term}]_{total} = [Tris^{Lead}]_{total} + [glv]_{total} \cdot \frac{m_{Tris}}{m_{glv}} - [Cl]_{total} \cdot \frac{m_{Tris}}{m_{Cl}} \qquad (3\text{-}3)$$

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[1] Derived in Appendix C as equation (C-15).

[2] Derived in Appendix C as equation (C-16).



Figure 22 Zone electrophoresis of polyAMPS spacers in a 12%T, 2.5%C polyacrylamide gel, TBE buffer pH 8.7. The procedure of electrophoresis and staining of the polyAMPS polymers is as for Figure 18. Lane 1: polyAMPS-10, lane 2: polyAMPS-15, lane 3: polyAMPS-30, lane 4: polyAMPS-50, lane 5: polyAMPS-100, lane 6: polyAMPS-200, lane 7: polyAMPS-300, lane 8: polyAMPS-500.



Figure 21 Zone electrophoresis of polyAMPS spacers in a 8%T, 2.5%C polyacrylamide gel, TBE buffer pH 8.7. The procedure of electrophoresis and staining of the polyAMPS polymers is as for Figure 18. Lane 1: polyAMPS-7.5, lane 2: polyAMPS 10, lane 3: polyAMPS-15, lane 4: polyAMPS-30, lane 5: polyAMPS-50, lane 6: polyAMPS-100, lane 7: polyAMPS-200, lane 8: polyAMPS-300.

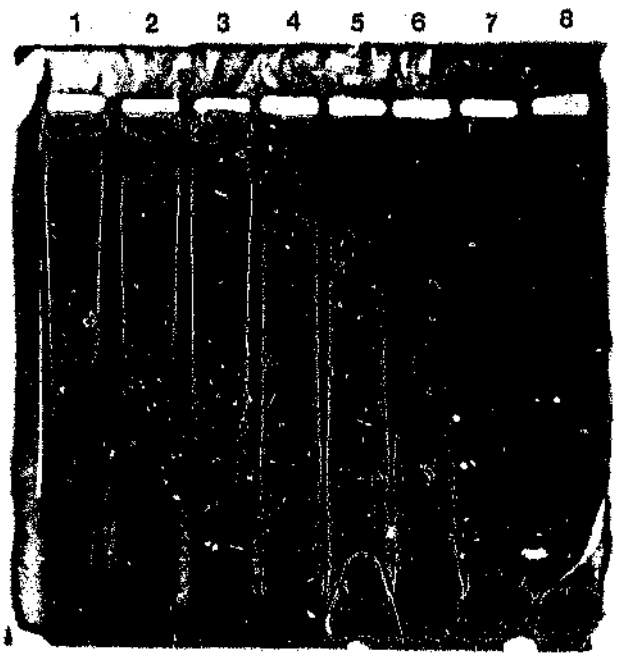


Figure 20 Zone electrophoresis of polyAMPS spacers in a 3.5%T, 4%C polyacrylamide gel, TBE buffer pH 8.7. The procedure of electrophoresis and staining of the polyAMPS polymers is as for Figure 18. Lane 1: polyAMPS-7.5, lane 2: polyAMPS-10, lane 3: polyAMPS-15, lane 4: polyAMPS-30, lane 5: polyAMPS-50, lane 6: polyAMPS-100, lane 7: polyAMPS-200, lane 8: polyAMPS-300.

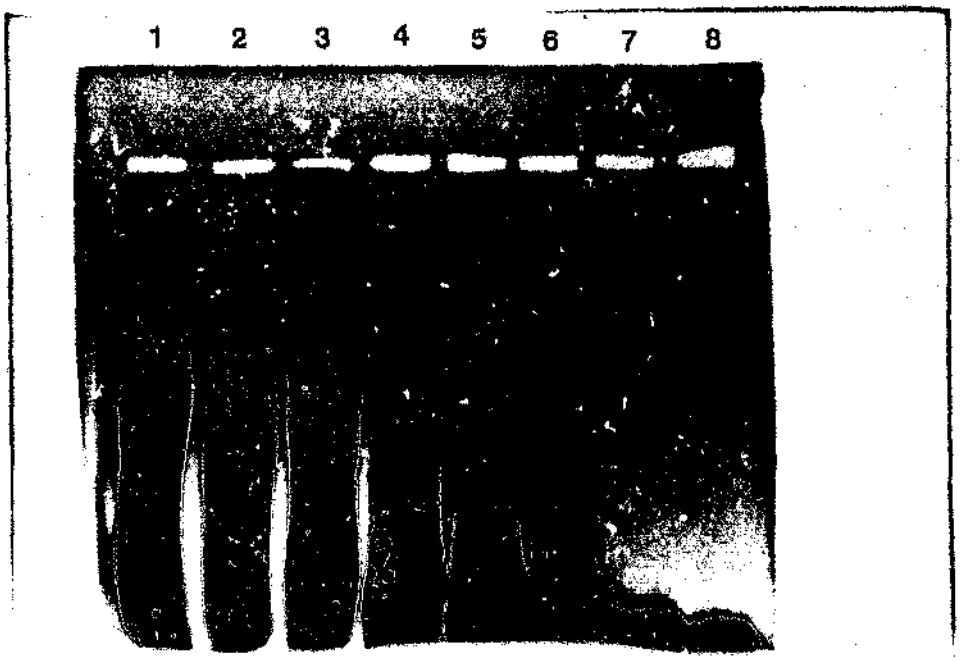


Figure 19 Zone electrophoresis of polyAMPS spacers in a 2% agarose gel, TBE buffer pH 8.7. The procedure of electrophoresis and staining of the polyAMPS polymers is as for Figure 18. Lane 1: polyAMPS-7.5, lane 2: polyAMPS-10, lane 3: polyAMPS-15, lane 4: polyAMPS-30, lane 5: polyAMPS-50, lane 6: polyAMPS-100, lane 7: polyAMPS-200, lane 8: polyAMPS-300.

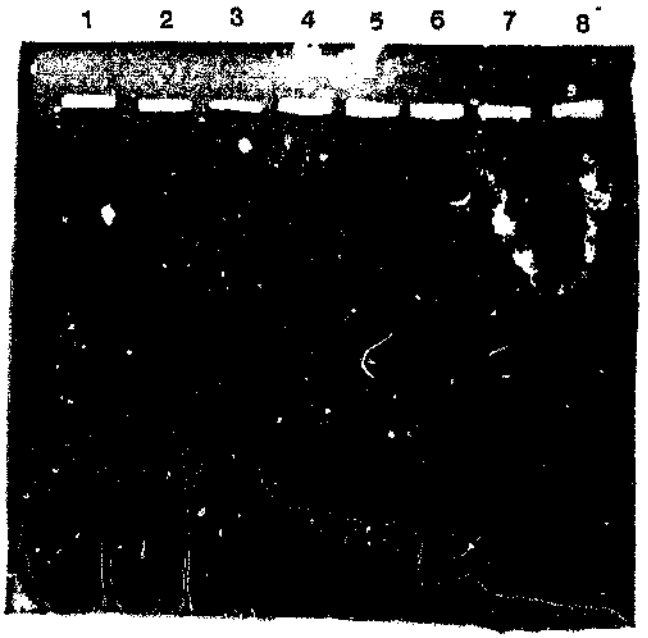


Figure 18 Zone electrophoresis of polyAMPS spacers in a 1% agarose gel, TBE buffer pH 8.7. The filter paper electrode strips were soaked in 10 ml of 16x TBE (62.5 mM Tris, 52 mM boric acid and 0.6 mM Na_2-EDTA) buffer, pH 8.7. Each well contained 20 µl of a 30 mM solution of polyAMPS spacers in TBE buffer containing bromophenol blue and xylene cyanol as markers. The samples were run at 300-400 V at room temperature until the bromophenol blue band was 1 cm from the anode strip. The polyAMPS spacers were stained in a 0.05% solution of methylene blue. The bromophenol blue and xylene cyanol bands diffused out of the gel during staining. Lane 1: polyAMPS-7.5, lane 2: polyAMPS-10, lane 3: polyAMPS-15, lane 4: polyAMPS-30, lane 5: polyAMPS-50, lane 6: polyAMPS-100, lane 7: polyAMPS-200, lane 8: polyAMPS-300.

Table 11: Efficient range of separation of linear DNA molecules by agarose and polyacrylamide gels in TBE buffer

Gel concentration	Efficient range of separation of linear DNA[a]	Dye comigration value[b]	
		Bromophenol blue	Xylene cyanol FF
0.75% agarose	1 000 - 15 000 bp	720 bp	9 200 bp
1% agarose	500 - 10 000 bp	400 bp	4 100 bp
1.25% agarose	400 - 5 000 bp	280 bp	2 500 bp
1.5% agarose	200 - 4 000 bp	200 bp	1 800 bp
2% agarose	100 - 2 500 bp	70 bp	850 bp
3.5% polyacrylamide	100 - 1 000 bp	100 bp	460 bp
5% polyacrylamide	80 - 500 bp	65 bp	260 bp
8% polyacrylamide	60 - 400 bp	45 bp	160 bp
12% polyacrylamide	40 - 200 bp	20 bp	70 bp
15% polyacrylamide	25 - 150 bp	15 bp	60 bp
20% polyacrylamide	10 - 100 bp	12 bp	45 bp

[a] Agarose gel data for Boehringer Mannheim Agarose MP. Polyacrylamide gel data from FMC Bioproducts Catalogue 1994.

[b] Agarose gel data from FMC Bioproducts Catalogue 1994. Data is that for SeaKem® LE and GTG® all purpose agaroses, which would be most similar in performance to Boehringer Mannheim Agarose MP. Polyacrylamide gel data from FMC Bioproducts Catalogue 1994.

Hence the bpe values of the polyAMPS spacers could be estimated by subjecting them to zone electrophoresis in agarose and polyacrylamide gels of various porosities (Figures 18 to 23), using bromophenol blue and xylene cyanol FF as migration markers. The polyAMPS fractions containing 7.5 mM thioglycolic acid (polyAMPS-7.5) to 500 mM thioglycolic acid (polyAMPS-500) were run since these fractions were fluid enough to pipette.

3.3.3 Evaluation of polyAMPS polymers as spacers for ITP

Characterization of the polyAMPS polymers by zone electrophoresis

In order to determine precisely the molecular mass ranges of each of the polyAMPS fractions, gel filtration upon Sephadex G-15 or G-25, calibrated with anionic polymers such as polyacrylic acid of known molecular masses, would be necessary. However, in the case of spacers for isotachophoretic separations, it is not the molecular masses of the spacers, but their apparent ionic mobilities[1], that are important. As the ionic mobilities of the polyAMPS spacers are dependent upon the degree of gel sieving, zone electrophoresis in agarose and polyacrylamide gels of various porosities was used as a facile method of characterizing the various polyAMPS fractions.

Since DNA is also a polyanionic polymer whose ionic mobility is dependent upon the degree of molecular sieving, it was decided that a convenient method of categorizing the polyAMPS spacers was by expressing their apparent ionic mobilities in terms of "DNA base pair equivalents" (bpe values). The molecular mass ranges of linear DNA separated by gels of varying porosity are listed in Table 11.

---

[1] The absolute ionic mobilities of molecules are the ionic mobilities of the molecules in free solution. However, since the charge/mass ratios of the polyAMPS molecules (like DNA and RNA) are identical no matter their individual lengths, the individual polyAMPS species can only be discriminated in gel media exerting a sieving effect. Hence it is more useful to measure their "apparent ionic mobilities" in gel media of varying porosity.

Table 10: Effect of thioglycolic acid concentration on viscosity of polyAMPS solution

Concentration of thioglycolic acid (mM)	Viscosity, and ease of subsequent dissolution in water, of resulting polyAMPS polymers
0 (control)	gel, impossible to dissolve
0.1	gel, impossible to dissolve
0.15	gel, impossible to dissolve
0.3	gel, impossible to dissolve
0.4	gel, impossible to dissolve
0.5	gel, impossible to dissolve
0.6	gel, impossible to dissolve
0.75	gel, impossible to dissolve
1.0	gel, impossible to dissolve
1.2	gel, impossible to dissolve
1.5	gel, impossible to dissolve
2	gel, impossible to dissolve
3	extremely viscous fluid, impossible to dissolve
4	extremely viscous fluid, extremely difficult to dissolve
5	very viscous fluid, very difficult to dissolve
6	very viscous fluid, very difficult to dissolve
7.5	viscous fluid, dissolves slowly on vigorous agitation of solution
10	viscous fluid, dissolves slowly on vigorous agitation of solution
15	viscous fluid, dissolves on vigorous agitation of solution
30	moderately viscous fluid, dissolves readily on vigorous agitation of solution
60	moderately viscous fluid, dissolves easily on vigorous agitation of solution
100	slightly viscous fluid, dissolves easily on gentle agitation of solution
200	very slightly viscous fluid, dissolves easily
300	fluid
500	fluid

As can be seen, thiol compounds are incredibly active chain transfer agents, and the addition of very small amounts to a polymerization reaction can drastically reduce the average chain length. Hence I decided to synthesize polyAMPS polymers of a uniform distribution of molecular mass by the addition of various amounts of thiol chain transfer agents such as thioglycolic acid (mercaptoacetic acid) to the polymerization reaction mixture. It was noted that the free radical-induced polymerization of a 60% (3 M) solution of AMPS[1] resulted in the formation of a firm gel. Hence the decrease in average chain length of polyAMPS caused by the addition of increasing concentrations of thioglycolic acid to a 3 M solution of AMPS could be qualitatively measured by the decrease in viscosity of the polyAMPS solution, as detailed in Table 10.

The polyAMPS solutions resulting from the 7.5 mM to 500 mM thioglycolic acid reactions were fluid enough to permit pipetting, and so these fractions were subjected to further testing. The effect of thioglycolic acid concentration on the resulting average chain lengths was gauged by subjecting the polyAMPS fractions to zone electrophoresis on agarose and polyacrylamide gels.

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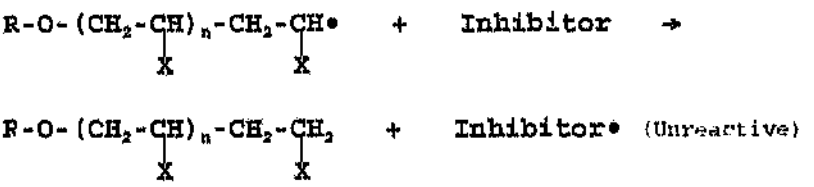
[1] The hydrogen peroxide-$FeSO_4$-ascorbic acid system (Uri, 1950; Blackshear, 1984) commonly known as Fenton's reagent was used since the normal persulfate-TEMED or riboflavin free radical initiator systems do not work at low pH.

The solvent molecules in which the polymerization reaction is conducted may act as chain transfer agents, leading to lower average chain length than would be expected from the initiator to monomer ratio. (Water, however, is unreactive.) The chain transfer constants for poly(methyl methacrylate) radicals with various solvents is given below in Table 9.

Table 9: **Chain transfer constants for poly(methyl methacrylate) radicals with various solvents (from Kine and Novak, 1981)**

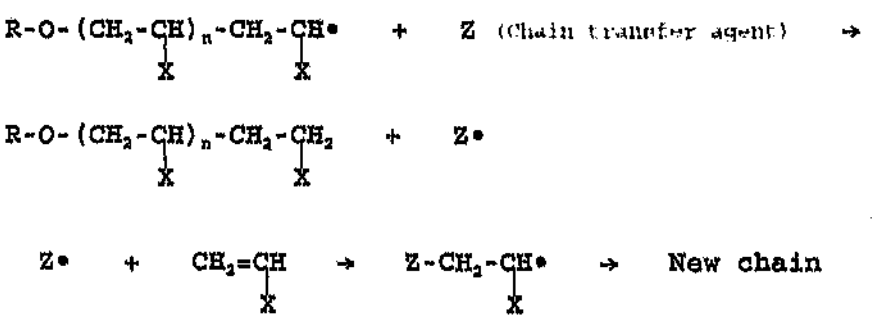
Solvent	Chain transfer constant x 10^5	
	at 60°C	at 80°C
benzene	0.4	0.8
chlorobenzene	0.74	2.1
isobutanol	1.0	2.3
toluene	2.6	5.3
chloroform	4.5	11.3
isopropanol	5.8	19.1
3-pentanone	8.3	17.3
carbon tetrachloride	9.3	24.2
2-mercaptoethanol	66 000	
thiophenol	270 000	

Inhibition



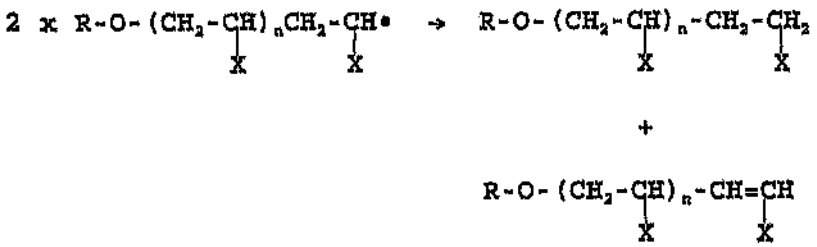
Chain transfer agents also react with the terminal free radicals of growing polymer chains to generate a new free radical that can initiate a new polymerization chain (Barson, 1989). Since one reaction chain is replaced by another, the rate of polymerization is unaffected, and complete consumption of the monomers occurs.

Chain transfer



Chain transfer agents thus compete with the monomer for the growing radicals. The ratio of rate constants for chain transfer and for normal chain propagation, $k_{transfer}/k_{polymerization}$, is called the *transfer constant*; it is a measure of how effective the chain transfer agent is at lowering the molecular mass of the polymer.

B. Disproportionation



The chain propagation step is generally much faster than the chain initiation step, since no time is required for decomposition of the initiator to form peroxy radicals to occur. Thus the formation of new polymer chains occurs continuously during the polymerization, resulting in a Gaussian distribution of chain lengths, with the average chain length being proportional to the ratio of monomer to initiator. The average polymer chain length may be decreased by the addition of *inhibitors* or *chain transfer agents*.

Inhibitors are compounds that react with the growing polymer free radical to generate a new free radical that is not reactive enough to add to monomer. Thus not only is the average chain length decreased, but an incomplete consumption of monomer also results, as all the free radicals are rapidly mopped up. Examples of inhibitors are phenols, quinones and aromatic amines.

Four isomers are expected from the reaction of Remazol Red RB with BAPEDA. Since the two tetramines are similar in size, differences in ionic mobility of the ampholytic dye isomers was expected to occur solely on the basis of net charge.

The ampholytic dye products were synthesized by reacting the various ratios of tetramines with Remazol Red RB in aqueous solution at room temperature for 24 hr. The ampholytic dye fractions were purified as described in Section 2.2.3 of the Materials and Methods, and stored as aqueous solutions at 4°C.

Estimation of the pI ranges of the ampholytic dyes by PAGE

A rough idea of the pI ranges of the individual ampholytic dye fractions was obtained by PAGE in a 4.5% polyacrylamide gel, with 15 mM HCl-ethanolamine, pH 9.5, as the gel buffer (Figure 27). The ampholytic dyes were fixed by placing the gel in a saturated solution of picric acid (Knisley and Rodkey, 1992) containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol. The function of the acetic acid in the first fixing step was to acidify the gel so that all the ampholytic dye molecules possessed a net positive charge, and so would form an insoluble complex with the picric acid. As expected from the p*K* values of the tetramines, the ampholytic dye fractions containing BAPEDA are the most basic.

(MCT) group and one vinyl sulfonate (VS) group. Moreover Remazol Red RB contains three sulfonic acid groups[1]. Therefore a 25x molar excess of polyamine was used in order to ensure that the two reactive groups did not both react with one polyamine molecule.

Two tetramines were used:

***N,N'*-bis(3-aminopropyl)ethylenediamine (BAPEDA)**
This is available as an isomerically pure substance.
Structure: H_2N-$[CH_2]_3$-NH-$[CH_2]_2$-NH-$[CH_2]_3$-NH_2
p*K* values[2] at 25°C: 5.6, 8.3, 9.8, 10.5

Triethylenetetramine (TETA)
This is available as a mixture of structural isomers.
Structure 1: H_2N-$[CH_2]_2$-NH-$[CH_2]_2$-NH-$[CH_2]_2$-NH_2
p*K* values at 25°C: 3.3, 6.6, 9.1, 9.7

Structure 2 (Tris(2-aminoethyl)amine): N-($[CH_2]_2$-NH_2)$_3$
p*K* values at 25°C: <2, 7.9, 9.1, 10.0

Bergstedt and Widmark (1970) have found commercially available TETA to contain the above two isomers, as well as another five isomers containing piperazine rings. Hence the heterogeneity of the product could be varied by altering the ratios of BAPEDA and TETA[3], with the 100% BAPEDA reaction product being the least heterogeneous.

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[1] Personal communication from Dr. U. Reiber of DyStar, the dyestuff subsidiary company of Bayer and Hoechst.

[2] The p*K* values of BAPEDA, and the isomers of TETA, were obtained from Smith and Martell (1975).

[3] The various tetramine ratios were designated in the following manner: 100% BAPEDA = 10B fraction, 90% BAPEDA/10% TETA = 9B1T fraction...etc....to 100% TETA = 10T fraction.

3.3.4 TEP of ampholytic dyes using polyAMPS spacers

It was suspected that ionic interactions would occur between the polyanionic polyAMPS spacers and ampholytic biomolecules such as proteins. Proteins may possess positively-charged areas on their surface, even if their net charge is negative. These anomalous interactions can be suppressed in ion-exchange chromatography by the use of buffers of moderate ionic strength, but this solution would not work in ITP since the introduction of extraneous ions must absolutely be avoided. Hence I decided to synthesize heterogeneous ampholytic dyes (as synthetic substitutes for natural ampholytic biomolecules) and subject them to TEP with polyAMPS spacers. Since the ampholytic dye species were coloured, they provided a simple method for monitoring the degree of ionic interaction.

Synthesis of ampholytic dyes

Basic ampholytic dyes were synthesized by reacting the reactive dye[1] Remazol Red RB (Hoechst) with an excess of polyamines. Since the exact structure of Remazol Red RB is a trade secret, the molecular mass was arbitrarily taken to be 1000 daltons, assuming the dye to be of a related structure to other commercially available reactive dyes of known structure, such as Cibacron Brilliant Red 3B-A (995 Da). It is, however, known from the literature (von der Eltz, 1982) that Remazol Red RB contains two reactive groups - one monochlorotriazine

[1] The chemistry of the Remazol family of reactive dyes is given in Heyna (1962,1963) and von der Eltz (1982). Reviews of reactive dye chemistry are given by Elliott and Yeung (1979), Stead (1987) and Smith (1993).

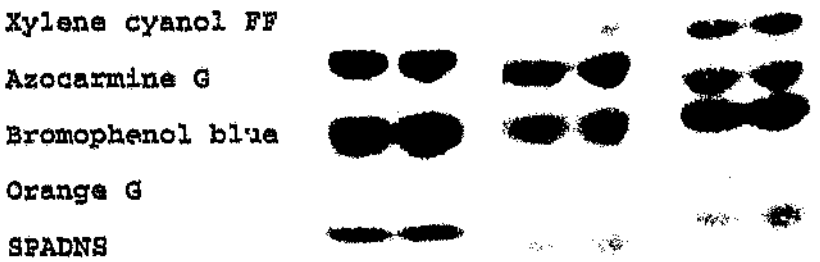


Figure 26 Comparison of the resolution obtained by TEP of a mixture of dyes in 3.5%, 5% and 7.5% polyacrylamide gels (left-hand, centre and right-hand lanes, respectively). The sample dyes, and the conditions of electrophoresis are the same as for Figure 25. The 3.5% and 5% polyacrylamide gels contained 4% crosslinker, and the 7.5% polyacrylamide gel contained 2.5% crosslinker. The spacing between the dye bands is decreased as the density of the polyacrylamide gel is increased.

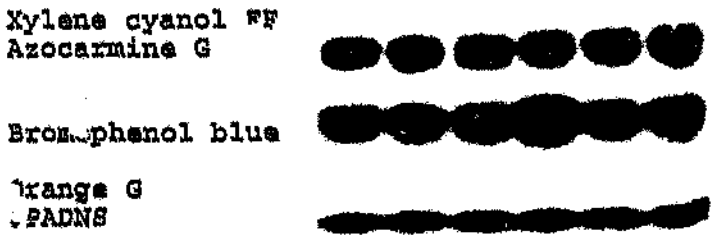


Figure 25 TEP of a mixture of dyes (in order of decreasing ionic mobility: SPADNS, orange G, bromophenol blue, azocarmine G and xylene cyanol FF) in a 3.5%T, 4%C polyacrylamide gel. The gel buffer was composed of a 10 mM solution of polyAMPS-50 spacer polymers adjusted to pH 9.5 with ethanolamine (p*K*=9.5). The anolyte was composed of 3 M ethanolamine titrated to pH 9.5 with boric acid. The catholyte (terminating electrolyte) was composed of 3 M EACA (p*K* 10.80) titrated to pH 9.5 with ethanolamine. Thin filter paper strips soaked in the anolyte and catholyte solutions respectively were applied to the extremities of the TEP gel, upon which the electrodes were laid. Solutions (15 µl) of the sample dyes (dissolved in 15 mM of terminating electrolyte) were applied to shallow wells on the gel surface. The gel was run at 200 V at room temperature.

On the contrary, an excellent resolution of the sample dyes has been obtained by TEP (see Figure 25). All the zones are sharply focused; moreover close observation of the gel shows the presence of an orange minor band between the orange G and bromophenol blue zones, and a pink minor band between the bromophenol blue and azocarmine G zones, caused by the presence of impurities in the orange G and azocarmine G products. The sharpness of the dye zones obtained in the TEP experiment are due to the counteraction of diffusion by the electric field strength gradient.

TEP was repeated in 5% and 7.5% polyacrylamide gels. A comparison of the dye patterns obtained by TEP in the 3.5%, 5% and 7.5% polyacrylamide gels (left-hand, centre and right-hand lanes, respectively, of Figure 26) shows a progressive compression of the orange G, bromophenol blue and azocarmine G bands on increasing the density of the polyacrylamide gel. The decreased spacing in the 5% and 7.5% polyacrylamide gels indicates that a depletion in polyAMPS spacers of that range of apparent ionic mobilities has occurred. This is because the apparent ionic mobilities of the polyAMPS spacers has decreased due to the increased molecular sieving effect.

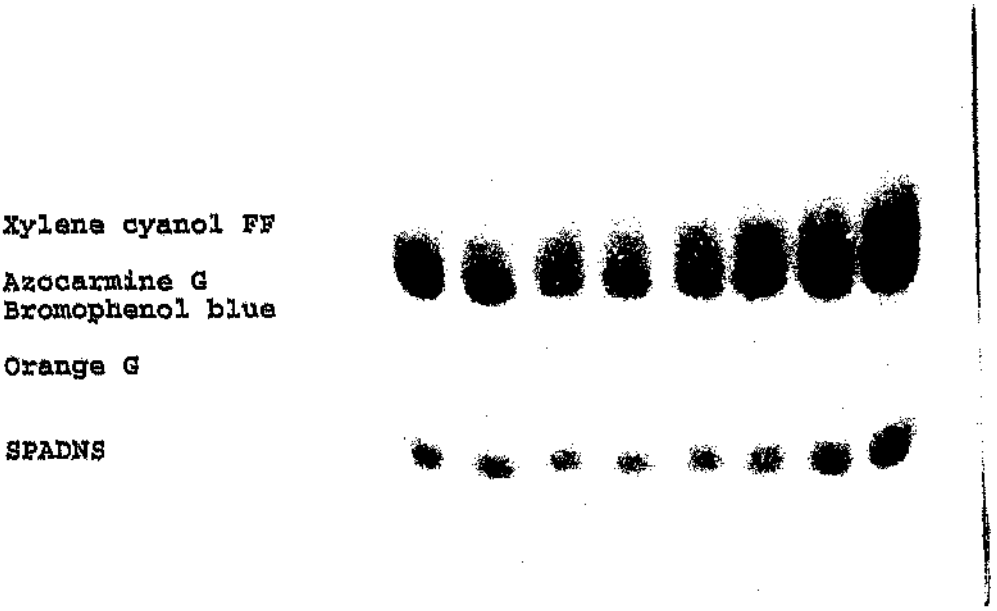


Figure 24 Zone electrophoresis of a mixture of dyes (in order of decreasing ionic mobility: SPADNS, orange G, bromophenol blue, azocarmine G and xylene cyanol FF) in a 3.5%T,4%C polyacrylamide gel. The gel buffer was composed of 15 mM HCl titrated to pH 9.5 with ethanolamine. The electrode strips were soaked in a 1.5 M solution of the same buffer. Solutions (15 µl) of the sample dye mixture (dissolved in gel buffer) were applied to shallow wells on the gel surface. The gel was run at 200 V at 10°C.

TEP gel spacers. The TEP and zone electrophoresis gels were both run at 200 V in 3.5% polyacrylamide gels, and identically sized sample wells (0.8 mm x 0.4 mm) were used so that the degree of resolution obtained was solely dependent upon the electrophoretic process.

The gel buffer for zone electrophoresis was composed of 15 mM HCl titrated to pH 9.5 with ethanolamine. The electrode buffers were composed of a 1.5 M solution of the same buffer. The TEP gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers[1] adjusted to pH 9.5 with ethanolamine (p*K*=9.5). The anolyte was composed of 3 M ethanolamine titrated to pH 9.5 with boric acid. The catholyte (terminating electrolyte) was composed of 3 M EACA (p*K*=10.80) titrated to pH 9.5 with ethanolamine.

The resolution obtained by zone electrophoresis is shown in Figure 24. The SPADNS and orange G zones are clearly resolved, but the bromophenol blue, azocarmine G and xylene cyanol FF zones have not been resolved. Zone spreading, particularly of the xylene cyanol FF zone, has occurred due to diffusion.

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[1] It was found that using a higher concentration of polyAMPS resulted in deformation of the gel. This was possibly due to the mechanical stress caused by molecular sieving, or else due to entanglement of longer polyAMPS species with the gel fibres, leading to immobile negative charges on the gel matrix, with consequent electroosmosis.

The intermediate molecular mass polyAMPS-30 and polyAMPS-50 fractions are suitable spacers for performing ITP in low-percentage acrylamide gels; the lower molecular mass polyAMPS-100 spacers being optimal for separations in high-percentage polyacrylamide gels. (None of the other polyAMPS fractions provided an adequate range of apparent ionic mobilities.) Thus the various porosities of polyacrylamide gels each have a corresponding optimal size range of polyAMPS spacers.

Testing the suitability of polyAMPS as spacers for ITP - separation of dyes

The suitability of the polyAMPS fractions for use as spacers for isotachophoretic separations was then evaluated by:

(i) Seeing if an electric field strength gradient was generated in a telescope electrophoresis (TEP) system[1], ie. there was a focusing effect,

(ii) Comparing the resolution obtained with that of ordinary zone electrophoresis.

Dyes were chosen as markers of resolving power since the separation process and the final resolution obtained could be directly visualised. The following sample dyes were used in order of decreasing ionic mobility: SPADNS, orange G, bromophenol blue, azocarmine G and xylene cyanol FF. The polyAMPS-50 fraction was chosen for the

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[1] These and all subsequent ITP experiments were performed using the TEP format. The isotachophoretic steady state is immediately attained in TEP. (Section 1.2.3 of the Introduction.) The TEP gels are also more convenient to prepare since there are no two separate leading electrolyte and spacer zones, as in conventional ITP.

The polyAMPS fractions were graded for suitability as spacers for ITP according to the range of ionic mobilities covered (Table 13):

Table 13: Apparent bpe ranges of the various polyAMPS fractions

PolyAMPS fraction	Estimated bpe range[a]	Porosity of gel for which the polyAMPS fraction would be suitable as ITP spacers
PolyAMPS-7.5	100 – 2000	4.5% – 8% polyacrylamide
PolyAMPS-10	100 – 1500	4.5% – 8% polyacrylamide
PolyAMPS-15	100 – 1500	4.5% – 8% polyacrylamide
PolyAMPS-30	100 – 1200	4.5% – 8% polyacrylamide
PolyAMPS-50	50 – 1000	3.5% – 12% polyacrylamide
PolyAMPS-100	20 – 200	12% – 15% polyacrylamide
PolyAMPS-200	<85	None
PolyAMPS-400	<70	None
PolyAMPS-800	<40	None

[a] Obtained from Table 12.

Lanes 1 – 3 of Figures 20 and 21 show the polyAMPS 7.5, 10 and 15 fractions to be only suitable for use as ITP spacers for sample ions of medium to low ionic mobility.

Lanes 4 and 5 of Figures 20 and 21 show the polyAMPS 40 and 50 fractions to be suitable for use as ITP spacers for sample ions of a wide range of ionic mobilities.

Lane 6 of Figures 22 and 24 shows the polyAMPS 100 fraction to be suitable for use as ITP spacers for sample ions of a wide range of ionic mobilities.

The estimated DNA base pair equivalent (bpe) ranges of the polyAMPS fractions, determined by zone electrophoresis (Figures 18-23), are summarised in Table 12:

Table 12: Estimated bpe ranges of polyAMPS fractions

PolyAMPS fraction[a]	Agarose (%)		Polyacrylamide (%)			
	1	2	3.5	8	12	15
7.5	<3000	<2000	>100	>100		
10	<2500	<1500	>100	>80	>100	>60
15	<2500	<1500	>100	>70	>70	>60
30	<2000	<1200	>100	>50	>40	>30
50	<2000	<1000	<1000	>50	>40	>20
100	<700	<700	<700	<400	<200	>20
200	<500	<300	<200	<200	<120	<85
400	<500	<200	<100	<100	<100	<70
500					<40	<30

[a] Value refers to the concentration of thioglycolic acid (mM) in the polymerization reaction mixture.

It was noted that in all the gels the polyAMPS fractions exhibited streaking patterns, moreover precipitation of polyAMPS polymers occurred in the polyacrylamide gels near the sample wells. This was taken to be caused by entanglement of the longer polyAMPS polymers with the gel fibres.



Figure 23 Zone electrophoresis of polyAMPS sp··ers in a 15%T, 2.5%C polyacrylamide gel, TBE buffer pH 8.7. The procedure of electrophoresis and staining of the polyAMPS polymers is as for Figure 18. Lane 1: polyAMPS-10, lane 2: polyAMPS-15, lane 3: polyAMPS-30, lane 4: polyAMPS-50, lane 5: polyAMPS-100, lane 6: polyAMPS-?00, lane 7: polyAMPS-300, lane 8: polyAMPS-500.

TEP of DNA molecular mass markers using polyAMPS spacers

TEP was conducted in 5% polyacrylamide gels. The gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers adjusted to pH 9.5 with ethanolamine. Both the anolyte and the catholyte (terminating electrolyte) were composed of 400 ml each of 100 mM EACA titrated to pH 9.5 with ethanolamine. The extremities of the gel were connected to the electrode buffer by filter paper strips soaked in electrode buffer. The same DNA molecular mass markers were used as for PAGE.

The polyAMPS-50 and -30 fractions were used at first as spacers for TEP (left-hand and centre sections, respectively, of Figure 34). Nine DNA bands were resolved by the polyAMPS-50 spacers (as opposed to 12 bands with PAGE as shown in Figure 32). The larger DNA molecules (1033, 1230, 1766 and 2176 bp molecules) were not resolved, and appeared together as a single band. These results are corroborated by the apparent bpe range of the polyAMPS-50 fraction: 100 - 1000 (Table 13), so that DNA molecules greater than 1000 bp in size would not be spaced.

The resolution of the larger DNA molecules was slightly better with the polyAMPS-30 spacers - the 1033 bp DNA molecule was resolved this time, with the 1230, 1766 and 2176 bp DNA molecules migrating together as a single band. Once again, these results are corroborated by the apparent bpe range of the polyAMPS-30 fraction: 100 - 1200 (Table 13), so that DNA molecules greater than 1200 bp in size would not be spaced.

The results of the TEP experiments using the polyAMPS-50 and -30 fractions as spacers seemed to

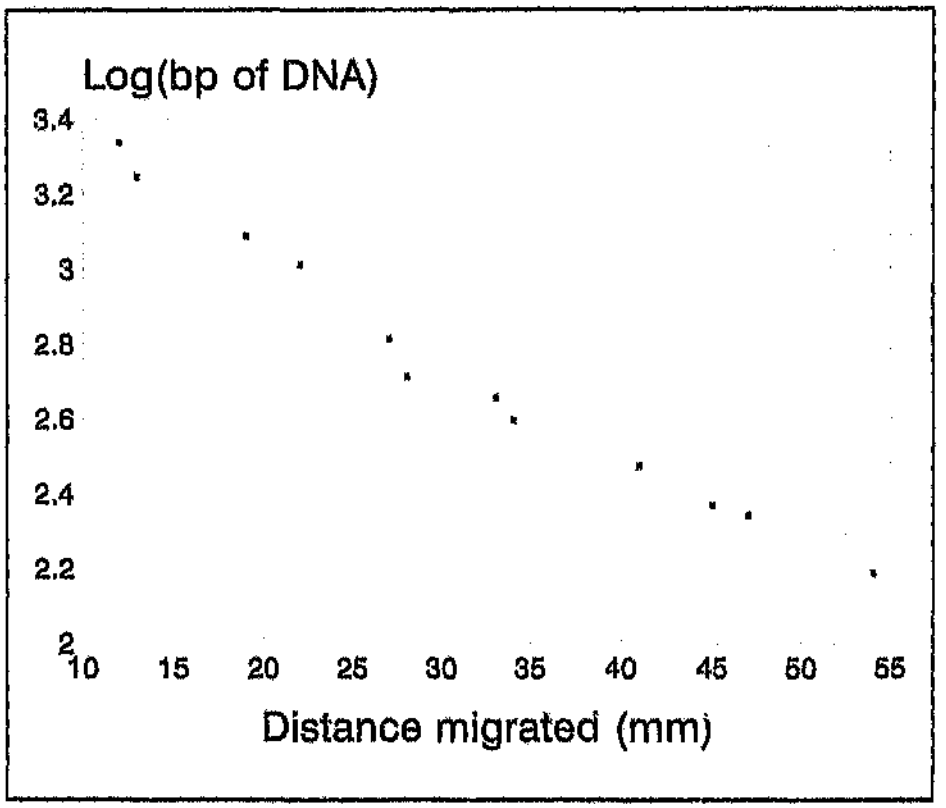


Figure 33 Plot of the logarithm of the size (in bp) of each DNA molecule versus its migration distance, for the PAGE experiment of Figure 33.

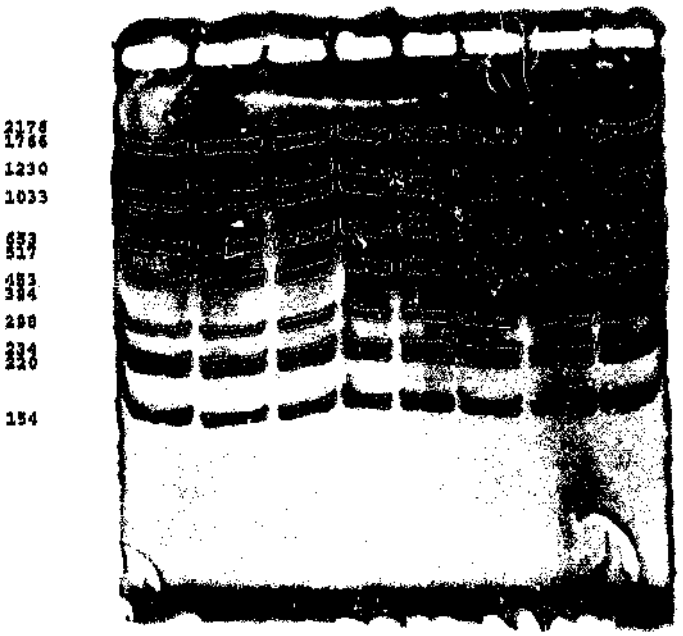


Figure 32 Zone electrophoresis of DNA on a horizontal 5%T, 4%C polyacrylamide gel in TBE buffer (62.5 mM Tris, 52 mM boric acid and 0.6 mM Na$_2$-EDTA, pH 8.7). The filter paper electrode strips were soaked in a 16X solution of the same buffer. Boehringer Mannheim molecular weight marker VI was used as sample DNA. The mixture contains 15 fragments with the following number of base pairs (1 base pair ≈ 660 daltons): 2176, 1766, 1230, 1033, 653, 517, 453, 394, (2x 298), (2x 234), 220, (2x 154), but only 12 bands are resolved. Samples (5 µl) of a 40 µg/ml solution of DNA (≈ 200 ng DNA per well) in 15 mM of EACA-ethanolamine buffer, pH 9.5 were loaded in shallow wells on the polyacrylamide gel surface. The sample buffer contained bromophenol blue as a migration marker. The gels were run at 200 V at room temperature until the bromophenol blue was 1 cm away from the filter paper electrode strip. The DNA bands were detected with the silver staining method of Sanguinetti *et al.* (1994).

3.3.5 TEP of DNA using polyAMPS spacers

TEP of DNA was attempted since DNA, like polyAMPS, is a polyanion, and should not undergo any ionic interactions with polyAMPS polymers. In fact polyAMPS polymers, being similar in structure to DNA, should be ideal as spacers for the isotachophoretic separation of DNA molecules, since they should exhibit similar electrophoretic behaviour. As far as I know, ITP has never been used before for the separation of polynucleotides.

PAGE of DNA molecular mass markers

Separation of standard DNA molecular mass markers by PAGE was first performed in order to compare the resolution of PAGE and TEP. Boehringer Mannheim molecular mass marker VI was used as sample DNA. On standard PAGE in a 5% polyacrylamide gel in TBE buffer 12 bands were resolved (Figure 32). The DNA bands were detected with the silver staining method of Sanguinetti *et al.* (1994). A plot of the logarithm of the size (in bp) of each DNA molecule *versus* its migration distance is portrayed in Figure 33.

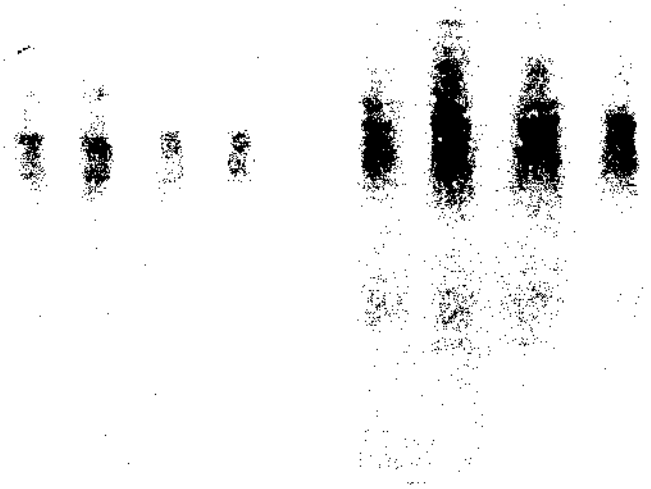


Figure 31 TEP, in the presence of urea, of the 3B7T ampholytic dye fraction in a 5%T, 4%C polyacrylamide gel, using polyAMPS-15 (left) and polyAMPS-10 (right) spacers. The gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers containing 9.5 M urea, adjusted to pH 9.5 with ethanolamine (p*K*=9.5). The anolyte and catholyte (terminating electrolyte) were both composed of 400 ml of 100 mM EACA titrated to pH 9.5 with ethanolamine. Solutions (20 μl) of the 3B7T fraction were applied to shallow wells on the gel surface. The gels were run at 200 V at room temperature. The ampholytic dyes were fixed by placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol.

The polyAMPS-15 and -10 fractions were then tried in an attempt to get better spacing between the individual ampholytic dye species. The faster ampholytic dye species were well resolved, but distortion of the bands of the slower species was obtained (results not shown). This problem was not alleviated by increasing the concentration of the urea to 9.5 M (Figure 31), indicating that the problem was not due to interactions between the ampholytic dye molecules and the polyAMPS spacers, but due to distortions in the electric field strength gradient. It is most probable that such a distortion would be caused by entanglement of the longer polyAMPS polymers in the polyacrylamide matrix, as noticed during zone electrophoresis of the various polyAMPS fractions (Figures 18 to 23).

Nevertheless the faster ampholytic dye isomers have been well resolved. The introduction of polymeric spacers possessing a lower ionic charge would lower their ionic mobility, and hence allow the use of polymeric spacers of shorter length. This should overcome the entanglement problem, and allow the resolution of slower-moving sample ions. Most probably monoanionic polymeric spacers would be the best solution. Moreover, the use of monoanionic polymeric spacers would solve the problem of ionic interactions with ampholytic sample ions, such as polypeptides.

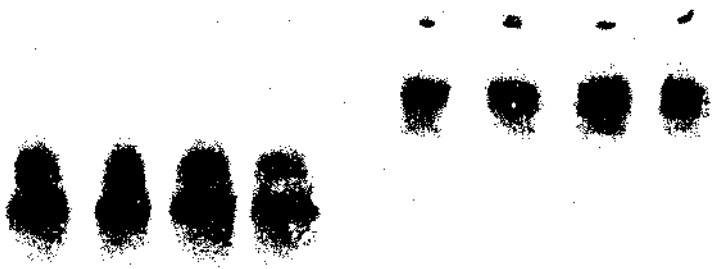


Figure 30 TEP, in the presence of urea, of the 3B7T ampholytic dye fraction in a 5%T, 4%C polyacrylamide gel, using polyAMPS-50 (left) or polyAMPS-30 (right) spacers. The gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers containing 8 M urea, adjusted to pH 9.5 with ethanol-amine (p*K*=9.5). The anolyte and catholyte (terminating electrolyte) were both composed of 400 ml of 100 mM EACA titrated to pH 9.5 with ethanol-amine. Solutions (20 µl) of the 3B7T fraction were applied to shallow wells on the gel surface. The gels were run at 200 V at room temperature. The ampholytic dyes were fixed by placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol.

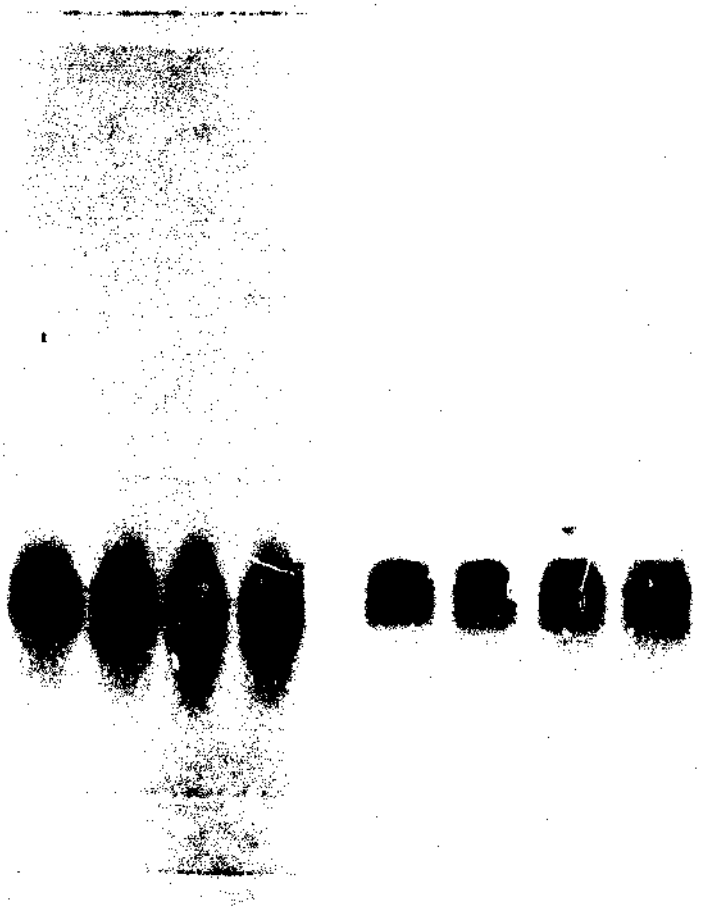


Figure 29 TEP of the 3B7T ampholytic dye fraction in a 5%T, 4%C polyacrylamide gel, using polyAMPS-50 (left) or polyAMPS-30 (right) spacers. The gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers adjusted to pH 9.5 with ethanolamine (p*K*=9.5). The anolyte and catholyte (terminating electrolyte) were both composed of 400 ml of 100 mM EACA titrated to pH 9.5 with ethanolamine. Solutions (20 µl) of the 3B7T fraction were applied to shallow wells on the gel surface. The gels were run at 200 V at room temperature. The ampholytic dyes were fixed by placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol.

TEP of the ampholytic dyes using polyAMPS spacers

Although the 5B5T to 2B8T ampholytic dye fractions were judged to have an equally good spread of individual ampholytic dye species (Figure 28), the 3B7T ampholytic dye fraction was chosen for the TEP experiments because of its greater concentration - allowing high band intensity without the necessity of loading large volumes. TEP was conducted in 5% polyacrylamide gels. The gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers adjusted to pH 9.5 with ethanolamine. Both the anolyte and the catholyte (terminating electrolyte) were composed of 400 ml each of 100 mM EACA titrated to pH 9.5 with ethanolamine.

The polyAMPS-50 and -30 fractions were tried at first (Figure 29). The resolution is improved with the polyAMPS-30 spacers, although both gels show slight smearing of the ampholytic dye bands due to ionic interactions with the polyAMPS spacers. These interactions could be inhibited (Righetti et al., 1977) by the inclusion of 8 M urea into the gel mixture (Figure 30), with an increase in resolution. TEP with the polyAMPS-30 spacers shows nearly as many bands as the pH 8-10.5 IEF gel (Figure 28), but these are not as easily distinguished as the bands are very close together. (The photographs in Figure 30 do not show the bands with as much clarity as the original gels.)

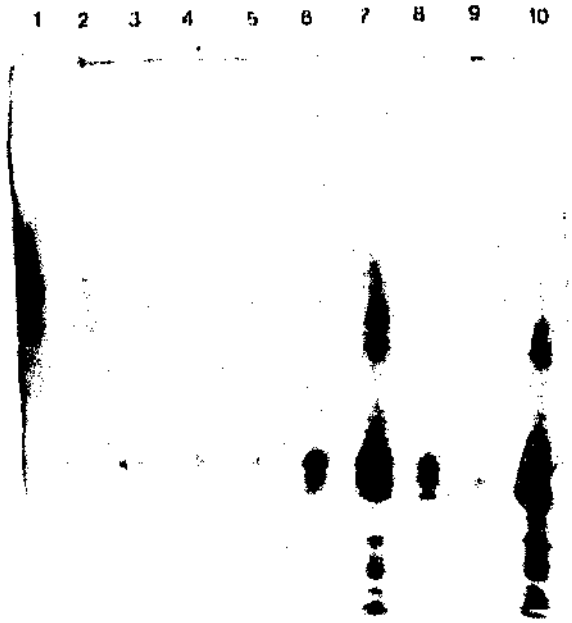


Figure 28 IEF of ampholytic dyes in a pH 6-10,5 gradient. The gel was composed of 10%T, 4% polyacrylamide, and contained 2% Pharmalyte pH 6-10,5, 10% sorbitol, 8 M urea and 30% DMF. The ampholytic dye solutions were loaded directly on the gel surface. Since the various ampholytic dye fractions varied in concentration, the volumes loaded (20-40 µl) were accordingly adjusted in an effort to obtain an equal band intensity of the various fractions. However, the 8%T and 10% fractions are slightly overloaded. The anode and cathode consisted of thin filter paper strips soaked in 1 M H_3PO_4 or NaOH, respectively, upon which were laid the platinum electrodes. Focusing was performed at 10°C at an initial voltage of 200 V, with the voltage gradually increased to a final value of 1,200 V. Focusing was continued at 1,200 V for a further 4 hr. The focused ampholytic dyes were fixed by placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol. The Pharmalytes were also fixed by the picric acid, and appear as opaque bands. Lane 1: 10%, Lane 2: 20%T, Lane 3: 8%T, Lane 4: 4%T, Lane 5: 6%T, Lane 6: 4%T, Lane 7: 8%T, Lane 8: 20%T, Lane 9: 10%T, Lane 10: 10%T. The anode is at the bottom.

IEF of the ampholytic dyes

IEF of the ampholytic dye fractions was performed in a pH 8-10.5 gradient (Figure 28) in order to determine the number of the individual ampholytic dye species of each fraction. It was found that the ampholytic dye molecules interacted strongly with carrier ampholytes, resulting in smearing (results not shown). Fortunately, these interactions could be inhibited by including 8 M urea and 30% dimethylformamide (DMF) in the gel solution (Righetti *et al.*, 1977). The IEF gels were composed of 10%T, 4%C polyacrylamide, since DMF has an inhibitory effect on polymerization.

The focused ampholytic dyes were fixed by placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol. The carrier ampholytes were also precipitated by the picric acid, appearing as opaque bands (Knisley and Rodkey, 1992).

The pH 8-10.5 IEF shows the 10B ampholytic dye fraction to possess, as expected, four major isomers with pI values greater than 9.5. However, there are small quantities of other less basic isomers present, perhaps due to reaction of both the MCT and VS groups with one BAPEDA molecule. The TETA-containing ampholytic dye fractions contain at least 15 isomers of pI values between 8 and 9.5. The 5B5T to 2B8T ampholytic dye fractions (lanes 5-8 of Figure 28) seem to possess the best spread of isomers.

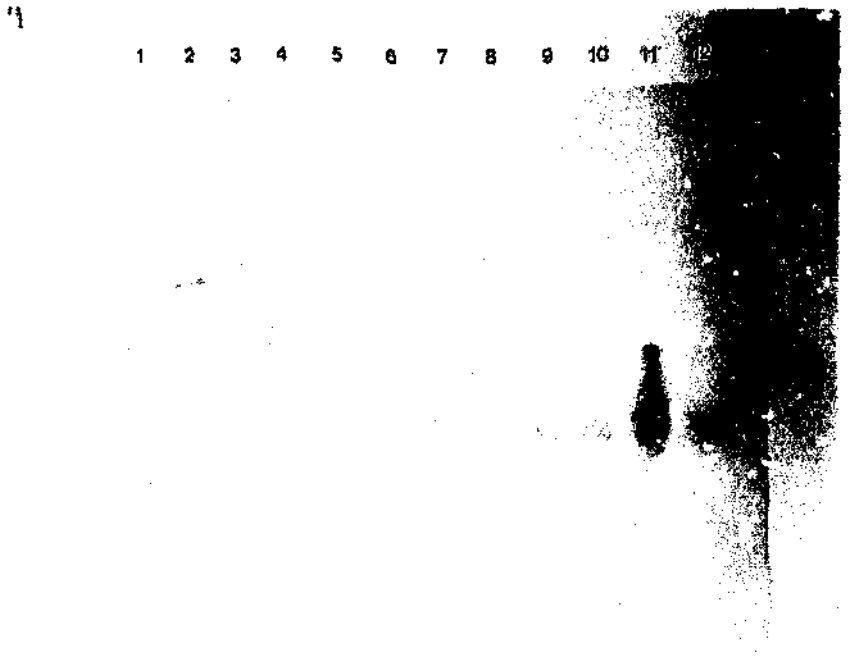


Figure 27 Estimation of the pI range of the ampholytic dye fractions by PAGE in a 4.5%T, 4%C polyacrylamide gel, with 15 mM HCl-ethanolamine, pH 9.5, as the gel buffer. Thin filter paper strips soaked in a 1.5 M solution of the same buffer were applied to the extremities of the gel, upon which the platinum electrodes were placed. 15 µl of the ampholytic dye fractions were loaded in narrow wells in the centre of the gel. Lanes 1 and 12: bromophenol blue and xylene cyanol FF dissolved in gel buffer. Lane 2: 10B, lane 3: 8B2T, lane 4: 7B3T, lane 5: 6B4T, lane 6: 5B5T, lane 7: 4B6T, lane 8: 3B7T, lane 9: 2B8T, lane 10: 1B9T, lane 11: 10T. Electrophoresis was conducted at 100 V at 5°C until the bromophenol blue had migrated 5 cm. The ampholytic dyes were fixed by placing the gel in a saturated solution of picric acid containing 10% acetic acid and 2% glycerol, followed by washing in saturated picric acid containing 2% glycerol. The bromophenol blue cannot be seen because it is in its acidic form (yellow). The 10B to 3B7T fractions (lanes 1 to 8) contain a significant proportion of ampholytic dye isomers with pI values greater than 9.5.

Using their experimental data, Bundgaard and Johansen (1981) were able to predict kinetically the half-lives of decomposition of *N*-Mannich bases of morpholine and piperidine in neutral to basic aqueous solutions as a function of the p*K* of the parent amide as shown in Table 15.

Table 15: **Predicted half-lives of decomposition of *N*-Mannich bases of morpholine and piperidine in neutral to basic aqueous solutions (37°C) as a function of the p*K* of the parent *NH*-acidic compound (from Bundgaard and Johansen, 1981)**

p*K* of parent *NH*-acidic compound	$t_{0.5}$	
	Morpholine *N*-Mannich base	Piperidine *N*-Mannich base
15	24.4 h	54 min
14	1.7 h	2.4 min
13	7.1 min	5.4 s
12	0.5 min	0.3 s
10	0.2 s	4×10^{-3} s

Acrylamide would be expected to have a p*K* similar to acetamide, ie. *ca.* 15. So the half-life of *N*-(*N'*-morpholinomethyl)acrylamide at 37°C would only be about 24 h. However, running an IPG gel at 5-10°C should increase the $t_{0.5}$ to about a week[1] rendering the percentage decomposition of the acrylamido buffer

[1] In organic chemistry a general rule of thumb is that the rate of a reaction doubles for every 10 C rise in temperature. Hence decreasing the temperature from 37 C to 7 C would increase the $t_{0.5}$ by a factor of eight.

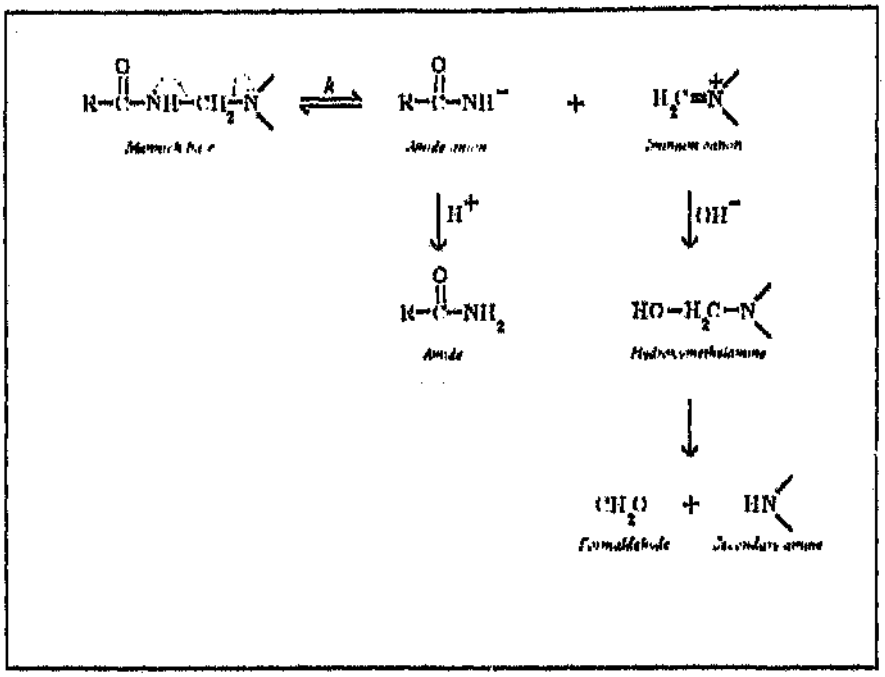


Figure 37 Mechanism of basic hydrolysis of aminomethylated amides.

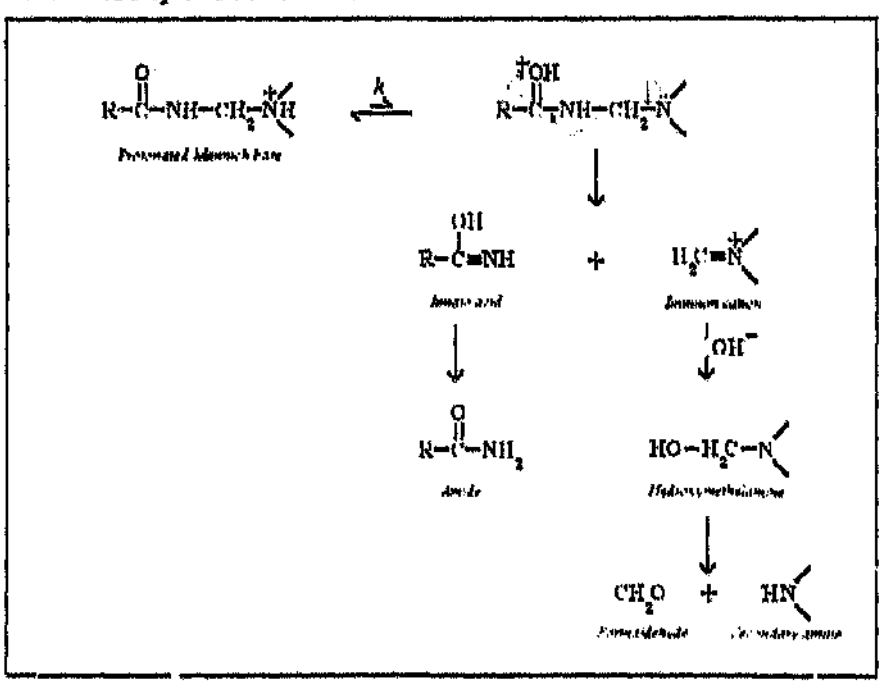


Figure 38 Mechanism of acidic hydrolysis of aminomethylated amides.

11.2 for piperidine). It can also be seen that the rate of hydrolysis is increased with increasing acidity (caused by electron-withdrawing substituents) of the amide (Bundgaard and Johansen, 1980*b*):

Amide	p*K* (25°C)
Acetamide	15.1
Benzamide	14
Thiobenzamide	12.8
Trichloroacetamide	12.4
p-Toluenesulfonamide	10.2

Loudon *et al.* (1981) were able to elucidate the kinetic mechanism of both the acidic and basic hydrolysis reactions. The mechanism of hydrolysis in basic solution seems to be best described as an unimolecular solvolysis with an amide anion as a leaving group as shown in Figure 37.

This reaction mechanism fits the experimental results obtained by Bundgaard and Johansen (1980*a*,*b*) where hydrolysis is favoured by increased acidity of the amide (and hence increased ability to form an amide anion), and by increased basicity of the amine (and hence facilitating the acceptance of a proton with subsequent formation of an iminium ion). In acidic solution the most likely mechanism of hydrolysis appears to involve expulsion of an amide enol (imidic acid) as shown in Figure 38.

Table 14: Rates of the decomposition of various *N*-Mannich bases in aqueous solution (37°C) (Data from Bundgaard and Johansen 1980a,b)

Compound	p*K*	k_1 (× 10^{-4} min^{-1})[a]	k_2 (× 10^{-4} min^{-1})[b]
N (piperidinomethyl)-acetamide	8.1	0.55	ND
N-(piperidinomethyl)-benzamide	7.8	5.1	0.004
N (piperidinomethyl)-nicotinamide	7.5	17	0.01
N (piperidinomethyl)-thiobenzamide	6.6	1300	ND
N-(piperidinomethyl)-trichloroacetamide	6.4	3500	ND
N (morpholinomethyl) benzamide	5.6	0.05	0.06
N-(morpholinomethyl) thiobenzamide	4.4	52	0.6
N-(morpholinomethyl) trichloroacetamide	4.1	72	1.1
N (morpholinomethyl) p-toluenesulfonamide	4.4	6000	150

ND Not determined.
[a] Rate constant for decomposition of unprotonated Mannich base.
[b] Rate constant for decomposition of protonated Mannich base.

It can be seen from the above data that the rate of hydrolysis of the piperidine derivatives of benzamide, thiobenzamide and trichloroacetamide is much higher than that of the corresponding morpholine derivatives. Thus the rate of hydrolysis is decreased by decreasing the basicity of the amine (p*K* of morpholine is 8.6 versus



Figure 36 pH 5.1 6.1 IPG using 20 mM crude *N*-[*N'*,*N'*-bis (2 hydroxyethyl)aminomethyl]acrylamide, with AMPS as the strong acidic titrant in concentrations determined using the Henderson Hasselbalch equation. Left lane: bovine carbonic anhydrase. Right lane : crude L-amino acid oxidase from *Crotalus adamanteus*. The anodic and cathodic filter paper electrode strips were soaked in 10 mM glutamic acid and 10 mM histidine respectively. Prefocusing was performed at 500 V until the salt fronts had migrated into the electrode strips. The sample proteins were then applied to the gel surface as 10 mg/ml solutions in water absorbed in filter paper separates. Focusing was performed at 5°C at 1500 V for two hr, then at 2500 V overnight, followed by 4500 V for a further 2 hr. The gel was stained with Coomassie R-250 according to Reisner (1984). The major band is the pI 5.9 carbonic anhydrase isozyme. The anode is at the bottom.

isozyme, but further bands of more acidic pI are also visible. Focusing of crude L-amino acid oxidase from *Crotalus adamanteus* venom (M_r 135 000, 17 isozymes with major isozyme bands of pI 5.71 and 5.87 at 4°C[1]) were used as pI markers. The sample proteins (2 mg of protein dissolved in 200 µl of 10 mM glutamic acid) produced numerous faint bands.

Problems with instability of acrylamido buffers

After I had already invested a considerable amount of time and effort into the synthesis of aminomethylated acrylamide derivatives, I came across papers that showed that the Mannich bases of amides are unstable in aqueous solution and slowly decompose to the original amide, formaldehyde and secondary amine (McDonald and Beaver, 1979; Bundgaard, 1985). Pelton (1984), upon prolonged dialysis of dimethylaminomethylated polyacrylamide against distilled water, obtained a quantitative removal of the tertiary amine groups.

Bundgaard and Johansen (1980a,*b*) measured the decomposition rates of various *N*-Mannich bases in aqueous solution. The pH-dependent hydrolysis rate profiles followed a sigmoidal curve with plateaus in the acidic and alkaline regions with the inflexion point centred at the p*K* of the Mannich base; the rates of hydrolysis in the alkaline region (ie. for the free Mannich bases) being somewhat faster as can be seen in Table 14.

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[1] pI values obtained from Righetti and Caravaggio (1976). For the interest of the reader, the most comprehensive compilations of protein pI and molecular mass values to date are Righetti and Caravaggio (1976) and Righetti *et al.* (1981a). An extensive compilation of subunit molecular masses alone is given in Darnall and Klotz (1975).

3.4 ISOELECTRIC FOCUSING IN IMMOBILISED pH GRADIENTS

3.4.1 Preparation of immobilised pH gradients using the acrylamido buffer *N*-[bis(2-hydroxyethyl)-aminomethyl]acrylamide

Electrofocusing results obtained

N-[bis(2-hydroxyethyl)aminomethyl]acrylamide (BHEAMA) was not successfully purified. However it was possible to determine the concentration of BHEAMA in the crude product by titration. The reaction product was then diluted with distilled water to give a 0.2 M solution of BHEAMA. A pH 5.1 to 6.1 IPG gel was prepared, using 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) as the titrant, in the necessary ratios as determined by the Henderson-Hasselbalch equation. Contaminants present in the BHEAMA solution, such as unreacted formaldehyde and diethanolamine, did not affect the IPG since they were removed from the IPG gel after polymerization by the washing step in distilled water. Possible copolymerizable contaminants in the crude BHEAMA solution such as unreacted acrylamide, and *N* hydroxymethylacrylamide, were considered to have a negligible effect on the IPG since they were uncharged.

Figure 36 shows that the isozymes of bovine erythrocyte carbonic anhydrase[1] are well resolved in the pH 5.1 to 6.1 gradient. The major band is the pI 5.9

[1] Bovine carbonic anhydrase is present as multiple isozymes with pI values ranging from 5 to 6.5 (Daniels and Landers, 1996). The major isozyme has a pI of 5.9 (Righetti and Caravaggio, 1976).

Monoionic polymeric spacers are expected to have similar ionic mobilities as shown by the following homologous series of carboxylic acids:

Carboxylic acid	Ionic mobility ($\times 10^{-5}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$)
Formate	-51
Acetate	-38
Propionate	-34
Butanoate	-31
Pentanoate	-29
Hexanoate	-27

Monoanionic polymers would be similar in structure to ionic surfactants such as SDS, except they would bear a hydrophilic "tail". It seems logical to base the synthesis of monoanionic spacers for ITP on those of surfactants, such as the Triton series, which contain poly(ethylene glycol) chains. Such spacers would be synthesized by reacting phenolsulfonic acids with ethylene oxide under alkaline conditions.

The introduction of monoanionic polymeric spacers for ITP is expected to improve the poor results obtained with DNA. The resolution obtained was slightly inferior to that of PAGE. However, this is due to deficiencies in the polyAMPS spacers leading to distortions in the electric field strength gradient and insufficient spacing of the DNA, rather than due to the technique itself. In fact, it is expected that ITP of DNA molecules should yield superior results to PAGE, due to the counteraction of diffusion by the electric field strength gradient.

3.3.6 The problems occurring with polyAMPS spacers and their possible solution

Certain deficiencies in the use of polyAMPS as polymeric spacers were noted:

I. The longer polyAMPS species became entangled in the gel fibres, leading to smearing and precipitation (Figures 18-23). This had two deleterious consequences:

(i) Distortion of the electric field strength gradient occurred, with subsequent loss of resolution (Figures 31 and 34).

(ii) The polymer matrix was, in effect, given a negative charge, leading to severe electroosmosis and resultant deformation of the gel. However, this phenomenon could be prevented by using low concentrations (10 mM) of polyAMPS spacers.

II. In the absence of disaggregating agents such as urea, ionic interactions occurred between the polyAMPS spacers and amphoteric sample species, leading to smearing and loss of resolution (Figure 29).

Fortunately all of the above problems may be solved by the introduction of monoanionic polymeric spacers. The problem of ionic interactions with amphoteric sample species would naturally no longer occur. Moreover, these polymeric spacers, having only a single charge, would have to be very much shorter than polyAMPS spacers in order to have similar net ionic mobilities. Hence entanglement of the polymeric spacers in the gel fibres should no longer be a problem.

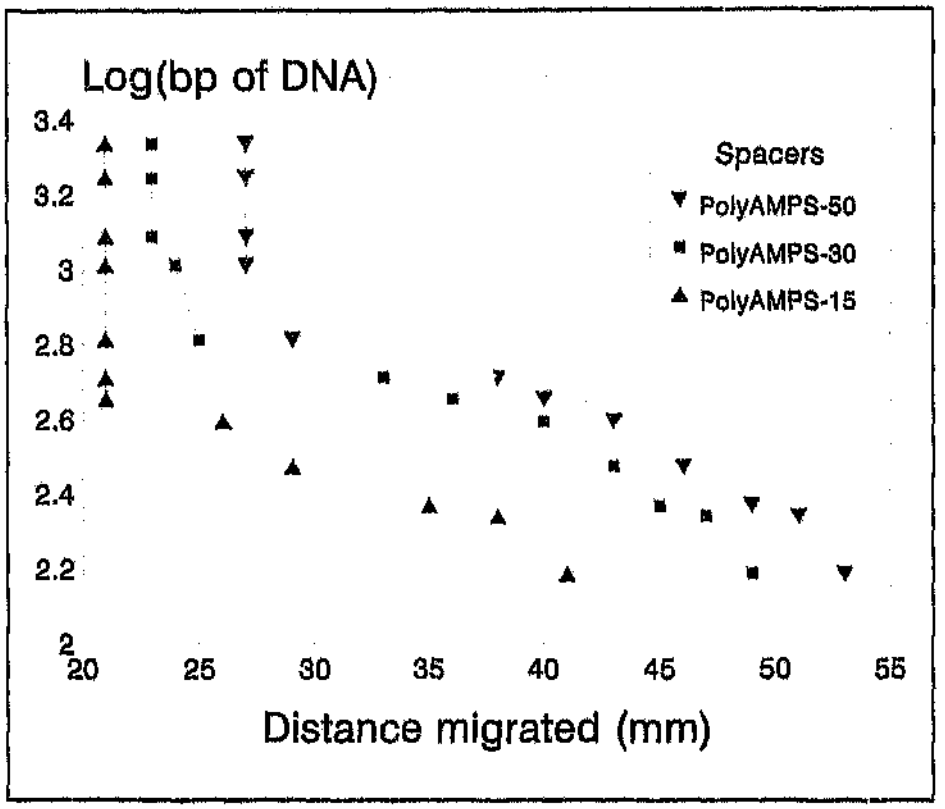


Figure 35 Plot of the logarithm of the size (in bp) of each DNA molecule *versus* its migration distance, for the TEP experiment of Figure 35.



Figure 34 TEP of P. falciparum on a 7ET, 4% polyacrylamide gel, using the polyAMPS 50 (left), 40 (centre) and 15 (right) fractions as spacers. The gel buffer was composed of a 10 mM solution of polyAMPS spacer polymers adjusted to pH 9,5 with ethanolamine (pK 9,5). Both the anolyte and the catholyte (terminating electrolyte) were composed of 400 ml each of 100 mM EACA titrated to pH 9,5 with ethanolamine. The extremities of the gel were connected to the electrode buffer by filter paper strips soaked in electrode buffer.Boehringer Mannheim molecular weight marker VI was used as sample DNA. Nine, ten and six DNA bands are resolved by TEP using the polyAMPS 50, 40 and 15 spacers, respectively, compared to 12 by PAGE (Figure 33). Samples (5 µl) of a 40 µg/ml solution of DNA (200 ng DNA per well) in 15 mM of EACA ethanolamine buffer, pH 9,5 were loaded in shallow wells on the polyacrylamide gel surface. The sample buffer contained bromophenol blue as a migration marker. The gels were run at room temperature at constant voltage (8,5 W), with an initial voltage of 200 V, and a final voltage of 450 V. Electrophoresis was stopped when the bromophenol blue was 1 cm away from the filter paper electrode strip. The DNA bands were detected with the silver staining method of Sanguinetti *et al.* (1994).

indicate that the larger DNA molecules should be resolved by the use of slower polyAMPS spacers, leading to increased spacing between the slower-moving DNA molecules. Thus TEP was tried using the polyAMPS-15 fraction as spacers (right-hand section of Figure 34). However, contrary to expectations, only the five fastest-moving DNA molecules were resolved - the 453 bp to 2176 bp DNA molecules comigrated. According to the apparent bpe range of the polyAMPS-15 fraction (100 - 1500, Table 13), only DNA molecules greater than 1500 bp should not have been spaced.

Apart from the anomalous spacing effect, it was noticed that the DNA bands had a distorted appearance - indicating some disturbance in the electric field strength gradient, as occurred similarly for TEP of ampholytic dyes using polyAMPS-15 and -10 spacers. As explained before, this distortion is most probably caused by entanglement of the longer polyAMPS polymers (see Figures 18 to 23), so that the degree of resolution was actually decreased.

A plot of the logarithm of the size (in bp) of each DNA molecule *versus* its migration distance is portrayed in Figure 35. A regular distribution of the faster DNA molecules is obtained for all the polyAMPS fractions; however when a certain size of DNA is reached (1030, 1230 and 453 bp for the polyAMPS-50, -30 and -15 spacers, respectively) the DNA molecules are no longer resolved, and comigrate as a single band.

upon crystallization from hot acetone/2-propanol. These crystals absorbed water from the atmosphere, but did not deliquesce like α-(*N*-morpholinomethyl)acrylic acid. The hygroscopic nature of these two α-aminomethylated acrylic acids may explain why none of the α-aminomethylated acrylic acid derivatives from the previous experiments in aqueous solution could be crystallized.

The pK_a values of 10 mM solutions of the pure α-(*N*-morpholinomethyl)acrylic acid and α-[*N*-methyl-*N*-(2-hydroxyethyl)aminomethyl]acrylic acid derivatives were determined to be 7.59 ± 0.03 (23°C) and 9.22 ± 0.08 (22°C) respectively, using the method recommended by Albert and Serjeant (1984).

Preparation of IPG gels using α-(*N*-morpholinomethyl)-acrylic acid as the immobilized buffer

A pH 6.9-7.9 IPG gel was prepared using the DoctorpH program (obtainable from Hoefer) to calculate the ratios of α-(*N*-morpholinomethyl)acrylic acid (weak acid, p*K* 7.6) and strong basic titrant (in this case the p*K* 9.3 Immobiline') required. Equine myoglobin (pI = 7.4 at 25°C) was used as the marker. An excellent resolution of myoglobin and various contaminants (presumably myoglobin degraded in some way) was obtained (Figure 46). However, lateral compression of the proteins bands in the cathodal half of the gradient was obtained. This was found to be due to distortion of the electric field by the sample wells being too deep. Another pH 6.9-7.9 IPG was prepared with longer, more shallow sample wells. This resulted in a better focusing pattern, although some distortion of the bands still occurred (see Figure 47). Comparison of the two gels show their focusing patterns to be identical

Improved protocol for the synthesis of α-aminomethylated acrylic acid derivatives

During the course of my work a paper was published (Krawczyk, 1995) detailing the synthesis of the α-aminomethylated acrylic acid derivatives of various dialkylamines such as diethylamine, dibutylamine and dipentylamine. However, the reactions were performed in the absence of water: paraformaldehyde was used as the source of formaldehyde, and absolute ethanol or dioxane were used as the reaction solvents. Heating was necessary for the reaction to occur. (Presumably because of the use of organic solvents rather than water - my earlier experiments had shown the use of water as a solvent to have a catalytic effect on the reaction.) All the α-aminomethylated acrylic acid derivatives were obtained as oils which solidified on standing. Recrystallization from acetone gave the pure products.

I repeated the synthesis of the α-aminomethylated acrylic acid derivatives shown in Figure 44 using Krawczyk's protocol. Only the oil of α-(*N*-morpholinomethyl)acrylic acid readily crystallized on standing. The crude solid was found to be extremely deliquescent - liquifying within minutes if left exposed to the atmosphere. The pure product obtained after recrystallization from acetone was also deliquescent - a sample left in an open container became liquid after a few days.

After standing at room temperature for several weeks, the oil of α-[*N*-methyl-*N*-(2-hydroxyethyl)aminomethyl]acrylic acid was found to have solidified. The crude product was successfully used as seed crystals for a second synthesis. Small white crystals of α-[*N*-methyl-*N*-(2-hydroxyethyl)aminomethyl]acrylic acid were obtained

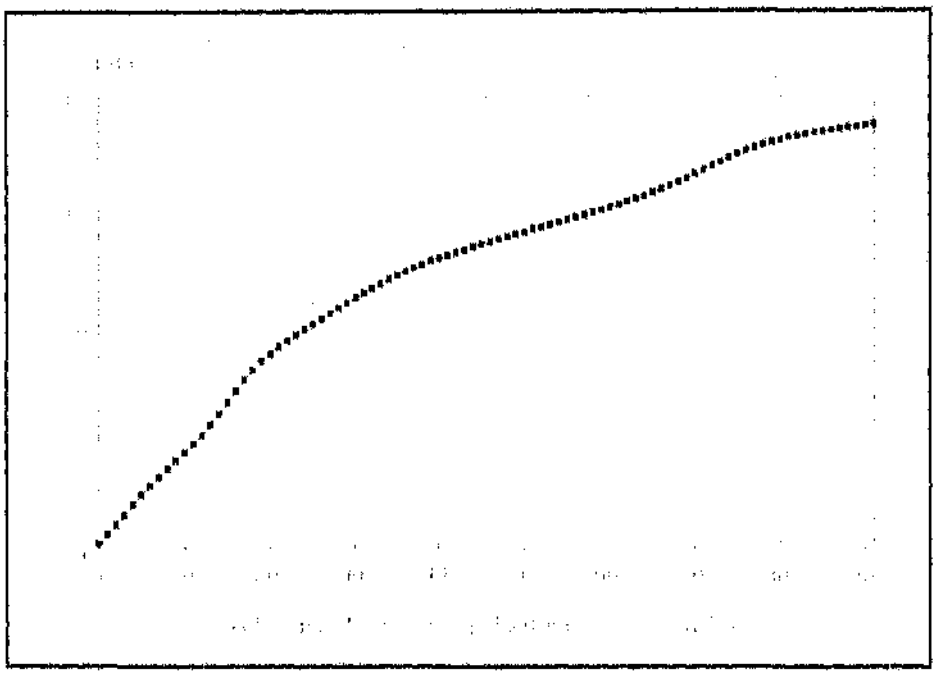


Figure 45 Titration curve of crude α-(*N*,*N*-dimethylaminomethyl)acrylic acid. The α-(*N*,*N*-dimethylaminomethyl)acrylic acid was synthesized by dissolving 0.25 mole of malonic acid in 26% (w/v) dimethylamine solution (dimethylamine content: 0.25 mole), followed by the slow addition of 0.5 mole formaldehyde (commercial 37% solution), with cooling in ice-water. The reaction was conducted at room temperature. A 0.5 ml sample of reaction mixture was diluted in 35 ml of distilled water, and titrated with 0.1 N NaOH at 20°C. The titration curve shows the product to be impure. The major contaminant is probably bis(*N*,*N*-dimethylaminomethyl)acetic acid, as obtained by Pelletier and Franz (1952).

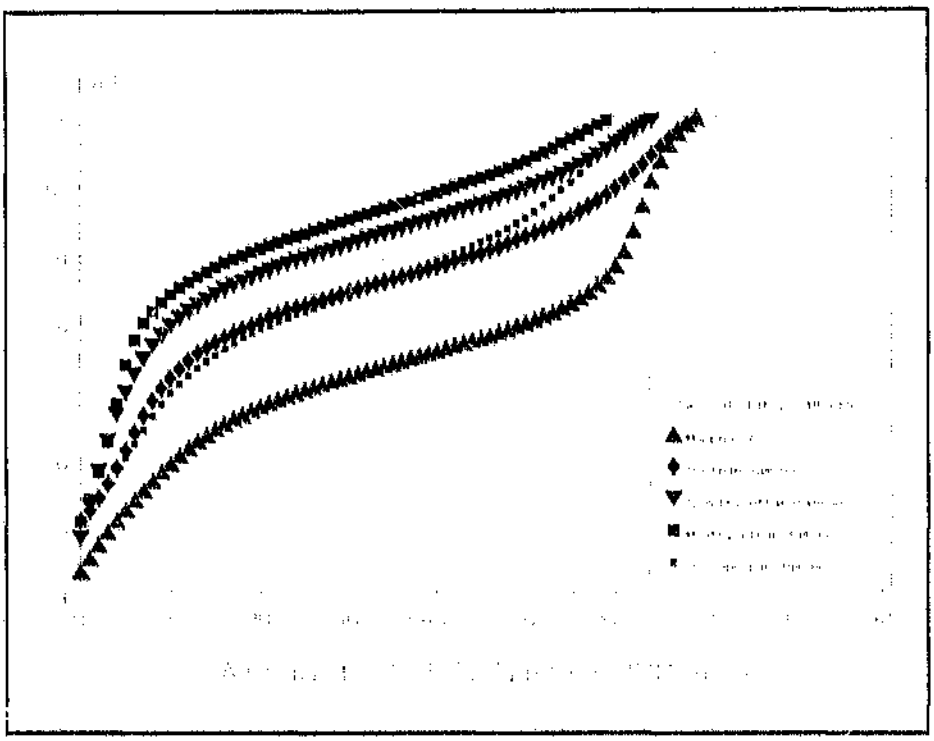


Figure 44 Titration curves of various α-aminomethylated acrylic acids. The α-aminomethylated acrylic acids were synthesized by dissolving 0.25 mole of malonic acid and 0.25 mole of secondary amine in 50 ml of distilled water, followed by the slow addition of 0.5 mole formaldehyde (commercial 37% solution), with cooling in ice-water. The reaction was conducted at room temperature. A 0.5 ml sample of reaction mixture was diluted in 35 ml of distilled water, and titrated with 0.1 N NaO^{-} at 20°C. The titration curves show the presence of only one buffering group in each reaction mixture, differing in *pK* from the original secondary amine, ie. the products are essentially pure.

The improved protocol was used to synthesise a range of α-aminomethylated acrylic acids. Their titration curves are shown in Figure 44. The titration curves of the derivatives of morpholine, diethanolamine, di-*iso*-propanolamine, *N*-(methyl)ethanolamine and *N*-(ethyl)-ethanolamine show them to be substantially pure (Figure 44). The morpholine, *N*-(methyl)ethanolamine and diethanolamine derivatives are the most promising since they can be used as hydrophilic alternatives to the rather hydrophobic basic Immobiline buffers; Figure 44 shows their p*K* values to be approximately 7.6, 8.7 and 9.2 respectively.

However, it can be seen from the titration curve of α-(*N*,*N*-dimethylaminomethyl)acrylic acid (Figure 45) that a major contaminant is present with p*K* values of about 7.5 and 9.5, suggesting that it is a diamine[1]. In spite of the high degree of purity of the other α-amino-methylated acrylic acid derivatives, none of them could be successfully crystallized.

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[1] α-(*N*,*N*-Dimethylaminomethyl)acrylic acid is expected to have a p*K* value similar to that of β-alanine; at least 10.3 .

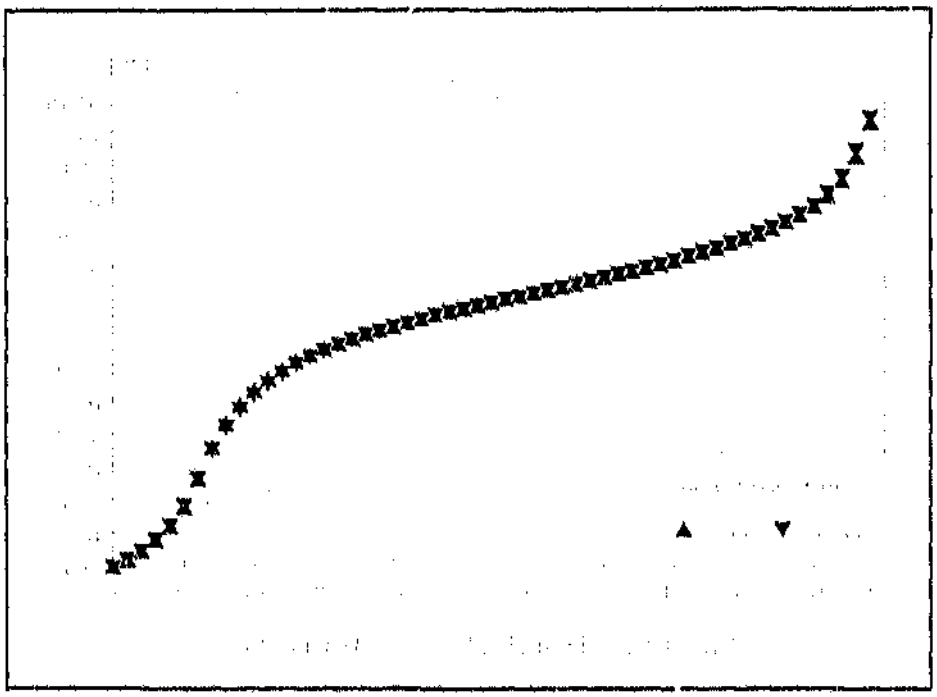


Figure 43 The reaction of malonic acid, formaldehyde and morpholine (molar ratio 1:2:1) to form a single product of *pK* 7.6, showing the catalytic effect of water as a solvent. The malonic acid (0.25 mole) and morpholine (0.25 mole) were dissolved in 50 ml of distilled water, followed by the slow addition of 37% formaldehyde solution (formaldehyde content: 0.5 mole), with cooling in ice-water. The reaction was conducted at room temperature. A 200 µl sample of reaction mixture was diluted in 35 ml of distilled water, and titrated with 0.1 *N* NaOH at 22°C. The reaction is essentially complete after 1 hr, compared to at least 50 hr for the reaction with no water added (Figure 40).

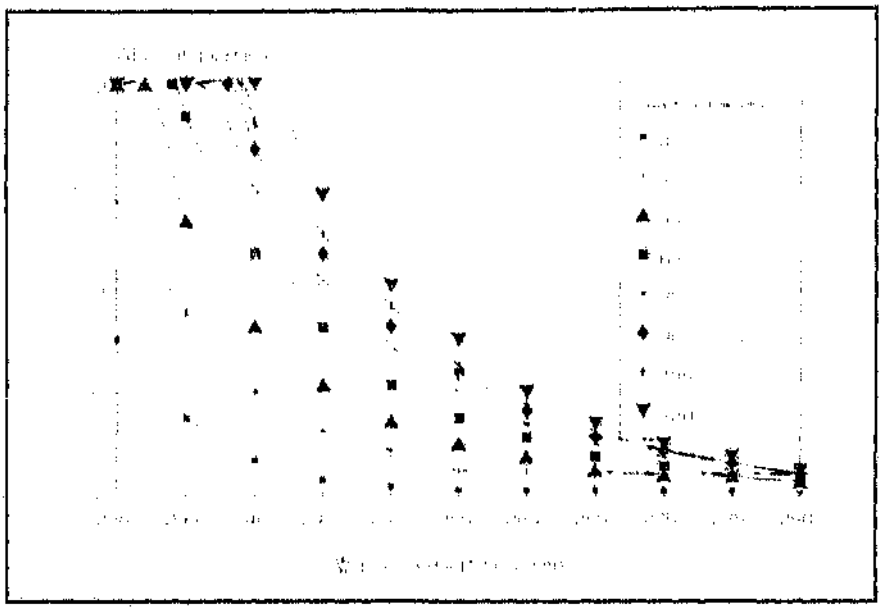


Figure 41 Progress of the reaction at room temperature between a 1:1:1 (by volume) mixture of 1 N malonic acid, 2 N formaldehyde, and 1 N morpholine. The reaction was followed spectrophotometrically by the increase in UV absorbance.

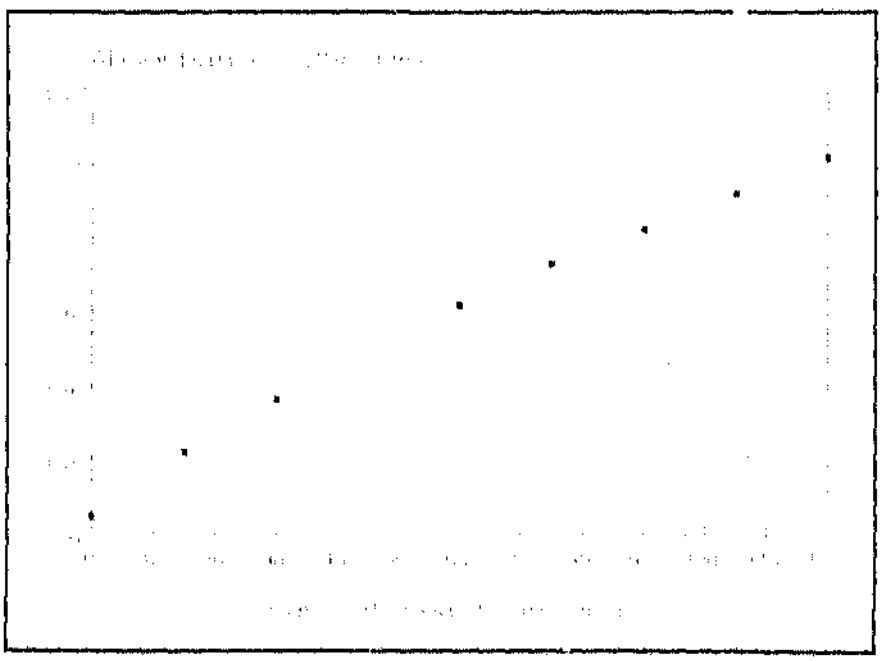


Figure 42 Reaction kinetics for the above reaction, determined by increase in $A_{\lambda_{max}}$.

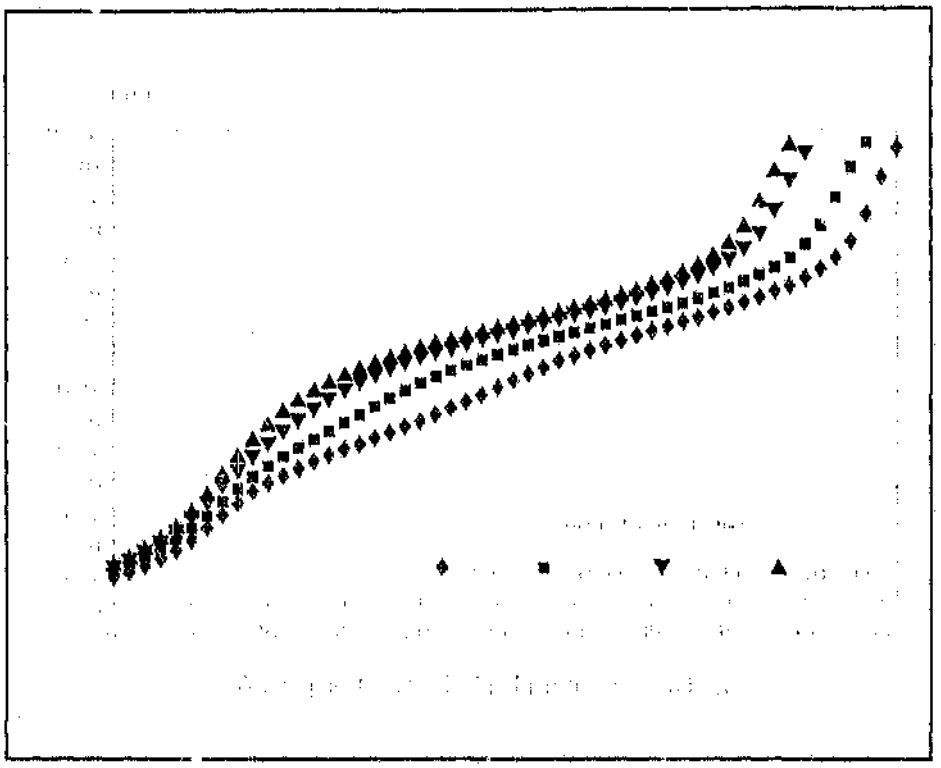


Figure 40 Progress of the reaction between malonic acid, formaldehyde and morpholine (molar ratio 1:2:1), showing the consumption of malonic acid (pK_1 = 5.7) with time and the formation of a single product of pK 7.6. The malonic acid (0.25 mole) was dissolved directly in 37% formaldehyde solution (formaldehyde content: 0.5 mole), followed by the slow addition of morpholine (0.25 mole), with cooling in ice-water. The reaction was conducted at room temperature. A 200 μl sample of reaction mixture was diluted in 35 ml of distilled water, and titrated with 0.1 N NaOH at 22°C.

In my first experiments I dissolved the malonic acid directly in the 37% formaldehyde solution, followed by the slow addition of morpholine, with cooling of the flask in ice-water. The progress of the reaction, determined by titration, is shown in Figure 40. It can be seen that the reaction at room temperature is quite slow, requiring about 50 hr to reach completion. I decided to monitor more closely the reaction rate by measuring the increase in UV absorbance (due to the double bond present in α-(*N*-morpholinomethyl)acrylic acid[1]) of a 1:1:1 mixture of 1 M malonic acid, 2 M formaldehyde and 1 M morpholine with the course of time. However, the reaction now appeared to be substantially faster, as shown in Figures 41 and 42.

It was reasoned that this increase in the reaction rate must be due to the presence of water as a solvent. The above reaction was repeated in the presence of water. It was found that the addition of 50 ml water to the reaction mixture significantly accelerated the reaction (which became very exothermic with vigorous release of CO_2), so that it was substantially complete within 2-3 hours (see Figure 43).

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[1] IUPAC name: 2-(*N*-morpholinomethyl)propenoic acid.

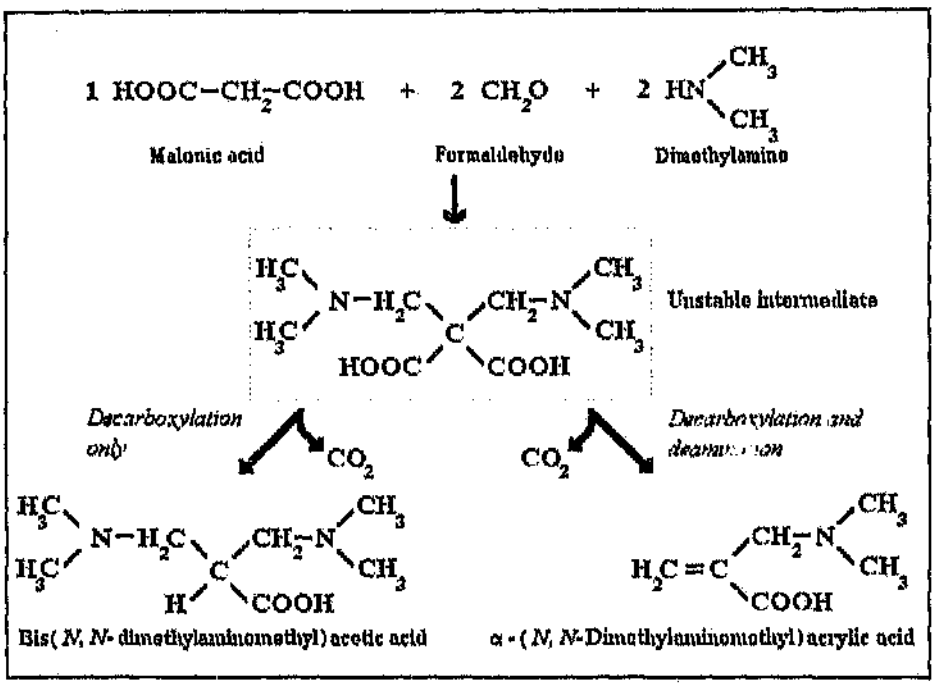


Figure 39 Reaction between malonic acid, formaldehyde and dimethylamine in aqueous solution (Pelletier and Franz, 1952).

3.4.2 Preparation of immobilized pH gradients by incorporation of acrylic acid buffers into polyacrylamide gels via copolymerization

Synthesis of α-aminomethylated acrylic acid derivatives

While investigating the chemistry of the Mannich reaction, I came across a review article (Tramontini *et al.*, 1988) which stated that 1,3-dicarbonyl compounds (such as cyanoacetic acid (Adrian, 1971)) could react with formaldehyde and secondary amines with the formation of α-aminomethylated-α,β-unsaturated carbonyl compounds, which could be polymerized to form ionic polymers. I wondered whether malonic acid could also be used to synthesize α-aminomethylated acrylic acids.

These compounds would be in effect zwitterionic buffers, in which the zwitterionic state would correspond to its "unionized" form. Such buffers could act as weak acids ($pK_2 = pK_{amine}$) or weak bases ($pK_1 = pK_{carboxylic\ acid}$). Since the decarboxylation-deamination step that occurs during the course of the reaction is irreversible, the α-aminomethylated acrylic acid derivatives would be expected to be stable. A more detailed literature search revealed that malonic acid has been used in this type of Mannich reaction for a long time (Mannich and Kather, 1920), but only as a means of synthesizing bis-aminomethylated acetic acids! It appears that the decarboxylation step is not always accompanied by deamination. For example, Pelletier and Franz (1952) obtained a 25% yield of bis(*N,N*-dimethylaminomethyl)acetic acid and a 35% yield of α-(*N,N*-dimethylaminomethyl)acrylic acid from the Mannich reaction of malonic acid, formaldehyde and dimethylamine, as shown in Figure 39.

negligible during the run itself (10 h maximum usually). In fact satisfactory focusing of carbonic anhydrase isozymes was obtained using BHEAMA (Figure 36) which is a slightly stronger base than the morpholine compound (p*K* of diethanolamine is 8.8 versus 8.6 for morpholine) and hence is expected to be slightly more susceptible to hydrolysis. However, it appears that acrylamido derivatives of more basic amines will be too unstable to use as IPG buffers as can be seen from the predicted hydrolysis rates of piperidine derivatives.

This prediction is unexpected since it appears to contradict the results obtained by Bartoli *et al.* (1975) who synthesised *N*-[*N'*,*N'*-dialkylaminomethyl]acrylamides for the preparation of anion-exchange resins. Although the secondary amines used were strongly basic (p*K* ca. 10.5) the resulting anion-exchange resins seemed to be quite stable. Moreover Pelton (1984) found that *N*-[*N'*,*N'*-dimethylaminomethyl]acrylamide required about two weeks for complete hydrolysis of the Mannich base. The apparently slow rate of hydrolysis is most probably due to re-reaction of the formaldehyde-dimethylamine adducts with free amides in their vicinity. However, hydrolysis during the course of an IPG run would result in a slow irreversible acidification of the pH gradient since the split-off secondary amines would be rapidly removed electrophoretically.

Since it seemed unlikely that these *N*-Mannich base derivatives of acrylamide would be as stable as the commercially available Immobilines', it was decided to search for alternative vinyl monomers to acrylamide.

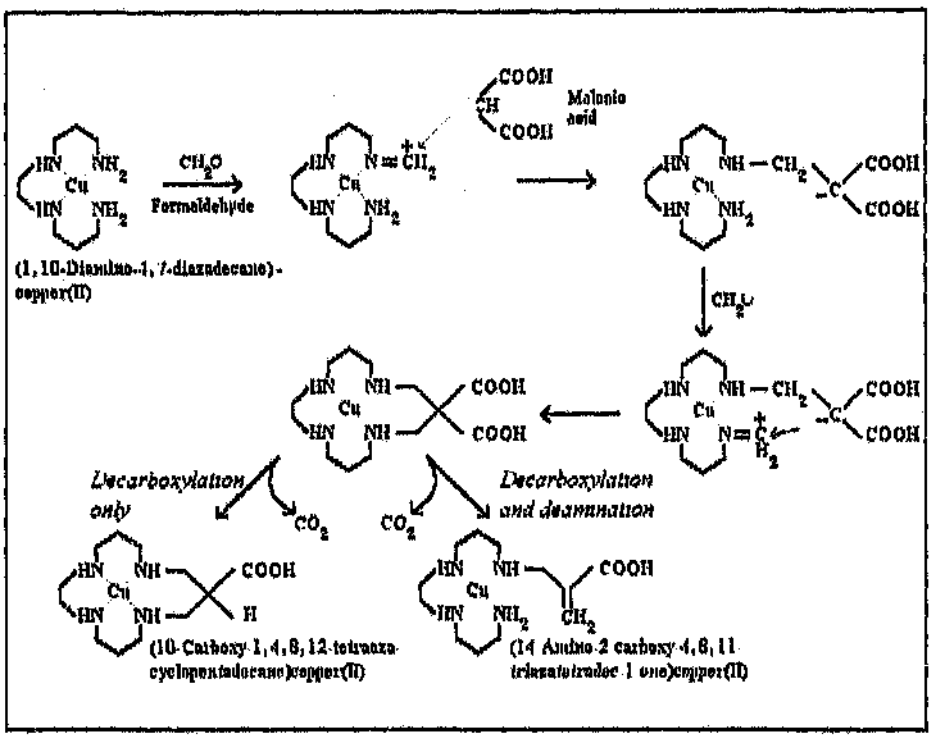


Figure 52 Proposed pathway for the synthesis of 14-amino-2-carboxy-4,8,11-triazatetradec-1-ene.

I decided to try maloric acid in a similar reaction. I envisaged a scheme (set out in Figure 52) in which the cyclic Cu^{2+}-tetramine-formaldehyde-malonic acid product would decompose by the decarboxylation and decarboxylation-deamination pathways to give a cyclic tetramino carboxylic acid and a linear α-tetraminomethylated acrylic acid respectively. These products could be subsequently easily separated from each other by gel filtration on Sephadex G-15. Unfortunately, I did not take into account the fact that the electron pairs of the nitrogen atoms of the terminal amino groups will also become "locked" kinetically into coordination with the Cu^{2+} ion once the cyclic structure has been completed, and hence rendered unreactive, and unable to undergo the deamination step, so that only decarboxylation of the Mannich intermediate occurs upon acidification as shown in Figure 53.[1] Thus this approach had to be abandoned.

---

[1] Decarboxylation of *gem*-carboxylic acids occurs spontaneously under acidic conditions due to the strong electron-withdrawing effect of the undissociated *gem*-carboxylic acid groups (Lawrence *et al.*, 1991). However decarboxylation can also occur under alkaline conditions with heating (Xin *et al.*, 1992).

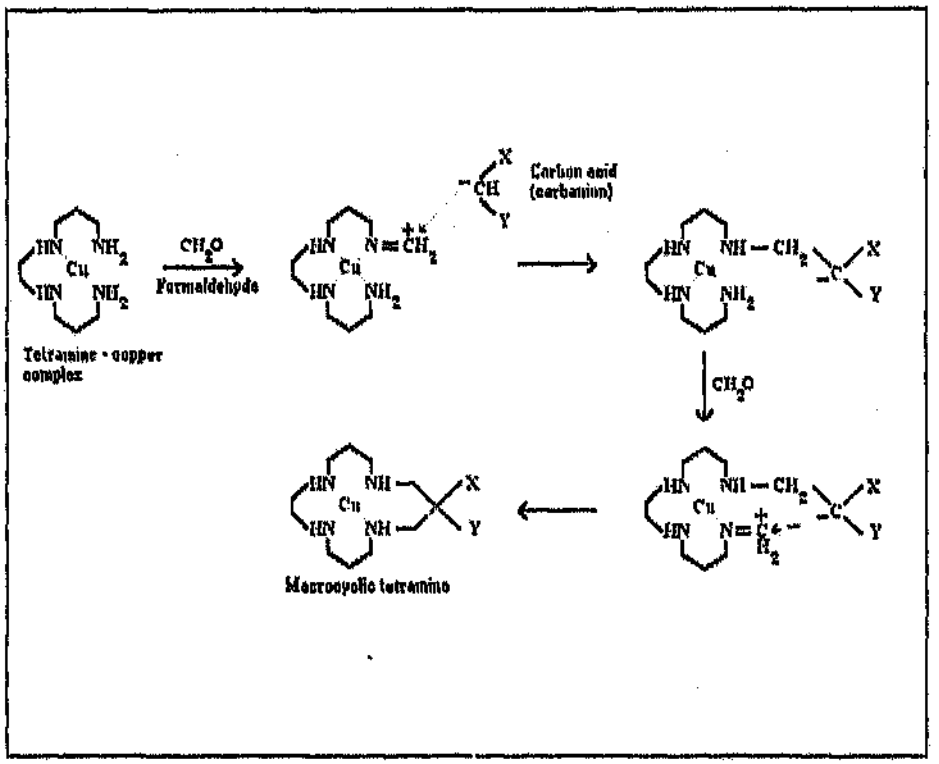


Figure 51 Reaction of carbon acids and formaldehyde with a tetramine-copper complex. The *X* and *Y* groups of the carbon acid are carbonyl groups such as carboxylic acids and their esters, ketones and nitriles.

Attempted syntheses of polyamine derivatives of acrylic acid

In my search for an acrylic acid derivative that buffered over the range pH 4-10, it occurred to me that polyamines were the logical chemicals to derivatize. I decided to try *N,N'*-bis(3-aminopropyl)piperazine and *N,N'*-bis(3-aminopropyl)ethylenediamine as these were the only polyamines that I could find that were available in a single isomeric form. Unfortunately, a resinous mixture of polyamino-polyacrylic acids was obtained, which was only soluble in fairly strong acidic or basic solutions (ie. it was insoluble in its isoelectric state).

I wondered if it was possible to react polyamines in such a way so as to get substitution at only one nitrogen atom. This would cut down the number of side-products enormously. I found an interesting paper by Lawrence and O'Leary (1987) in which the authors formed cyclic Cu^{II}-tetramine complexes. The terminal amino groups of these complexes were found to react specifically in a Mannich reaction with formaldehyde and *H*-acidic compounds such as nitroethane and diethylmalonate, to produce cyclic products, as shown in Figure 51. The specificity of the reaction lies in the fact that the electron pairs of the nitrogen atoms of the interior aza groups are "locked" into coordination with the Cu^{II} ion, and are hence rendered unreactive, while the terminal amino groups are free to "wiggle", so that the electron pairs of the terminal nitrogen atoms become available for reaction.



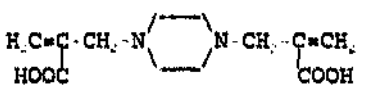
Figure 50 Focusing of bovine carbonic anhydrase isozymes on a pH 5-7 IPG (basic pH extreme 8.2 in reality, but the pH 7-8.2 portion of the gel is very small) prepared with 20 mM *N,N'*-bis(2-carboxyprop-2-enyl)piperazine titrated manually with the strongly basic p*K* 9.3 Immobiline®. Filter paper electrode strips soaked in distilled water were applied, and prefocusing of the gel performed at a maximum power of 8.5 W until the salt fronts has migrated into the electrode strips. Fresh filter paper electrode strips were then applied - the anodic strip was soaked in 10 mM glutamic acid and the cathodic strip in 10 mM arginine. Carbonic anhydrase was applied in shallow wells on the gel surface as 75 µl samples of approximately 10 mg/ml solutions in 20% glycerol. The gel was run at 5°C and stained with Coomassie G-250 according to Reisner (1984). The resolution was impaired in the acidic region due to rupturing of the gel surface caused by overswelling during the washing of the gel in distilled water. The anode is at the bottom.

9.3 Immobiline® as the strong titrant. Bovine carbonic anhydrase isozymes were successfully resolved as shown in Figure 50. However the resolution was impaired by rupturing of the surface of the IPG gel in the acidic portion of the pH gradient, due to excessive swelling during the washing step in distilled water. (The overswelling was caused by the higher-than-normal local ionic strength on the polyacrylamide matrix, due to the presence of multivalent BCPP ions.)

The insolubility of BCPP in its free acid form, although conducive to easy purification, made the preparation of the acidic and basic solutions for the gradient maker a very laborious task. This was due to the fact that the solutions had to be manually titrated to the desired pH values with the p*K* 9.3 Immobiline®, since the dissociation constants could not be determined via titration, because of overlapping of buffering ranges. The problem of overswelling of the acidic region of the gel during the washing step in distilled water, with consequent damage to the gel surface, might be overcome by the addition of additives such as glycerol which would reduce the water potential.

Synthesis of a diamine derivative of acrylic acid

The difficulty in obtaining pure products from the previous reaction led me to investigate the synthesis of α-aminomethylated acrylic acids of a structure facilitating their purification. I remembered that the crystallization of double zwitterionic buffers is facilitated by their insolubility at their isoelectric points (Good *et al.*, 1966; Jermyn, 1967). Hence the Mannich reaction of malonic acid was tried for the synthesis of *N,N'*-bis(2-carboxyprop-2-enyl)piperazine[1] (BCPP):



BCPP was very easy to synthesize since, being a double zwitterion, it was not very soluble in its free acid form, spontaneously crystallizing out of solution (like PIPES, in the synthesis of Good *et al.*, 1966). This chemical is bifunctional, and can act as a crosslinker as well as a buffer.

Unfortunately, it does not buffer between pH 5 and 8 (see Figure 13 in the Material and Methods) - it had been hoped that the p*K* values of the carboxylic acids and the nitrogen atoms would be reasonably spaced apart so as to provide linear buffering over the pH range pH 4 to 9. An acidic IPG over the pH range 5 to 7 was prepared using

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[1] IUPAC name: 2-[4-(2-carboxyprop-2-enyl)-piperazinylmethyl]propenoic acid.



Figure 49 Focusing of myoglobin from horse heart on a pH 7.3-7.5 IPG, prepared using α-(*N*-morpholinomethyl)acrylic acid as the buffer and the p*K* 9.3 Immobiline® as the titrant, in concentrations calculated using the DoctorpH program. The same procedure for prefocusing and loading of the IPG gels was used as for Figure 48. Focusing was performed at 5°C overnight at an initial field strength of 1000 V. The next day the electric field strength was gradually raised over the course of seven hours to a final value of 4900 V. Focusing was continued at 4900 V for a further 56 hr. The gel was stained with Coomassie Blue G-250 according to Reisner (1984). The major band is the myoglobin. The anode is at the bottom.

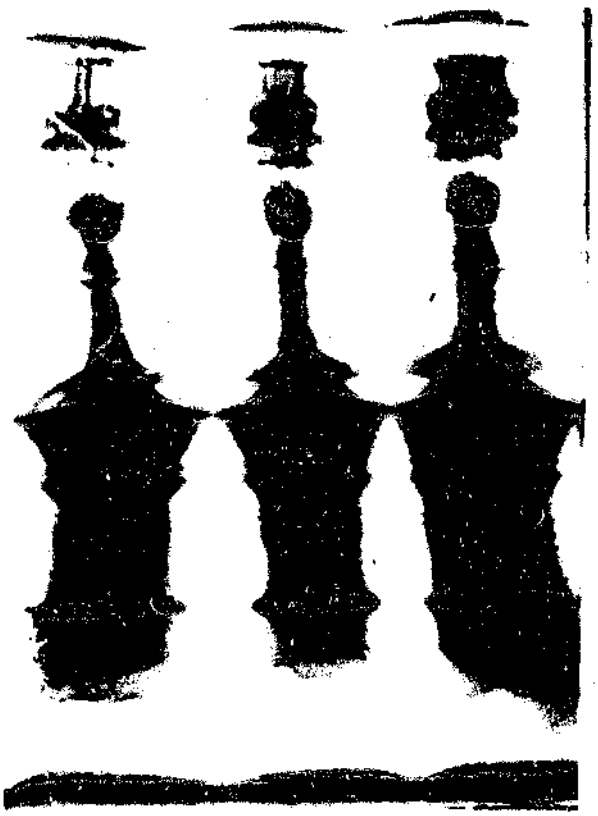


Figure 49 Focusing of myoglobin from horse heart on a pH 7.2-7.6 IPG, prepared using α-(*N*-morpholinomethyl)acrylic acid as the buffer and the p*K*, 9.3 Immobiline as the titrant, in concentrations calculated using the DoctorpH program. Filter paper electrode strips (distilled water) were applied and prefocusing of the gel performed at a maximum power of 15 W until the salt fronts had migrated into the electrode strips. The same electrode strips were used for the rest of the electrofocusing procedure. The sample protein (about 10 mg/ml of equine myoglobin in 20% glycerol) was applied to shallow wells (75 µl in each well) on the surface of the gel. Focusing was performed at 5°C for 2 hr at an initial field strength of 1300 V, which was gradually raised to a final value of 4500 V. Focusing was performed at 4500 V for a further 2 hr. The gel was stained with Coomassie Blue G-250 according to Reisner (1984). The major band is the myoglobin. The anode is at the bottom.

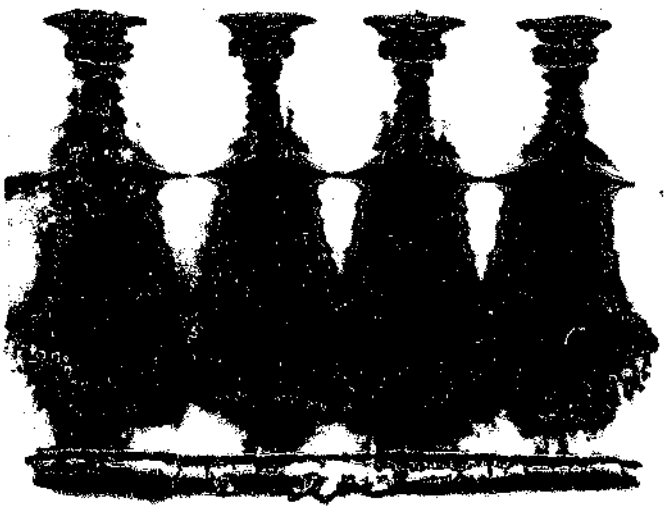


Figure 47 Focusing of myoglobin from horse heart on a pH 6.9-7.9 IPG, prepared using α-(*N*-morpholinomethyl)acrylic acid as the buffer and the p*K* 9.3 Immobiline' as the titrant, in concentrations calculated using the DoctorpH program. The procedure of electrofocusing and staining is as for Figure 48, with the difference that the myoglobin sample was loaded on long shallow wells (1 mm deep) on the gel surface. The major band is the myoglobin. The anode is at the bottom.

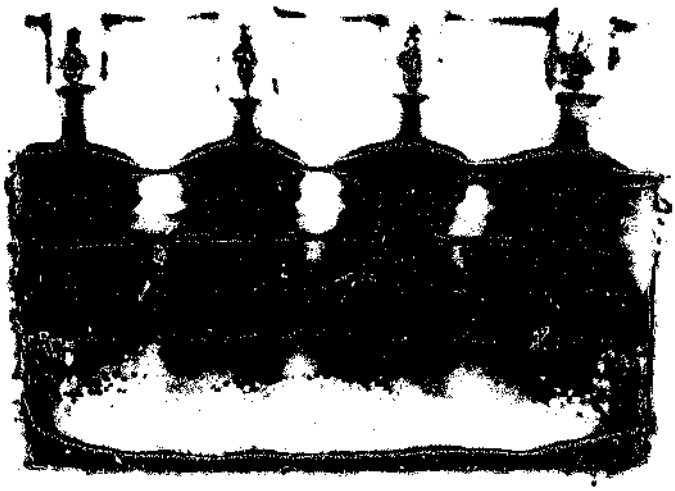


Figure 46 Focusing of myoglobin (pI 7.4) from horse heart on a pH 6.9-7.9 IPG, prepared using *α*-(*N*-morpholinomethyl)-acrylic acid as the buffer and the p*K* 9.3 Immobiline as the titrant, in concentrations calculated using the DoctorpH program. Filter paper electrode strips (distilled water) were applied and prefocusing of the gel performed at a maximum power of 15 W until the salt fronts had migrated into the electrode strips. Fresh electrode strips were then applied (anode - 10 mM glutamic acid, cathode - 10 mM histidine). The myoglobin sample was loaded on shallow wells (2 mm deep) on the gel surface as 100 µl samples of approximately 10 mg/ml solutions in 20% glycerol. Focusing was performed at 5°C for 2 hr at an initial field strength of 2000 V, which was gradually raised to a final value of 5000 V. The gel was stained with Coomassie Blue G-250 according to Reisner (1984). The major band is the myoglobin. The anode is at the bottom.

- an example of the excellent reproducibility of the IPG technique.

An even narrower IPG (pH 7.2-7.6) was prepared with the same chemicals, in order to see whether resolution of the main myoglobin band could be increased. This was indeed the case - proteins with very close pI values focusing as single bands on the pH 6.9-7.9 IPG were resolved in the pH 7.2-7.6 IPG (see Figure 48).

Finally, a pH 7.3-7.5 IPG was prepared. An extremely long focusing time was required in order to obtain sharp bands. Polypeptides differing by a little as 0.005 pH unit were successfully resolved (see Figure 49). This is an example of the possibilities of pH gradient engineering that is one of the main advantages of the IPG technique.

generated pH gradient not to be distorted by the addition of the protein sample. Hence an ampholyte such as glycine (pI = 6.1) is useless for isoelectric focusing as it hardly possesses any buffering capacity at its pI (pI-pK = 3.7).

The conductivity of focused carrier ampholytes

Moreover a poor carrier ampholyte such as glycine (pK_1 = 2.4, pK_2 = 9.8) will possess a net charge of zero from about pH 5 to 7, i.e. it will not focus in a sharp zone at a pH corresponding to its pI (6.1) but in a broad zone from pH 5 to 7. Such a distribution not only results in poor buffering of pH but also in poor conductivity of the current. If carrier ampholytes were perfectly stationary once they reached their isoelectric points, then the current would become extremely low, since only water would be carrying the current (*via* hydronium and hydroxyl ions).

However, as shown in Figure 54, a focused zone of a carrier ampholyte is in a dynamic state, with the inward electrophoretic migration of charged species balanced by the outward diffusion of ampholyte species possessing a net charge of zero. The charged forms of the ampholyte species entering their isoelectric zones either lose or gain a proton to acquire a net overall charge of zero. These neutral species become ionized once more upon diffusing into adjacent carrier ampholyte zones of slightly higher or lower pI (i.e. lose or gain a proton respectively), hence continuing the cycle. Since all the carrier ampholyte zones overlap, there is a net movement of protons throughout the whole system which carries the current. A good conductivity is essential for satisfactory migration of proteins to their isoelectric points.

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It must be stressed that *adjacent zones of focused ampholytes always overlap*. For example adjacent focused zones of aspartic acid (pH = pI = 2.8) and glutamic acid (pH = pI = 3.2) cannot be separated by a zone of pure water (pH = 7) as this would violate the law of pH monotony which states that the pH increases from the anode to the cathode when a solution of salts or ampholytes is subjected to electrolysis (Rilbe, 1976).

The concept of carrier ampholytes

Ampholytes that provide even conductivity and pH gradient stability

The main requirements of ampholytes suitable for generating pH gradients for isoelectric focusing are:

(i) a good buffering capacity at their isoelectric points, and

(ii) good conductivity in their focused states.

In other words, the ampholytes must be able to *"carry" the electrical current and the pH (i.e. to provide a stable pH gradient)*, hence the term carrier ampholyte (Rilbe, 1976; Vesterberg, 1976; Righetti, 1983).

The buffering capacity of focused carrier ampholytes

In order for an ampholyte to possess a good buffering capacity at its pI, its pI must lie within at least 1.5 pH units of the p*K* of a buffering group; preferably within 0.9 units at which there would still be 10% of the maximum buffering capacity. A good buffering capacity is required in order for the carrier ampholyte-

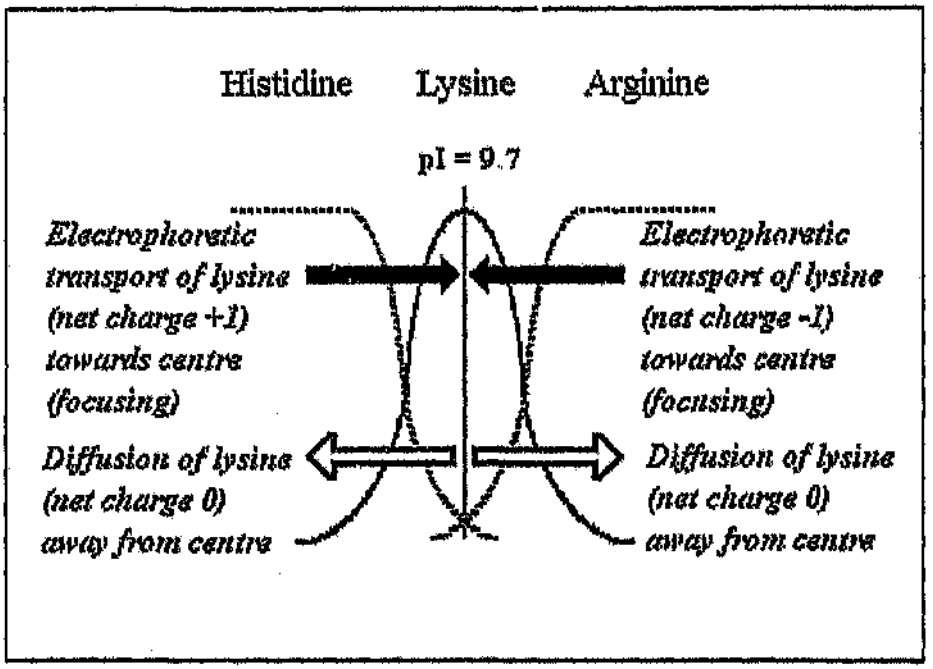


Figure 54 Illustration of a focused zone of lysine, represented as a symmetrical Gaussian peak about its pI. The electrophoretic inward migration of lysine is balanced by the outward movement due to diffusion.

histidine molecules (net charge zero) move by diffusion into the lysine zone, they will immediately become negatively charged and will migrate back into their own zone. Similarly if any lysine molecules (net charge zero) move by diffusion into the histidine zone, they will immediately become positively charged and will migrate back into their own zone. This principle is displayed in Figure 4. As can be seen, the shape of the focused lysine zone will be in the form of a Gaussian distribution about its pI, with the inward electrophoretic migration of lysine balanced by the outward diffusion of the lysine molecules.

The formation of linear pH gradients by heterogeneous mixtures of ampholytes

This concept can be extrapolated to a system comprising a complex mixture of acidic, neutral and basic ampholytes. Electrolysis of this mixture will result in an anodal migration of the acidic ampholytes and a cathodal migration of the basic ampholytes, resulting in acidification and alkalization of the anodal and cathodal regions respectively. The most acidic and the most basic ampholytes will collect at the electrodes, but all the other ampholytes will migrate until they reach an ambient pH equal to their pI values. Eventually all the ampholytes will condense in bell-shaped zones, overlapping with adjacent zones of ampholytes of slightly higher or lower pI. The pH of each ampholyte zone will be equal to its pI. Thus an even distribution of pI values from, say, 3 to 11 will result in the formation of a linear pH gradient extending from pH 3 to 11.

remain neutral, but the pH at the anodal end will rapidly fall to about 1 or 2, while the pH at the cathodal end will rise to about 12 or 13. If a salt of a *weak acid* and a *weak base*, such as Tris acetate, is subjected to electrolysis, then the pH values at the anodal and cathodal regions will not be as extreme - about 3 and 11 respectively.

If the constituents of the salt used are both *ampholytes*, for example aspartic acid and arginine, then the pH values of the anodal and cathodal regions will be equal to the pI values of aspartic acid (pI = 2.8) and arginine (pI = 11.2) respectively. If lysine (pI = 9.7) is added to this system, the lysine molecules will migrate towards the cathode. As the lysine molecules approach the cathode, they will encounter a progressively higher pH due to the buffering effect of arginine. As soon as the ambient pH rises to its pI value (9.7), the lysine molecules will possess a net charge of zero and will become electrophoretically immobile. If any lysine molecules (net charge zero) move by diffusion into the arginine zone, they will immediately become negatively charged and will migrate back into their own zone. Similarly if any arginine molecules (net charge zero) move by diffusion into the lysine zone, they will immediately become positively charged and will migrate back in o their own zone.

The focusing of ampholytes in a pH gradient

Consider what would happen if another, very weakly basic ampholyte, histidine (pI = 7.5) was added to the above system. It too would migrate towards the cathode, but would condense in a zone of pH equal to its pI (7.5) just before the more basic lysine zone (pH 9.7). If any

3. Hydrazides are unreactive towards functional groups present on proteins, such as primary amines and thiols.
4. Hydrazides react specifically and quantitatively with another functional group present on a chromatographic matrix (aldehydes), to which the functional groups present on the proteins would be unreactive[1]. Aldehyde groups can be easily introduced onto any polysaccharide chromatographic matrix by oxidation with periodic acid.

3.5.2 Background to IEF - the formation of a pH gradient

The formation of pH gradients during electrolysis

If a salt solution such as sodium sulphate is subjected to electrolysis, the sodium ions will migrate towards the anode and the sulphate ions will migrate towards the anode. Due to the law of electroneutrality, the total number of positive charges and negative charges in solution must be equal. Hence the ionization equilibrium of water will be shifted by Le Chatelier's principle so as to neutralize the negative sulphate ions at the anode (with hydronium ions, H_3O^+) and the positive sodium ions at the cathode (with hydroxyl ions, OH^-). The pH of the central part of the electrolysis chamber will

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[1] If a mixture of proteins and hydrazide group-containing carrier ampholytes are passed through such a matrix at a pH below 5, the carrier ampholytes will quantitatively bind to the matrix *via* hydrazone linkages. The proteins, however, will not bind since the adducts formed between aldehyde groups and amines or thiols are stable only under neutral or basic conditions (Ogata and Kawasaki, 1970).

that guarantees their quantitative removal[1] afterwards from the proteins of interest. For this reason the FDA has never permitted the preparative purification of pharmaceutical proteins using isoelectric focusing in carrier ampholyte-generated pH gradients. Carrier ampholytes must often be added to analytical IPG's to overcome problems such as protein precipitation and poor conductivity (see Appendix D). Hence, there is a pressing need for carrier ampholytes that can be quantitatively removed from proteins in some manner.

Up until this time all my efforts had been directed to the search for alternative monomers to the acrylamido buffers required for the IPG technique. I realised that a modification to my idea of attaching buffering groups to polysaccharide matrices might provide a potential solution to the problem of carrier ampholytes in IPG technology. Carrier ampholytes bearing hydrazide groups could be specifically and quantitatively removed from focused proteins by passing a mixture of the focused protein with the contaminating carrier ampholytes through a chromatographic matrix bearing aldehyde groups.

Hydrazides seem to be the ideal functional group for this kind of "affinity carrier ampholytes" because of their physicochemical properties (Paulsen and Stoye, 1970; Smith, 1983):

1. Hydrazides are hydrophilic.
2. Hydrazides are stable over the pH range 2 to 12.

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[1] Although carrier ampholytes can be "quantitatively" removed from proteins by techniques such as gel filtration and electrodialysis in buffers of high ionic strength (to disrupt ionic interactions), there is no guarantee that carrier ampholytes (especially the basic species) are not still bound to the proteins of interest *via* some type of hydrophobic interaction.

The problem of carrier ampholytes in IPG's

Although these technical difficulties rendered the chemical approach to the preparation of IPG's very problematic, I was loathe to abandon the idea due to the interesting concepts that had already been brought up. Perhaps other areas of electrophoresis would benefit. Some of the deficiencies of current IPG technology which require remedying are represented below:

1. **The preparation of IPG matrices is restricted to polyacrylamide matrices:** At present no other vinyl monomers capable of forming hydrogels have been tried for IPG's. The recent introduction of hydrolysis-resistant acrylamide derivatives (see Section D.4 of Appendix D) will solve a lot of the remaining problems of polyacrylamide IPG's, and will probably cement the position of acrylamides as the monomers of choice for this technique. The chemical attachment of buffering groups to gels does not have the reproducibility that copolymerization of acrylamido buffers with acrylamide gives.

2. **Carrier ampholytes are still required:** The use of preparative isoelectric membrane electrofocusing (PrIME) allows for the large-scale preparation of pure proteins suitable for use in pharmaceutical applications (Righetti *et al.*, 1988b; Wenisch *et al.*, 1992; Righetti *et al.*, 1992, Daniels and Landers, 1996). Unfortunately, a lot of proteins cannot be purified by this technique due to insolubility problems. Carrier ampholytes cannot be added to improve the solubility of proteins at their isoelectric points since there is no technique available

Among the favourable properties of hydrazides[1] are:

1. Hydrazides, like amides, are generally solids at room temperature, allowing for purification via crystallization.
2. Hydrazides are easily synthesized by reacting hydrazine hydrate with anhydrides or esters at room temperature.
3. Hydrazides are uncharged above pH 3, so the acidic and basic hydrazide buffers can be titrated against each other.
4. Hydrazides react quantitatively and rapidly with aldehydes above pH 3 to form stable hydrazone linkages. The hydrazone linkages are uncharged between pH 2 and 12, so the IPG is unaffected.

Hydrazides have already been used in biochemistry for attaching biomolecules bearing aldehyde groups to polymer supports in affinity chromatography. For example, the sugar moieties of glycoproteins can be oxidized with periodic acid to give free aldehyde groups, which can then be covalently attached to polymer supports bearing hydrazide groups (O'Shannessy and Wilchek, 1990). Ion exchange cellulose papers have been prepared by reacting charged hydrazide derivatives with periodate-oxidized cellulose filter paper (Jermyn and Thomas, 1953).

The only problem with this approach is that the degree of oxidation of the polysaccharide matrices would be rather hard to control, so that the concentration of aldehyde groups would vary quite a lot between batches. This would obviously adversely affect the reproducibility of the IPG's obtained.

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[1] The chemistry of hydrazides has been reviewed by Paulsen and Stoye (1970) and Smith (1983).

3.5 SYNTHESIS OF IMMOBILIZABLE CARRIER AMPHOLYTES SPECIFICALLY DESIGNED FOR USE IN IMMOBILIZED pH GRADIENTS

3.5.1 The evolution of the concept of immobilizable carrier ampholytes

Background

I wondered whether it would be possible to generate IPG's in a system in which buffering groups were directly attached to a polymer matrix by a chemical reaction, such as used in the field of affinity chromatography, instead of by a copolymerization reaction. Ideally such a system would make use of matrix-activating chemicals that were non-toxic, non-moisture sensitive and inexpensive.

Investigation of functional groups capable of reacting with aldehydes

Aldehyde groups can be easily introduced onto polysaccharide matrices by oxidation with periodic acid (Dyer, 1956). However, the Schiff base linkages formed upon reaction of these aliphatic aldehyde groups with amines are acid-labile. A search was made for functional groups that would react with aliphatic aldehydes to form stable, non-chromophoric products. Hydrazides seemed to be the functional group of choice for attaching buffering groups to oxidized polysaccharide matrices *via* their aldehyde groups.

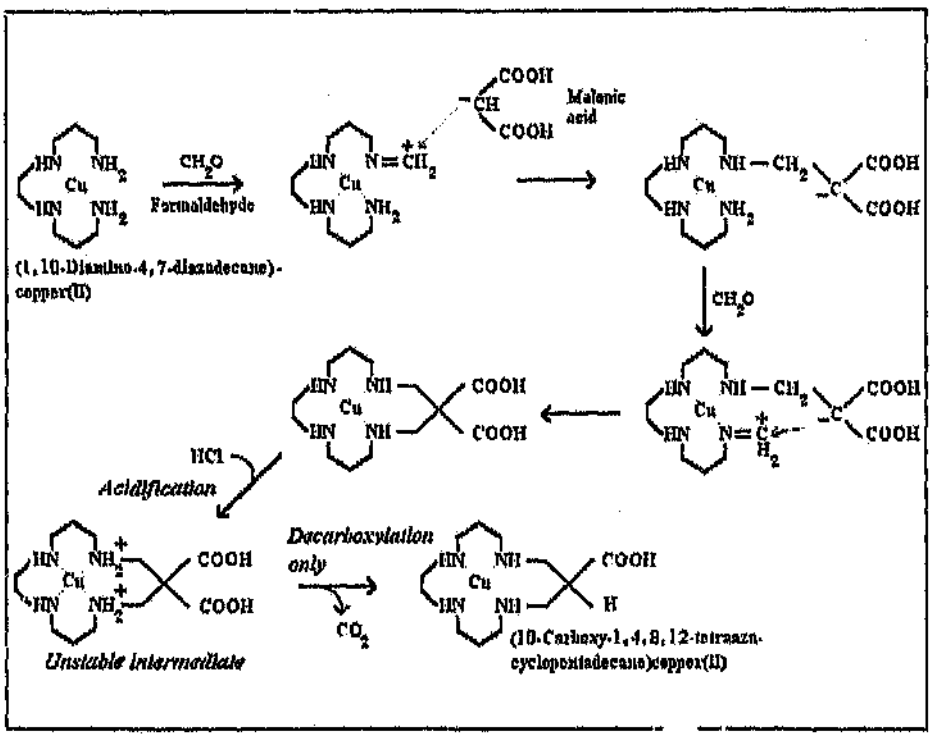


Figure 53 Result of attempted synthesis of 14-amino-2-carboxy-4,8,11-triazatetradec-1-ene.

bis(3-carboxyacryloyl)hydrazine caused it to hydrolyse to maleic acid and MAMH, which however spontaneously underwent a thermally induced cyclization (even in aqueous solution) *via* an internal condensation to maleic hydrazide. However, I hoped that the Michael reaction of MAMH with PEHA would be fast enough to avoid the necessity of heating the reaction mixture; especially since 1,2-bis(3-carboxyacryloyl)hydrazine had been previously shown to react readily with amines (Liu *et al.*, 1974b).

Before attempting the synthesis of hydrazide group-containing carrier ampholytes on a preparative scale, I decided to measure the rate of reaction at room temperature of MAMH with polyamines in methanol and in water. It was found that a red-shift in the UV absorbance spectrum of MAMH occurred upon transition from a water to a methanol environment[1] (A_{max} of MAMH in methanol = 320 nm), as shown in Figure 58.

The rate of reaction of MAMH with TEPA at room temperature was unfortunately found to be very low for both the water and methanol solvents - the reaction had still not reached completion after 500 hr (see Figures 59-61). Hence it appeared that heating of the reaction mixture was unavoidable for the practical large-scale preparation of hydrazide-containing carrier ampholytes - I hoped however that the thermal cyclization of MAMH would be slower than the Michael reaction with PEHA.

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[1] According to Mashima and Ikeda (1972) the degree of hydrogen bonding of the hydrazide group with the solvent affects the electron-donating effect by the electron pairs of the hydrazine on the π electrons of the double bond, and hence leading to a shift in the UV absorbance spectrum.

The removal of the various protective groups leaves a carbazic acid derivative R-NH-NHCOOH, which is immediately hydrolysed in aqueous solution to a hydrazide and CO_2. However the carbazate anion is relatively stable in aqueous solutions at low temperature and high alkalinity (Frahn and Mills, 1964; Caplow, 1968).

I noticed that the evolution of CO_2 upon acidification of solutions of carbazate salts in organic solvents was very slow, due to the fact that there were few water molecules present to react with the carbazic acid formed. It occurred to me that, provided the reaction mixture was kept cool and as anhydrous as possible, the synthesis of the carbazic derivative of MAMH (HOOC-CH=CH-CONHNH-COOH) could be accomplished by the direct reaction of maleic anhydride with carbazic acid, since the synthetic reaction would be much faster than the hydrolysis reaction of carbazic acid. The carbazic acid derivative of MAMH could in turn be hydrolysed to MAMH (HOOC-CH=CH-CONHNH$_2$) and CO_2 by the addition of water. The product obtained by this reaction was found to be indeed MAMH, as shown from the ^1H and ^{13}C NMR spectra (see p.80). This novel and simple method for the synthesis of mono *N*-substituted hydrazides seems not to have been used before.

The Michael reaction of maleic acid monohydrazide with polyamines

Feuer *et al.* (1958) discovered that reacting maleic anhydride and hydrazine at room temperature gave 1,2-bis(3-carboxyacryloyl)hydrazine. However, heating maleic anhydride and hydrazine, or heating 1,2-bis(3-carboxyacryloyl)hydrazine, gave maleic hydrazide (1,2-dihydro-3,6-pyridazinedione). It was reasoned that heating 1,2-

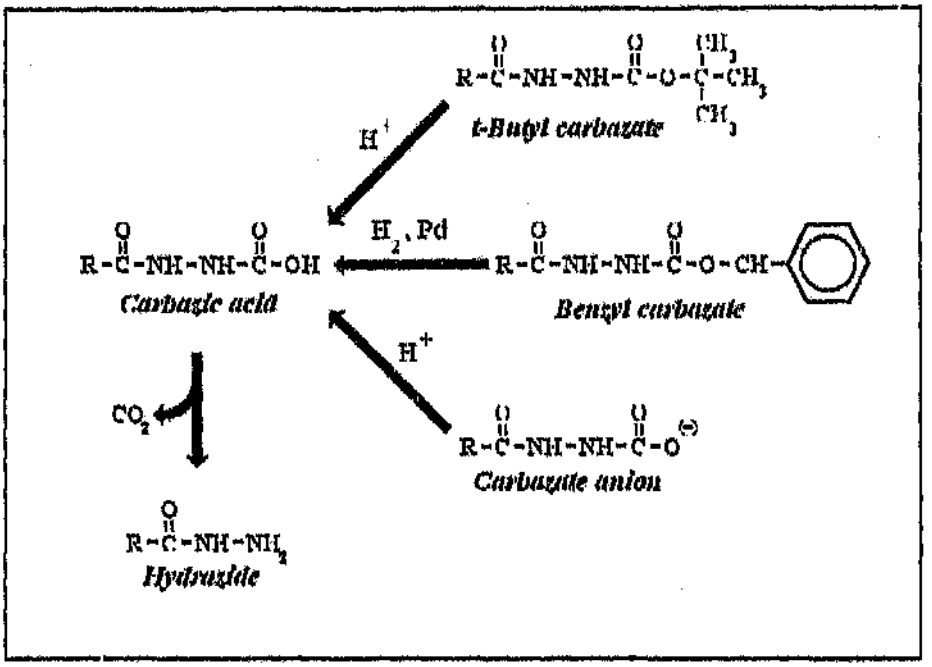


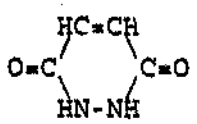
Figure 57 Different methods of protecting of one of the nitrogen atoms of hydrazine so as to get acylation of only one nitrogen by maleic anhydride.

The synthesis of maleic acid monohydrazide

A comprehensive search of *Beilstein* and *Chemical Abstracts* revealed no published syntheses of maleic acid monohydrazide[1] (MAMH). The normal products of the reaction between maleic anhydride and hydrazine are 1,2-dimaleic acid hydrazine (Feuer *et al.*, 1958):

HOOC-CH=CH-CO-HN-NH-CO-CH=CH-COOH

and maleic hydrazide (1,2-dihydro-3,6-pyridazinedione) (pp. 130-131 of Flett and Gardner, 1952; Feuer *et al.*, 1958):



The reaction of maleic anhydride with hydrazine is complicated by the fact that acylation of both the nitrogens of hydrazine can occur. Therefore it is necessary to protect one of the nitrogens in some way, as demonstrated in Figure 57. Protection of one of the hydrazine nitrogens can be achieved using the tert-butyloxycarbonyl, benzyloxycarbonyl or 9-fluorenylmethyl-oxycarbonyl groups which are the principal means used of protecting amine groups in peptide synthesis (Gordon *et al.*, 1980).

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[1] A synthesis of MAMH by the reaction of hydrazine hydrate with the monomethyl ester of maleic acid was published by Barnett and Morris (1946). However, repetition of their protocol showed the true product of this reaction to be methyl 3-carboxy-3-hydrazino-propanoate.

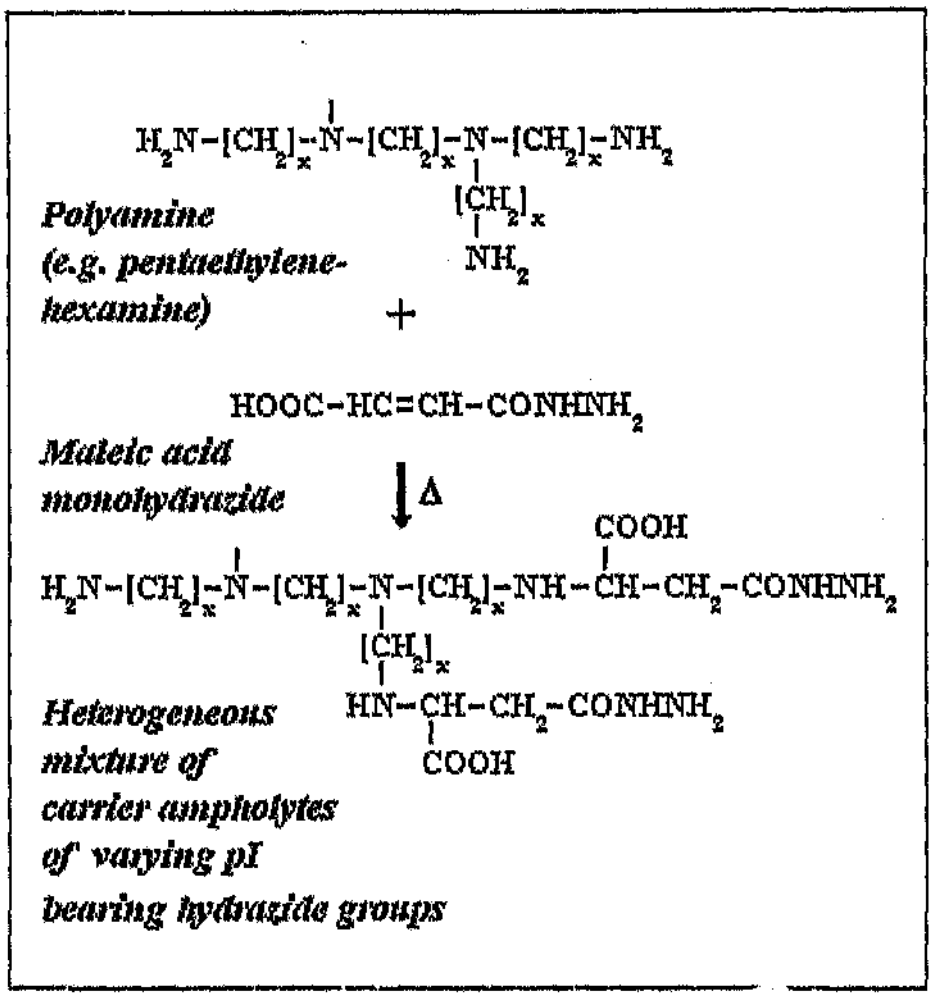


Figure 56 Attempted synthesis of carrier ampholytes bearing hydrazide groups by reacting polyamines such as PEHA with maleic acid monohydrazide.

3.5.5 The synthesis of carrier ampholytes bearing hydrazide groups

The reaction of PEHA with maleic acid monohydrazide

Synthetic approach

The Michael reaction is the addition of nucleophilic reagents to a double bond that has been polarized by a neighbouring electron-withdrawing group, e.g. α,β-unsaturated carbonyl compounds such as acrylates and acrylonitriles (Friedman and Wall, 1966; Shenhav *et al.*, 1970). *N*-Alkylation of amines by a Michael-type addition with maleic acid also occurs, but prolonged heating is necessary, as the symmetrical arrangement of the carboxylic acid groups on either side of the double bond renders the double bond relatively unpolarized and hence not very reactive (van Westrenen *et al.*, 1990). The polarity (and hence reactivity) of maleic acid can be increased by converting one of the carboxylic acids to an ester or an amide (Zilkha and Bachi, 1959). The Michael reaction of amines with derivatives of maleamic acid ($HOOC\text{-}CH{=}CH\text{-}CONH_2$) has been used for the synthesis of substituted asparagines (Liwschitz *et al.*, 1956; Zilkha and Bachi, 1959; Liu *et al.*, 1974*a*). I wondered whether the hydrazide counterpart of maleamic acid, maleic acid monohydrazide ($HOOC\text{-}CH{=}CH\text{-}CONHNH_2$), would react similarly with polyamines such as PEHA as a facile means of synthesizing carrier ampholytes bearing hydrazide groups, as portrayed in Figure 56.

The design of carrier ampholytes that could be easily separated from polypeptides

A logical answer to this last problem is to synthesize carrier ampholytes modified in some way that allows their facile removal from small polypeptides. The obvious approach is to increase the size of the carrier ampholytes. Righetti and Hjertén in 1981 synthesized high molecular mass carrier ampholytes (50-90 kDa) by reacting polyethylenimine (40-60 kDa) with a linear gradient of acrylic acid in a flow-through system. The giant carrier ampholytes appeared to interact only weakly and reversibly with polypeptides, and proved useful in the preparative fractionation of model peptides, allowing recoveries up to 85% with no contamination of the recovered peptides. However these high molecular mass species had a severe drawback: they tended to aggregate by ionic interactions (multipoint attachment) giving heavy precipitates, especially among acidic and alkaline compounds.

Carrier ampholytes bearing special functional groups which interact quantitatively with a derivatized chromatographic matrix seem to be a more promising approach. This would allow facile purification of polypeptides of any molecular mass from contaminating carrier ampholytes *via* a kind of affinity chromatography. Hydrazides, as explained previously, seem to fulfill all the necessary criteria.

3.5.4 The synthesis of carrier ampholytes specifically designed for use in immobilized pH gradients

Problems experienced with the removal of carrier ampholytes from polypeptides

Various techniques have been used to separate the carrier ampholytes from the focused proteins after preparative isoelectric focusing (Righetti, 1983). Since carrier ampholytes have a M_r below 1000, it is possible to separate them from proteins *via* techniques such as gel filtration and electrodialysis, provided that the buffer used is of a high ionic strength (0.1 - 0.5 M) in order to disrupt weak complexes which might form between carrier ampholytes and proteins by electrostatic interactions. Carrier ampholytes are soluble in concentrated solutions of ammonium sulphate (as high as 100% saturated) at which most proteins precipitate, hence allowing their removal. Hydrophobic interaction chromatography and mixed-bed ion exchange chromatography have also been successfully used.

However, none of the above techniques work for small polypeptides (M_r < 1500) which include many important biomolecules such as growth factors, hormones such as oxytocin, endorphins, neuropeptides and enzyme inhibitors. This is because carrier ampholytes and small polypeptides have quite similar physicochemical properties, both in terms of net charge and mass, making them almost impossible to separate from each other.

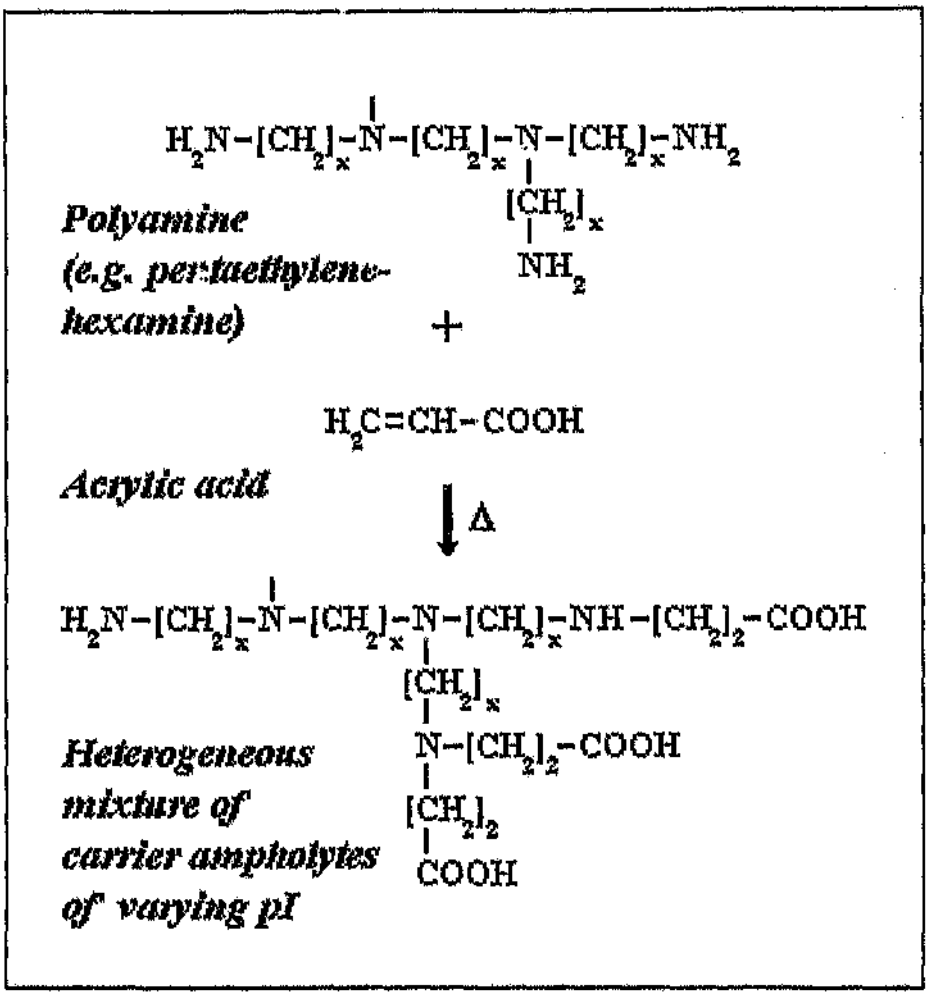


Figure 55 Synthesis of carrier ampholytes by reaction of a polyamine with acrylic acid to produce a highly heterogeneous mixture of polyamino-polycarboxylic acid buffers of varying pI.

Table 16: Dissociation constants for polyamines (from Smith and Martell, 1975)

Amine	pK_1	pK_2	pK_3	pK_4	pK_5	Temp (°C)
Ethylenediamine	7.1	9.9				25
Diethylene-triamine	4.2	9.1	9.8			25
Triethylene-tetramine	3.3	6.6	9.1	9.7		25
Tetraethylene-pentamine	3.0	4.7	8.1	9.1	9.7	25

The dissociation constants given in Table 16 are for the linear isomeric forms of the polyamines. Obviously other isomeric forms will have slightly different values. Vesterberg determined *via* titration that pentaethylene-hexamine (PEHA) has the more even distribution of p*K* values over the p*H* range 3 to 10 than tetraethylene-pentamine (TEPA). This is important as it ensures carrier ampholytes possessing good buffering capacity and conductivity at their isoelectric points (pI-p*K* < 1.5). Moreover, PEHA possesses more isomeric forms than TEPA - analysis of commercial polyamine preparations (Bergstedt and Widmark, 1970) has shown 20 isomeric forms of PEHA to be present (out of a possible 23) compared to only 12 for TEPA. This is important because it increases the heterogeneity of the final carrier ampholyte product.

(iv) minimal degree of interaction with proteins, ie. no formation of stable complexes

(v) low UV absorbance (for preparative column isoelectric focusing)

(vi) low molecular mass, facilitating subsequent separation *via* gel filtration or electrodialysis from focused protein of interest.

The synthesis of heterogeneous mixtures of carrier ampholytes

An ingenious synthesis of carrier ampholytes meeting the above criteria was developed by Vesterberg in the 1960's (Vesterberg, 1969) and was subsequently elaborated by LKB into their Ampholine' range of carrier ampholytes (Haglund, 1975; Vesterberg, 1976; Righetti, 1983). Vesterberg noticed that polyamines containing four or more amino groups have p*K* values distributed throughout the pH range 3 to 10, as shown in Table 16. By coupling to these polyamines one or more residues containing a carboxylic acid group, many ampholytes with different pI values can be obtained. After much experimentation, it was found that reacting acrylic acid with polyamines using a 1:2 ratio of carboxylic acid groups to amine groups would give a highly heterogeneous mixture of carrier ampholytes with pI values from 3 to 10.

Subsequent published protocols for the synthesis of carrier ampholytes have usually just been slight modifications of the above concept (Vinogradov *et al.*, 1973; Righetti *et al.*, 1975; Just, 1980; Binion and Rodkey, 1981; Just, 1983; Righetti, 1983). The protocol for the synthesis of carrier ampholytes for IEF is displayed diagrammatically in Figure 55.

The necessity for carrier ampholytes with closely-spaced pI values

In order for the conductivity across the system to be even, there must be a good deal of overlapping between adjacent focused carrier ampholyte zones, ie. the carrier ampholytes must have closely-spaced pI values. It may be added here that water may also be considered as an ampholyte, although an extremely poor one, with a pI of 7. Thus in order to prevent the formation of a zone of pure water at pH 7 (which would have an extremely low conductivity compared to the rest of the system, leading to a local increase in electric field strength with subsequent overheating), it is essential to have good carrier ampholytes present with pI values as close to 7 as possible.

3.5.3 The synthesis of carrier ampholytes in general

The required physicochemical properties of carrier ampholytes

The required properties of carrier ampholytes suitable for isoelectric focusing of proteins may be summarized thus:

(i) good buffering and current-carrying capacity at the pI, i.e. $(pI-pK) < 1.5$,

(ii) highly heterogeneous mixture of carrier ampholytes required with closely spaced pI values,

(iii) carrier ampholytes must be hydrophilic and must possess high solubility at their isoelectric points,

4.1.2 Conclusions

The general rules given by Williams and Reisfeld (1964) are inadequate for the design of new discontinuous buffer systems - it is necessary to calculate the necessary ionic parameters from first principles, using the isotachophoretic steady state equations. However, theoretical papers discussing these steady state equations are generally too detailed to be readily assimilated by non-experts in the field of ITP (Everaerts and Routs, 1971; Jovin 1973*a,b,c*; Schafer-Nielsen *et al.*, 1980). Although some authors have described computer programs for the design of isotachophoretic systems (Schafer-Nielsen et al., 1980), to my knowledge copies of these programs have never been published.

I have therefore, for the benefit of the reader, given in Appendix B a comprehensive treatment of the isotachophoretic steady state equations, preceded in Appendix A by a general introduction to the basic equations of electrophoresis. The steady state equations are applied in Appendix C to the analysis of the Ornstein-Davis and Tamura-Ui discontinuous buffer systems. These applied equations can be made use of for the design, from first principles, of new discontinuous buffer systems.

Chapter 4 - Conclusions

4.1 THE DESIGN OF NEW DISCONTINUOUS BUFFER SYSTEMS

4.1.1 Recapitulation

The stacking effect in discontinuous electrophoresis is based on isotachophoretic principles, in which the sample ions are focused in a moving boundary formed between a leading ion of high electrophoretic mobility, and a terminating ion of low electrophoretic mobility. The net ionic mobility of the terminating ion is dictated by its net ionic charge, and hence by the pH of the terminating zone. The ionic parameters of the terminating zone are set by those of the leading zone, by a relationship known as the Kohlrausch regulating function (Ornstein, 1964). In order to calculate the exact ionic parameters, the equations governing the mass balance of the buffering counterion, and the electroneutrality equations must also be taken into account (Everaerts and Routs, 1971; Everaerts *et al.*, 1973; Jovin 1973a,b,c; Schafer-Nielsen *et al.*, 1980).

An anionic alkaline discontinuous buffer system designed by Tamura and Ui (1972) using a general guideline given by Williams and Reisfeld (1964) did not succeed in stacking rat and fruit bat LDH-5 isozymes, even though the stacking pH was high enough to ensure that the LDH-5 isozymes possessed a net negative charge. Calculation of the ionic parameters in the terminating zone using isotachophoretic steady state equations showed that the ionic mobility of the glycine terminating ion to be too high.

Possible alternative routes to the synthesis of "affinity" carrier ampholytes

As no simple and inexpensive methods of protecting the hydrazide groups could be found, it was decided to abandon the synthesis of hydrazide-containing carrier ampholytes, thus bringing to an end my research work in the field of immobilized pH gradients. It is possible that alternative functional groups may be found in future investigations which allow for the synthesis of "affinity carrier ampholytes".

An alternative, yet more elegant approach may be the synthesis of superparamagnetically-labelled carrier ampholytes. These could be instantaneously and quantitatively separated from peptides after focusing by placing the mixture in a magnetic field. Moreover these superparamagnetic carrier ampholytes could be recovered and reused indefinitely, making them extremely cost-effective in the long term, even if they are initially expensive to synthesize.

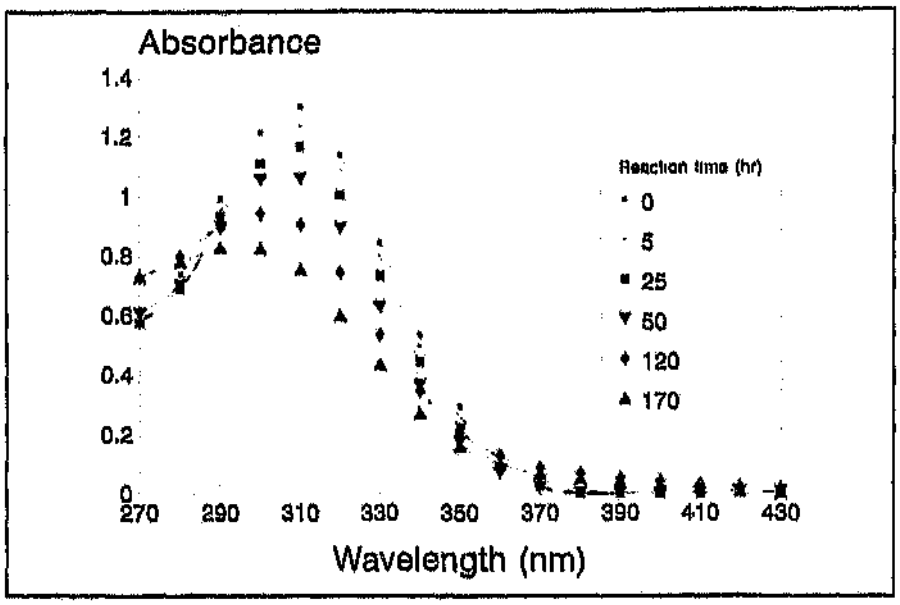


Figure 63 Reaction of MABH with PEHA in methanol at room temperature by following the decrease in A_{310}.

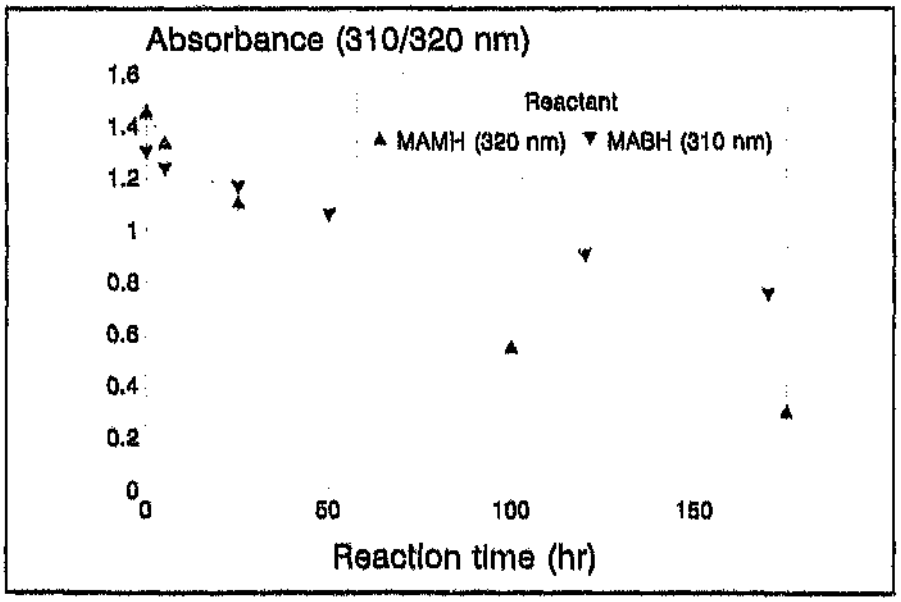


Figure 64 Comparison of the rates of reaction of MAMH and MABH with a polyamine at room temperature in methanol.

The reaction of MABH with PEHA

Before attempting the large scale synthesis of hydrazide-containing carrier ampholytes, the rate of reaction of MABH with PEHA in methanol at room temperature was investigated. A reaction did indeed occur, but at a very slow rate - notably slower than the rate of reaction of MAMH with TEPA (see Figures 63 and 64). This may have be due to steric hinderance caused by the benzylhydrazone groups which made it difficult for additional molecules of MABH to react with the polyamine. However, this was not considered a problem as extended heating of the reaction mixture was now possible due to the protection of the hydrazide groups.

Attempted synthesis of hydrazide-containing carrier ampholytes

MABH and PEHA (1:2 ratio of carboxylic acid groups to amine groups) were refluxed in methanol for 20 hr. Unfortunately, subsequent debenzylation of the hydrazide groups could not be effected by the Pd-formate reagent, even on prolonged reaction.

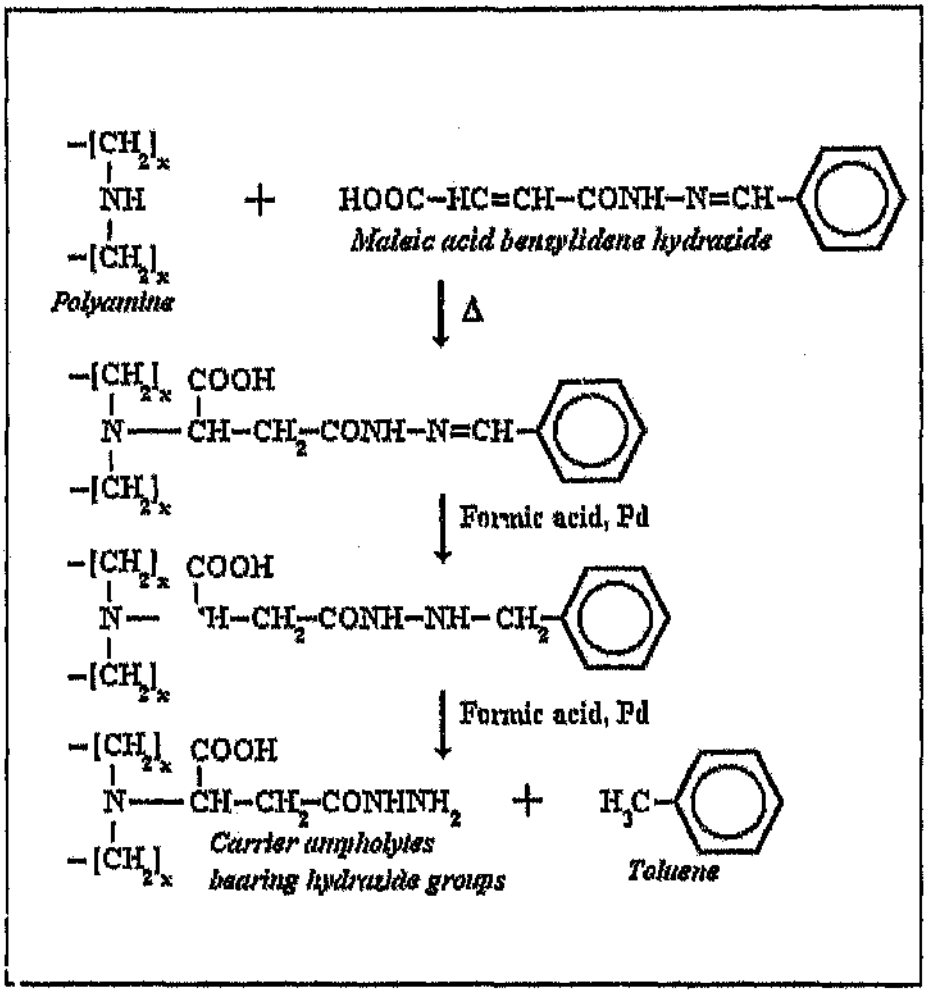


Figure 62 Attempted synthesis of carrier ampholytes bearing hydrazide groups by reacting polyamines such as PEHA with MABH. (The hydrazide group is protected in the form of its benzyl hydrazone.)

blocking groups are generally used to protect hydrazides as are used for amines (Gordon et al., 1980).

Hydrazide groups react rapidly and quantitatively with benzaldehyde to form the corresponding benzyl hydrazones. These in turn can be reduced to give *N'*-benzylhydrazides. It occurred to me that the same catalytic transfer hydrogenation with Pd-formate could be used to effect consecutively both the reduction of the benzyl hydrazones to their corresponding *N'*-benzylhydrazides, and the subsequent debenzylation of the *N'*-benzylhydrazides to their corresponding hydrazides. Hence, it was reckoned that hydrazide-containing carrier ampholytes could be simply and elegantly synthesized by reacting the pre-synthesized benzyl hydrazone derivative of MAMH (maleic acid benzylidene hydrazide) with PEHA, followed by catalytic transfer hydrogenolysis (Figure 62).

The synthesis of maleic acid benzylidene hydrazide

Benzylhydrazone was facilely synthesized by the reaction of benzaldehyde with an excess of hydrazine hydrate according to the method of Pross and Sterrhall (1970). The reaction of benzylhydrazone with maleic anhydride in chloroform gave a quantitative yield of maleic acid benzylidene hydrazide (MABH). MABH was found to be insoluble in water and in most organic solvents in its free acid form (Snyder et al., 1938), but dissolved in aqueous and alcoholic solutions that had been previously alkalized, eg. by the addition of polyamines. This was fortunate as it allowed for the reaction of MABH with PEHA in methanol, the preferred solvent for catalytic transfer hydrogenolysis (Adger et al., 1987; Ram and Spicer, 1987).

This cyclization reaction is surprising considering that Feuer *et al.* (1951) were unable to prepare the cyclic form of succinic hydrazide upon prolonged reaction of hydrazine hydrate with equimolar amounts of succinic anhydride, diethyl succinate or succinimide. Hence some means of protection of the hydrazide group was considered necessary, since there seemed to be no other way of avoiding the cyclization side reaction.

The reaction of PEHA with maleic acid benzylidene hydrazide

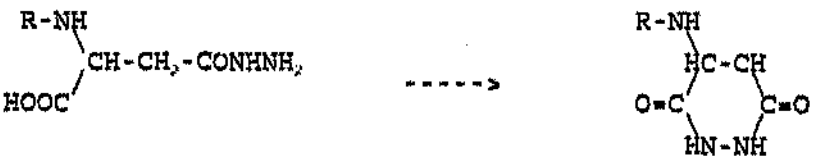
The search for a simple and economical means of protecting the hydrazide groups

Hydrazides may be protected with the protecting groups customarily used in peptide synthesis for the protection of amines (Gordon *et al.*, 1980). Unfortunately these protection methods require the use of expensive reagents, which would render the synthesis of hydrazide-containing carrier ampholytes far too costly. Therefore a search was made for a simple and inexpensive means of protecting the hydrazide group of MAMH.

A common method of protection of amines is in the form of their *N*-benzyl derivatives. Debenzylation is effected by hydrogenolysis, eg. *via* catalytic transfer hydrogenolysis using formate as the *H*-donor and palladium as the catalyst (ElAmin *et al.*, 1979; Anwer and Spatola, 1980; Adger *et al.*, 1987; Pam and Spicer, 1987). However, a meticulous search of the chemical literature failed to show examples of hydrazides protected in this way. Nevertheless, I decided to attempt it since the same

Attempted synthesis of hydrazide-containing carrier ampholytes

The reaction between MAMH and PEHA (1:2 ratio of carboxylic acid groups to amine groups) in water at 60°C was followed by diluting an aliquot of the reaction mixture in methanol and measuring the decrease in absorbance at 320nm. The absorbance at 320 nm was found to decrease very rapidly until it reached 20% of its original value, at which it then stayed constant over time. This indicated that roughly 80% of the MAMH underwent a Michael reaction with PEHA - the rest undergoing cyclization to maleic hydrazide, which also absorbs strongly at 320 nm[1]. However, the final pH of the reaction mixture was found to be over 9, whereas a neutral to slightly acidic pH was expected[2]. The only probable reason is that condensation of the carboxylic acid and hydrazide groups of the carrier ampholyte molecules occurred to give cyclic succinic hydrazides:



[1] Maleic hydrazide is a weak acid with a p*K* of 5.65. The free acid has an A_{max} at 300 nm, and the ionized form has an A_{max} at 340 nm (Miller and White, 1956).

[2] Apart from the 1:2 ratio of carboxylic acid groups to amino groups which lower the pH of the reaction mixture below 8 (acidity of MAMH expected to be similar to that of maleamic acid, which has a p*K* of 3.6 - Dahlgren and Simmerman, 1965), the reaction of MAMH with PEHA would lead to a further decrease in the pH of the reaction mixture. This is because the electron-withdrawing properties of the hydrazide groups would lower the basicity of the PEHA amine groups.

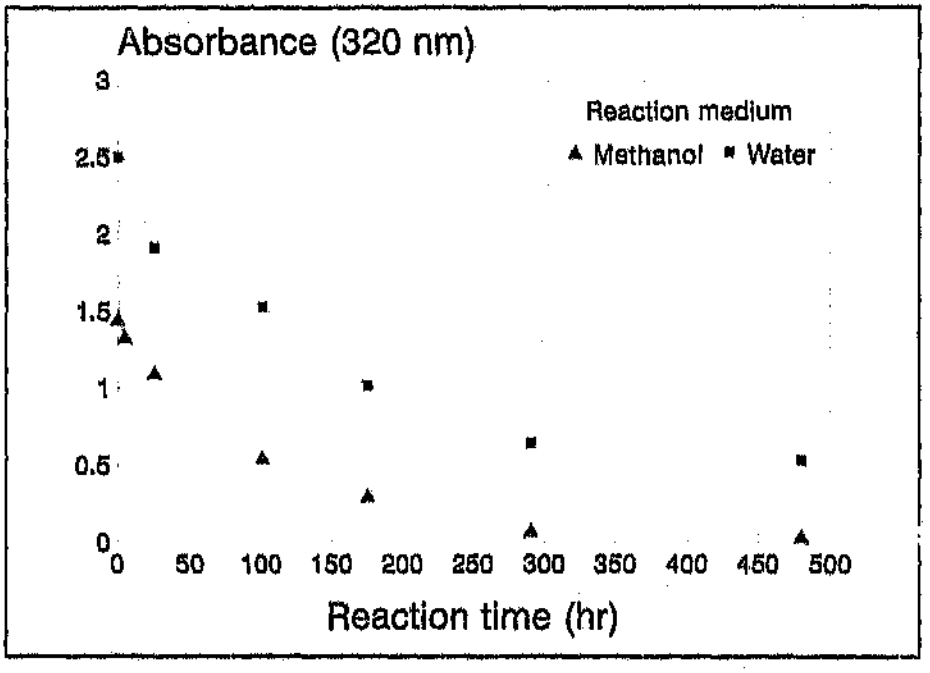


Figure 61 Comparison of the rates of reaction of maleic acid monohydrazide with TEPA at room temperature in methanol and water.

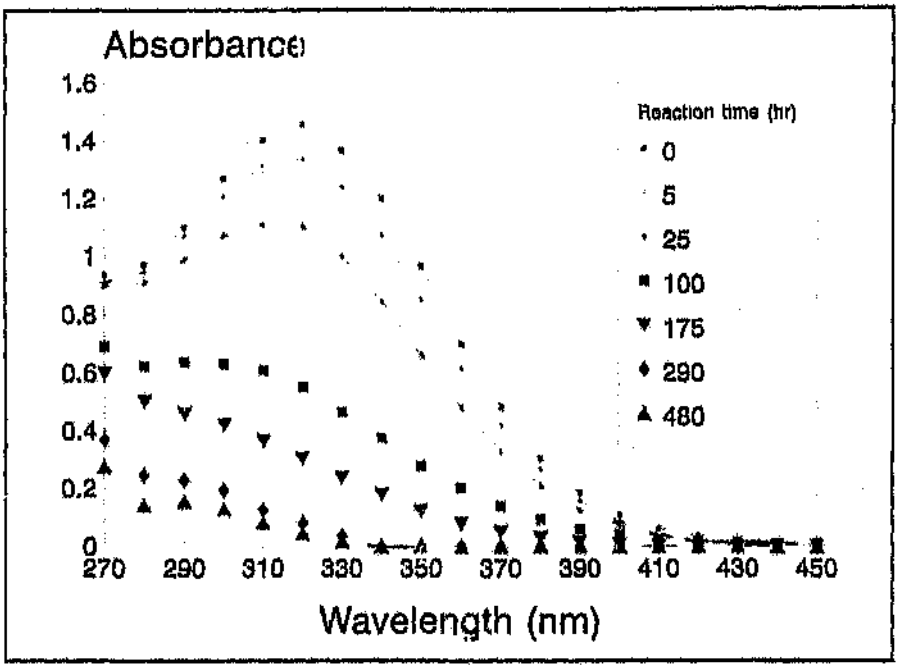


Figure 59 Reaction of maleic acid monohydrazide with TEPA at room temperature in methanol followed by measuring the decrease in A_{420}.

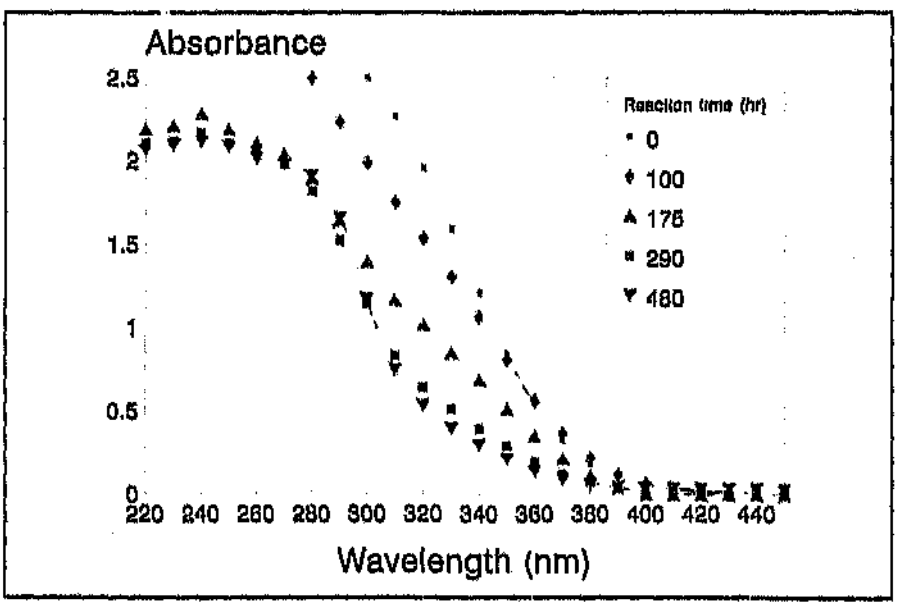


Figure 60 Reaction of maleic acid monohydrazide with TEPA at room temperature in water followed by measuring the decrease in A_{420}.

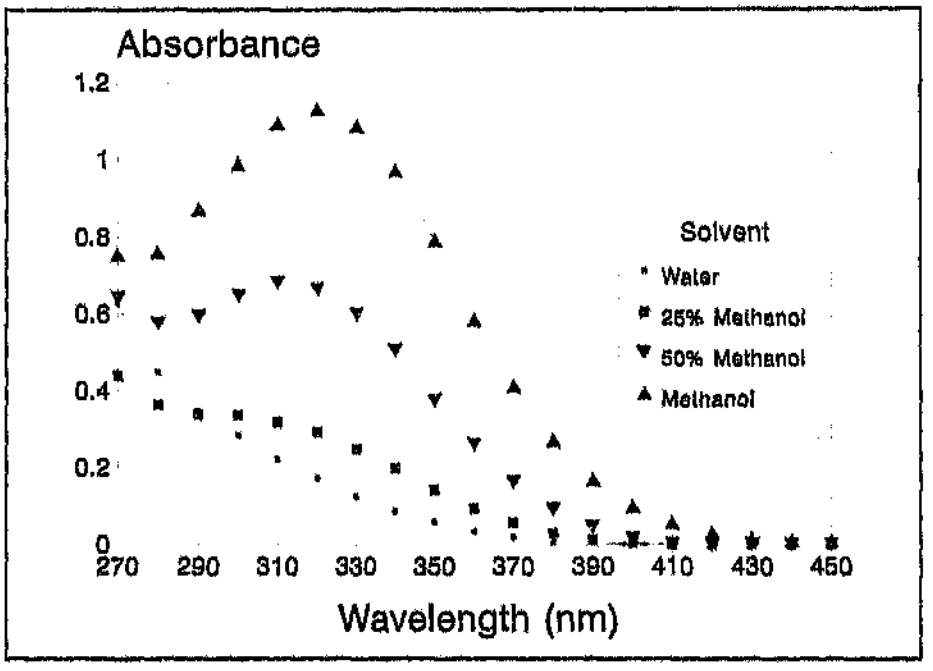


Figure 58 Shift in the UV absorbance spectrum of maleic acid monohydrazide on going from a water to a methanol environment.

v_i Linear velocity of ionic constituent i. Signed quantity, of the same sign as charge of i ($cm \cdot s^{-1}$).

$v_{i,j}$ Linear velocity of ionic subspecies i^j. Signed quantity, of the same sign as charge of i, ($cm \cdot s^{-1}$).

$\bar{v}_i$ Net linear velocity of ionic constituent i, representing the average of the individual linear velocities of the various subspecies of i. Signed quantity, of the same sign as the charge of i ($cm \cdot s^{-1}$).

V Potential difference (V).

z_i Charge or valency of ionic subspecies i.

Z_i Maximum charge or valency of ionic subspecies i.

η Viscosity of a solution (centipoise, cP).

κ Conductivity or specific conductance ($S \cdot cm^{-1}$).

Λ Molar conductivity ($S \cdot cm^2 \cdot mol^{-1}$) or equivalent conductivity ($S \cdot cm^2 \cdot equiv^{-1}$).

ρ Resistivity or specific resistance ($\Omega \cdot cm$).

F Faraday constant where $F = eN_A$ (9.65×10^4 $C \cdot mol^{-1}$).

i Arbitrary ionic species which may exist in solution as an ensemble of subspecies of various valency.

i^z Subspecies of ionic constituent i of valency z.

L Length (cm).

m_i Ionic mobility of ionic constituent i. Signed quantity, given the same sign as the charge of i ($cm^2 \cdot V^{-1} \cdot s^{-1}$).

$m_{i,z}$ Ionic mobility of ionic subspecies i^z. Signed quantity, given the same sign as the charge of i, ($cm^2 \cdot V^{-1} \cdot s^{-1}$).

$\overline{m}_i$ Net or effective ionic mobility of ionic constituent i, representing the average of the individual ionic mobilities of the various subspecies of i. Signed quantity, of same sign as charge of i ($cm^2 \cdot V^{-1} \cdot s^{-1}$).

n Number of different kinds of ionic species in solution.

N_A Avogadro's number (6.02×10^{23} mol^{-1}).

Q Electric charge (C).

R Resistance (Ω).

t Time (s).

Appendix A - Introduction to the physical chemistry of electrophoresis[1]

List of symbols

A	Cross-sectional area (cm^2).
c_i	Concentration of the ionized fraction of ionic constituent i ($mol \cdot cm^{-3}$).
$c_{i,z}$	Concentration of the ionic subspecies i of valency z ($mol \cdot cm^{-3}$).
c_i^{total}	Total concentration of ionic constituent i ($mol \cdot cm^{-3}$).
e	Charge of an electron (1.60×10^{-19} C).
E	Electric field strength ($V \cdot cm^{-1}$).
$\mathcal{F}$	Force (N).
f	Frictional coefficient.

[1] An in-depth discussion of the physical chemistry of electrophoresis may be found in Longsworth (1959a) and Rilbe (1983). However, nearly all the review articles dealing with the physical theory of electrophoresis, and of ITP in particular, are quite advanced and presume the reader to be already familiar with the equations governing ion motion in electric fields. Thus it has seemed a good idea to me to include a chapter on the basic theory of electrophoresis as an introduction to the following chapter on the physical chemistry of ITP. The core of the material in this chapter has been extracted from Atkins (1990), but has been substantially augmented with additions from other sources to improve the intelligibility of the concepts involved.

An alternative, yet more elegant approach may be the synthesis of superparamagnetically-labelled carrier ampholytes. These could be instantaneously and quantitatively separated from peptides after focusing by placing the mixture in a magnetic field. Moreover these superparamagnetic carrier ampholytes could be recovered and reused indefinitely, making them extremely cost-effective in the long term, even if they were expensive to synthesize.

protein/carrier ampholyte mixture through a chromatographic matrix bearing aldehyde groups.

Carrier ampholytes are normally synthesized *via* the Michael reaction of polyamines such as PEHA with acrylic acid. The Michael reaction of amines can also be performed with derivatives of maleamic acid (HOOC-CH=CH-$CONH_2$) (Liwschitz *et al.*, 1956; Zilkha and Bachi, 1959; Liu *et al.*, 1974a). I wondered whether the hydrazide counterpart of maleamic acid, maleic acid monohydrazide (HOOC-CH=CH-$CONHNH_2$), would react similarly with polyamines such as PEHA as a facile means of synthesising carrier ampholytes bearing hydrazide groups. However cyclization of maleic acid monohydrazide to 1,2-dihydro-3,6-pyridazinedione was found to occur during the carrier ampholyte synthesis. A simple and inexpensive means of protecting the hydrazide group of maleic acid monohydrazide was searched for, but one could not be found that worked.

4.4.2 Conclusion

It is possible that alternative functional groups may be found in future investigations which allow for the synthesis of "affinity carrier ampholytes". However it is difficult to find functional groups that interact specifically with another functional groups and none other. This task is complicated by the plethora of different functional groups present in proteins - apart from the normal functional groups that are present on amino acid sidechains - carboxylic acids, amides, amines, hydroxyls, phenols, indoles and thiols - post-translational modifications of polypeptides also introduces groups such as phosphates and carbohydrate moieties.

hydrophilic, as well as being stable in the presence of alkali. (The amide group of the basic Immobilines is susceptible to alkaline hydrolysis.) The α-aminomethylated acrylic acids can also be used in the acidic pH range, due to the buffering effect of their carboxylic acid groups.

However, α-aminomethylated acrylic acids cannot be used in wide-range IPG's (eg. pH 3-10), since, in order to obtain a linear pH gradient, the concentration of each immobilized buffer must be independently optimized with the help of computer programs such as DoctorpH. The concentrations of the two buffering groups of α-aminomethylated acrylic acids must necessarily always be the same, and cannot be independently optimized as is possible for monoprotic immobilized buffers. However, it could be possible to form linear wide-range IPG's with the use of a single polyprotic immobilized buffer. Problems might be experienced, though, with overswelling of the IPG gels, due to the high local ionic strength, as did occur with BCPP.

4.4 ATTEMPTED SYNTHESIS OF CARRIER AMPHOLYTES BEARING HYDRAZIDE GROUPS

4.4.1 Recapitulation

It was noted that carrier ampholytes were often required to be present in IPG's to improve the conductivity of the system, and maintain the solubility of polypeptides. The synthesis of carrier ampholytes bearing hydrazide groups was proposed as these could be specifically and quantitatively removed from focused proteins after electrofocusing by passing the

they are zwitterionic, they act in effect as weak acids buffering in the neutral to alkaline pH regions.

Since α-aminomethylated acrylic acids are amino acids, they are solids at room temperature. Hence it was expected that their purification would be simple. However, only two compounds were successfully crystallized - α-(*N*-morpholinomethyl)acrylic acid (pK_2 = 7.59 ± 0.03 at 23°C) and α-[*N*-methyl-*N*-(2-hydroxyethyl)aminomethyl]acrylic acid (pK_2 = 9.22 ± 0.08 at 22°C). α-(*N*-morpholinomethyl)acrylic acid was successfully used to focus equine myoglobin (pI = 7.4 at 25°C).

Acrylic acid derivatives of di- and tetramines

An attempt was made to synthesize acrylic acid derivatives of di- and tetramines, in order to obtain compounds that buffered over the pH 3-10 region. *N,N'*-Bis(2-carboxyprop-2-enyl)piperazine (BCPP) was facilely synthesized by the reaction of piperazine with formaldehyde and malonic acid. It shows a sinusoidal buffering profile over the pH range 3 to 10. The synthesis of acrylic acid derivatives of the tetramines *N,N'*-bis(3-aminopropyl)ethylenediamine and 1,4-bis(3-aminopropyl)piperazine was attempted in an effort to get a linear buffering profile. However these efforts were unsuccessful.

Conclusion

α-Aminomethylated acrylic acids can be successfully used to prepare IPG's in the neutral to alkaline portion of the pH gradient. They have a number of advantages over the basic Immobiline' in that they are much more

Conclusion

It appears that BHEAMA is stable enough to be used as an acrylamido buffer for IPG runs. However, the slow hydrolysis of *N*-aminomethylated acrylamides in water means that aqueous solutions of these compounds cannot be stored. It may possible, though, that their stability would be improved if stored as methanol or ethanol solutions. Moreover their synthesis is simple, and the stock chemicals required are inexpensive.

So it can be concluded that *N*-aminomethylated acrylamides are potentially useful for the preparation of IPG's. However, the many problems experienced during the attempted purification of BHEAMA and other acrylamido buffers persuaded me to drop these chemicals for the time being and to pursue a more promising avenue of research.

4.3.2 The synthesis of α-aminomethylated acrylic acids and their use in immobilized pH gradients

Recapitulation

Acrylic acid derivatives of monoamines

A number of α-aminomethylated acrylic acids were facilely synthesized by the Mannich reaction of malonic acid, formaldehyde and secondary amines. These compounds are much more stable than the corresponding acrylamido derivatives since the reaction is irreversible. Since

4.3 THE SYNTHESIS OF NOVEL ACRYLAMIDO AND ACRYLIC ACID BUFFERS FOR ISOELECTRIC FOCUSING IN IMMOBILISED pH GRADIENTS

4.3.1 The synthesis *N*-aminomethylated acrylamides and their use in immobilised pH gradients

Recapitulation

N-Aminomethylated acrylamides such as *N*-[bis(2-hydroxyethyl)aminomethyl]acrylamide (BHEAMA) can be facilely synthesized by the Mannich reaction of acrylamide, formaldehyde and a secondary amine. BHEAMA has a p*K* of 5.6, making it ideal to fill the gap in buffering power between the p*K* 4.6 and 6.2 Immobilines'.

However, the Mannich reaction of formaldehyde and secondary amines with amides is reversible (Bundgaard and Johansen, 1980*a*,*b*), and studies by Pelton (1984) have shown that a quantitative removal of the attached amine groups of *N*-dimethylaminomethylated polyacrylamide is achieved upon prolonged dialysis against distilled water. Using the experimental data of Bundgaard and Johansen (1981), it is expected that the half-life of BHEAMA at 37°C would only be about 24 h. However, since IEF-IPG is normally conducted at 5-10°C, it appears that the percentage decomposition of BHEAMA should be negligible during a normal IPG run. In fact satisfactory focusing of carbonic anhydrase isozymes was obtained using BHEAMA as the immobilized buffer.

However it is expected that all of the above problems may be solved by the introduction of monoanionic polymeric spacers. The problem of ionic interactions with amphoteric sample species would naturally no longer occur. Moreover, these polymeric spacers, having only a single charge, would have to be very much shorter than polyAMPS spacers in order to have similar net ionic mobilities. Hence entanglement of the polymeric spacers in the gel fibres should no longer be a problem.

ITP of nucleic acids

These polyanionic spacers seem ideally suited to the separation of the linear polyanionic species, in particular, nucleic acids. It seems probable that this has been the first time that DNA has been analyzed by ITP. However, the resolution obtained was slightly inferior to that obtained by PAGE, due to the compression of the DNA bands. This was most likely caused by an insufficient spacing of the DNA in those regions, and not due to the technique itself. In fact, it is expected that ITP of DNA molecules should yield superior results to PAGE, due to the counteraction of diffusion by the electric field strength gradient. The development of monoanionic polymeric spacers will probably help in this regard, since distortions of the electric field strength gradient should no longer occur. This work could have important ramifications in techniques such as DNA sequencing.

4.2.2 Conclusions

The advantage of ITP spacers whose ionic mobilities are independent of the ambient pH

As far as it is known to the author, this work is the first example of the application of polymeric species for use as spacers in ITP. The advantage of polymeric spacers lies in the fact that differences in ionic mobility are governed solely by the molecular masses of the spacers, and not by the pH of the milieu, ie. ITP spacers. Hence spacer mixtures can be formulated to give the optimum range of ionic mobilities for any particular concentration of polyacrylamide. These ionic mobilities are intrinsic to the polymeric spacers, and are not affected by the pH at which the isotachophoretic experiment is conducted.

Problems occurring with polyAMPS spacers and their solution

The longer polyAMPS species became entangled in the gel fibres during electrophoresis, causing distortion of the electric field strength gradient, as well as leading to severe electroosmosis and resultant deformation of the gel. For this reason only low concentrations (10 mM) of the polyAMPS spacers could be used, so that the isotachophoretic system was poorly buffered. Moreover ionic interactions occurred between the polyAMPS spacers and amphoteric sample species, leading to smearing and loss of resolution.

4.2 NOVEL POLYMERIC SPACERS FOR ISOTACHOPHORETIC SEPARATIONS

4.2.1 Recapitulation

Carrier ampholytes have certain deficiencies which make them non-optimal spacers for isotachophoresis:

(i) their ionic mobilities change with ambient pH,

(ii) they exert a buffering effect of their own, affecting the pH within the isotachophoretic stack.

It was reasoned that the use of anionic polymers of a uniform distribution of molecular mass as spacers for isotachophoresis would eliminate these problems. Polymers of 2-acrylamido-2-propane-1-sulfonic acid (polyAMPS) of various size ranges were synthesized for this purpose.

TEP of dyes conducted with polyAMPS spacers gave a superior resolution to zone electrophoresis, since the electric field strength gradient generated within the isotachophoretic system counteracted the effect of diffusion. However TEP of DNA gave inferior results due to band compression. TEP of ampholytic species was problematic due to ionic interactions with the polyAMPS spacers.

It was found that low concentrations of the polyAMPS spacers had to be used, or else deformation of the polyacrylamide gel occurred. Smearing and precipitation of the longer polyAMPS species was noted on analysis by zone electrophoresis.

c_i / c_t — Concentration of the ionized form of the leading and terminating constituents, respectively (mol·cm^{-3}).

$c_{i,z}$ / $c_{t,z}$ — Concentration of an ionized subspecies of valency z of the leading and terminating constituents, respectively (mol·cm^{-3}).

$c_{OH(L)}$ / $c_{OH(T)}$ — Concentration of hydroxyl ions OH^- in the leading and terminating zones, respectively (mol·cm^{-3}).

$c_{b(L)}^{total}$ / $c_{t(T)}^{total}$ — Total concentration of the buffering counterion constituent in the leading and terminating zones, respectively (mol·cm^{-3}).

c_l^{total} / c_t^{total} — Total concentration of the leading and terminating constituents, respectively (mol·cm^{-3}).

E — Electric field strength (V·cm^{-1}).

F — Faraday constant where $F = eN_A$ (9.65×10^4 C·mol^{-1}).

I^0 — Unionized fraction of an arbitrary ionic species.

I^z — An arbitrary ionic subspecies of charge z.

I — Electric current (A).

$m_{b,z}$ / $m_{i,z}$ / $m_{t,z}$ — Ionic mobility of an ionized subspecies of valency z of the buffering counterion,

Appendix B - Derivation of the isotachophoretical steady state equations[1]

List of symbols

$c_{b(L)}$ / $c_{b(T)}$ — Concentration of the ionized form of the buffering counterion constituent in the leading and terminating zones, respectively (mol·cm^{-3}).

$c_{b(L),z}$ / $c_{b(T),z}$ — Concentration of an ionized subspecies of valency z of the buffering counterion constituent in the leading and terminating zones, respectively (mol·cm^{-3}).

$c_{H(L)}$ / $c_{H(T)}$ — Concentration of hydronium ions H_3O^+ in the leading and terminating zones, respectively (mol·cm^{-3}).

c_i — Concentration of the ionized fraction of an arbitrary ionic species i (mol·cm^{-3}).

---

[1] Theoretical treatments of the steady state boundaries existing in ITP may be found in Ornstein (1964), Everaerts and Routs (1971), Jovin (1973a) and Everaerts *et al.* (1973). I have found the paper of Everaerts and Routs (1971) to be the most lucid in its treatment of the various equations. Therefore I have based this chapter on their paper, but have altered some of their equations which failed to take into account the signed nature of quantities such as the ionic charge, ionic mobility and linear velocity of ions. Moreover I have filled in gaps in their paper where they neglected to show the complete derivatization of certain equations. The notation has also been altered in a manner which I hope makes the equations clearer.

Since

$$F = eN_A \qquad \text{(A-28)}$$

where F is the Faraday constant, equation (A-27) can be rewritten as:

$$I = AF\sum_{i=1}^{n}\sum_{z=1}^{Z} z_i c_{i,z} v_{i,z} \qquad \text{(A-29)}$$

Substituting equation (A-7) results in:

$$I = AFE\sum_{i=1}^{n}\sum_{z=1}^{Z} z_i c_{i,z} m_{i,z} \qquad \text{(A-30)}$$

where $m_{i,z}$ is the ionic mobility of an ionic subspecies i^z. Hence a definition for the conductivity of an electrolyte solution may be obtained by inserting equation (A-30) into equation (A-17) to give:

$$\kappa = F\sum_{i=1}^{n}\sum_{z=1}^{Z} z_i c_{i,z} m_{i,z} \qquad \text{(A-31)}$$

Likewise equation (A-31) may be inserted into equation (A-20) to provide a definition for the molar conductivity of an electrolyte solution:

$$\Lambda = F\sum_{i=1}^{n}\sum_{z=1}^{Z} z_i m_{i,z} \qquad \text{(A-32)}$$

interval Δt is equal to the number of charges in the volume:

$$v_{i,z}A\Delta t \qquad \text{(A-24)}$$

Therefore the total number of charges ΔQ carried by all the various ionic subspecies of the ionic constituent i passing through a given point of cross-sectional area A in a time interval Δt is equal to the number of charges in the volume:

$$A\Delta t\sum_{i=1}^{n}\sum_{z=1}^{Z}v_{i,z} \qquad \text{(A-25)}$$

The numerical concentration of charge within this volume is given by equation (A-22). Hence ΔQ can be determined by multiplying equations (A-22) (numerical concentration of Q) and (A-25) (volume containing ΔQ):

$$\Delta Q = AeN_A\Delta t\sum_{i=1}^{n}\sum_{z=1}^{Z}z_iC_{i,z}v_{i,z} \qquad \text{(A-26)}$$

Substituting equation (A-26) into (A-23) gives:

$$J = AeN_A\sum_{i=1}^{n}\sum_{z=1}^{Z}z_iC_{i,z}v_{i,z} \qquad \text{(A-27)}$$

A.3 THE RELATIONSHIP BETWEEN THE IONIC MOBILITIES OF IONS AND THE CONDUCTIVITIES OF SOLUTIONS

The *molar concentration* of charge Q in an electrolyte solution is equal to:

$$e\sum_{i=1}^{n}\sum_{z=1}^{Z} z_i C_{i,z} \qquad \text{(A-21)}$$

where z_i is the charge of an individual ionic subspecies i' in solution. The *actual numerical concentration* of charge Q can be found by inserting Avogadro's number N_A:

$$eN_A\sum_{i=1}^{n}\sum_{z=1}^{Z} z_i C_{i,z} \qquad \text{(A-22)}$$

The electric current I is defined as the net amount of charge Q that passes a given point per unit time:

$$I = \frac{\Delta Q}{\Delta t} \qquad \text{(A-23)}$$

The number of charges carried by an ion i of valency z (i') passing through a given point of cross-sectional area A in a time interval Δt is equal to the number of ions i' that have migrated a distance d in that time interval Δt, and hence to the number of charges in the volume dA. Since $d = v_{i'}\Delta t$, where $v_{i'}$ is the velocity of the ionic subspecies i', the number of charges carried by the ionic subspecies i' passing a given point in a time

Hence the total molar conductivity Λ of an electrolyte is the sum of the molar conductivities λ_i of the individual ionic species i (including λ_{H^+} and λ_{OH^-}):

$$\Lambda = \sum_{i=1}^{n} \lambda_i \qquad \text{(A-19)}$$

If an individual ionic species i happens to be a weak electrolyte possessing one or more protolytic groups with a pK value within two pH units of the pH of the electrolyte, then a number of subspecies of i of various valency z will be simultaneously present in solution. In this case the total molar conductivity will be expressed as:

$$\Lambda = \kappa / \sum_{i=1}^{n} \sum_{z=1}^{Z} c_{i,z} \qquad \text{(A-20)}$$

where $c_{i,z}$ is the molar concentration of a particular ionic subspecies i^z, and Z is the maximum degree of ionization.

where κ is expressed[1] in $S \cdot cm^{-1}$. Substituting (A-1) and (A-14) into (A-16) results in:

$$\kappa = \frac{I}{EA} \qquad \text{(A-17)}$$

Since the conductivity of a solution depends on the number of ions present, it is logical to introduce a quantity known as the molar conductivity Λ, which is defined as:

$$\Lambda = \kappa \,/\, \sum_{i=1}^{n} c_i \qquad \text{(A-18)}$$

where Λ is expressed[2] in $S \cdot cm^2 \cdot mol^{-1}$, and n is the total number of different ionic species in solution. There is always more than one kind of ionic species i in solution, since the law of electroneutrality demands that the number of positive and negative charges in solution be equal:

$$[i^+] + [H_3O^+] = [i^-] + [OH^-]$$

---

[1] The units of κ are $\Omega^{-1} \cdot cm^{-1}$, which was often written in the past as $mho \cdot cm^{-1}$. However the reciprocal of the ohm has now been officially designated as the siemens, S, so that the units of κ are $S \cdot cm^{-1}$.

[2] If c_i is expressed in equivalents per cm^3, Λ is defined as the equivalent conductivity, and is expressed in $S \cdot cm^2 \cdot equiv^{-1}$.

A.2 THE CONDUCTIVITY OF AN ELECTROLYTE

The resistance of a conductor is defined by Ohm's law:

$$R = \frac{V}{I} \qquad \text{(A-14)}$$

where R is expressed in ohms (Ω). The resistance of a conductor increases as the cross-sectional area A decreases and as the length L increases. In order to eliminate this effect of shape, and to obtain a resistance which is characteristic of a given conductor, a quantity known as the resistivity, or specific resistance ρ, is defined. This is the resistance of a section of a conductor which is 1 cm^2 in cross-sectional area and 1 cm in length. It is related to the actual resistance by the formula:

$$\rho = \frac{RA}{L} \qquad \text{(A-15)}$$

where ρ is expressed in $\Omega \cdot cm$. The conductivity, or specific conductance κ, of a conductor is defined as the reciprocal of the resistivity:

$$\kappa = \frac{L}{RA} \qquad \text{(A-16)}$$

$$\overline{m}_i = \left(\sum_{z=0}^{z} c_{i,z} m_{i,z}\right) / c_i^{total} \qquad \text{(A-10)}$$

Equation (A-10) can be rewritten in the form:

$$\overline{m}_i = \left(c_{i,0} m_{i,0} + \sum_{z=1}^{z} c_{i,z} m_{i,z}\right) / c_i^{total} \qquad \text{(A-11)}$$

where $c_{i,0}$ and $m_{i,0}$ are the concentration and ionic mobility respectively of the unionized subspecies of i. Since $m_{i,0}$ is expected to be zero, equation (A-11) can be simplified to:

$$\overline{m}_i = \left(\sum_{z=1}^{z} c_{i,z} m_{i,z}\right) / c_i^{total} \qquad \text{(A-12)}$$

Substituting equation (A-12) into (A-8) provides an expression for the net velocity $\overline{v}_i$ of the ionic constituent i:

$$\overline{v}_i = \frac{E}{c_i^{total}} \sum_{z=1}^{z} c_{i,z} m_{i,z} \qquad \text{(A-13)}$$

$$v_{i,z} = m_{i,z}E \tag{A-7}$$

Since we are interested in the overall velocity $\bar{v}$ of the ionic species i and not the individual velocities of the subspecies, we introduce a term known as the net or effective ionic mobility $\bar{m}$:

$$\bar{v}_i = \bar{m}_i E \tag{A-8}$$

The net ionic mobility $\bar{m}_i$ of an ionic species i is:

$$\bar{m}_i = \frac{\sum_{z=0}^{Z} c_{i,z} m_{i,z}}{\sum_{z=0}^{Z} c_{i,z}} \tag{A-9}$$

where $\bar{m}_i$ is the average of the all the individual ionic mobilities $m_{i,z}$ of all the ionic subspecies i^z, and Z is the maximum degree of ionization. Since:

$$c_i^{total} = \sum_{z=0}^{Z} c_{i,z}$$

where c_i^{total} is the total concentration of the ionic constituent i, equation (A-9) can be simplified to:

$$v_i = \frac{z_i e E}{f} \qquad \text{(A-4)}$$

By substituting the Stoke's formula for the coefficient of friction of a sphere of radius r (in this case the radius of the ion plus its hydration shell) in a solvent of viscosity η, we can write:

$$v_i = \left(\frac{z_i e}{6\pi\eta r}\right) E \qquad \text{(A-5)}$$

The term $z_i e/6\pi\eta r$ is a constant for any ionic species i moving in a solvent of viscosity η. Hence it can be replaced by a coefficient of proportionality known as the ionic mobility m:

$$v_i = m_i E \qquad \text{(A-6)}$$

where m is expressed in $cm^2 \cdot V^{-1} \cdot s^{-1}$. Since, by convention, the electric current I is defined as the flow of positive charge towards the cathode, v_i and m_i are signed quantities, of the same sign as the charge of the ionic species i.

If i happens to be a weak electrolyte possessing one or more protolytic groups, then a number of subspecies of i of various valency z will be simultaneously present in solution in rapid equilibrium. In this case each individual ionic subspecies i of charge z and ionic mobility $m_{i,z}$ will migrate with a linear velocity $v_{i,z}$:

A.1 THE MOTION OF IONS IN AN ELECTRIC FIELD

When two electrodes a distance L apart are at a potential difference V, the ion in the solution between them experiences a uniform electric field E ($V \cdot cm^{-1}$) of magnitude:

$$E = \frac{V}{L} \qquad \text{(A-1)}$$

In such a field an ionic species i of charge $z_i e$ (where e is the charge of an electron) experiences a force $\mathcal{F}$ of magnitude:

$$\mathcal{F} = z_i eE \qquad \text{(A-2)}$$

A cation will accelerate towards the negative electrode (cathode) and an anion will accelerate towards the positive electrode (anode). However as an ion moves through the solvent it experiences a frictional retarding force $\mathcal{F}'$ that is proportional to its electrophoretic velocity v_i ($cm \cdot s^{-1}$):

$$\mathcal{F}' = fv_i \qquad \text{(A-3)}$$

where f is the frictional coefficient. The two forces act in opposite directions, and the ion will reach a maximum constant velocity when the accelerating force $\mathcal{F}$ is balanced by the viscous drag $\mathcal{F}'$. The net force is zero when:

In the older literature the ionization constants of bases refer to the equilibrium

$$B + H_2O \rightleftharpoons BH^+ + OH^-$$

with the ionization constant, K_b, given by

$$K_b = \frac{[BH^+]\,[OH^-]}{[B]}$$

Today the use of K_b is avoided because it does not touch on the heart of the matter, namely: an acid produces hydrogen ions and a base receives them. Thus both acids and bases are best related in terms of a single quantity, their affinity for the hydrogen ion. Such a relationship requires the use of the acidic constant K_a for both acids and bases.

B.5.2 Equilibria equations for acids

Imagine a weakly acidic, polyprotic ionic species i. In solution i will exist, depending on the pH, in a number of subspecies of various degrees of ionization, all in rapid equilibrium with each other. The equilibrium reaction of the first ionization reaction is:

$$i^0 \rightleftharpoons i^{-1} + H^+$$

and the dissociation constant K_1 is:

$$K_1 = \frac{C_{i,-1}\,C_H}{C_{i,0}}$$

B.5 THE EQUILIBRIA EQUATIONS

B.5.1 Note on the modern usage of acidic dissociation constants to describe the ionization of both acids and bases[1]

The Brønsted-Lowry theory is the most useful and widely accepted description of the ionization of both acids and bases. Its underlying concept is the definition of an acid as any substance that can ionize to give a solvated hydrogen ion (ie. a proton stabilized by interaction with the solvent). Conversely a base is substance which can accept a hydrogen ion. Brønsted was the first to show the advantage of having the ionization of both bases (ie. conjugate acids) expressed on the same scale. For acids the ionization process is

$$HA + H_2O \rightleftharpoons H_3O^+ + A$$

and the ionization constant, K_a, is given by

$$K_a = \frac{[H_3O^+]\,[A^-]}{[HA]}$$

For bases the ionization is

$$BH^+ + H_2O \rightleftharpoons H_3O^+ + B$$

and the ionization constant, K_a, is given by

$$K_a = \frac{[H_3O^+]\,[B]}{[BH^+]}$$

---

[1] From Albert and Serjeant (1984).

$$C_{b(T)}^{total} \quad C_{b(L)}^{total} \quad C_i^{total}, \frac{\sum_{z=1}^{z} C_{b(T),z} m_{b,z}}{\sum_{z=1}^{z} C_{i,z} m_{i,z}} \quad C_i^{total}, \frac{\sum_{z=1}^{z} C_{b(L),z} m_{b,z}}{\sum_{z=1}^{z} C_{i,z} m_{i,z}} \tag{B-22}$$

This is the extended form of the mass balance equation of the buffering counterion *b* in the isotachophoretic steady state.

B.4 THE ELECTRONEUTRALITY EQUATIONS

The law of electroneutrality states that the overall charge of an electrolyte solution is zero. Therefore the electroneutrality equations for the leading and terminating zones are:

$$C_{OH(L)} z_{OH} + C_{H(L)} z_H + \sum_{z=1}^{z} C_{i,z} z_i + \sum_{z=1}^{z} C_{b,z(L)} z_b = 0 \tag{B-23}$$

$$C_{OH(T)} z_{OH} + C_{H(T)} z_H + \sum_{z=1}^{z} C_{i,z} z_i + \sum_{z=1}^{z} C_{b,z(T)} z_b = 0 \tag{B-24}$$

$$V_T C_{b(T)}^{total} - V_L C_{b(L)}^{total} = E_T \sum_{x=1}^{\infty} C_{b(T),x} m_{b,x} - E_L \sum_{x=1}^{\infty} C_{b(L),x} m_{b,x}$$

(B-20)

From equation (B-10) $V_L = V_T$, so:

$$V_T (C_{b(T)}^{total} - C_{b(L)}^{total}) = E_T \sum_{x=1}^{\infty} C_{b(T),x} m_{b,x} - E_L \sum_{x=1}^{\infty} C_{b(L),x} m_{b,x}$$

$$\therefore C_{b(T)}^{total} - C_{b(L)}^{total} = \frac{E_T \sum_{x=1}^{\infty} C_{b(T),x} m_{b,x}}{V_T} - \frac{E_L \sum_{x=1}^{\infty} C_{b(L),x} m_{b,x}}{V_T}$$

From equation (B-10) $V_L = V_T$, so:

$$C_{b(T)}^{total} - C_{b(L)}^{total} = \frac{E_T \sum_{x=1}^{\infty} C_{b(T),x} m_{b,x}}{V_T} - \frac{E_L \sum_{x=1}^{\infty} C_{b(L),x} m_{b,x}}{V_L}$$

(B-21)

Inserting equation (B-11) results in:

The total mass transport of *b* into the terminating zone is therefore:

$$E_t \sum_{z=1}^{z} C_{b(m),z} m_{b,z} - v_t C_t^{(total)} \qquad \text{(B-17)}$$

If the zone of terminating ions t was followed by another zone of terminating ions t*, where $m_t > m_{t^*}$, then at the isotachophoretic steady state the total mass transport of *b* ions out of the terminating zone into the terminating* zone would be:

$$E_T \sum_{z=1}^{z} C_{b(m),z} m_{b,z} - v_T C_T^{(total)} \qquad \text{(B-18)}$$

The concentration of the buffering counterion *b* within a zone is constant in the isotachophoretic steady state. Therefore the amount of *b* that is transported into the terminating zone is equal to amount that leaves the zone. Therefore:

$$E_t \sum_{z=1}^{z} C_{b(m),z} m_{b,z} - v_t C_{b(t)}^{(total)} = E_T \sum_{z=1}^{z} C_{b(m),z} m_{b,z} - v_T C_T^{(total)}$$

(B-19)

Equation (B-19) may be rearranged to:

This is the extended form of the Kohlrausch regulating function which gives a relation between the ion concentration in the leading and terminating zone (Ornstein, 1964; Karol and Karol, 1978).

B.3 THE BALANCE OF MASS

Let us consider the total mass balance of the buffering counterion *b* in the terminating zone. If the boundary between the leading and terminating zones is moving forward with a speed v_t, this is equivalent to *b* ions moving into the terminating zone with a speed $-v_t$[1]. If the total concentration of the buffering counterion in the leading zone is $c_{b(L)}^{total}$, then the mass transport of *b* (in mol·cm^{-2}·s^{-1}) into the terminating zone due to this movement is:

$$-v_t c_{b(L)}^{total} \qquad \text{(B-15)}$$

Furthermore there is an electrophoretic transport of *b* ions (in mol·cm^{-2}·s^{-1}), which is equal to:

$$E_L \sum_{z=1}^{z} c_{b(L),z} m_{b,z} \qquad \text{(B-16)}$$

---

[1] Since, by convention, electric current is defined as the flow of positive charge towards the cathode, the velocity of an ion is a signed quantity - positive for cations and negative for anions. Since the movement of the *b* ions is in the opposite direction to that of the *l* and *t* ions, its velocity is of an opposite sign.

$$\vec{v}_i = \frac{E}{C_j^{total}} \sum_{x=1}^{z} c_{i,x} m_{i,x} \qquad \text{(B-11)}$$

Combining equations (B-10) and (B-11) gives:

$$\frac{E_L}{C_j^{total}} \sum_{x=1}^{z} c_{l,x} m_{l,x} = \frac{E_T}{C_j^{total}} \sum_{x=1}^{z} c_{t,x} m_{t,x} \qquad \text{(B-12)}$$

Equation (B-12) may be rearranged to give:

$$\frac{C_t^{total}}{C_j^{total}} \quad \frac{E_T}{E_L} \cdot \frac{\sum_{x=1}^{z} c_{t,x} m_{t,x}}{\sum_{x=1}^{z} c_{l,x} m_{l,x}} \qquad \text{(B-13)}$$

Substituting equation (B-9) into equation (B-13) results in:

$$\frac{C_t^{total}}{C_j^{total}} \quad \frac{\sum_{x=1}^{z} c_{t,x} m_{t,x}}{\sum_{x=1}^{z} c_{l,x} m_{l,x}} \cdot \frac{c_{OH(T)} m_{OH} z_{OH} + c_{H(T)} m_H z_H + \sum_{x=1}^{z} c_{l,x} m_{l,x} z_l + \sum_{x=1}^{z} c_{b,x(T)} m_{b,x} z_b}{c_{OH(T)} m_{OH} z_{OH} + c_{H(T)} m_H z_H + \sum_{x=1}^{z} c_{t,x} m_{t,x} z_t + \sum_{x=1}^{z} c_{b,x(T)} m_{b,x} z_b} \qquad \text{(B-14)}$$

$$E_L K_L = E_T K_T \qquad \text{(B-7)}$$

Substitution of equation (B-3) into (B-7) results in:

$$E_L\left(C_{OH(L)}m_{OH}z_{OH} + C_{H(L)}m_H z_H + \sum_{z=1}^{n} C_{l,z}m_{l,z}z_l + \sum_{z=1}^{n} C_{b,z(L)}m_{b,z}z_b\right) = E_T\left(C_{OH(T)}m_{OH}z_{OH} + C_{H(T)}m_H z_H + \sum_{z=1}^{n} C_{t,z}m_{t,z}z_t + \sum_{z=1}^{n} C_{b,z(T)}m_{b,z}z_b\right) \qquad \text{(B-8)}$$

Equation (B-8) may be rearranged as:

$$\frac{E_T}{E_L} = \frac{C_{OH(L)}m_{OH}z_{OH} + C_{H(L)}m_H z_H + \sum_{z=1}^{n} C_{l,z}m_{l,z}z_l + \sum_{z=1}^{n} C_{b,z(L)}m_{b,z}z_b}{C_{OH(T)}m_{OH}z_{OH} + C_{H(T)}m_H z_H + \sum_{z=1}^{n} C_{t,z}m_{t,z}z_t + \sum_{z=1}^{n} C_{b,z(T)}m_{b,z}z_b} \qquad \text{(B-9)}$$

In an isotachophoretic system all zones will migrate at the same speed:

$$v_L = v_T \qquad \text{(B-10)}$$

The net linear velocity $\bar{v}_i$ of an arbitrary ionic species i is defined[1] as:

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[1] Derived in Appendix A as equation (A-13).

$$\kappa = F\sum_{i=1}^{n}\sum_{z=1}^{Z} z_i c_{i,z} m_{i,z} \qquad \text{(B-2)}$$

where Z is the maximum valency of an ionic subspecies. Therefore the conductivity of the leading zone will be:

$$\kappa_L = F(c_{OH(L)} m_{OH} z_{OH} + c_{H(L)} m_H z_H + \sum_{z=1}^{Z} c_{l,z} m_{l,z} z_l + \sum_{z=1}^{Z} c_{b,z(L)} m_{b,z} z_b) \qquad \text{(B-3)}$$

Ohm's law[1] gives:

$$I_L = E_L \kappa_L A \qquad \text{(B-4)}$$

$$I_T = E_T \kappa_T A \qquad \text{(B-5)}$$

where A is the cross-sectional area of the conductor. Substituting equations (B-4) and (B-5) into (B-1) gives:

$$E_L \kappa_L A = E_T \kappa_T A \qquad \text{(B-6)}$$

Since the cross-sectional area of the conductor is assumed to be constant:

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[1] Derived in Appendix A as equation (A-17).

Equilibrium equations. The acidic and basic equilibria determine the degree of dissociation and hence the partial ion concentrations of the constituent ionic species.

Moreover, in order to simplify the calculations it is assumed that:

(i) The concentrations of the constituent ionic species are low enough that no activity coefficients have to be included (ie. < 20 mM).

(ii) An identical temperature is maintained in the leading and terminating zones, so that the ionic mobilities of those constituent ionic species common to both zones remain constant.

B.2 THE BALANCE OF ELECTRIC CURRENT

The current in the leading zone is equal to the current in the terminating zone:

$$I_L = I_T \qquad \text{(B-1)}$$

The conductivity κ^i of a solution of an arbitrary ionic species i is a function of the concentrations of its constituent ionic subspecies c_i, their individual ionic mobilities m_i and their individual charges z_i, such that:

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[1] Derived in Appendix A as equation (A-31).

B.1 PHYSICOCHEMICAL PRINCIPLES OF ITP

Let us consider an electrolyte system composed of two zones - a leading zone containing an ion *l*, and a terminating zone containing an ion *t*, where $m_l > m_t$, and a common buffering counterion *b*.

The concentration of *l* and the pH of the leading zone are preset. When a potential difference is applied across the system, the concentrations of *t* and *b* in the terminating zone will adjust to the concentrations of *l* and *b* in the leading zone, in such a way that they will create a voltage gradient large enough to give the terminating zone the same speed as the leading zone. In order to calculate the concentrations of *t* and *b* in the terminating zone, one makes use of the following electrophoretic principles:

The balance of electric current. Since each part of the system are electrically in series, the electric current is constant throughout the system, assuming that the cross-sectional area through which the current passes remains constant. The current in the leading zone is therefore the same as the current in the terminating zone.

The balance of mass. The concentration of the buffering counterion *b* within one zone is constant in the steady state. This means that the amount of *b* transported into a zone is equal to the amount that moves out the zone.

The electroneutrality principle. The amounts of positive and negative charges are equal in the same zone.

leading and terminating constituents, respectively. Signed quantity, given the same sign as the charge of the constituent ($cm^2 \cdot V^{-1} \cdot s^{-1}$).

m_i Ionic mobility of arbitrary ionic species i. Signed quantity, given the same sign as the charge of i ($cm^2 \cdot V^{-1} \cdot s^{-1}$).

v_i Linear velocity of ionic species i. Signed quantity, of the same sign as charge of i ($cm \cdot s^{-1}$).

$\bar{v}_i$ Net linear velocity of ionic species i, representing the average of the individual linear velocities of the various subspecies of i. Signed quantity, of the same sign as the charge of i ($cm \cdot s^{-1}$).

z_b /z_l /z_t Charge or valency of the ionized form of the buffering counterion, leading and terminating constituents, respectively.

z_i Charge or valency of an arbitrary ionic species i.

Z_i Maximum charge or valency of an arbitrary ionic species i.

κ Conductivity or specific conductance ($S \cdot cm^{-1}$).

C.2 ADAPTATION OF THE ITP EQUATIONS FOR THE CALCULATION OF THE IONIC PARAMETERS IN THE LEADING AND TERMINATING ZONES

If the total molar concentration of the leading ion, $c_l^{(total)}$, in the leading zone, and the pH of the leading zone, are set by the experimenter, then all the other physicochemical parameters of the leading and terminating zones can be calculated from the fundamental ITP equations.

The calculations of the various ionic parameters are ordered as follows:

1. Calculation of c_l.
2. Calculation of $c_{b(l)}$.
3. Calculation of $c_{b(l)}^{(total)}$.
4. Calculation of $c_l^{(total)}$.
5. Calculation of $c_{b(t)}^{(total)}$.
6. Calculation of c_t.
7. Calculation of $c_{b(t)}$.
8. Calculation of pH_t.
9. Calculation of $\bar{m}_t$.
10. Calculation of κ_l and κ_t.
11. Calculation of I_l and I_t.

1. Calculation of c_l

The equilibrium equation (C-7) may be rewritten in a form which allows the for the calculation of c_l from $c_l^{(total)}$ and pH_t:

$$c_l = (c_l^{(total)} - c_l) \cdot 10^{(pH_t - pK_l)}$$

Equilibria equations[1]

$$c_i = (c_i^{total} - c_i) \cdot 10^{(pH_i - pK_i)} \qquad \text{(C-7)}$$

$$c_i = (c_i^{total} - c_i) \cdot 10^{(pH_i - pK_i)} \qquad \text{(C-8)}$$

$$c_b = (c_b^{total} - c_b) \cdot 10^{(pK_b - pH)} \qquad \text{(C-9)}$$

It may be noted that the simplified equations (C-7) and (C-8) are simply rearrangements of the Henderson-Hasselbalch equation for acids:

$$pH = pK_i + \log_{10} \frac{c_i}{c_i^{total} - c_i} \qquad \text{(C-10)}$$

and that the simplified equation (C-9) is simply a rearrangement of the Henderson-Hasselbalch equation for bases:

$$pH = pK_i + \log_{10} \frac{c_i^{total} - c_i}{c_i} \qquad \text{(C-11)}$$

---

[1] Equations (C-7) and (C-8) are derived from equation (B-36) of Appendix B, and equation (C-9) is derived from equation (B-46) of Appendix B. These equations assume that the concentrations of the ionic constituent are expressed in mol·dm^{-3} (M) and not mol·cm^{-3} (kM).

Electroneutrality equations[1]

$$c_l z_l + c_{b(1)} z_b = 0 \qquad \text{(C-3)}$$

$$c_l z_l + c_{b(2)} z_b = 0 \qquad \text{(C-4)}$$

Equation (C-3) may be simplified as follows:

$$c_l z_l + c_{b(1)} z_b = 0$$

$$\therefore c_l z_l = -c_{b(1)} z_b$$

Since z_l, the charge of Cl^-, is -1, and z_b, the charge of $Tris^+$, is +1:

$$c_l(-1) = -c_{b(1)}(+1)$$

Therefore:

$$c_l = c_{b(1)} \qquad \text{(C-5)}$$

In a similar fashion equation (C-4) is simplified to:

$$c_l = c_{b(2)} \qquad \text{(C-6)}$$

---

[1] Equations (C-3) and (C-4) derived from equations (B-23) and (B-24), respectively, of Appendix B.

C.1 MODIFICATION OF THE FUNDAMENTAL EQUATIONS OF ITP FOR DISCONTINUOUS ELECTROPHORESIS SYSTEMS

In the discontinuous electrophoresis system of Ornstein and Davis, chloride is the leading ion, glycine the terminating ion, and Tris is the buffering counter-ion. The composition of the various buffers is:

Stacking gel buffer: 60 mM HCl - 62.5 mM Tris, pH 6.8
Resolving gel buffer: 60 mM HCl - 375 mM Tris, pH 8.8
Electrode buffer: 192 mM glycine - 25 mM Tris, pH 8.3

Since the pH of the electrophoresis buffers lie between pH 4 and pH 10, the concentrations of H_3O^+ ions and OH^- ions are negligible, and can be ignored. Moreover, since all the constituent ions are monovalent, the isotachophoretic equations are greatly simplified, and take the form:

Kohlrausch regulating function[1]

$$\frac{c_i^{total}}{c_j^{total}} = \frac{c_i m_i}{c_j m_j} \cdot \frac{c_j m_j z_j + c_{buf,j} m_b z_b}{c_i m_i z_i + c_{buf,i} m_b z_b} \qquad \text{(C-1)}$$

Mass balance equation of buffering counterion[2]

$$c_{buf,i}^{total} - c_{buf,j}^{total} = c_i^{total} \cdot \frac{c_{buf,i} m_b}{c_i m_i} - c_j^{total} \cdot \frac{c_{buf,j} m_b}{c_j m_j} \qquad \text{(C-2)}$$

---

[1] Derived from equation (B-14) of Appendix B.

[2] Derived from equation (B-22) of Appendix B.

sign as charge of constituent ionic species ($cm^2 \cdot V^{-1} \cdot s^{-1}$).

$\bar{m}_t$ — Net or effective ionic mobility of the terminating ion (glycine), representing the average of the individual ionic mobilities of the various charged and uncharged subspecies of glycine. Signed quantity, of same sign as charge of the glycine terminating ion ($cm^2 \cdot V^{-1} \cdot s^{-1}$).

z_b / z_l / z_t — Charge or valency of the buffering counterion (Tris or AMPD), leading ion (chloride) and terminating ion (glycine), respectively ($cm^2 \cdot V^{-1} \cdot s^{-1}$).

κ_l, κ_t — Conductivity or specific conductance of leading or terminating zones, respectively ($S \cdot cm^{-1}$).

Appendix C - Practical application of the isotachophoretical steady state equations to discontinuous electrophoresis

List of symbols

$c_{b(L)}$ / $c_{b(T)}$	Concentration of the ionized fraction of the buffering counterion constituent (Tris or AMPD) in the leading and terminating zones, respectively (mol·dm^{-3}).
c_L / c_T	Concentration of the ionized fraction of the leading ionic constituent (chloride) and the terminating ionic constituent (glycine), respectively (mol·dm^{-3}).
$c_{b(L)}^{total}$ / $c_{b(T)}^{total}$	Total concentration of the buffering counterion constituent (Tris or AMPD) in the leading and terminating zones, respectively (mol·dm^{-3}).
c_L^{total} / c_T^{total}	Total concentration of the leading ionic constituent (chloride) and the terminating ionic constituent (glycine), respectively (mol·dm^{-3}).
I_L, I_T	Ionic strength of the leading or terminating zone, respectively (mol·dm^{-3}).
m_b / m_L / m_T	Absolute ionic mobility of the buffering counterion (Tris or AMPD), leading ion (chloride) and terminating ion (glycine), respectively. Signed quantity, of same

Equation (B-42) may be reexpressed as:

$$c_{i,z} = \frac{(c_i^{total} - \sum_{z=1}^{z} c_{i,z}) \prod_{z=1}^{z} c_H}{\prod_{z=1}^{z} 10^{-pK_{i,z}}} \qquad \text{(B-43)}$$

which is more simply written as:

$$c_{i,z} = (c_i^{total} - \sum_{z=1}^{z} c_{i,z}) \prod_{z=1}^{z} \frac{c_H}{10^{-pK_{i,z}}} \qquad \text{(B-44)}$$

The variable c_H can be reexpressed by substituting equation (B-34) into equation (B-44) to give:

$$c_{i,z} = (c_i^{total} - \sum_{z=1}^{z} c_{i,z}) \prod_{z=1}^{z} \frac{10^{-pH}}{10^{-pK_{i,z}}} \qquad \text{(B-45)}$$

Equation (B-45) can be further simplified to:

$$c_{i,z} = (c_i^{total} - \sum_{z=1}^{z} c_{i,z}) \prod_{z=1}^{z} 10^{(pK_{i,z} - pH)} \qquad \text{(B-46)}$$

where all the concentrations are expressed in mol·cm^{-3}.

$$c_{i,2} = \frac{c_{i,0}\, c_H^2}{K_1\, K_2} \qquad \text{(B-39)}$$

If this process is continued to i^z, the subspecies of i to the highest degree of protonation z, then equation (B-39) becomes:

$$c_{i,z} = c_{i,0}\, c_H^z \,/\, \prod_{z=1}^{z} K_{i,z} \qquad \text{(B-40)}$$

Substituting equation (B-29) into equation (B-40) results in:

$$c_{i,z} = \frac{\left(c_i^{total} \sum_{z=1}^{z} c_{i,z}\right) c_H^z}{\prod_{z=1}^{z} K_{i,z}} \qquad \text{(B-41)}$$

Inserting equation (B-31) allows equation (B-41) to be rewritten as:

$$c_{i,z} = \frac{\left(c_i^{total} \sum_{z=1}^{z} c_{i,z}\right) c_H^z}{\prod_{z=1}^{z} 10^{pK_{i,z}}} \qquad \text{(B-42)}$$

B.5.3 Equilibria equations for bases

Imagine a weakly basic, polyprotic ionic species i. In solution i will exist, depending on the pH, in a number of subspecies of various degrees of protonation, all in rapid equilibrium with each other. The equilibrium reaction of the first ionization reaction is:

$$i^{+1} \rightleftharpoons i^{0} + H^{+}$$

and the dissociation constant K_1 is:

$$K_1 = \frac{C_{i,0}\, C_H}{C_{i,+1}}$$

$$\therefore C_{i,+1} = \frac{C_{i,0}\, C_H}{K_1} \qquad \text{(B-37)}$$

In a similar fashion:

$$C_{i,+2} = \frac{C_{i,+1}\, C_H}{K_2} \qquad \text{(B-38)}$$

Substituting (B-37) into (B-38) gives:

$$c_{i,z} = (c_i^{total} - \sum_{z=1}^{Z} c_{i,z}) \cdot \prod_{z=1}^{Z} \frac{10^{pK_{i,z}}}{c_H} \qquad \text{(B-33)}$$

If the concentrations of the ionic constituents are expressed in mol·dm^{-3} (M) instead of mol·cm^{-3} (kM), then c_H can be reexpressed as:

$$c_H = 10^{-pH} \qquad \text{(B-34)}$$

Substituting equation (B-34) into (B-33) gives:

$$c_{i,z} = (c_i^{total} - \sum_{z=1}^{Z} c_{i,z}) \cdot \prod_{z=1}^{Z} \frac{10^{pK_{i,z}}}{10^{-pH}} \qquad \text{(B-35)}$$

Equation (B-35) can be further simplified[1] to:

$$c_{i,z} = (c_i^{total} - \sum_{z=1}^{Z} c_{i,z}) \cdot \prod_{z=1}^{Z} 10^{(pH - pK_{i,z})} \qquad \text{(B-36)}$$

where all the concentrations are expressed in mol·cm^{-3}.

---

[1] Since $a^x/a^y = a^{x-y}$ for $a > 0$ and $x \in \mathbb{R}$ and $y \in \mathbb{R}$.

equation (B-28) becomes:

$$C_{i,z} = \frac{C_i^{total} - \sum_{z=1}^{z} C_{i,z}}{C_H^z} \cdot \prod_{z=1}^{z} K_{i,z} \qquad \text{(B-30)}$$

Since:

$$K_{i,z} = 10^{-pK_{i,z}} \qquad \text{(B-31)}$$

equation (B-30) may be rewritten as:

$$C_{i,z} = \frac{C_i^{total} - \sum_{z=1}^{z} C_{i,z}}{C_H^z} \cdot \prod_{z=1}^{z} 10^{-pK_{i,z}} \qquad \text{(B-32)}$$

Equation (B-32) may be reexpressed as:

$$C_{i,z} = \frac{C_i^{total} - \sum_{z=1}^{z} C_{i,z}}{\prod_{z=1}^{z} C_H} \cdot \prod_{z=1}^{z} 10^{-pK_{i,z}}$$

which is more simply written as:

$$\therefore C_{i,-1} = \frac{C_{i,0}\, K_1}{C_H} \qquad \text{(B-25)}$$

In a similar fashion:

$$C_{i,-2} = \frac{C_{i,-1}\, K_2}{C_H} \qquad \text{(B-26)}$$

Substituting (B-25) into (B-26) gives:

$$C_{i,-2} = \frac{C_{i,0}\, K_1\, K_2}{C_H^2} \qquad \text{(B-27)}$$

If this process is continued to i^Z, the subspecies of i to the highest degree of ionization Z, then equation (B-27) becomes:

$$C_{i,Z} = \left(C_{i,0} \prod_{z=1}^{Z} K_{i,z}\right) \;/\; C_H^Z \qquad \text{(B-28)}$$

Since:

$$C_i^{total} = C_{i,0} + \sum_{z=1}^{Z} C_{i,z} \qquad \text{(B-29)}$$

Ionic parameters of leading and terminating zones during separating phase

The following ionic parameters were calculated for the separating phase of discontinuous electrophoresis, using the equations in section C.2. The total molar concentration of the chloride leading ion is 0.060 M, and the pH of the leading zone (pH_l) is 8.8.

1. $c_{chloride}$ = 0.060 M
2. $c_{Tris(+1)}$ = 0.060 M
3. $c_{Tris(+1)}^{total}$ = 0.375 M
4. $c_{glycine}^{total}$ = 0.046 M
5. $c_{Tris(+1)}^{total}$ = 0.371 M
6. $c_{glycine}$ = 0.015 M
7. $c_{Tris(+1)}$ = 0.015 M
8. pH_t = 9.5
9. $\bar{m}_{glycine}$ = -11.9×10^{-5} $cm^2 \cdot V^{-1} \cdot s^{-1}$
10. κ_l = 6.29 $mS \cdot cm^{-1}$
    κ_t = 1.57 $mS \cdot cm^{-1}$
11. I_l = 0.060 M
    I_t = 0.015 M

Composition of the leading zone:
0.060 M HCl - 0.375 M Tris, pH 8.8

Composition of the terminating zone:
0.046 M glycine - 0.361 M Tris, pH 9.5

When the chloride-glycine steady state moving boundary crosses the stationary pH boundary between the stacking and separating gels, the composition of the leading electrolyte is changed from 0.060 M HCl - 0.063 M Tris, pH 6.8 to 0.060 M HCl - 0.375 M Tris, pH 8.8.

concentration of the chloride leading ion is 0.060 M, and the pH of the leading zone (pH_l) is 6.8.

1.	$c_{chloride}$ = 0.060 M	8.	pH_t	=	8.9
2.	$c_{Tris(l)}$ = 0.060 M	9.	$\bar{m}_{glycine}$	=	-4.7×10^{-4} $cm^2 \cdot V^{-1} \cdot s^{-1}$
3.	$c_{Tris(l)}^{total}$ = 0.063 M				
4.	$c_{glycine}^{total}$ = 0.046 M	10.	κ_l	=	6.29 $mS \cdot cm^{-1}$
			κ_t	=	0.61 $mS \cdot cm^{-1}$
5.	$c_{Tris(t)}^{total}$ = 0.049 M				
6.	$c_{glycine}$ = 0.006 M	11.	I_l	=	0.060 M
			I_t	=	0.006 M
7.	$c_{Tris(t)}$ = 0.006 M				

Composition of the leading zone:
0.060 M HCl - 0.063 M Tris, pH 6.8

Composition of the terminating zone:
0.046 M glycine - 0.049 M Tris, pH 8.9

Electrode buffer:
0.192 M glycine - 0.025 M Tris, pH 8.3

Although the initial composition of the terminating zone (electrode buffer) is 0.192 M glycine - 0.025 M Tris, pH 8.3, once electrophoresis is commenced the concentrations of glycine and Tris in the terminating zone become regulated against the concentrations of chloride and Tris in the leading zone, so that the composition of the terminating zone becomes 0.046 M glycine - 0.049 M Tris, pH 8.9. The net mobility of the glycine terminating ions is -4.7×10^{-4} $cm^2 \cdot V^{-1} \cdot s^{-1}$.

C.3 PRACTICAL CALCULATION OF THE IONIC PARAMETERS OF THE ORNSTEIN-DAVIS AND TAMURA-UI SYSTEMS[1]

The following physicochemical data were obtained from Hirokawa *et al.* (1986):

Table 17: Ionic characteristics of constituent ions

Constituent ion	pK	Charge	Mobility (m)
Chloride (l)	fully ionized	-1	-79.1
Glycine (t)	9.78	-1	-27.4
Tris (b)	8.08	+1	+29.5
AMPD (b)	8.80	+1	+42.0

[a] Values from Martell and Smith (1974) and Smith and Martell (1975).

[b] Units in $\times 10^{-9}$ $m^2 V^{-1} s^{-1}$.

C.3.1 Ornstein-Davis system

Ionic parameters of leading and terminating zones during stacking phase

The following ionic parameters were calculated for the stacking phase of discontinuous electrophoresis, using the equations in section C.2. The total molar

[1] The composition of the Ornstein-Davis system is given in Blackshear (1984). The composition of the Tamura-Ui system is given in Tamura and Ui (1972).



for the [illegible]

7. Calculation of $c_{b,r}$

The electroneutrality equation (C-6) shows $c_{\ } \cdot c_{\cdot\cdot}$

8. Calculation of pH_r

The value of pH_r may be calculated from $c_i^{(*)}$ and c_r using the Henderson-Hasselbalch equation (C-17).

9. Calculation of $\bar{m}_i$

The formula[1] for the net mobility of an arbitrary ionic species i is:

$$\bar{m}_i = \left(\sum_{j=1}^{\nu} c_{i,j} m_{i,j}\right) / c_i^{(*)} \qquad \text{(C-25)}$$

ies such as glycine, equation

(C-26)

Equation (C-22) may be rearranged into the form of the quadratic equation $ax^2 + bx + c = 0$:

$$\log_{10} \frac{c_{b(f)}^{(total)} c_i^{(total)} - (c_{b(f)}^{(total)} + c_i^{(total)}) c_i + c_i^2}{c_i^2} = pK_i - pK_i'$$

$$\frac{c_{b(f)}^{(total)} c_i^{(total)} - (c_{b(f)}^{(total)} + c_i^{(total)}) c_i + c_i^2}{c_i^2} = 10^{(pK_i - pK_i')}$$

$$c_{b(f)}^{(total)} c_i^{(total)} - (c_{b(f)}^{(total)} + c_i^{(total)}) c_i + c_i^2 = c_i^2 \cdot 10^{(pK_i - pK_i')}$$

Therefore:

$$c_{b(f)}^{(total)} c_i^{(total)} - (c_{b(f)}^{(total)} + c_i^{(total)}) c_i + (1 - 10^{(pK_i - pK_i')}) c_i^2 = 0$$

(C-23)

The value of c_i is therefore found by the formula:

$$c_i = \frac{(c_{b(f)}^{(total)} + c_i^{(total)}) \pm \sqrt{(c_{b(f)}^{(total)} + c_i^{(total)})^2 - 4 c_{b(f)}^{(total)} c_i^{(total)} (1 - 10^{(pK_i - pK_i')})}}{2(1 - 10^{(pK_i - pK_i')})}$$

(C-24)

$$pH_T = pK_b + \log_{10}\frac{c_{b(T)}^{total} - c_{b(T)}}{c_{b(T)}} \qquad \text{(C-18)}$$

Combining equations (C-17) and (C-18) gives:

$$pK_b + \log_{10}\frac{c_{b(T)}^{total} - c_{b(T)}}{c_{b(T)}} = pK_t + \log_{10}\frac{c_t}{c_t^{total} - c_t} \qquad \text{(C-19)}$$

Rearranging equation (C-19) results in:

$$\log_{10}\frac{c_{b(T)}^{total} - c_{b(T)}}{c_{b(T)}} - \log_{10}\frac{c_t}{c_t^{total} - c_t} = pK_t - pK_b \qquad \text{(C-20)}$$

It can be seen from the electroneutrality equation (C-6) that $c_{b(T)} = c_t$. Therefore equation (C-20) becomes:

$$\log_{10}\frac{c_{b(T)}^{total} - c_t}{c_t} - \log_{10}\frac{c_t}{c_t^{total} - c_t} = pK_t - pK_b \qquad \text{(C-21)}$$

Equation (C-21) may be simplified[1] to:

$$\log_{10}\frac{(c_{b(T)}^{total} - c_t)(c_t^{total} - c_t)}{c_t^2} = pK_t - pK_b \qquad \text{(C-22)}$$

---

[1] Since $\log_a(M/N) = \log_a M - \log_a N$ for $M > 0$, $N > 0$, $a > 0$.

$$c_{b(T)}^{total} - c_{b(L)}^{total} = c_t^{total} \cdot \frac{c_{b(T)} m_b}{c_t m_t} - c_l^{total} \cdot \frac{c_{b(L)} m_b}{c_l m_l}$$

$$\therefore c_{b(T)}^{total} - c_{b(L)}^{total} = c_t^{total} \cdot \frac{c_t m_b}{c_t m_t} - c_l^{total} \cdot \frac{c_l m_b}{c_l m_l}$$

$$\therefore c_{b(T)}^{total} - c_{b(L)}^{total} = c_t^{total} \cdot \frac{m_b}{m_t} - c_l^{total} \cdot \frac{m_b}{m_l}$$

Therefore:

$$c_{b(T)}^{total} = c_{b(L)}^{total} + c_t^{total} \cdot \frac{m_b}{m_t} - c_l^{total} \cdot \frac{m_b}{m_l} \qquad \text{(C-16)}$$

6. Calculation of c_t

The Henderson-Hasselbalch equation (C-10) relates pH_T to pK_1, c_t and c_l^{total}:

$$pH_T = pK_1 + \log_{10} \frac{c_t}{c_l^{total} - c_t} \qquad \text{(C-17)}$$

Likewise the Henderson-Hasselbalch equation (C-11) relates pH_T to pK_2, c_{l-} and $c_{l(-)}^{total}$:

$$\therefore \frac{c_t^{total}}{c_l^{total}} = \frac{c_t m_t}{c_l m_l} \cdot \frac{c_l (m_l z_l + m_b z_b)}{c_t (m_t z_t + m_b z_b)}$$

$$\therefore \frac{c_t^{total}}{c_l^{total}} = \frac{m_t}{m_l} \cdot \frac{m_l z_l + m_b z_b}{m_t z_t + m_b z_b}$$

Therefore:

$$c_t^{total} = c_l^{total} \cdot \frac{m_t}{m_l} \cdot \frac{m_l z_l + m_b z_b}{m_t z_t + m_b z_b} \qquad \text{(C-14)}$$

Since z_l and z_t, the charges of Cl^- and glycine$^-$ respectively, are -1, and z_b, the charge of Tris$^+$ is +1, equation (C-14) can be further simplified to:

$$c_t^{total} = c_l^{total} \cdot \frac{m_t}{m_l} \cdot \frac{m_b - m_l}{m_b - m_t} \qquad \text{(C-15)}$$

5. Calculation of $c_{b(t)}^{total}$

The buffering counterion mass balance equation (equation (C-2)) is used to calculate the value of $c_{b(t)}^{total}$ from $c_{b(l)}^{total}$, c_l^{total} and c_t^{total}. The electroneutrality equations (C-5) and (C-6) can be used to simplify equation (C-2):

$$\therefore \frac{C_{b(L)}^{total} - C_{b(L)}}{C_{b(L)}} = 1 / 10^{(pK_b - pH_L)}$$

$$\therefore \frac{C_{b(L)}^{total}}{C_{b(L)}} - 1 = 1 / 10^{(pK_b - pH_L)}$$

$$\therefore \frac{C_{b(L)}^{total}}{C_{b(L)}} = 1 + (1 / 10^{(pK_b - pH_L)})$$

Therefore:

$$C_{b(L)}^{total} = C_{b(L)} + (C_{b(L)} / 10^{(pK_b - pH_L)}) \qquad \text{(C-13)}$$

4. Calculation of I_b^{total}

One can use the Kohlrausch regulating function (equation (C-1)) to calculate C_i^{total} from C_j^{total}. The electroneutrality equations (C-5) and (C-6) can be used to simplify equation (C-1):

$$\frac{C_i^{total}}{C_j^{total}} = \frac{C_i m_i}{C_j m_j} \cdot \frac{C_i m_i z_i + C_{b(L)} m_b z_b}{C_i m_i z_i + C_{b(L)} m_b z_b}$$

$$\therefore \frac{(c_l^{total} - c_l)}{c_l} = 1 / 10^{(pH_l - pK_l)}$$

$$\therefore \frac{c_l^{total}}{c_l} - 1 = 1 / 10^{(pH_l - pK_l)}$$

$$\therefore \frac{c_l^{total}}{c_l} = 1 + (1 / 10^{(pH_l - pK_l)})$$

Therefore:

$$c_l = \frac{c_l^{total}}{1 + (1 / 10^{(pH_l - pK_l)})} \qquad \text{(C-12)}$$

2. Calculation of $c_{b(L)}$

It can be seen from the electroneutrality equation (C-5) that $c_{b(L)} = c_l$.

3. Calculation of $c_{b(L)}^{total}$

The equilibrium equation (C-9) may be rewritten in a form which allows for the calculation of $c_{b(L)}^{total}$ from $c_{b(L)}$ and pH_l:

$$c_{b(L)} = (c_{b(L)}^{total} - c_{b(L)}) \cdot 10^{(pK_l - pH_l)}$$