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Ross L. Prestidge[a]; Milton T. W. Hearn[a]
[a] Immunopathology Research Unit, Medical Research Council of New Zealand, University of Otago Medical School, Dunedin, New Zealand

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THE APPLICATION OF BUFFER ELECTROFOCUSING TO GRANULATED FLAT BED MEDIA.

Ross L. Prestidge and Milton T.W. Hearn*

Immunopathology Research Unit,
Medical Research Council of New Zealand,
University of Otago Medical School,
P.O. Box 913, Dunedin,
New Zealand.

ABSTRACT

The characteristics of a series of natural pH gradients, generated in layers of granulated gels using mixtures of amphoteric or amphoteric-non-amphoteric buffer reagents, are described. Because of the favourable properties of these flat bed systems, high voltage gradients (20-50V/cm) can be used for the electrofocusing experiments. Linear or step pH gradients can be formed depending on the choice of buffer system used. Buffer systems, suitable for the preparative electrofocusing of proteins over the pI ranges of 4.0-6.0, 4.5-8.0, 5.0-8.5 and 6.0-8.5 are reported. In contrast to results obtained with polyacrylamide gel supports, non amphoteric buffers form very steep and unstable pH gradients on beds of granulated gels.

INTRODUCTION

The electrolysis of carrier ampholyte mixtures can be used to generate natural pH gradients in inert supports like

* To whom correspondence should be addressed.

polyacrylamide gels or sucrose density gradients. The physico-chemical basis for the formation of the pH gradient with a group of ampholytes has been generally discussed[1] in terms of dissociation theory for polyprotic substances. In this treatment, an ideal steady state system is assumed to form, i.e. at equilibrium, the electromigration of the ampholytes towards the electrodes and the opposing diffusion flow are balanced. If the ampholyte mixture is initially distributed uniformly throughout the support, then during the electrolysis individual ampholytes will migrate and accumulate in zones corresponding to their respective isoelectric points[2]. The shape of each zone will be dependent on the diffusion coefficient, the nature of the surrounding ionogenic components and the conductance or field strength maintained by the ampholyte at its pI. As a consequence of its buffering capacity, each focused ampholyte generates a pH gradient upon the immediate vicinity in the supporting medium with the pH increasing monotonically from anode to cathode. This principle of pH monotony and the associated assumption that a zone of pure water separating two ampholytes would be incompatible with pH monotomy have lead to the conclusion that ampholytes can only be fully separated from each other when a third, with intermediate pI, is present. The electromigration-diffusion hypothesis thus predicts that the various zones of an ampholyte mixture will not be completely separated but will necessarily extend to some extent into adjacent zones. As adjacent isoelectric points become more similar in magnitude then greater will be the overlap of two ampholytes. Obviously, to maintain a stable natural pH-gradient, each ampholyte must have sufficient buffer capacity so that the buffering properties of adjacent ampholytes do not adversely influence the pH gradient shape. In addition, all ampholytes must have approximately similar conductances at their pIs.

Svensson[3,4] demonstrated that any amphoteric molecule could be focused by stationary electrolysis to a position corresponding to its isoelectric point. Since conductance is proportional to the ion concentration, he concluded that the conditions for good buffering capacity and conductance of an ampholyte were similar, namely a low value of pI-pK_1. It was inferred on theoretical grounds that pH gradients of any desired range and shape could be generated by proper choice of simple ampholytes and their concentration but, at that time, a sufficient number of suitable simple amphoteric reagents was not available. In particular, Svensson proposed[4] that all ampholytes with pI-pK_1 values bigger than 2.5 units, which includes all the neutral amino acids, were useless as carrier reagents and those with pI-pK_1 values between 1.5 and 2.5 units were poor carriers. Based on these criteria, Vesterberg[5] prepared synthetic polyamino-polycarboxylic acids - the Ampholines - which had apparent advantages as carrier ampholytes compared to substances examined earlier. However, the Svensson electro-migration-diffusion theory has proved unable to explain several common observations, the most important being the time dependent instability of pH gradients, formed with Ampholines, in all media[6,7]. Under normal electrofocusing conditions, the decay of the pH gradient is generally accompanied by a progressive acidification of the support medium as the carrier ampholytes predominantly migrate into the cathode chamber. Several studies[8-10] have concluded that the cathodal drift is independent of the type of supporting medium, the hydration water flow and the nature of the ampholytes. Nguyen et al.[11] have shown however that the pH drift can be either cathodic or anodic depending on the acid/base strength ratio of the catholytes and anolytes used.

Recently, several reports have described the formation of stable pH gradients under isoelectric focusing conditions in polyacrylamide gels[12-17] and granular polysaccharide gels[19,20] using mixtures of simple, amphoteric buffers in place of Ampholines. It has been suggested[11] that under these conditions the pH gradient forms by steady-state stacking with the stack confined within the gel by the use of strong acid and base electrolyte reservoirs. A close similarity exists between electrofocusing under these conditions and multiphasic zone electrophoresis. In preliminary studies on flat-bed buffer electrofocusing with granulated gels we demonstrated[20] the suitability of this system for focusing proteins to sharp boundaries and found that the relative positions of protein bands were in accord with their pI values. We have now investigated the generation of a number of different pH gradients and the relative stability of the focused zone positions, using a range of multicomponent buffer systems primarily designed for preparative applications, in flat beds of granulated gels subjected to electrofocusing conditions. This paper deals with the behaviour of these buffer systems which also provides useful information on the mechanism of isoelectric focusing per se.

MATERIALS AND METHODS

(i) Procedure of focusing experiments: Focusing was carried out on a Pyrex flat bed apparatus using washed Sephadex G75 or G200 superfine (3-5g), suspended in the appropriate buffer, as described previously[19,20]. The dimensions of the gel bed were 240 x 110 x 7mm. Generally the anolyte and catholyte buffers, 0.2M H_2SO_4 and 0.2M KOH, were used unless otherwise indicated. The temperature of the gel was maintained during the electrophoresis at ca. 18^{o}, using a water cooled contact plate. The experiments were usually conducted with the voltage regulated such that the power dissipated in the gel

was limited at all times to ca 15W[21]. At the time intervals indicated under the Results, the gel bed was sectioned and the pH of each section was, after the addition of water ($2cm^3$), measured with a glass electrode on a Radiometer PHM64 meter. Alternatively, the pH was measured directly with a surface combination pH electrode (model 2117-111 from LKB-Produkter AB, 161-25 Bromma, Sweden). For convenience and reproducibility in measuring the pH and voltage gradients the gel bed was divided into 30 equivalent sections using a calibrated grid. Voltage, current and resistivity were determined during and at the completion of the focusing experiments as reported previously[20]. In experiments utilising proteins, these were visualised in focused gel stabs by the paper print technique of Radola[22].

(ii) Reagents: All the buffer compounds and commercial protein samples were obtained from Sigma Chemicals Co., St Louis, Miss. Buffer mixtures were made up in distilled water ($\chi = 10^{-7}$ ohm^{-1} cm^{-1}) to the appropriate concentration, the pH adjusted where indicated with 1M NaOH or 1M H_2SO_4. The buffer mixtures can be stored at 4^{o} for at least one month.

RESULTS

The following examples are representative of the behaviour of simple buffer mixtures, formed from components listed in Table I, under electrofocusing conditions in flat beds of granulated gels. Most experiments were carried out with focusing times in the range 5-48hr. Under these conditions the pH gradients were relatively stable although a progressive migration of the gradient with time became apparent with focusing times greater than 60hr. In addition, band resolution obtained with standard protein mixtures appeared independent of voltage for up to 48hrs following the formation of stable isoelectric end points. It was apparent from a number of experiments that the high conductivity of the amphoteric

buffer mixtures minimised local heating effects which usually result in protein denaturation[23]. Although this is of minor important for analytical separation, it is however a significant consideration for preparative work.

Amphoteric Buffers.

(i) A 10mM solution of each of the following buffers was made: Glu, Gly-Gly, His and Lys, (see Table 1 for abbreviations and pI values). A Sephadex G200 superfine (3.0g) gel bed was prepared in this buffer and focusing was carried out at an initial power setting of 15W. After 5 hours a constant current of 3.0mA was reached. Figure 1 shows the pH gradient formed in the gel containing this buffer mixture (system 1) and the Volts/cm change along the gel after 2.5, 3.5 and 5 hours.

(ii) (a) Figure 2 depicts the pH gradient formed in a Sephadex G200 superfine (4.0g) gel containing the amphoteric buffer mixture: 10mM each of Glu, Tau, Gly, GABA, Lys, (system 2). After ca. 20 hours a steady state was reached with the current constant at 2mA. The pH and voltage gradients were measured at 22.5 hours.
(b) Figure 3 shows a similar focusing experiment except that Gly was deleted from the buffer system 2. The pH and voltage gradients of this new buffer (system 3) were determined at 20 hours. The current had decayed from an initial 50mA to a constant 2mA at the completion of the experiment.

(iii) (a) The pH and voltage gradient generated on a Sephadex-G5 superfine (3g) gel bed with a narrow range amphoteric buffer mixture composed of 10mM each of MES, ACES, TES, Tricine and Bicine and 5mM of Tau, pH adjusted from the natural value of 4.33 to 7.1 with 1M KOH (system 4) is depicted in Figure 4. The rate of decrease of current at constant voltage was asymptotically limited (from

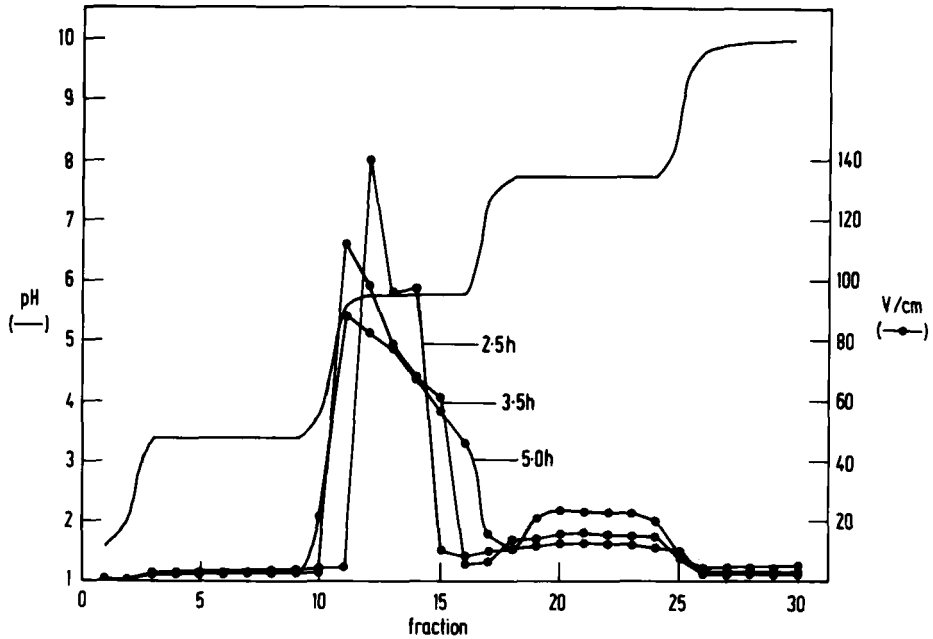

Figure 1.

pH and voltage gradient formed with a mixture of amphoteric buffers (buffer system 1) as a function of time of focusing at 15W. Buffer concentration: 10mM for each constituent buffer Glu, Gly-Gly, His and Lys. Anolyte: 0.2M H_2SO_4; catholyte 0.2M KOH; gel support; Sephadex G200 superfine.

an initial value of ca. 8mA to ca. 2mA after 5 hrs). Using the same buffer component system, but (a) at its natural pH all other conditions held constant and (b) at its natural pH but replacing the anolyte and catholyte with sufficient IM HCl or IM KOH to titrate all the buffers, resulted in only a small compression of the pH gradient along the gel bed. The value of the pH plateau (ca. pH 4.5-5.0) and the gradient stability were not affected by these changes. The effect of reducing the concentration of the acidic components of buffer system 4 from 10mM to 2mM was to uniformly lower the level of

Table I. Amphoteric and Non-Amphoteric Reagents Arranged in Order of Increasing pKa.

Reagent	Abbreviation	pKa	pI
Glutamic acid	Glu	2.2	3.2
Lactic acid	LA	3.8	-
Propionic acid	PA	4.9	-
Pyridine	Pyr	5.5	-
2-(N-Morpholino)-ethane sulphonic acid	MES	6.4	3.8
N-2-Acetamidoiminodi-acetic acid	ADA	6.6	4.5
Bis-(2-Hydroxyethyl)-imino-tris-hydroxymethyl methane	Bistris	6.8	-
3-(N-Morpholino) propane-sulphonic acid	MOPS	7.2	4.3
N-(2-Acetamido)-2-amino ethanesulphonic acid	ACES	7.3	4.2
Imidazole	Imid	7.46	-
N-2-Hydroxyethylpiper-azine-N'-2-ethane sulphonic acid	HEPES	7.55	4.5
N-tris (Hydroxymethyl) methyl-2-aminoethane sulphonic acid	TES	7.9	4.5
N-2-Hydroxyethylpiper-azinepropanesulphonic acid	EPPS	8.0	4.7
Triethanolamine	TEA	8.35	-
N-tris (Hydroxymethyl)methyl-aminopropanesulphonic acid	TAPS	8.4	4.9
Glycinylglycine	Glygly	8.4	5.6
N-tris (Hydroxymethyl)-methylglycine	Tricine	8.6	5.2

Table I cont'd.

Reagent	Abbreviation	pKa	pI
N,N-bis(2-Hydroxyethyl)-glycine	Bicine	8.74	5.3
Asparagine	Asn	8.8	5.4
N-tris(Hydroxymethyl)amino-methane	Tris	8.8	-
2-Amino-2-ethyl-1,3-prop-anediol	AEPD	8.8	-
2-Amino-2-methyl-propanol	AMP	8.8	-
Ammonia	NH_3	9.25	-
Histidine	His	9.3	7.6
Glycine	Gly	9.6	6.0
2-Aminoethanesulphonic acid	Tau	9.7	4.8
β-Alanine	β-Ala	10.2	6.9
Ethanolamine	EA	10.4	-
Lysine	Lys	10.5	9.8
Triethylamine	TA	10.7	-
ε-Aminocaproic acid	GACA	10.8	7.6
γ-Aminobutyric acid	GABA	11.3	7.5

Data available from references 4, 12, 36, 37.

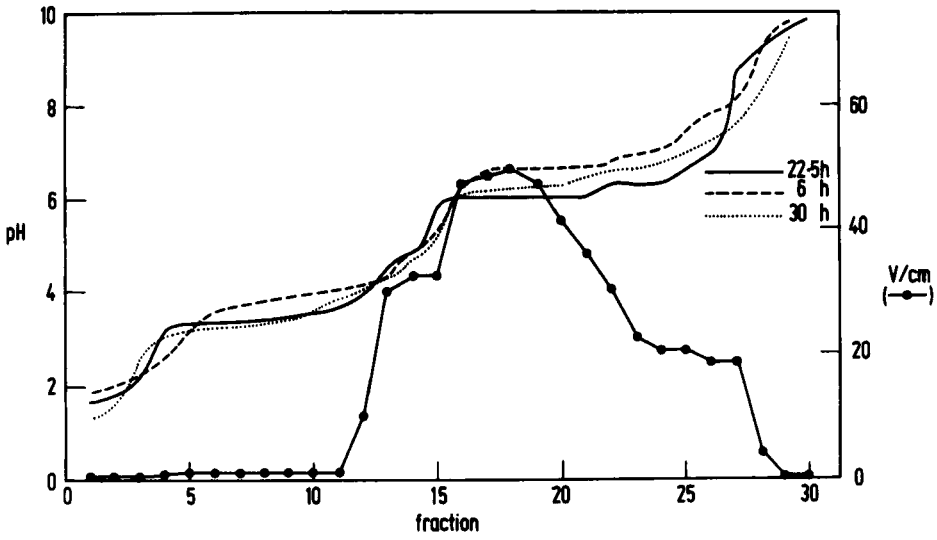

Figure 2.

pH and voltage gradient formed in a Sephadex G200 superfine gel bed containing a mixture of amphoteric buffers (buffer system 2). Buffer concentration: 10mM for each constituent buffer, Glu, Tau, Gly, GABA and Lys. Anolyte: 0.2M H_2SO_4; catholyte 0.2M KOH. The gradients were measured after 22.5 hours at a steady state current of 2mA. Also shown are the pH gradients generated after focusing for 6 and 30 hours.

the pH plateau to pH 4.3-4.6.

(b) A sample of crude ovalbumin (100mg) was dissolved in the narrow range buffer mixture comprising 10mM each of MES, ACES, TES, Tau, Tricine and Bicine (system 5) and loaded onto a Sephadex G200 superfine (3g) gel bed containing the same buffer mix. After 24 hours, (Figure 5), the protein bands were visualised by the Radola techniques[22]. On recovery of the protein bands, ovalbumin (61mg) was obtained from the focused gel in the pH range 4.6-4.8(cf. pI 4.52-4.98[24]). A minor protein was recovered from the gel in the pH range 4.9-5.0 of the gradient.

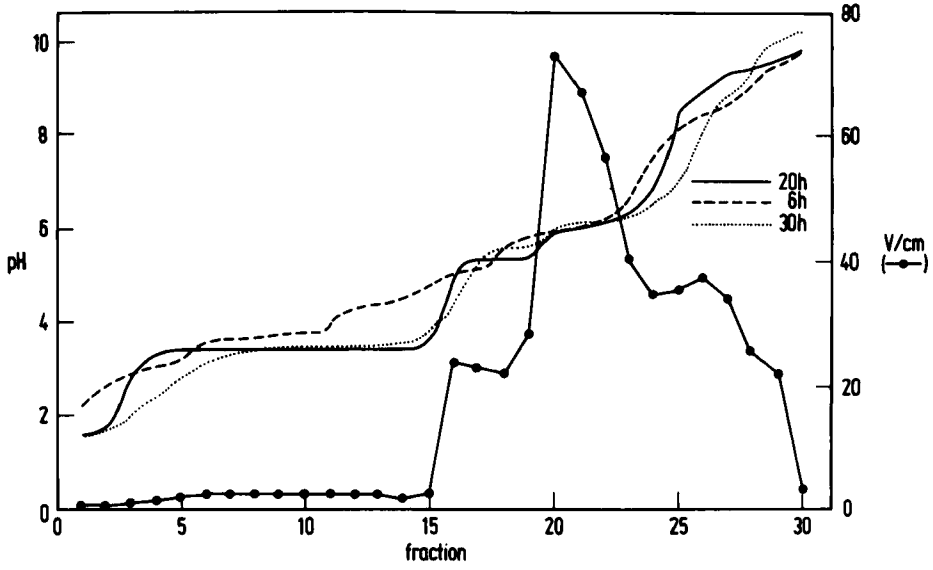

Figure 3.

pH and voltage gradient formed in a Sephadex G200 superfine gel bed using buffer system 3. The conditions were identical to those listed in Figure 2 except that Gly was deleted from buffer system 2. The gradients were measured after focusing for 20 hours at a steady state current of 2mA. Also shown are the pH gradients generated after focusing for 6 and 30 hours.

(iv) The amphoteric buffer system: Glu, MES, ACES, MOPS, HEPES, TES, EPPS, TAPS, Tau, Tricine, Bicine, Asn, GlyGly, at a concentration of 10mM except Tau (5mM) containing β-mercaptoethanol (10mM) (system 6): generated, on a Sephadex G200 superfine (3g) gel bed, the pH and voltage gradient shown in Figure 6. The effect of pH adjustment of the buffer mixture from 4.3 to 7.0 is also depicted in Figure 6. The gradient was stable for at least 48 hours.

(v) In figure 7, the pH gradient formed in a Sephadex G200 superfine gel bed by electrofocusing a buffer mixture

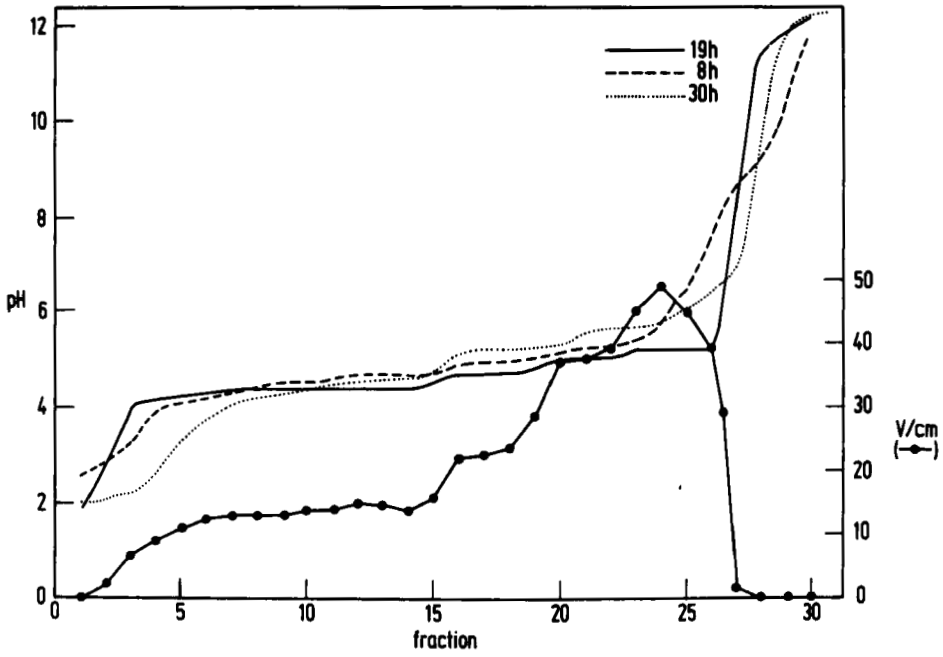

Figure 4.

pH and voltage gradients generated on a Sephadex G75 superfine flat bed with the narrow range amphoteric buffer system 4 after focusing for 19 hours at a constant power at 15W. Buffer concentrations: 10mM for each constituent buffer; MES, ACES, TES, Tricine, Bicine; 5mM for Tau; pH 7.1; anolyte: 0.2M H_2SO_4; catholyte: 0.2M KOH. Also shown are the pH gradients generated after focusing for 8 and 30 hours.

of 10mM each of GlyGly, Gly, β-Ala, GABA, GACA, His and Lys, pH 7.1 (system 7) is shown. The voltage gradient determined after 20 hours is super-imposed. A mixture of haemoglobin and myoglobin (20mg each) was resolved into discrete bands at the positions indicated. This buffer system provides a useful focusing range from pH 6 to 8.5.

(vi) In an attempt to extend the pH range of system 7, the effect of a combination of basic non-amphoteric buffers

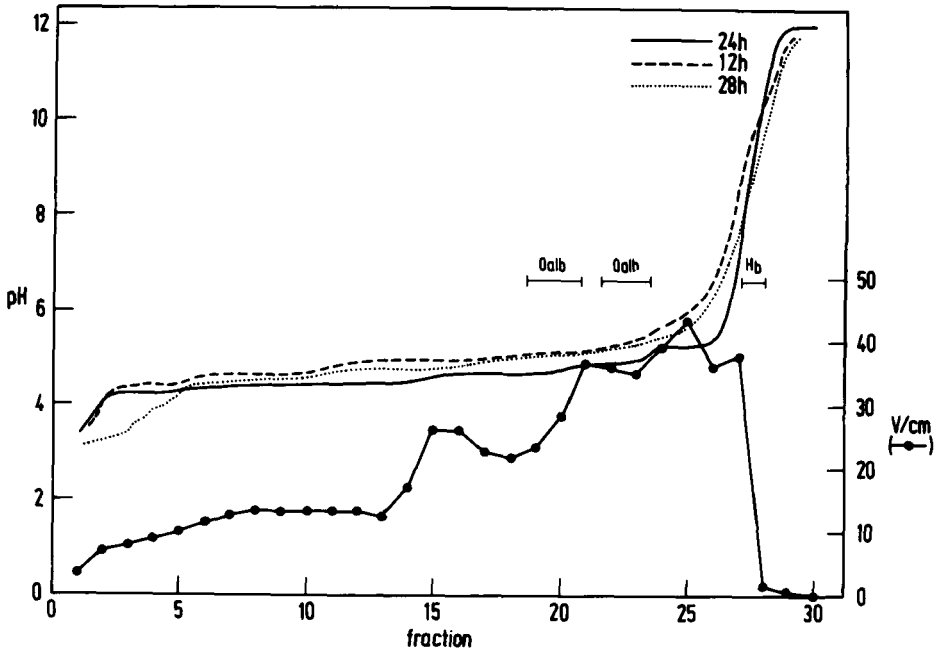

Figure 5.

Focusing of a crude ovalbumin preparation (100mg) containing haemoglobin on a Sephadex G200 superfine (3g) flat bed using a 10mM amphoteric buffer mixture (buffer system 5) under the standard conditions described above at 15W. Buffer components: MES, ACES, TES, TAU, Tricine and Bicine, focusing time 24 hours. The positions of the protein bands, visualised by the Radola print technique (17), are indicated above. pH and voltage gradients were measured after focusing for 24 hours. Also shown are the pH gradients generated after focusing for 12 and 28 hours.

was examined. The pH and voltage gradient formed on a Sephadex G200 superfine gel bed with a buffer mixture containing 10mM of system 7 together with Tris, AEPD, NH_3, AMP and TEA (system 8) is shown in Figure 8. Although a mixture of haemoglobin and myoglobin were fully resolved with this buffer system, the low conductivity below the pH 6.2 range limited the usefulness of

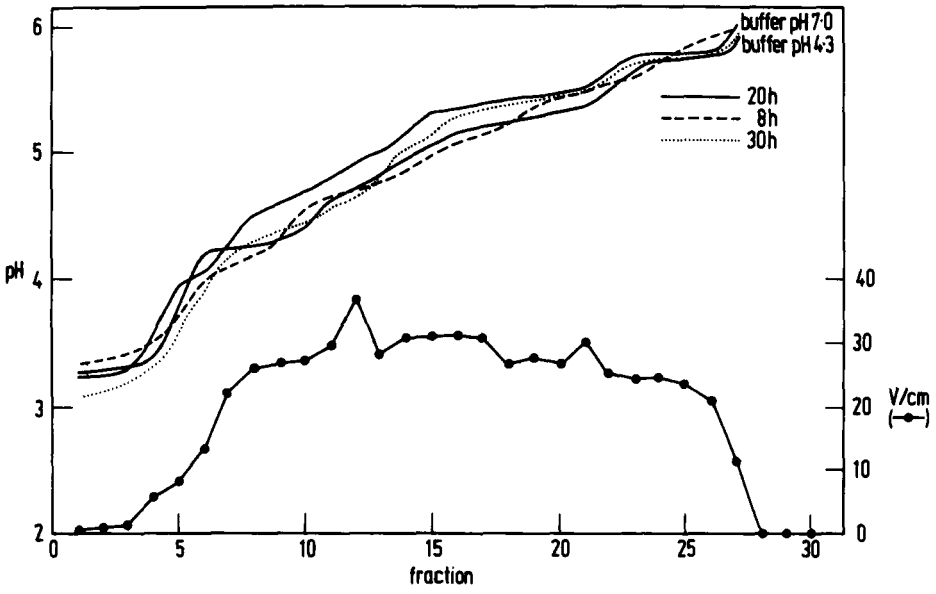

Figure 6.

pH gradients formed with the narrow range buffer system 6, with a Sephadex G200 superfine gel bed after focusing at 8W for 20 hours, as a function of pH of the buffer. Buffer concentrations: 10mM of each constituent buffer: Glu, MES, ACES, MOPS, HEPES, TES, EPPS, TAPS, Tricine, Bicine, Asn, GlyGly, 5mM for Tau, 10mM β-mercaptoethanol; anolyte: 0.2M H_2SO_4, catholyte 0.2M KOH. Also shown is the voltage gradient generated at a steady state current of 4mA, 1000V input with this buffer at pH 4.3 after 20 hours and the pH gradients generated with this carrier system after focusing for 8 and 30 hours.

of this buffer system to basic proteins.

(vii) The previous narrow range buffer systems cover the pH ranges 4-6 and 6-8.5. Buffer systems which could be used to establish a stable pH gradient over the range 4.5-8.0 were also examined ie. a 'standard' buffer adequate for the resolution of many acidic and neutral proteins.

(a) The buffer system employing amphoteric and non-

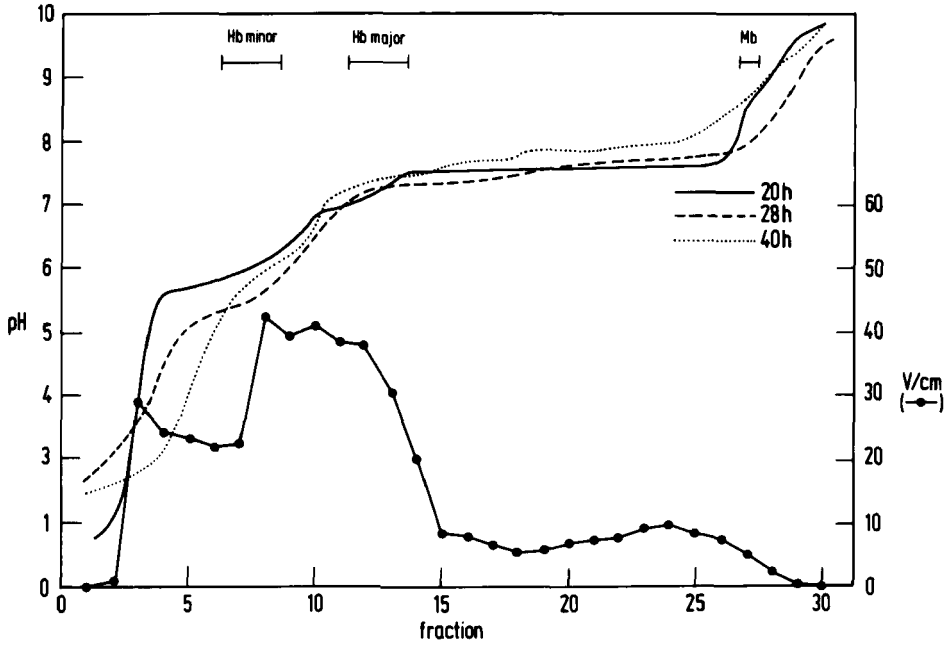

Figure 7.

pH and voltage gradients generated using the narrow range basis amphoteric buffer system 7 after focusing for 20 hours. Buffer concentration: 10mM of each constituent buffer GlyGly, Gly, β-Ala, GABA, GACA, His and Lys, pH 7.1: anolyte 0.2M H_2SO_4, catholyte 0.2M KOH. Also shown are the focused band positions of a mixture of haemoglobin and myoglobin (20mg each), and the pH gradients formed after focusing for 28 and 40 hours.

amphoteric components: MES, ACES, TES, Tricine, Bicine, Asn, Tau, Gly, GABA, GlyGly, Lactic and Propionic acid, His and Bistris, 20mM each component (system 9): generated the pH gradient shown in Figure 9. Under conditions of voltage regulation such that the total electrophoretic power dissipated in the gel was held at ca. 15W, the voltage gradient change along the gel versus time showed the anticipated increase. The voltage gradient measurements at time T = 0,1,3 and 9 hours are shown superimposed on the pH gradient in Figure 9. After

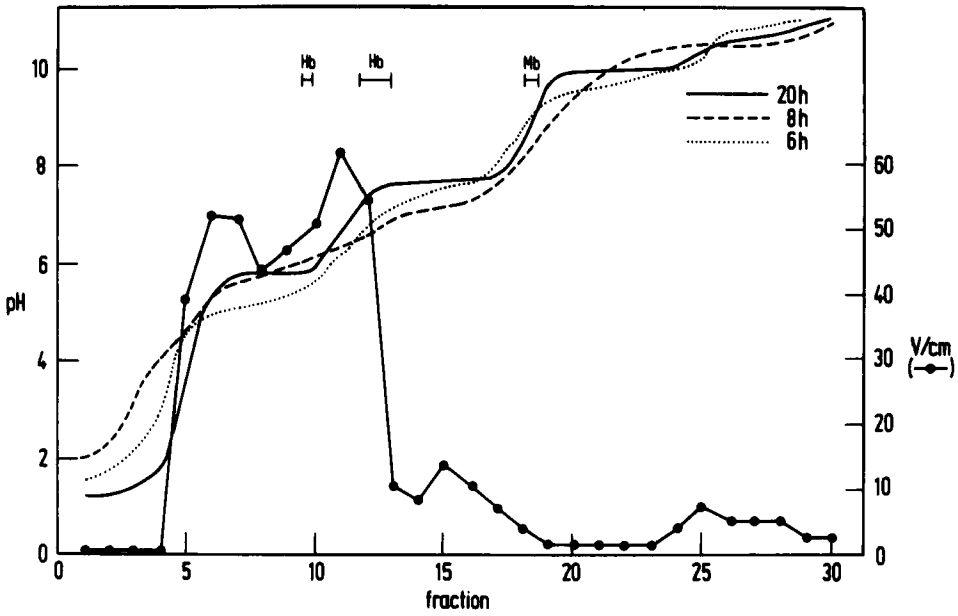

Figure 8.

pH and voltage gradient formed with a mixture of amphoteric and non-amphoteric buffers (system 8) in a Sephadex G200 gel bed after focusing for 20 hours at ca. 10W. Buffer concentration: 10mM of each constituent buffer, GlyGly, Gly, β-Ala, GABA, GACA, Hist, Lys, Tris, AEPD, NH_3, AMP, TEA and β-mercaptoethanol. The pH of the buffer mixture was adjusted from pH 9.3 to pH 8.0 with 1M H_2SO_4. Also shown are the focused positions of a mixture of haemoglobin and myoglobin after 20 hours focusing and the pH gradients formed after focusing for 6 and 8 hours.

9 hours at 1000V, no further change in the gradients occurred under these conditions with the pH remaining stable for at least 48 hours. The stability and shape of this pH gradient makes this buffer system suitable for preparative focusing experiments. Protein separations employing this buffer have been reported by us elsewhere[20].

(b) When the non-amphoteric reagents, Tris, TEA and EA, were included with the buffer system 9, a pH gradient

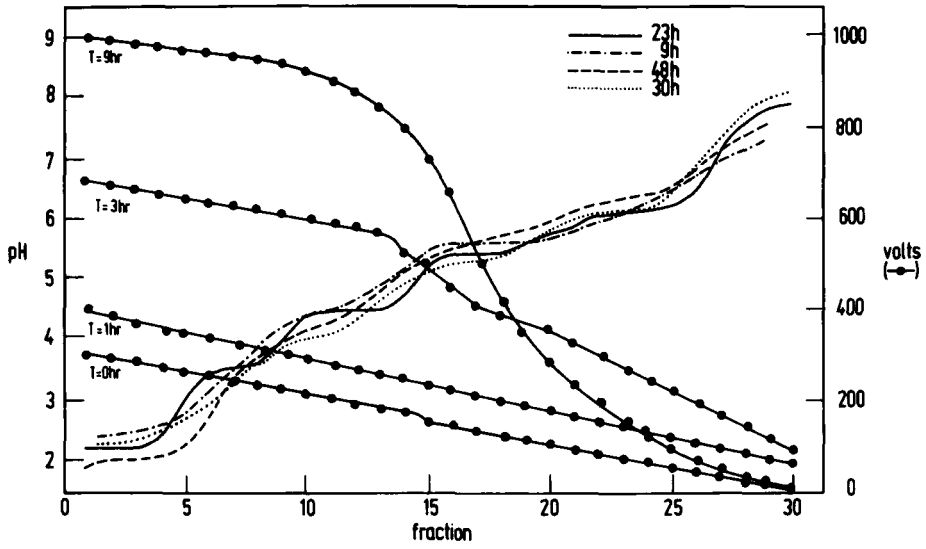

Figure 9.

pH and voltage gradients formed with a mixture of amphoteric and non-amphoteric buffers (butter system 9) as a function of time of focusing on a Sephadex G75 superfine gel bed. Buffer concentration: 20mM for each constituent buffer, MES, ACES, TES, Tricine, Bicine, Asn, Tau, Gly, GABA, GlyGly, LA, Pa, His, Bistris. The voltage gradients were measured at T = 0,1,3 and 9 hours. After 9 hours at constant voltage of 1000V, no further change in the voltage gradient was evident.

useful over the range 5-8.5 was generated (Figure 10). The pH gradient of this buffer combination (system 10) remained essentially constant for the duration of electrofocusing for at least 48 hours.

When the 0.2M H_2SO_4 anolyte was changed to 0.1M lactic acid in an attempt to circumvent the rapid pH change below pH 5 found with buffer system 10 only a small effect was witnessed similar to what is found with 0.01M phosphoric acid as anolyte in related systems.

Non Amphoteric Buffers.

In view of recent reports[13,25] that stable pH gradients can be formed in polyacrylamide gels containing simple non-

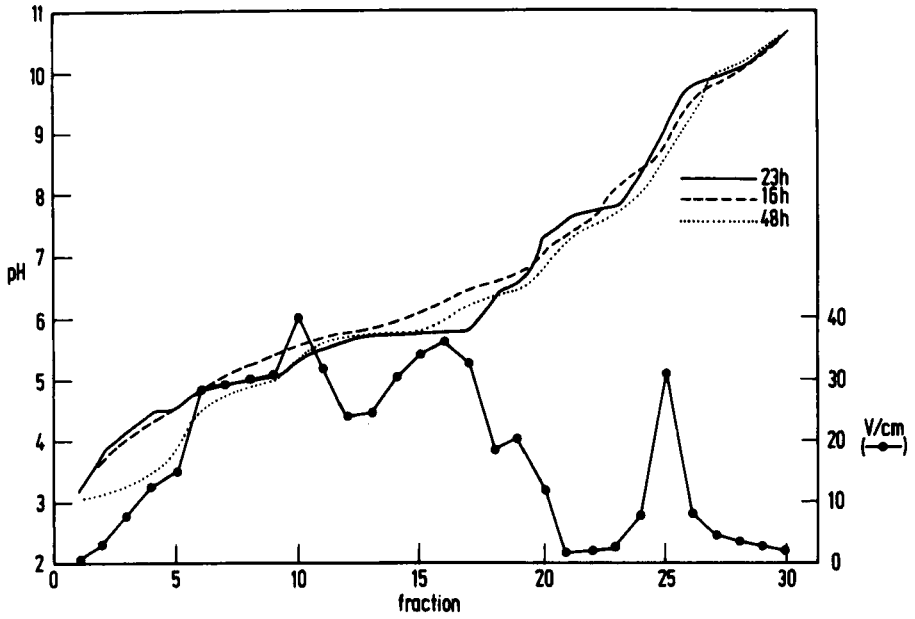

Figure 10.

pH and voltage gradients generated by the wide range buffer system 10. Buffer concentration: 20mM for each constituent buffers used in buffer system 12 together with 20mM of the non-amphoteric reagents Tris, TEA and EA, anolyte 0.2M H_2SO_4 catholyte 0.2M KOH, focusing conditions 23 hours at 15W with a Sephadex G75 superfine gel bed. Also shown are the pH gradients formed after focusing for 16 and 48 hours.

amphoteric reagents, a range of non-amphoteric buffer systems were examined under electrofocusing conditions in gradulated gel flat beds.

(i) A 20mM solution of each of the following reagents was prepared: LA, PA, Bistris, Tris, EA, Pyr, Tea (system 11). Electrofocusing was carried out using a Sephadex G75 gel bed and 0.2M H_2SO_4 anolyte and 0.2M KOH catholyte were used. After 6 hours the resistivity reached a constant value. The pH and voltage gradient generated along the gel measured after 10 hours at 1000V is shown

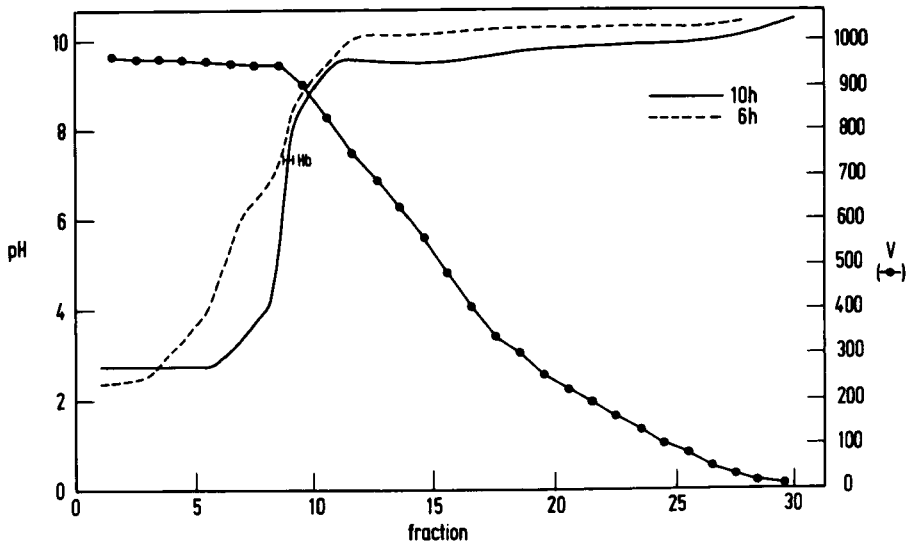

Figure 11.

The pH and voltage gradient generated by buffer system 12 after focusing for 10 hours in a Sephadex G75 superfine (4g) flat bed containing a mixture of non-amphoteric buffers (buffer system 14). Buffer concentration: 20mM of each constituent buffer, LA, PA, Bistris, Tris, EA, Pyr, TEA; anolyte 0.2M H_2SO_4, catholyte 0.2M KOH. Also superimposed is the position of a focused haemoglobin sample and the pH gradient generated after focusing for 6 hours.

in Figure 11. In a duplicate experiment haemoglobin (20mg) focused to a tight band at pI = 7.5 but the steepness of the pH gradient over the range 4-9.5 limits the usefulness of this buffer system, ie. fails to discriminate between proteins with isoelectric points less than one pH unit apart.

(ii) The pH and voltage gradient produced in a Sephadex G200 superfine gel bed by a basic non-amphoteric buffer is shown in Figure 12. The buffer mixture contained the following reagents: 10mM each, Imid, TEA, Tris, AEPD,

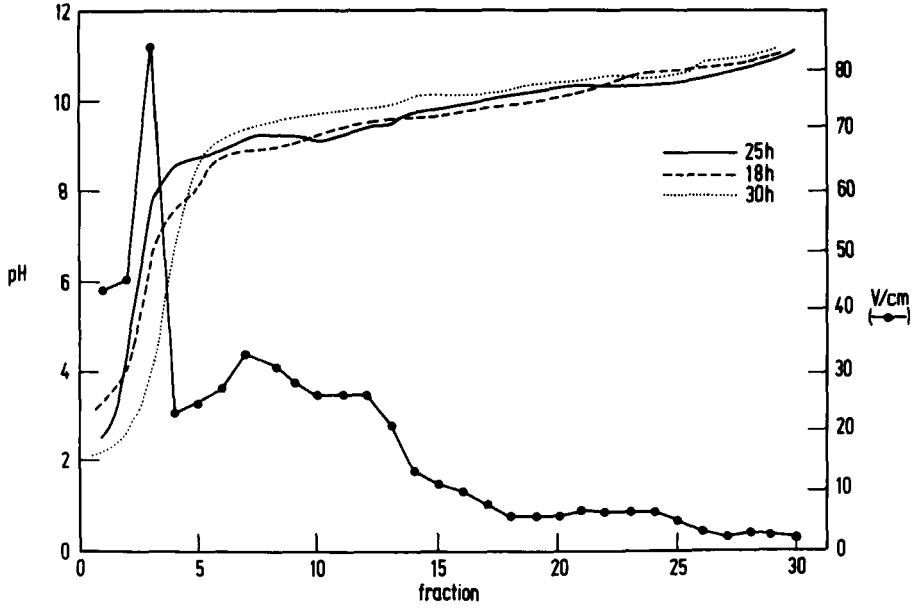

Figure 12.

The pH and voltage gradient formed by the basic non-amphoteric buffer system 13 in a Sephadex G200 superfine flat bed after focusing for 25 hours at 10W. Buffer concentration: 10mM of each constituent buffer, Imid, TEA, Tris, AEPD, NH_3, AMP, TA; anolyte 0.2M H_2SO_4, catholyte, 0.2M KOH. Also shown are the pH gradients formed after focusing for 18 and 30 hours.

NH_3, AMP and TA. This buffer system (system 12) proved unsuitable for focusing proteins. A haemoglobin sample reached a constant band position near the pH 7.8 zone after only 2 hours but denatured due to local heating.

DISCUSSION

This paper reports studies on a variety of buffer systems, containing mixtures of simple amphoteric and non-amphoteric reagents, designed to test whether stable pH gradients could be formed between a strongly acidic anolyte and a strongly basic catholyte in flat beds of granulated gels. The emphasis of these studies was on the development of buffer systems

suitable for preparative electrofocusing. Obviously, the requirements for preparative electrofocusing are different from those needed in analytical systems. For example, a linear pH range is not necessarily required with preparative loadings because the focused protein zones will not be at their theoretical narrowest width (diffusion increases with protein concentrations whereas the buffering capacity $\frac{dQ}{d(pH)}$ of the buffer system will not change significantly provided the protein concentration is not high enough to perturb the pH gradient). Hence, for preparative separations, a pH plateau near to the region of pI interest is preferred particularly if this creates a large physical separation between adjacent focused bands. Furthermore, for preparative electrofocusing as high a voltage gradient as possible is required, within limitations of overheating, in the region of interest to ensure that the focused bands are as sharp as possible. Similar criteria can be applied in analytical electrofocusing where resolution is also very dependent on the local slope of the pH gradient.

An examination of Table I reveals most of the amphoteric reagents examined in the present study have ($pI - pK_1$) values greater than 2.5 units. The amphoteric and non-amphoteric buffers used represent only a selection of available buffer reagents with suitable pI or pKa values for the isoelectric separation of proteins on the basis of differences in net charge. It has been our experience that suitable buffer concentration gradient zones, resulting in the formation of pH gradients, reasonably stable with time of electrofocusing, can be formed using buffer systems selected on the basis of criteria similar to those proposed[26,27] for multiphasic zone electrophoresis. The availability of narrow range buffer systems covering the pI ranges 4-6, 6-8 and 8-10 would cover a very comprehensive range of proteins (for a compendium of

protein pI values see ref. 28,29). The studies reported above are by no means exhaustive in determining buffer systems useful for preparative isoelectrofocusing over this range but they do provide several systems which can be used preparatively to discriminate between proteins differing in pI by ca. 0.2 units. Comparatively few amphoteric buffers with pI values above 8, suitable for use in electrofocusing experiments, are available. Mixed basic amphoteric-non amphoteric buffer systems for use with granulated gels may circumvent this deficiency and studies with these systems are curently under investigation. By the criteria of Svensson[4], most of the reagents listed in Table I should be ineffective as carrier ampholytes. Nevertheless, relatively stable linear and non linear pH gradients, as the case requires, can be generated using combinations of these compounds and granulated gels like Sephadex G75 as the supporting media.

The development of these electrofocusing systems with flat beds of granulated Sephadex gels thus extends the earlier demonstrations by Chrambach et al.[12-14] that suitable buffer systems could be used to generate stable pH gradients in polyacrylamide gels. Simple amphoteric buffers, based on amino acid or peptide mixtures[2,30,31] have also been used with limited success prior to the availability of the Ampholines. Taking into account the requirements for preparative separations the buffer systems 4,6,7,9 and 10 described above provided the most useful gradients, stable for at least 48 hours, following the transient state in which the gradient is formed in the granulated gel support. As have been noted before in polyacrylamide gels[14], progressive acidification of the granulated gel bed does however occur with time, starting from the anodic end, which limits the useful pH gradient lifetime to ca. 60-80 hours with a 0.2M H_2SO_4 anolyte. However, stabilisation of the pH gradient with time can be achieved[18]

by equilisation of the anolyte pH with the pI or pKa of the most acidic component in the ampholyte range, eg. by making the anolyte lactic acid in example (vii). It is apparent from these experiments that buffer ampholytes with pI-pK_1 values up to 3 can be used to form stable pH zones adjacent to their isoelectric points under focusing conditions of 60-80mW/cm^2 whereas buffers with pI-pK_1 values greater than 3, due to low conductance at their isoelectric point, have perturbed or unstable pH zones.

The pH gradient obtained with buffer system 1 (figure-1) provides an interesting insight into the mechanism involved in buffer electrofocusing in granulated gels. The pH values (3.4, 5.8, 7.7, 10.0) of the plateau steps in the pH gradient correspond closely to the respective pI values of the four buffer components (Glu, 3.2; Glygly, 5.6; His, 7.6; Lys, 9.8). Similar plateau regions, again corresponding to pI values of each component were observed with several other simple buffer systems. These observations suggest that the pH gradient is, in fact, generated by a series of step functions of pH. Multiphasic zone electrophoretic theory, predicts such a series of pH step functions for a mixture of amphoteric buffers with each step corresponding to a single buffer. In order to distinguish whether an isoelectric focusing, rather than a multiphasic stacking mechanism, operates under these granulated gel flat bed conditions, ie. pH steps follow the order of pIs rather than constituent mobilities, isotopically labelled buffers would be required. Evidence for mobility alignment within a spatially extended pH gradient has been obtained[31] with amino acid buffers, which were distributed at or near the anodic and cathodic pH gradient termini as well as in positions along the pH gradient approximating their respective isoelectric points. However, in this system pH gradient formation

and electrofocusing were carried out with buffer anolytes and catholytes at their isoelectric points thus confining the extended stack between the isoelectric 'leading' and 'trailing' constituents. Preliminary experiments addressed to this problem with the flat bed buffer systems favour a buffer component, as well as protein, alignment in order of respective isoelectric points, ie. an electrofocusing mechanism similar to that observed[2,32,33] with Ampholines under focusing conditions. Also consistent with this proposal is the observed unstable, steep pH gradients obtained with the non amphoteric buffer system 11 and 12 (figures 11, 12) despite the fact that the constituent components cover a range of pKa values from 3.8-4.9 and 6.9-9.5 for buffer system 11 and 7-11 for buffer system 12. This observation contrasts with earlier reports[13,25] that stable pH gradients can be formed in polyacrylamide gels with non-amphoteric buffers.

It was generally observed with the amphoteric buffer systems that the effect of increasing the pH of the buffer mixture from its initial average pH permitted higher starting currents, ie. since the electrophoretic mobility M_t of a buffer molecule is proportional to its degree of ionisation, pH titration can result in more rapid generation of the buffer concentration gradient zones. Once these are formed, however, the pH gradient was found not to be significantly different at comparable focusing times irrespective of initial buffer pH. This effect is clearly demonstrated by the pH gradients generated with the amphoteric buffer system 6 at two initial buffer pHs (Fig. 6) where the deviations from monotony are not significant and probably due to uneven sectioning of the gel bed. Furthermore, when an asymptotically constant current is reached, eg. 5½hr for buffer system 4, complete focusing of all components may not necessarily have occurred. In fact, all that is necessary for an asymptotically low and constant current to

be generated under the buffer focusing conditions in flat beds of granulated gels is that one component is focused to its isoelectric zone and the low conductivity of this zone would then govern the limiting current. At high initial currents H^+ and OH^- ions are generated at the anode and cathode respectively. Being uncoupled with counterions, they migrate into the gel slab, establish H^+ and OH^- ion fluxes, which are not necessarily of the same magnitude at equivalent ion concentrations, and titrate the buffer mixture which has no, or little, buffering capacity at its initial average pI. Titration of the buffer components by H^+ and OH^- ions, will increase the voltage gradient and in this increased field the various species can migrate towards their final position. When excess ion-paired H^+ and OH^- ions are available at the electrodes, ie. in example (iii(b)) were sufficient HCl and NaOH was available at the anode and cathode to titrate all the buffer compounds, no enhancement in the rate of focusing or significant change in the pH gradient was apparent. This would imply that the generation of sufficient H^+ and OH^- ion fluxes in these systems is still required before a suitable voltage gradient can be established. The formation of a proton flux greater than an OH^- ion flux would also account for the cathodal drift of the focused protein bands seen with long focusing times. Similar electrode effects have been observed with polyacrylamide gel systems in the presence and absence of ampholytes[8,10,18].

The results obtained with buffer systems 9 and 10 indicate that by increasing the number of amphoteric components with suitably different pI and pK_1 values in a buffer mixture, it is possible to create linear pH gradients comparable to those produced by the Ampholines. The narrow range pH gradients, eg. examples 6 and 7, would be expected to generally give better preparative resolution provided suitable buffers can

be selected to correspond to the pI region of interest. The various buffer systems described above have several additional advantages compared to Ampholines, and other proprietory carrier ampholytes, besides their low cost. Since they contain no high molecular weight polyionic species they are easily removed at the completion of preparative experiments. Furthermore, the buffer components do not bind to conventional protein dyes, cause staining artefacts or interfere with the detection of the immunological or enzymatic characteristics of the focused protein zones. The use of wide and narrow range buffer systems 6,7, 9 and 10 for the preparative separation of a range of proteins including thyroid glycoproteins and the pregnancy specific α2-glycoprotein will be described in associated papers[34,35].

REFERENCES

1. H. Rilbe, Ann. N.Y. Acad. Sci., 209, 11, (1973).

2. H. Haglund, Methods Biochem. Anal., 19, 1, (1971).

3. H. Svensson, Acta Chem. Scand., 15, 325, (1961).

4. H. Svensson, Acta Chem. Scand., 16, 456, (1962).

5. O. Vesterberg, Acta Chem. Scand., 23, 2653, (1969).

6. G. Baumann and A. Chrambach, in "Progress in Isoelectric Focusing and Isotachophoresis". P.G. Righetti, ed. Elsevier, Amsterdam, 1975, p.13.

7. J.S. Fawcett, in "Progress in Isoelectric Focusing and Isotachophoresis", P.G. Righetti, ed., Elsevier, Amsterdam, 1975, p.25.

8. L.E.M. Miles, J.E. Simmons and A. Chrambach, Anal. Biochem., 49, 109, (1972).

9. H. Rilbe, in "Electrofocusing and Isotachophoresis", B.J. Radola and D. Graesslin, eds., Walter de Gruyter, Berlin, 1977, p.35.

10. S. Pollack, Biochem. Biophys. Research Commun., 87, 1252, (1979).

11. N.Y. Nguyen, A.G. McCormick and A. Chrambach., Anal. Biochem., 88, 186, (1978).

12. N.Y. Nguyen and A. Chrambach, Anal. Biochem., 74, 145, (1976).

13. N.Y. Nguyen, D. Rodbard, P.J. Svendsen and A. Chrambach, Anal. Biochem., 77, 39, (1976).

14. N.Y. Nguyen, A. Salokangas and A. Chrambach, Anal. Biochem. 78, 287, (1977).

15. N.Y. Nguyen and A. Chrambach, Electrophoresis, 1, 14, (1980).

16. B. An der Lan and A. Chrambach, Electrophoresis, 1, 23, (1980).

17. A. Chrambach, L. Hjelmeland, N.Y. Nguyen and B. An der Lan, in "Electrophoresis '79" B.J. Radola, ed., W. de Gruyter, Berlin-New York, 1980, p.3.

18. N.Y. Nguyen and A. Chrambach, Anal. Biochem., 82, 54, (1977).

19. R.L. Prestidge and M.T.W. Hearn, Proc. Univ. Otago med. Sch., 56, 60, (1978).

20. R.L. Prestidge and M.T.W. Hearn, Anal. Biochem., 97, 95, (1979).

21. B.J. Radola, Biochim. Biophys. Acta, 386, 181, (1974).

22. B.J. Radola, Biochim. Biophys. Acta, 295, 412, (1973).

23. M.J. Swanson and B.E. Sanders, Anal. Biochem., 67, 520, (1975).

24. J.A. Beeley, S.M. Stevenson and J.C. Beeley, Biochim. Biophys. Acta, 285, 293, (1972).

25. B.E. Chidakel, N.Y. Nguyen and A. Chrambach, Anal. Biochem., 77, 216, (1976).

26. N.Y. Nguyen, D. Rodbard, P.J. Svendson and A. Chrambach, Anal. Biochem., 77, 39, (1976).

27. T.M. Jovin, Ann. N.Y. Acad. Sci., 209, 477, (1973).

28. P.G. Righetti and T. Caravaggio, J. Chromatogr., 127, 1, (1976).

29. D. Malamud, and J.W. Drysdale, Anal. Biochem., 86, 620, (1978).

30. H. Svensson, Arch. Biochem. Biophys. (Suppl. 1), 132, (1962).

31. M.L. Caspers and A. Chrambach, Anal. Biochem., 81, 28, (1977).

32. A. Chrambach and N.Y. Nguyen, in "Electrofocusing and Isotachophoresis", B.J. Radola and D. Graesslin, eds., W. de Gruyter, Berlin-New York, 1976, p.51.

33. H. Davies, Prot. Biol. Fluids, 17, 389, (1969).

34. M.T.W. Hearn, A.J. Paterson and R.L. Prestidge, Prep. Biochem., submitted for publication.

35. M.T.W. Hearn, R.L. Prestidge and F. Griffen, Prep. Biochem., submitted for publication.

36. N.E. Good, G.D. Winget, W. Winter, T.N. Connolly, S. Izawa, and R.M.M. Singh, Biochemistry, 5, 467, (1966).

37. D.D. Perrin, in "Dissociation Constants of Organic Bases in Aqueous Solution", Butterworths, London, 1965.