Chromatofocusing with Polybuffer and PBE

Handbook

18-1009-07
Edition AB

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Contents

1. Introduction

The separation of proteins according to their isoelectric points by isoelectric focusing (IEF) in ampholytes has proven to be a highly successful and popular technique. The resolution offered by analytical isoelectric focusing is amongst the highest available from present biochemical separation techniques.

Chromatofocusing is a unique new column chromatographic method for separating proteins according to their isoelectric points. Chromatofocusing offers the high resolution obtained by separations based on differences in isoelectric points, together with the high capacity of ion exchange techniques. Peak widths can be in the range of 0.04—0.05 pH unit, and samples containing several hundred milligrams of protein can be processed in one step.

Polybuffer™ and Polybuffer exchangers PBE 94 and PBE 118 have been specially designed by Amersham Pharmacia Biotech for chromatofocusing. They are inexpensive and very simple to use.

Chromatofocusing with Polybuffer and PBE allows the separation of proteins according to pI in a column chromatographic system characterized by

- self-generated pH gradients, which eliminate the need for gradient-making apparatus
- focusing effects, which produce sharp, well separated bands and very high resolution
- ease of use

This handbook is designed as an introduction to chromatofocusing and as a guide to the use of chromatofocusing products available from Amersham Pharmacia Biotech.

2. Chromatofocusing

Chromatofocusing was first described by *Sluyterman* and *co-workers* (1—3). They proposed that a pH gradient could be produced on an ion exchanger by taking advantage of the buffering action of the charged group of the ion exchanger. If a buffer, initially adjusted to one pH, is run through an ion exchange column, initially adjusted to a second pH, a pH gradient is formed just as if two buffers at different pH were gradually mixed in the mixing chamber of a gradient maker. If such a pH gradient is used to elute proteins bound to the ion exchanger, the proteins elute in order of their isoelectric points. Futhermore, focusing effects take place, resulting in band sharpening, sample concentration and very high resolution.

Linear gradients are required for optimal resolution, since non-linear pH gradients give poor resolution in certain areas of the chromatogram. In order to have a linear pH gradient, it is necessary that the eluent buffer and the ion exchanger should have an even buffering capacity over a wide pH range.

Polybuffer 96 and Polybuffer 74 are amphoteric buffers designed for chromatofocusing in the ranges pH 9—6 and pH 7—4 respectively. They contain a large number of buffering species to give even buffering capacity, and are used together with the Polybuffer exchanger PBE 94. For chromatofocusing in the range pH 11—8, Pharmalyte® pH 8—10.5 is used in conjunction with the Polybuffer exchanger PBE 118.

2.1 Description of chromatofocusing

Chromatofocusing is an extremely simple technique to set up and operate. The pH interval is chosen so that the isoelectric points of the proteins of interest fall roughly in the middle of the pH gradient. The appropriate Polybuffer exchanger is then equilibrated with start buffer, in much the same way as for a conventional ion exchanger. The pH of the start buffer is set slightly above the upper limit of the pH gradient, whilst the pH of the eluent, Polybuffer, is adjusted to the value chosen for the lower limit of the pH gradient. The sample is equilibrated with eluent and applied to the column. The column is eluted with Polybuffer and the pH gradient forms automatically. Proteins elute in order of their isoelectric points. The degree of resolution obtainable by chromatofocusing is shown in Figure 1, the fractionation of soluble proteins from elk muscle. For details, see Figure 21.

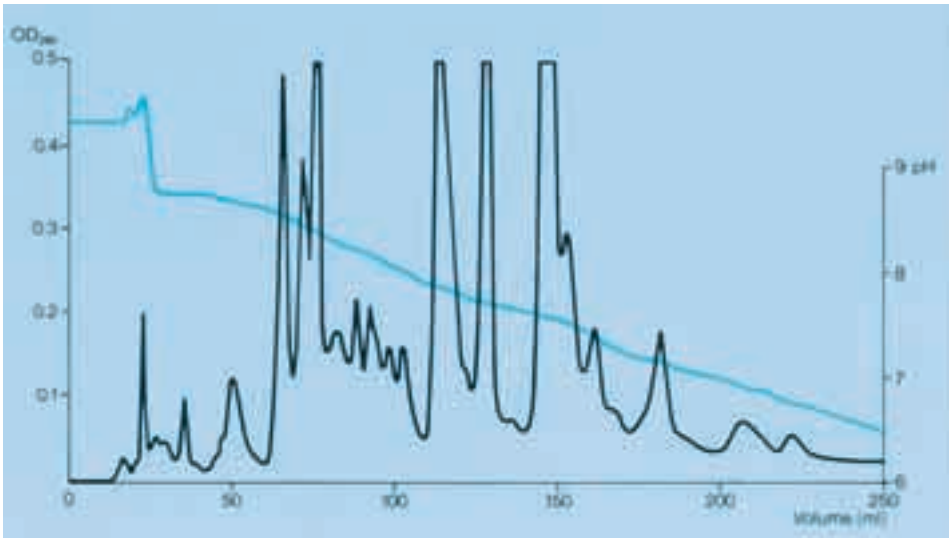

Fig. 1. Fractionation of soluble proteins extracted from elk muscle. Column C 10/40. Bed height: 35 cm. Sample: 5 ml of supernatant from elk meat homogenate. Elution conditions: Start buffer 0.025 M ethanolamine HCl, pH 9.4, elution buffer 0.0075 mmol/ pH unit/ ml Polybuffer 96, pH 6. Flow rate: 20 $cm.h^{-1}$.

2.2 Mechanism of chromatofocusing

1. Formation of the pH gradient

In ion exchange chromatography, a pH gradient is normally formed using a gradient mixer. For a descending gradient, the mixing chamber is filled with start buffer at high pH and the other chamber contains limit buffer at a lower pH. As the solution leaves the mixing chamber to pass onto a column, more limit buffer at low pH enters the mixing chamber and the pH of the eluent gradually decreases as shown in Figure 2a.

Chromatofocusing takes advantage of the buffering action of the charged group on the ion exchanger itself and the pH gradient is formed automatically as the eluting buffer titrates the ion exchanger. This is illustrated diagrammatically in Figure 2b. As an example of gradient formation, we can follow the development of a pH gradient from pH 9—6. For a descending pH gradient, the column is at a higher pH than the eluent. A basic buffering group is required on the ion exchanger so an anion exchanger is chosen. PBE 118 and PBE 94 are anion exchangers.

The column of PBE 94 is first equilibrated to pH 9. The pH of the eluent (corresponding to limit buffer) is set to pH 6. This eluent, Polybuffer 96, contains a large number of differently charged species. As these migrate down the column, the most acidic components bind to the basic anion exchanger. The pH in the solution leaving the column during the early stages of elution is close to the starting pH (fig. 3a). As elution progresses (stages b and c) the pH at each point in the column is gradually lowered as more Polybuffer is added to the column.

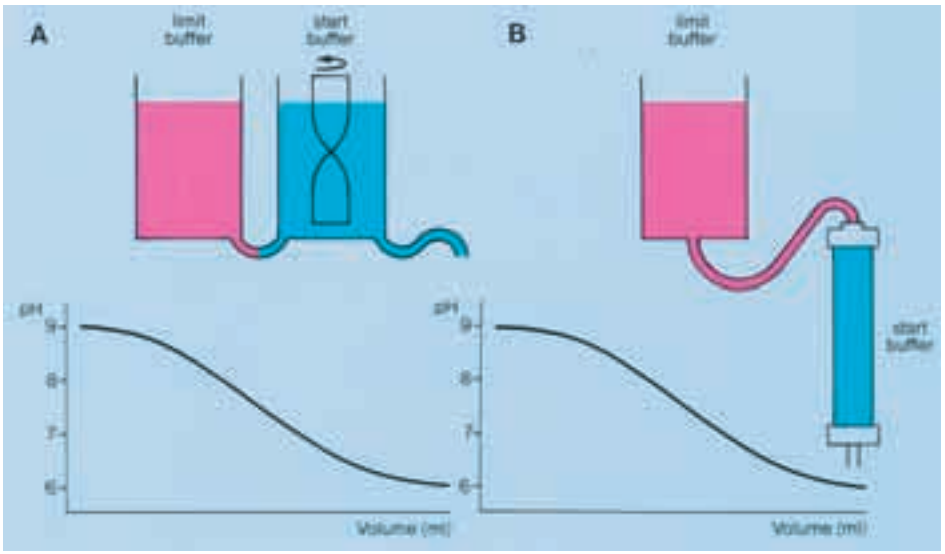

Fig. 2. Diagrammatic representation of gradient formation during (a) ion exchange chromatography and (b) chromatofocusing.

In the final stages of elution (d), almost all the column has reached equilibrium with the eluent, and the pH of the solution leaving the column is close to the pH of the original Polybuffer solution being added at the top. The pH gradient formed in the solution leaving the column is shown in the lower part of Figure 3.

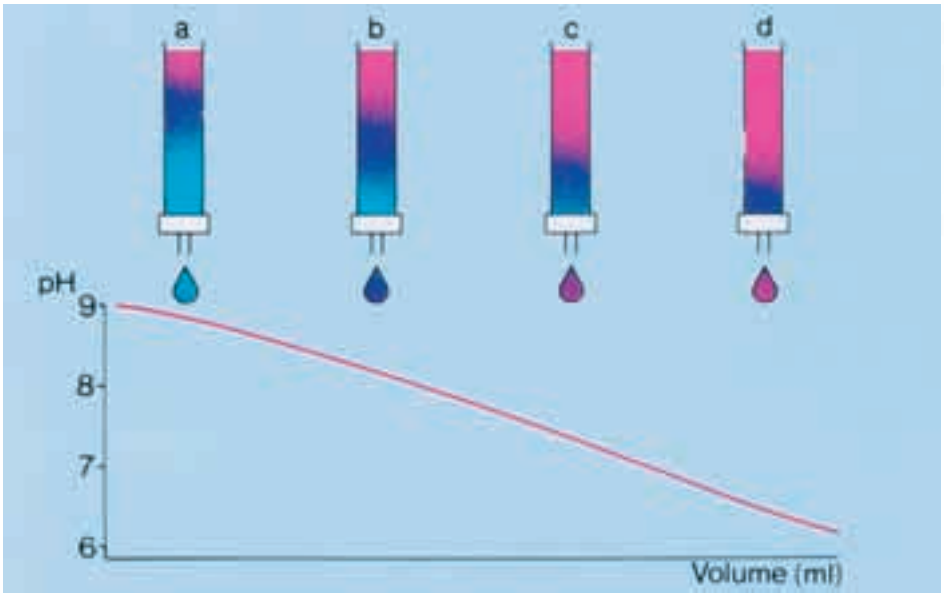

Fig. 3. Formation of a pH gradient (pH 9—6) in a column of PBE 94 eluted with Polybuffer 96. (a) (b) (c) (d) represent different stages in elution.

2. The behaviour of a protein

The charge on a protein depends on the pH and the isoelectric point of the protein. When the pH is less than its isoelectric point, a protein carries a positive charge and will migrate down the column of anion exchanger in the eluting buffer. However, as it migrates, the pH of the buffer surrounding the protein increases with the distance from the top of the column (fig. 3). When it has travelled sufficiently far down the column so that the pH is greater than the pI, the protein reverses its charge and binds to the ion exchanger. The molecule remains bound to the ion exchanger until the developing pH gradient causes the pH to drop below the pI of the protein. The protein is then carried along in the eluent buffer again until the pH rises above the pI and it rebinds. This process is repeated until the protein emerges from the column at its isoelectric point.

Proteins with different isoelectric points will migrate different distances on the column before binding. Elution is in order of their isoelectric points. This is shown diagramatically in Figure 4.

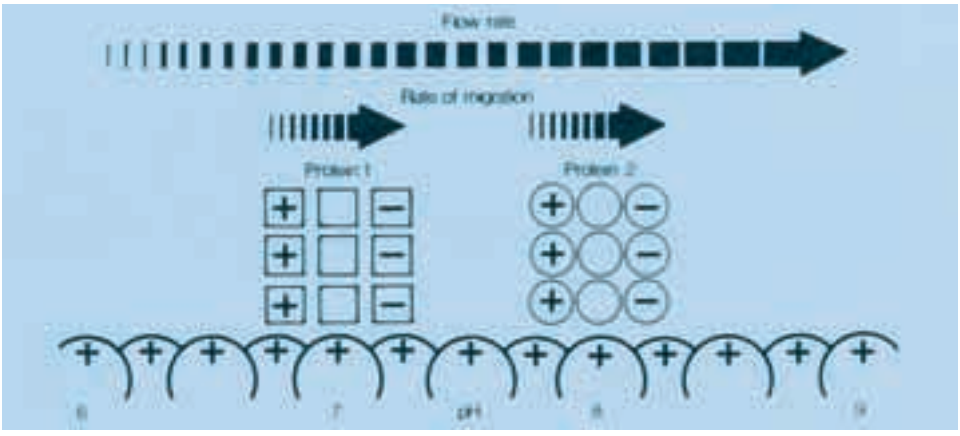

Fig. 4. Behaviour of protein molecules in chromatofocusing. The pH increases down the length of the column. Protein molecules above their isoelectric point in the gradient become negatively charged and bind to the gel. Molecules below their isoelectric point are repelled from the gel. Molecules at their isoelectric point are neither bound nor repelled. Protein 1 has a hypothetical pI = 7, protein 2 a pI = 8.

3. The focusing effect

The formation of an internal pH gradient also enables one to predict a focusing effect. If a protein is applied to the column it will migrate down the column in the eluent as far as its pI, from where it migrates more slowly until it elutes. If, during this process, a second sample of protein is applied, it will migrate down the column at the same speed as the eluent until it meets the slower moving first sample. The two samples then proceed down the column to the bottom where they co-elute. This is shown diagrammatically in Figure 5, where the length of the arrows above the zones represents the speed of migration. Note that all of the sample must be applied before the peak starts to elute, otherwise the peak will not have time to focus before it leaves the column. The focusing effect is shown in Figures 6 and 7.

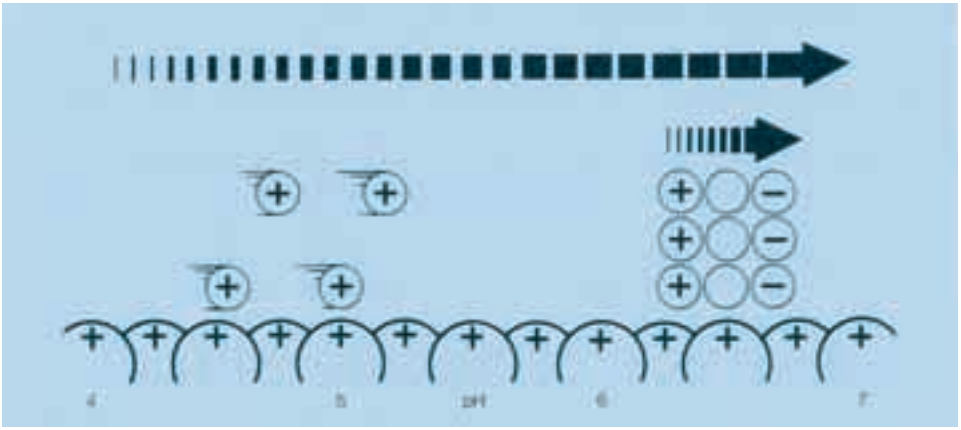

Fig. 5. Focusing effect in chromatofocusing. Protein molecules at the rear end of the zone are repelled from the gel and migrate more rapidly than proteins at the front. If a second sample of the same protein is applied to the column after the first, it will catch up and co-elute with the first. If a second sample of a protein with a higher pI is applied, it will pass through the first zone and elute before it.

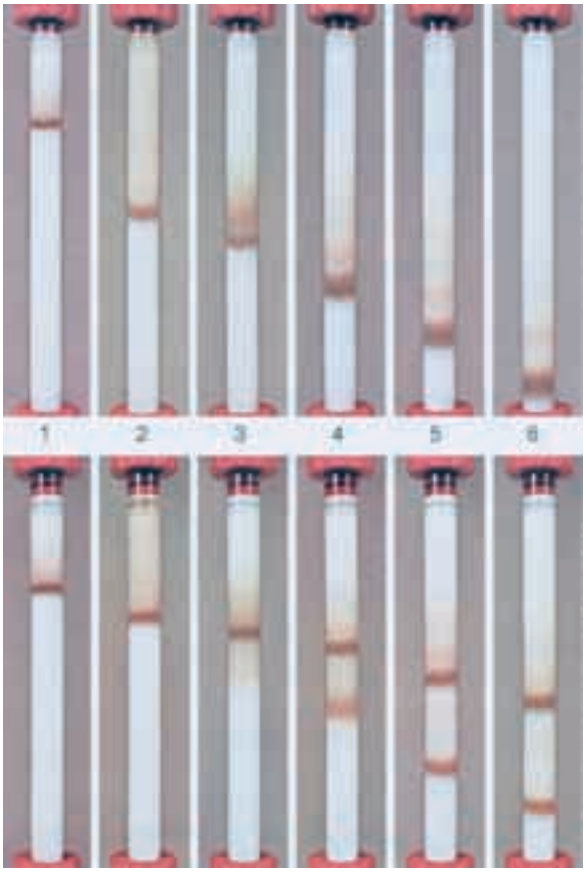

Fig. 6. Focusing effect in chromatofocusing. A sample of myoglobin (horse) is caught up by a second sample of the same protein on PBE 94. Photographs were taken at periodic intervals. The second sample is applied in frame 2 and appears as a diffuse zone until it starts to focus. The second sample was applied in frame two. Column: C 10/20. Bed height: 15 cm. Sample: 3 ml protein (2 mg/ml) applied in two aliquots. Elution conditions: Column was equilibrated to pH 9.5 with 0.025 M ethanolamine HCl and eluted with Polybuffer 96 (0.0075 mmol/pH unit/ml) equilibrated to pH 7. Flow rate: 18 cm.h^{-1}.

Fig. 7. Elution of myoglobin (sperm whale) and myoglobin (horse) in chromatofocusing. The sperm whale myoglobin not only catches up but overtakes the first sample on PBE 94. Horse myoglobin has a pI = 7.4, sperm whale myoglobin a pI = 8.2. Conditions as for Figure 6.

2.3 Requirements for chromatofocusing

Titrating one buffer with a second buffer at a different pH is not sufficient to create a linear pH gradient, since the buffering capacities of the systems produced are pH-dependent. In order to obtain a linear pH gradient, it is necessary that both buffers should have an even capacity over their working pH range. Polybuffers and Polybuffer exchangers PBE 118 and PBE 94 have been specially designed for chromatofocusing. PBE 118 and PBE 94 have extraordinarily even buffering capacities over a wide pH range. Polybuffer 96 and Polybuffer 74 are amphoteric buffers with even capacities over a broad pH range.

2.4 Resolution in chromatofocusing

Resolution in chromatofocusing, as in other column techniques, is related to the width of the zone as it elutes. The theoretical bandwith of a protein in chromatofocusing depends on several factors.

1. The slope of the pH gradient

To optimize the resolution by having well separated bands, it is best to have a fairly flat pH gradient. This is accomplished by having low buffer concentrations which give gentle pH changes and good separation between peaks. A flat gradient also results in a narrower bandwidth (2) in terms of pH units. The gradient should not be too flat since the bandwith in terms of volume becomes large and thus protein concentrations in the eluent will be low. A gradient volume equal to 10—15 bed volumes gives good results.

2. Charge on the ion exchanger

The charge difference between the ion exchanger and the surrounding medium contributes to zone sharpening in chromatofocusing, just as the electrical field strength contributes to zone sharpening in IEF. Polybuffer exchangers have a very high degree of substitution in order to give good focusing. In addition, the ionic strength and thus buffer concentration should be kept low. A low buffer concentration will also give a shallow pH gradient if the gradient is generated internally. Chromatofocusing with Polybuffer is thus a very economical technique.

3. Column packing

Since chromatofocusing produces extremely narrow bands of separated material, irregularities in column packing have a much more marked effect on resolution than can be detected with lower resolution techniques. It is thus important to use high quality media and columns.

In summary, the special features required for chromatofocusing are therefore:

- an ion exchanger with a high, even buffering capacity over a broad pH range.
- a buffer with an even buffering capacity over a wide pH range.

Additional desirable features include low UV adsorption, ease of removal from proteins, and low cost.

3. Product guide

3.1 Chromatofocusing media

Polybuffer 74, Polybuffer 96 (p. 11)
Specialised buffers for chromatofocusing characterised by an even buffering capacity over the specified pH range. Supplied as sterile solutions in packs of 250 ml.

Polybuffer exchangers PBE 94, PBE 118 (p. 13)
Bead formed gels for chromatofocusing with Polybuffer or Pharmalyte pH 8—10.5. Polybuffer exchangers have an even capacity over a wide pH range. They are supplied as a suspension in 24 % ethanol in packs of 200 ml.

Pharmalyte pH 8—10.5
Carrier ampholyte for isoelectric focusing. Pharmalyte pH 8—10.5 should be used with PBE 118 for chromatofocusing in the range pH 8—11. Available as a sterile solution in packs of 25 ml.

3.2 Equipment

Pharmacia columns (p. 18)
A full range of columns and accessories is available. Special attention is drawn to the C-series columns, an inexpensive range for chromatography in aqueous media and most organic solvents.

Peristaltic pump P-1
A compact single channel pump for applications which do not require a gradient-forming capability.

UV-Monitors UV-1
Sensitive and reliable dual beam UV-monitors for detecting proteins, nucleic acids peptides etc. at 280 and 254 nm.

Recorders REC 111, REC 112
Reliable and accurate chart recorders for use with Pharmacia monitors or other laboratory equipment to give a permanent record of your experiments.

Fraction Collector FRAC-200
A programmable fraction collector which allows sample collection as required by the separation problem.

Full details about equipment suitable for use for chromatofocusing can be found in the catalogue or the individual product brochures.

4. Polybuffer

4.1 Chemical and physical properties

Polybuffer is available in two different ranges, Polybuffer 96 for chromatofocusing in the range pH 9—6 and Polybuffer 74 for the range pH 7—4. Both are designed for use with the Polybuffer exchanger PBE 94. For chromatofocusing at pH values above pH 9, Pharmalyte pH 8—10.5 should be used in conjunction with PBE 118. Each Polybuffer consists of a selected range of constituents designed to give an even buffering capacity across a wide pH range, in order to provide a smooth linear pH gradient during chromatofocusing. The titration curves for Polybuffer 96 and Polybuffer 74 are shown in Figure 8.

The UV absorption of Polybuffer is shown in Figure 9. Polybuffer has very low absorption at 280 nm, but can be detected at 254 nm. The eluent from a chromatofocusing experiment should be monitored at 280 nm.

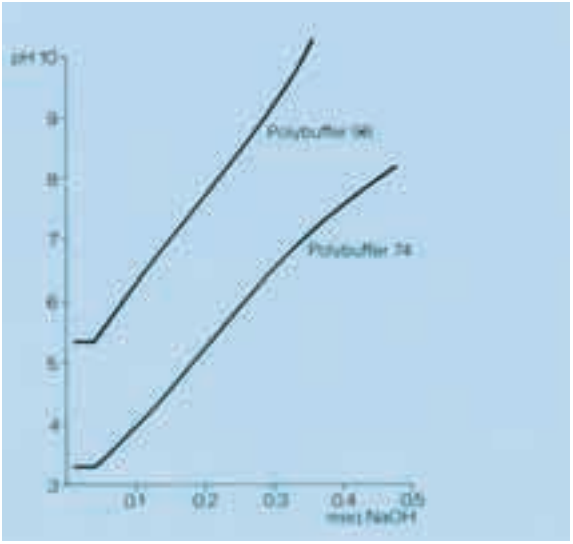

Fig. 8. Titration of 2 ml Polybuffer with 0.1 M NaOH.

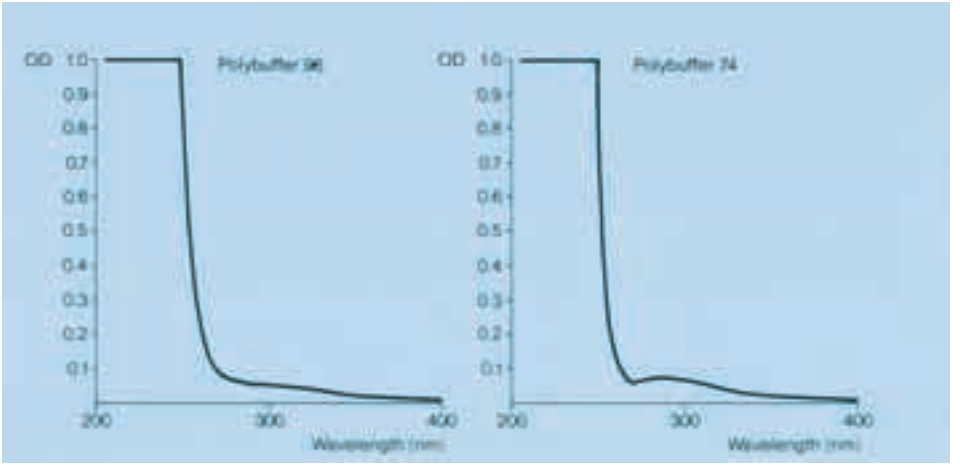

Fig. 9. UV absorption spectra of Polybuffer (1:5 dilution).

4.2 Availability

Polybuffer is supplied as a sterile filtered solution (0.075 mmol/pH unit/ml) in bottles of 250 ml, sufficient for 2—3 l of working solution. Polybuffer should be stored at 3—8° C in the dark.

5. Polybuffer exchangers

Polybuffer exchangers PBE 118 and PBE 94 are based on Sepharose® 6B, which has been cross-linked. Charged groups are then coupled to monosaccharide units in the gel by ether linkages.

5.1 Chemical and physical properties

Stability in aqueous media

Polybuffer exchangers are stable in water, salt solutions and organic solvents in the range pH 3—12. Polybuffer exchangers can be used with strongly dissociating solvents such as 8 M urea. The cross-linked nature of Polybuffer exchangers also gives them great physical stability, permitting high flow rates and preventing fluctuations in bed volume caused by electrostatic interactions at different pH values. Polybuffer exchangers can be used at elevated temperatures and can be sterilized repeatedly in the salt form by autoclaving at pH 7 at temperatures of 110—120° C.

Capacity

The charged groups in Polybuffer exchangers are specially chosen to give an even buffering capacity across a broad pH range. Capacity data for PBE 118 and PBE 94 are given in Table 1. Titration curves for each type of exchanger are given in Figure 10.

Table 1. Capacity data for Polybuffer exchangers PBE 94 and PBE 118.

Polybuffer exchanger	Total capacity meq/100 ml gel by pH interval							
	3—4	4—5	5—6	6—7	7—8	8—9	9—10	10—11
PBE 94	2.2	3.1	3.1	3.0	3.5	3.9	3.1	2.1
PBE 118	.6	.3	.9	1.7	2.8	3.7	4.6	4.9

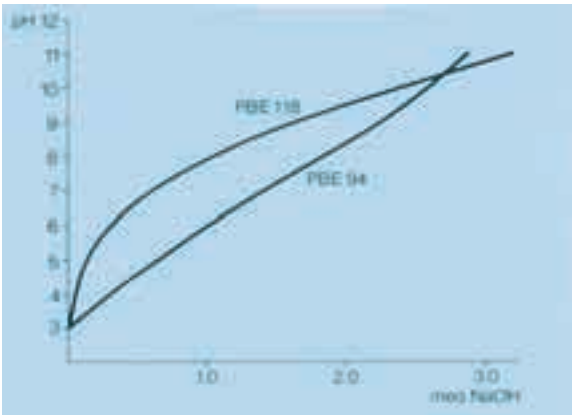

Fig. 10. Titration of 10 ml quantities of PBE 118 and PBE 94 in 1 M KCl.

Flow rates

The cross-linked nature of Polybuffer exchangers means that very high flow rates can be obtained. Figure 11 illustrates the variation of flow rate with pressure drop for PBE 118 and PBE 94 in different chromatography columns.

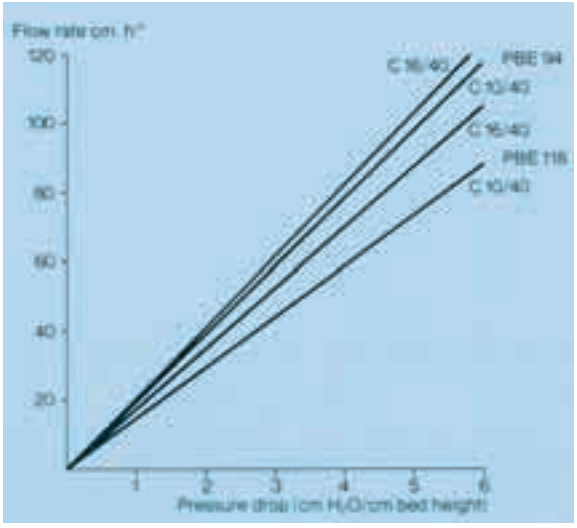

Fig. 11. The flow rate as a function of the pressure drop across a bed of PBE 94 and PBE 118.

The quality of separation in well packed columns in chromatofocusing is relatively independent of flow rate (2, 3), permitting the use of high flow rates. The relationship between flow and resolution is discussed further on page 21.

5.2 Availability

PBE 94 and PBE 118 are supplied in packs of 200 ml as a suspension in 24 % ethanol.

6. Experimental

Chromatofocusing, like IEF, is suitable for the separation of any amphoteric water-soluble molecule with an isoelectric point within the working range (pH 3—11). The technique is thus ideal for the separation of proteins and peptides. Other molecules which have been separated according to their pI include RNA.

The equipment required for chromatofocusing is very simple, and no more is required than a chromatography column. However, in order to take full advantage of the high performance of the technique, correctly designed monitors, recorders, pumps and fraction collectors should be used.

The critical stages in designing a chromatofocusing experiment are outlined in Figure 12. Further details are given in the relevant sections.

Fig. 12. Critical stages in planning a chromatofocusing experiment.

6.1 Choice of gel and buffer

Polybuffer and Polybuffer exchangers are designed to operate over intervals of a maximum of 3 pH units at a time.

If the isoelectric point of the sample is known, then the pH range of the experiment is chosen so that the component of interest elutes after ⅓—½ of the pH gradient (see fig. 13) in order to have optimal resolution with respect to time (see also p. 31).

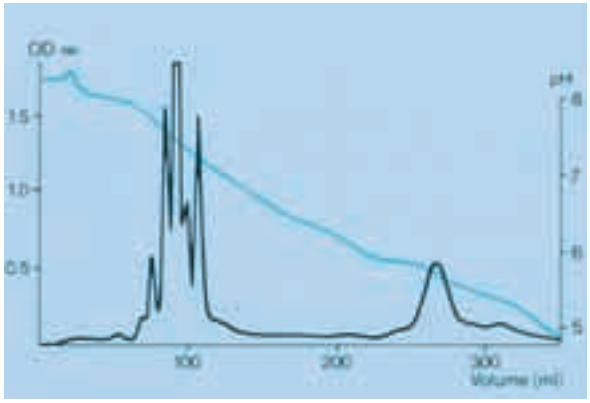

Fig. 13. Separation of a test mixture of carboxyhaemoglobin and transferrin on a column of PBE 94. Carboxyhaemoglobin elutes in the ideal position; transferrin elutes later. Column: SR 10/50. Bed height: 30 cm. Sample: 15 ml containing transferrin (8 mg) and carboxyhaemoglobin (10 mg). Elution conditions: Start buffer 0.025 M Tris HCl, pH 8.2, elution buffer 0.0075 mmol/pH unit/ml Polybuffer 96: Polybuffer 74 (1:2) pH 5. Flow rate: 40 cm.h^{-1}.

If the isoelectric point of the sample is unknown it can be determined by isoelectric focusing or by a simple test using ion exchangers (4, 5).

Alternatively, use chromatofocusing with Polybuffer 74 or Polybuffer 96, and PBE 94. This covers the range pH 9–4 and is thus sufficient for at least 85 % of characterised proteins (6). When working with an unknown sample, it is best to start with a gradient of Polybuffer 74, since if the sample does not have a pI in the range pH 7–4 it will pass straight through the column and can be recovered easily. If the first experiments are run at a pH above its pI, it will bind to the column and recovery will be more difficult since the column will need to be washed with salt and the sample re-equilibrated with eluent before re-application to the column. Furthermore, most proteins have isoelectric points in the range pH 7–4 (6). Once the pH range of the experiment is known, then the gel, start buffer and Polybuffer solutions can be chosen from the information given in Table 2.

Choice of gel quantity

The amount of gel required for a chromatofocusing run dependes on the amount of sample, the nature of the sample and contaminants, and the degree of resolution required. For most separations, a bed volume of 20—30 ml is sufficient for the separation of samples containing up to 1—200 mg of protein/pH unit in the gradient. However, the precise amount of gel used will depend on the resolution required from the experiment and can only be discovered empirically.

Table 2. Buffers and gels for chromatofocusing in different pH ranges.

pH range	Gel	Start buffer	Eluent	Dilution factor	Approximate volume (in column volumes) Dead volume	Gradient volume	Total volume
10.5—9[1]	PBE 118	pH 11	—	—	—	—	—
10.5—8	,,	0.025 M triethylamine-HCl	pH 8.0 Pharmalyte pH 8-10.5-HCl	1:45	1.5	11.5	13.0
10.5—7	,,	,,	pH 7.0 Pharmalyte pH 8-10.5-HCl	1:45	2.0	11.5	13.5
9—8[2]	PBE 94	pH 9.4 0.025 M ethanolamine-HCl	pH 8.0 Pharmalyte pH 8-10.5-HCl	1:45	1.5	10.5	12.0
9—7	,,	,,	pH 7.0 Polybuffer 96-HCl	1:10	2.0	12.0	14.0
9—6	,,	pH 9.4 0.025 M ethanolamine-CH_3COOH	pH 6.0 Polybuffer 96-CH_3COOH	1:10	1.5	10.5	12.0
8—7	PBE 94	pH 8.3 0.025 M tris-HCl	pH 7.0 Polybuffer 96-HCl	1:13	1.5	9.0	10.5
8—6	,,	pH 8.3 0.025 M tris-CH_3COOH	pH 6.0 Polybuffer 96-CH_3COOH	1:13	3.0	9.0	12.0
8—5[3]	,,	,,	pH 5.0 Polybuffer 96 (30%) + Polybuffer 74 (70%) — CH_3COOH	1:10	2.0	8.5	10.5
7—6	PBE 94	pH 7.4 0.025 M imidazole-CH_3COOH	pH 6.0 Polybuffer 96-CH_3COOH	1:13	3.0	7.0	10.0
7—5	,,	pH 7.4 0.025 M imidazole-HCl	pH 5.0 Polybuffer 74-HCl	1:8	2.5	11.5	14.0
7—4	,,	,,	pH 4.0 Polybuffer 74-HCl	1:8	2.5	11.5	14.0
6—5	PBE 94	pH 6.2 0.025 M histidine-HCl	pH 5.0 Polybuffer 74-HCl	1:10	2.0	8.0	10.0
6—4	,,	,,	pH 4.0 Polybuffer 74-HCl	1:8	2.0	7.0	9.0
5—4	PBE 94	pH 5.5 0.025 M piperazine-HCl	pH 4.0 Polybuffer 74-HCl	1:10	3.0	9.0	12.0

Degas all buffers before use

1. Gradients ending at pH 9 are not recommended since 9 is above the pH of Pharmalyte pH 8—10.5.
2. PBE 118 and Pharmalyte pH 8—10.5 also cover this range, as do PBE 94 and Polybuffer 96.
3. Mixing gives the best results. Polybuffer 74 works better than Polybuffer 96 when used alone.

The figures given for gradient volume are approximate and will vary with the exact conditions chosen. The dilution factor given is not critical, and the best conditions will be found by experience.

6.2 Choice of column

Correct column design is essential for the best results. Amersham Pharmacia Biotech provide a wide range of column designs to suit different applications and budgets. The best results are obtained on a long narrow column. Useful column types are the C10/40, SR 10/50, C16/70 and XK 16/70.

6.3 Preparation of the gel

The gel must be equilibrated with start buffer and packed into a suitable column before running.

A list of suitable start buffers is given in Table 2. The normal counter-ion is chloride. Monovalent anions other than chloride may be used as counter-ions, *but it is critical that these anions should have a* pKa *at least two pH units below the lowest point of the gradient chosen.* Bicarbonate ions cause flutuations in the pH gradient so *all buffers must be degassed before use.* Atmospheric carbon dioxide may cause a plateau in the region pH 5.5—6.5, depending on the conditions. These effects are most marked with Polybuffer 96 in pH gradients ending at pH 6, and can be avoided by using acetate as counter-ion. Alternatively, set the lower limit for gradients with Polybuffer 96 at pH 6.5.N.B. acetate is not usually recommended as a counter-ion with Polybuffer 74 because of its higher pKa. Multivalent anions are not recommended.

Polybuffer exchangers can be equilibrated in a sinter funnel or in a column after packing.

Funnel method

The chosen amount of Polybuffer exchanger is poured into a sintered glass funnel. Start buffer is then passed through the gel in the funnel, using aspiration, until the pH of the eluent is the same as that of the start buffer. The gel should be stirred gently from time to time to ensure complete equilibration. Equilibration usually requires 10—15 bed volumes of start buffer.

N.B. Speeding up the equilibration process by using concentrated buffer followed by 1—2 bed volumes of start buffer is not recommended, since the high capacity of Polybuffer exchangers can make equilibration to a lower ionic strength difficult.

After equilibration the gel can be packed into a suitable column.

Packing method

It is necessary to have a well packed column in order to get the full benefit of chromatofocusing. Extremely narrow zones (0.02 pH units) can be obtained in the best columns, as illustrated in Figure 14 for the separation of model proteins. Packing at high flow rates gives the best results and in our laboratories the following method has been used for columns of the XK- and SR-type. Columns of the C-type are best packed using the column packing reservoir RC10 or RC16. Instructions for use are supplied with the reservoir.

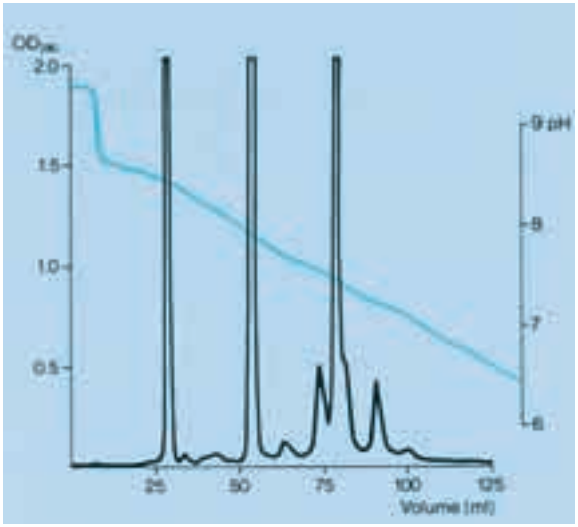

Fig. 14. Separation of a model mixture of proteins in the range pH 9—7. Column: SR 10/50. Bed height: 18 cm. Sample: 5 ml elution buffer containing sperm whale myoglobin (2 mg) horse myoglobin (2 mg) and carboxyhaemoglobin (2 mg). Elution conditions: start buffer 0.025 M ethanolamine, pH 9.5, elution buffer 0.0075 mmol/pH unit/ml Polybuffer 96, pH 7. Flow rate: 15 cm.h^{-1}.

1. The chosen amount of gel is dispersed in a small amount of start buffer (approx. 50 % of settled bed volume) to make a slurry. The slurry should then be degassed prior to packing in the selected column.
2. The column should be mounted vertically, and any air bubbles present should be flushed out from underneath the bed support nets. The tubing outlet should be greater than 50 cm below the top of the column. Ensure that the column outlet is closed.
3. Place 2—3 ml of start buffer in the column and pour in the gel slurry after first mixing it by swirling the slurry round the flask. If the volume of the slurry is greater than that of the column, a packing extension can be used.
4. Open the column outlet and allow the gel to settle rapidly into the column. When all the suspension has run into the column, the packing extension can be removed and the top piece connected to the column, taking care to exclude air bubbles.
5. Continue to pack the column at a linear flow rate of 100 cm.h^{-1} until the gel bed has completely settled. If the Polybuffer exchanger was not equilibrated prior to packing, then approximately 10—15 bed volumes of start buffer will be required for equilibration. Equilibration can be carried out at a lower flow rate.The column has reached equilibration when the pH and conductivity of the eluent match that of the start buffer.

Checking column packing

A well packed column is essential for high performance and good quality results so it is worth checking the packing carefully. Care must be taken in the choice of dye substances used for checking beds as they may be strongly charged. Bovine cytochrome *c* is recommended as a marker substance since it is coloured, readily available and is strongly basic (pI = 10.5) and therefore repelled from the gel.

6.4 Preparation of sample

The *amount* of sample applied depends on the amount of protein present in each zone. As a guideline 100 mg of protein can be applied for every 10 ml of gel bed volume, although this value will vary according to the number of proteins present. The *volume* of sample is unimportant so long as all of the sample is applied before the substance of interest is eluted from the column. Normally it is not necessary to exceed a volume of 0.5 bed volume. The *composition* of the sample is important and it should not contain large amounts of salts ($I<0.05$). Ideally the sample should be equlibrated with eluent buffer or start buffer, whichever is most suitable for the sample, by using gel filtration with Sephadex®G-25, expecially if the volume is greater than 10 ml. The sample pH is unimportant if the buffer concentration is low.

6.5 Sample application

Many methods are available for sample application (4). The most convenient and reproducible method is via a flow adaptor, using a syringe or sample applicator SA-5 or SA-50 (fig. 15). To ensure really even sample application, layer 1—2 cm Sephadex G-25 Coarse on top of the bed. This acts as a mixing chamber, and since sample volume is not critical, permits even sample application. The sample should be applied by first running on 5 ml of eluent, followed by the sample (in eluent buffer or start buffer), and then switching back to the eluent again. In this way the sample proteins are never exposed to extremes of pH.

6.6 Elution

No special gradient apparatus is required for elution since the gradient forms automatically. The upper limit of the gradient is defined by the pH of the column and the lower limit of the gradient is defined by the pH of the eluent buffer. Recommended buffer compositions are given in Table 2. The pH of the start buffer is normally set 0.4 pH units above the desired pH. This compensates for fluctuations in pH at the start of the run caused by slight differences in the conductivity of start buffer and eluent. The volume of the gradient is defined by the strength of the eluent solution. The recommendations in Table 2 are designed to give a gradient volume of approximately 10 column volumes for pH intervals of 3 pH units. Shorter pH gradients naturally have a smaller volume. This volume is optimal for most separations. Stronger buffers would give a steeper pH gradient, but some resolution would be lost since peaks would elute closer together. Buffer strengths much less than those recommended result in gradients which have large volume and long elution times, peaks are broader (in terms of volume) resulting in low sample concentration, and buffering capacity is low which may result in fluctuations in pH and poor gradient shape. Note that there is a dead volume of 1.5—2.5 bed volumes of buffer which pass through the column before the pH in the eluent starts to decrease, so the total amount of buffer required is approximately 12.5 bed volumes. Polybuffer is not suitable for intervals wider than 3 pH units.

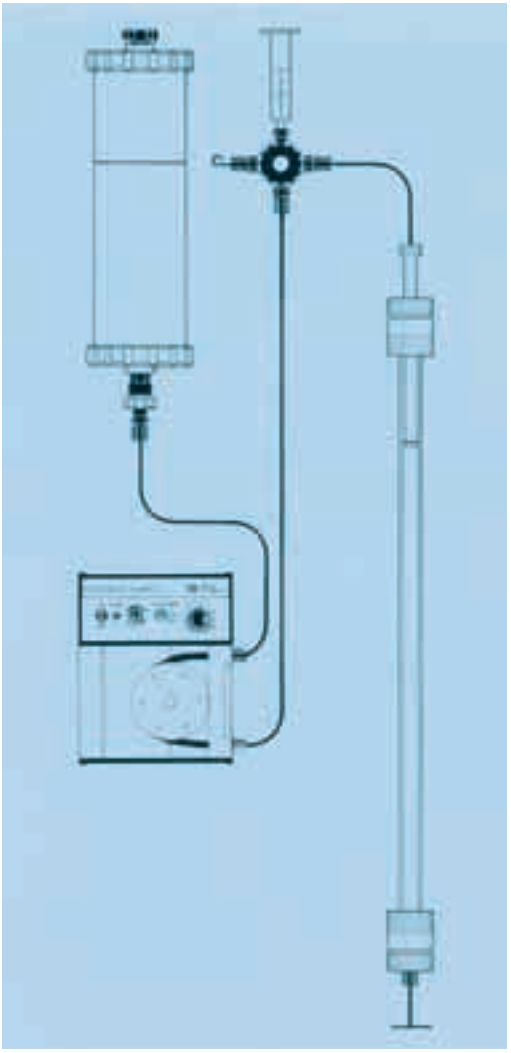

Fig. 15. Sample application using a syringe.

The choice of *flow rate* for elution depends on the degree of resolution required. The cross-linked nature of Polybuffer exchangers means that they are very resistant to pressure and give excellent flow rates (p. 14). The effect of flow rate on a separation is shown in Figure 16, which illustrates the separation of a mixture of standard proteins at a flow of 15 $cm.h^{-1}$ and 117 $cm.h^{-1}$. As can be seen, resolution deteriorated somewhat at the higher flow rate but was still excellent. A flow rate of 30—40 $cm.h^{-1}$ has been found to give consistently good results in our laboratories.

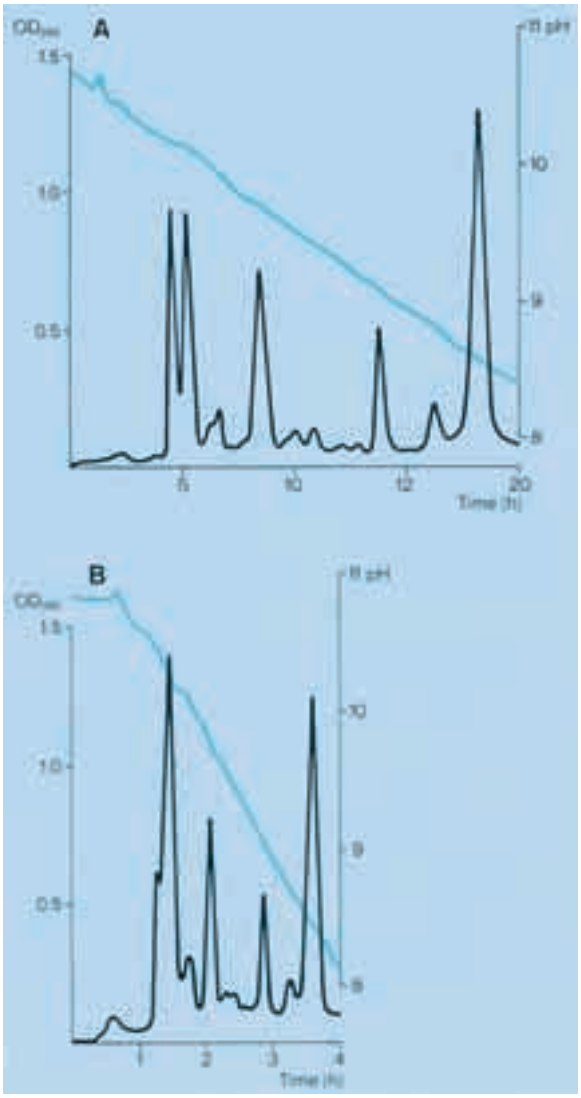

Fig. 16. Separation of a test mixture of proteins at different flow rates on a column of PBE 118. Pharmacia column SR 10/50. Bed height: 30 cm. Sample: 5 ml elution buffer containing cytochrome *c* (5 mg) ribonuclease (8 mg) and lentil lectin (10 mg). Elution conditions: Start buffer 0.025 M triethylamine HCl, pH 11.0, elution buffer 0.0075 mmol/pH unit/ml Pharmalyte pH 8—10.5 equilibrated to pH 8. Flow rate: A 15 $cm.h^{-1}$ B 117 $cm.h^{-1}$.

Proteins should be monitored at 280 nm since Polybuffer absorbs slightly at 254 nm. In addition to monitoring the protein content of the eluent, it is advisable to monitor the pH to ensure that there are no anomalies in the gradient. This can be done by measuring the pH in each fraction soon after it is collected so as to prevent disturbances caused by atmospheric CO_2, or better still by using a flowthrough pH electrode connected to one channel of a two-channel recorder such as the REC-112 whilst the other channel is connected to a UV-monitor such as the UV-1.

6.7 Separation of Polybuffer from protein

Polybuffer does not interfere with enzyme assays or amino acid analyses. Although Polybuffer does not interfere with the Coomassie Blue protein assay (7), it forms a complex with copper ions and interferes with the Lowry protein method.

There is a choice of methods for separating Polybuffer from proteins.

1. Precipitation

The simplest method is precipitation with ammonium sulphate. Solid ammonium sulphate is added to the relevant fractions to a suitable concentration (80—100 % saturated) and the sample is allowed to stand for 1—2 hours until the protein precipitates. This should be relatively simple since the protein is at its pI. The precipitate is collected by centrifugation in a bench centrifuge and washed a couple of times with saturated ammonium sulphate. Alternatively, the fractions of interest can be placed in dialysis tubing and dialysed against saturated ammonium sulphate. Precipitation has the advantages of concentrating and stabilizing the sample as well as being cheap to use.

2. Hydrophobic interaction chromatography (HIC)

HIC on Octyl- or Phenyl-Sepharose CL-4B can be used in conjunction with the above technique. Ammonium sulphate is added to the sample from chromatofocusing up to a level of 80 % saturation to favour hydrophobic interactions. This treatment may result in precipitation of the sample protein. If this happens, the purified sample can be harvested by centrifugation, washing with saturated ammonium sulphate.

Alternatively the sample can now be loaded onto a column of Phenyl-Sepharose CL-4B, equilibrated with 80 % saturated ammonium sulphate. If binding does not occur then the concentration of ammonium sulphate should be increased or the more hydrophobic gel Octyl-Sepharose CL-4B should be tried. Approximately 1 ml gel is required for 10 mg protein in the sample. The gel is washed with 2—3 bed volumes of 80 % saturated (for higher) ammonium sulphate, and then elution can be carried out with a suitable buffer at low ionic strength. If the sample is to be lyophilized, a suitable choice is a volatile buffer such as ammonium acetate.

Strongly bound sample can be removed by agents such as ethylene glycol. More information about hydrophobic interaction chromatography and the gels Octyl-Sepharose CL-4B and Phenyl-Sepharose CL-4B is available free on request from Pharmacia.

3. Gel filtration

Polybuffer can be removed from most proteins by gel filtration on Sephadex G-75 (fig. 17). If the molecular weight of the sample is less than 15,000, then some difficulties may be encountered in getting base-line separation in short columns.

4. Affinity chromatography

The sample of interest can easily be separated from Polybuffer by taking

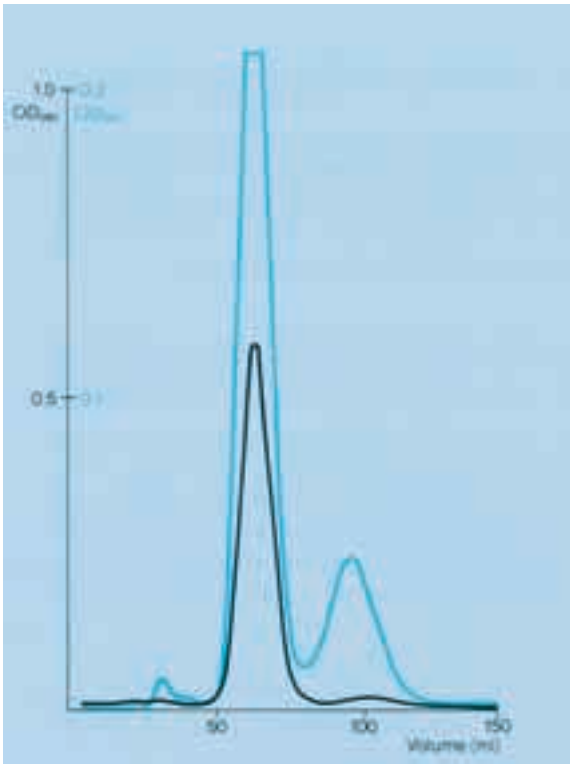

Fig. 17. Separation of protein from Polybuffer on Sephadex G-75. Pharmacia column K 26/40. Bed height: 20 cm. Sample: 5 ml Polybuffer 96 (0.0075 mmol/pH unit/ml) containing 5 mg chymotrypsinogen. Eluent: 0.9 % NaCl. Flow rate: 9.5 cm.h^{-1}. Monitor: UV-2 20 mm pathlength 1 OD FS, 280 nm (black curve): 1 mm pathlength 2 OD FS, 254 nm (coloured curve).

advantage of the biological activity of the sample. The fractions of interest are passed through a column containing a bioselective adsorbent which binds the sample and allows Polybuffer to elute unretarded. Further information about affinity chromatography is given in the booklet "Affinity Chromatography principles and methods" available free on request.

6.8 Regeneration

Regeneration of Polybuffer exchangers is particularly easy as this can be done in the column, without repacking. The gel should be washed (2—3 bed volumes) with 1 M NaCl to remove bound substances. Strongly bound proteins can be removed by washing with 0.1 M HCl. If HCl is used, the gel must be re-equilibrated to a higher pH as soon as possible after washing. The gel is then ready for re-equilibration with start buffer at the desired pH.

6.9 Storage

Polybuffer should be stored at 3—8° C in the dark. Polybuffer is sterile as supplied. Care should be taken to prevent microbial contamination. *Polybuffer exchangers* are stable in the wet state provided microbial growth is prevented. Due to the nature of the charged groups on PBE 118 and PBE 94, both should be stored in solutions containing 24 % ethanol as an antimicrobial agent. Agents containing charged groups should be avoided.

7. Applications

Chromatofocusing is a generally applicable technique available for the solution of separation problems in biochemistry.

Chromatofocusing, and the related technique ampholyte displacement chromatography, have been shown to be capable of solving a number of difficult separation problems such as the purification of β-acetylhexosaminidase isoenzymes (8), the purification of phospholipid transfer proteins (9), mouse α-foetoprotein (10, 11) the separation of human α-foetoprotein from albumin and other serum proteins (12—15) and the separation of haemoglobin variants (16—18). A comparison of chromatofocusing and ampholyte displacement chromatography is given on page 35.

Chromatofocusing has been shown to give separations in some cases which are at least as good as preparative isoelectric focusing (3) and in other cases separations which cannot be achieved by any other physicochemical method (8, 12, 15). The following section is devoted to examples illustrating the resolving power of chromatofocusing and its applicability to general separation problems.

7.1 Resolution in model systems

The high resolution available from chromatofocusing has already been illustrated for the ranges pH 9—6 (fig. 14) and pH 11—8 (fig. 16).

Figure 18 shows the separation of myoglobin, carbonic anhydrase, and albumin on a gradient of Polybuffer 74 on PBE 94.

The notable feature of chromatofocusing in these regions is the excellent symmetry of the protein peaks, due to the focusing effect of the pH gradient, and the very high resolution. The myoglobin samples in Figure 14 elute as zones with a bandwidth of only 0.02 pH units.

Polybuffer performs best over pH intervals of 3 pH units or less in length. For the highest resolution, short pH intervals should be chosen.

Several blood disorders, e.g. sickle cell anaemia, are due to the occurence of genetic variants of haemoglobin. Figure 19 shows the fractionation of carboxyhaemoglobin into a number of sub-components by chromatofocusing over a short pH interval (pH8—7) using a gradient of Polybuffer 96 on a column of PBE 94. Chromatofocusing is an excellent technique for studying the composition of genetic variants of haemoglobin.

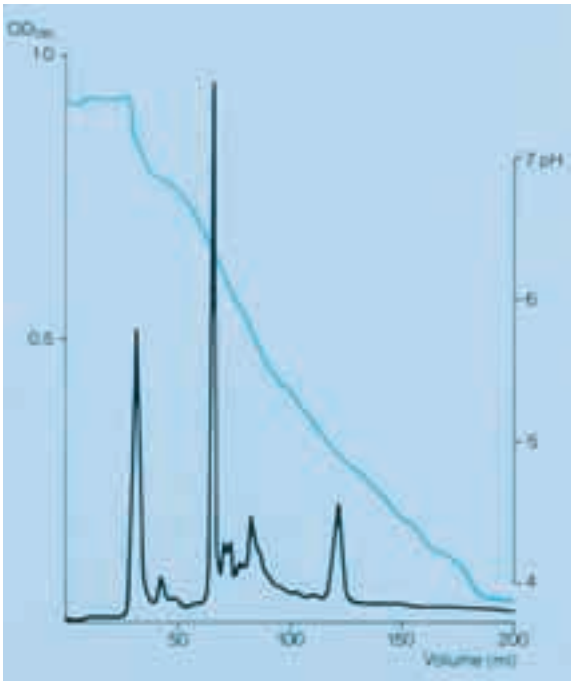

Fig. 18. Separation of a model mixture of proteins in the range pH 7—4. Column: C 10/20. Bed height: 15 cm. Sample: 4 ml elution buffer containing horse myoglobin (12 mg), carbonic anhydrase (8 mg) and albumin (12 mg). Elution conditions: Start buffer 0.025 M imidazole-HCl, pH 7.4; elution buffer 0.0075 mmol/pH unit/ml Polybuffer 74, pH 4. Flow rate: 12.5 cm.h^{-1}.

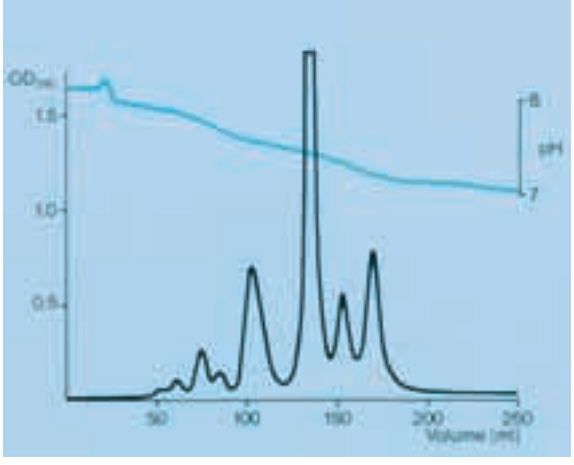

Fig. 19. Fractionation of carboxyhaemoglobin in a narrow pH interval (pH 8—7). Column: SR 10/50. Bed height: 30 cm. Sample: 15 ml elution buffer containing carboxyhaemoglobin (15 mg). Elution conditions: Start buffer 0.025 M Tris-methane sulphonic acid, pH 8.2; elution buffer 0.0075 mmol/pH unit/ml Polybuffer 96, pH 7. Flow rate: 40 cm.h^{-1}.

7.2 Resolution of complex samples

The resolution obtained with model systems suggests that chromatofocusing with Polybuffer should be capable of giving high resolution with relatively crude samples. Figure 20 shows the fractionation of crude egg white on a gradient from pH 7—4. The white of an egg was filtered on a glass sinter (G1) and diluted with Polybuffer 74, and was then centrifuged at 3000 g for 10 minutes before being

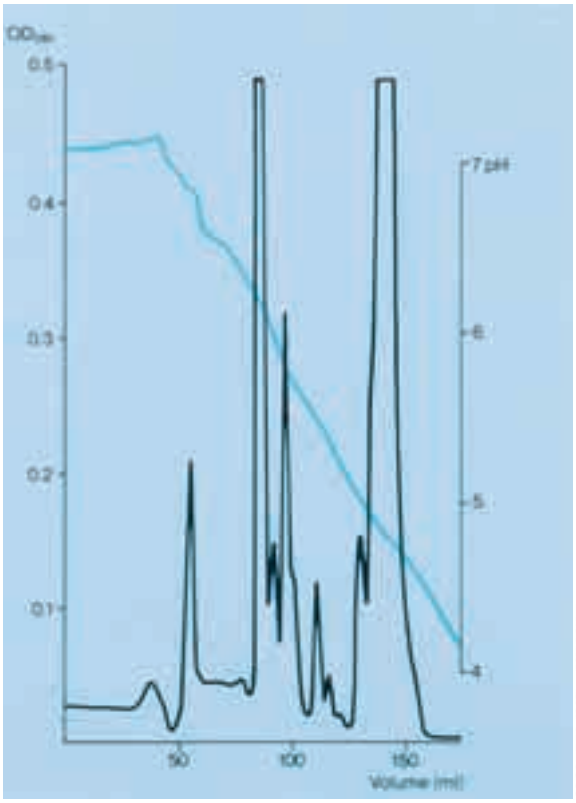

Fig. 20. Fractionation of egg white. Column: SR 10/50. Bed height: 30 cm. Sample: 5 ml elution buffer containing 0.5 ml egg white. Elution conditions: Start buffer 0.025 M imidazole-HCl, pH 7.5 elution buffer 0.0075 mmol/pH unit/ml Polybuffer 74, pH4. Flow rate: 40 cm.h^{-1}.

applied to a column of PBE 94 equilibrated to pH 7.2 with 0.025 M imidazole-HCl. The column was eluted with 1:10 Polybuffer 74, pH 4. The sample was separated into a large number of peaks with excellent resolution.

Resolution in the higher pH range is also excellent. Extracts from mammalian muscle are highly complex mixtures of proteins, and difficult to separate by a single step. Figure 21 shows the separation of proteins extracted from elk muscle. Approximately 10 g of elk muscle was homogenised with an equal volume of water, and the resultant mix was then centrifuged at 3000 g for 10 minutes. The supernatant was equilibrated with eluent and applied to a column of PBE 94 and eluted with Polybuffer 96. Again chromatofocusing was capable of resolving the sample into a large number of peaks, as shown in Figure 21. Analytical IEF showed that these peaks eluted in order of their isoelectric points. In addition to the proteins separated in this region several proteins remained bound to the column. These proteins had isoelectric points below pH 6 and could be separated with a gradient of Polybuffer from pH 6—4.

Chromatofocusing with Polybuffer is also a high capacity technique. *Trichoderma reesei* is a microorganism which produces large quantities of extracellular cellulases. Preliminary investigations indicated that these enzymes could be

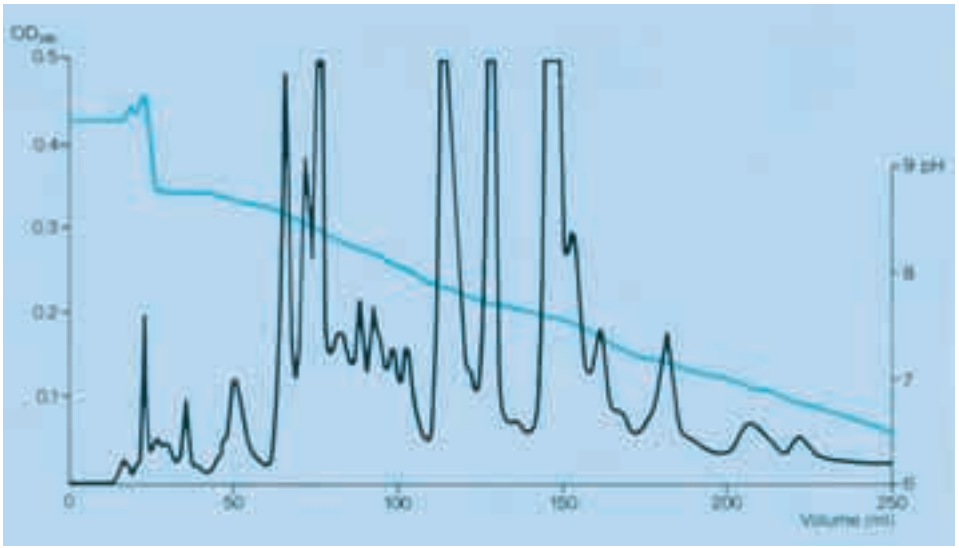

Fig. 21. Fractionation of soluble proteins extracted from elk muscle. Column: C 10/40. Bed height: 35 cm. Sample: 5 ml of supernatant from elk meat homogenate. Elution conditions: Start buffer 0.025 M ethanolamine HCl, pH 9.4, elution buffer 0.0075 mmol/pH unit/ml Polybuffer 96, pH 6. Flow rate: 20 $cm.h^{-1}$.

separated preparatively by chromatofocusing. Figure 22 shows the fractionation of 460 mg of freeze-dried culture supernatant on PBE 94 using a gradient of Polybuffer 74.

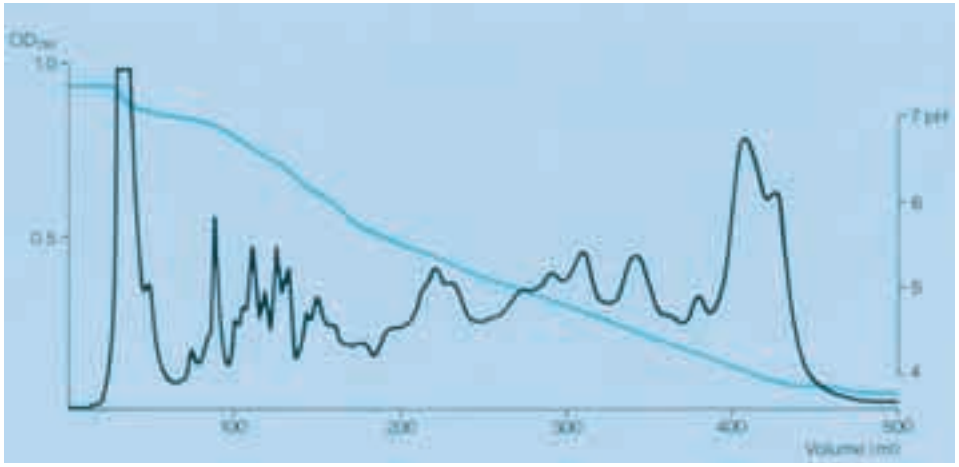

Fig. 22. Fractionation of extracellular proteins from *Trichoderma reesei*. Column: SR 10/50. Bed height: 30 cm. Sample: 5 ml elution buffer containing 460 mg freeze-dried culture supernatant. Elution conditions: Start buffer 0.025 M imidazole-HCl, pH 7.4, elution buffer 0.0075 mmol/pH unit/ml Polybuffer 74, pH 4. Flow rate: 30 $cm.h^{-1}$. The sample was supplied by courtsey of Dr. Göran Petterson.

7.3 Chromatofocusing in combination with other techniques

Although chromatofocusing gives excellent resolution of complex mixtures, the best results are obtained when chromatofocusing is used in combination with techniques which use other criteria for separation, such as gel filtration or affinity chromatography. The high resolving power means that chromatofocusing is most suitable for use in the intermediate or later stages of a separation. An example of the use of chromatofocusing in this way is shown in the separation of proteins from the potato.

Potato protein was chosen since it contains a number of proteins resembling each other in molecular weight but differing in isoelectric point. Potato (1 kg) was frozen, thawed and peeled prior to homogenization to a thick paste. 60 ml of a sodium sulphite: thiosulphate solution (5 g Na_2SO_3 + 3.75 g $Na_2S_2O_3$/100 ml) was added to prevent oxidation. The homogenate was centrifuged (15 000 g for 10 min) and the supernatant (750 ml) was dialyzed against 60 l distilled water for 72 h, and lyophilized.

The protein sample prepared in this fashion was examined by two-dimensional electrophoresis, using IEF in 5 % polyacrylamide in a gradient pH 3—10 in the first dimension, followed by SDS gradient gel electrophoresis (10—20 % polyacrylamide) in the second dimension. Figure 23 shows that there were several proteins with similar molecular weights, but which differed in charge properties in the sample.

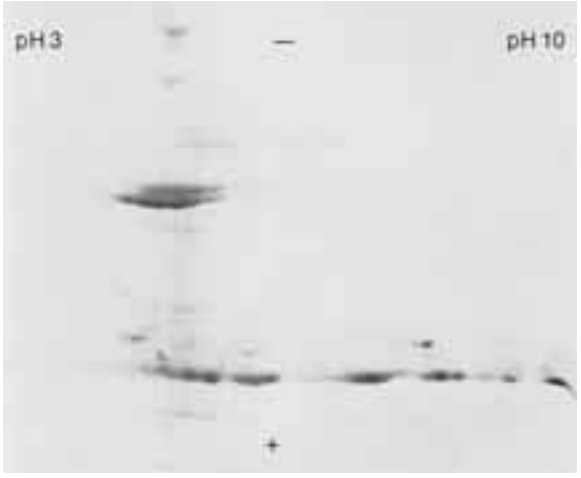

Fig. 23. IEF followed by SDS-gradient polyacrylamide gel electrophoresis of potato proteins. First dimension: Isoelectrofocusing for 20 h at 500 V in a gradient of Pharmalyte pH 3—10 in a 5 % polyacrylamide rod (150 mm × 2.7 mm). Second dimension: Electrophoresis in a gradient (10—20 %) of polyacrylamide in 0.2 % SDS. Sample size: 100 µl.

These proteins were partially purified by gel filtration on Sephadex G-100 in a Pharmacia column K 26/100. The eluent was analysed by gradient gel elctrophoresis and the relevant proteins were found to lie in the third peak. These fractions were pooled prior to further separation. The sample from gel filtration was then applied to a column of PBE 94, and eluted using a gradient of Polybuffer 74. The results are shown in Figure 24.

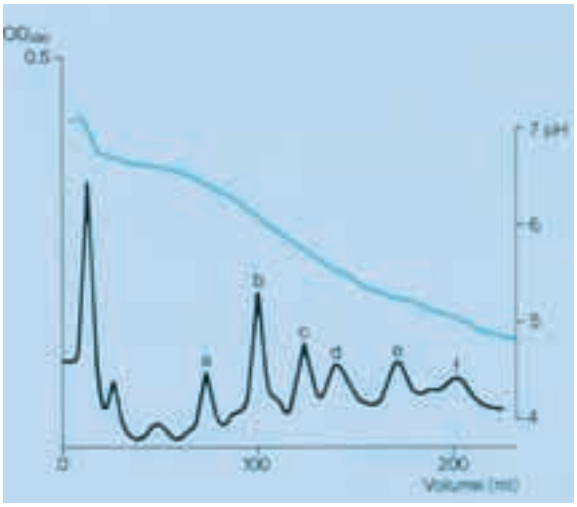

Fig. 24. Chromatofocusing of partially purified potato proteins on PBE 94. Column: SR 10/50. Bed height: 28.1 cm. Sample: 7 ml partially purified potato protein; Elution conditions: Start buffer 0.025 M imidazole-HCl, pH 7.0, elution buffer 0.0075 mmol/pH unit/ml Polybuffer 74, pH 4. Flow rate: 30 cm.h^{-1}.

The purity of these peaks was examined by isoelectric focusing. Figure 25 shows that the peaks eluted in order of their isoelectric points. Some of the peaks give more than one band on isoelectric focusing. This could be due either to the protein having charge isomers, or to proteins migrating in association with one another as complexes. The elution pH is determined by the isoelectric point of the complex.

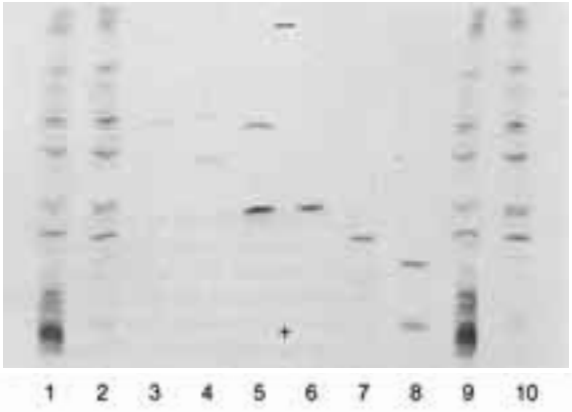

Fig. 25. Isoelectric focusing of peaks from the chromatofocusing run of partially purified potato proteins in polyacrylamide using Pharmalyte pH 3—10. Power maximum 30W, voltage maximum 2500V. Focusing was for 3000Vh. Sample: 1,9, crude sample; 2,10, peak from gel filtration; 3—8, peaks a—f respectively from fig 24.

The popularity and high resolution that isoelectric focusing combined with polyacrylamide gradient gel electrophoresis has as an analytical technique indicates the enormous potential chromatofocusing, coupled with gel filtration, has as a preparative analogue to the above method. Many interesting proteins in complex mixtures have previously only been identified using electrophoretic techniques but chromatofocusing may now permit these proteins to be obtained in quantities sufficient for further studies on their structure and biological function.

8. Interpretation of experimental results obtained by chromatofocusing

8.1 Effect of the composition of the eluent

One of the consequences of internal pH gradient formation is that the ionic strength in chromatofocusing increases throughout the separation. This is because during the early part of gradient formation, most of the Polybuffer ions bind to the ion exchanger and only the most basic species pass through the column. However, as more and more of the charged groups on the column are titrated and the pH of the column decreases, so can more Polybuffer species pass through the column unretarded. The ionic strength in the eluent thus increases gradually as elution progresses. The relationship between chloride ion concentration and pH during a run with Polybuffer is shown in Figure 26.

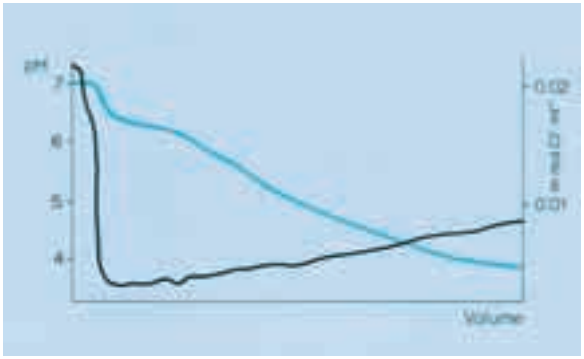

Fig. 26. The relationship between ionic strength, pH and volume during chromatofocusing with Polybuffer.

This increase in ionic strength during gradient formation will also affect the pH at which a protein elutes. This is discussed further in the next section, but in general the elution pH is closest to the pI of the protein when the ionic strength is low. Conditions in chromatofocusing should therefore be chosen so that the protein of interest elutes between 3 and 5 bed volumes from the start of the gradient, especially if the separation is designed using pI data from isoelectric focusing or ion exchange.

The composition of the buffer at any stage during the elution is such that each species follows the previous one as its "breakthrough" point is reached. At this point it becomes responsible for a large amount of the buffering capacity present. However it is not the only ionic species present, as is the case in isoelectric focusing, since Polybuffer species with higher isoelectric points will also be present. The elution profile of a pair of Polybuffer species is shown in Figure 27.

The concentration of each Polybuffer species in the eluent after its breakthrough point is the same as that in the Polybuffer mixture prior to elution.

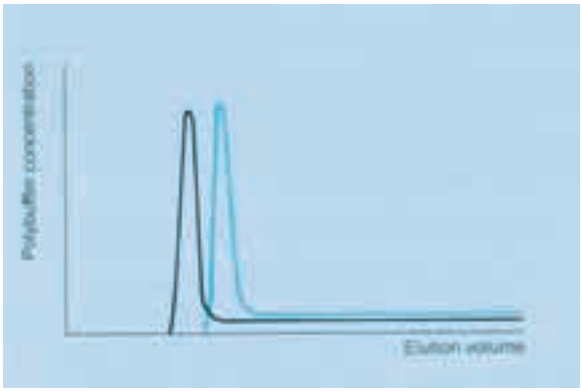

Fig. 27. Elution profiles of Polybuffer species.

8.2 Mechanism of protein elution

There are a number of factors which will influence the pH at which a protein elutes, and which will affect the interpretation of results in chromatofocusing.

1. Displacement effects

In addition to differing from each other by virtue of their isoelectric points, proteins also differ from each other by virtue of the shape of their titration curves. An example of this is shown in Figure 28, where the first protein (a) has a steep titration curve and the second protein (b) a much flatter titration curve. This means that if a protein molecule of the second type, b, diffuses a small distance away from its pI, the size of the charge it carries will only be very small and thus the electromotive force on the protein, requiring it to return to the point where it has a net charge of zero, will also be small. Proteins of this type are known to focus poorly in IEF, giving diffuse bands. Fortunately they are few in number.

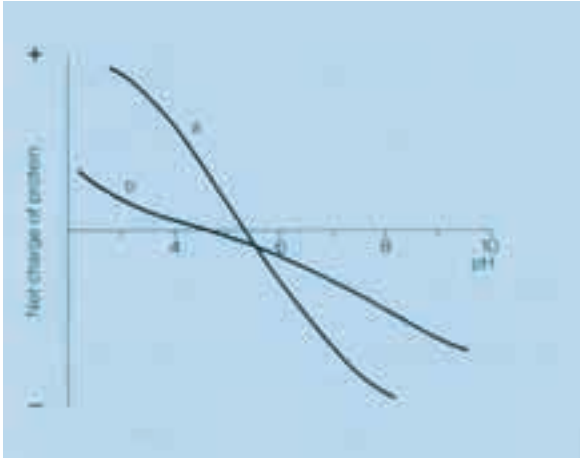

Fig. 28. Titration curves of two proteins Protein (a) has a steeper titration curve than protein (b) and will give a sharper band on focusing.

It may be expected that proteins of this type would also give diffuse bands in chromatofocusing. However, binding to an ion exchanger depends on the presence of competing ions as well as on the charge. A protein with a flat titration curve will be strongly bound to Polybuffer exchanger so long as the pH is well above the pI of the protein. However, as the pH decreases, so the binding becomes weaker, and the interaction between the protein and the gel becomes more sensitive to the presence of competing ions, such as Polybuffer ions. Thus the protein may elute from the gel via a process akin to ampholyte displacement, at a pH above its pI. This effect would be most pronounced in the latter portion of the pH gradient where Polybuffer concentration is higher (see fig. 26), and so it is advisable to design experiments so that elution occurs between 3 and 5 column volumes from the gradient start. This effect can mean a displacement as high as 1—2 pH units for certain proteins, but these are few in number. Furthermore, protein still elutes as a discrete band in chromatofocusing, and resolution is not impaired.

2. The Surface Potential

Since Polybuffer exchangers carry a strong charge, there exists an electrostatic field between the surface of the pore and the external liquid, which decreases with distance. This is similar to the well known electrochemical potential, set up across a semipermeable membrane by large, impermeable ionic species, known as the Donnan potential.

Since Polybuffer exchangers carry a posititive charge, cations, including hydrogen ions, are repelled by the matrix. As a result the pH inside the matrix is higher than the pH of the eluent immediately outside. It follows, therefore, that when the charge on a bound protein becomes zero (at its true pI) and it diffuses away from the interior surface of the pore, it will move into an area of lower pH. Here it will acquire a positive charge which will accelerate its migration out of the bead into the eluent. This happens throughout the column. The eluent in which the protein finds itself when it elutes will therefore be at a lower pH than the pI of the protein, and so the apparent pI is less than the pI measured by, say, isoelectric focusing. The opposite effect would be found for a cation exchanger used with ascending pH gradients.

3. The solubility of protein at its isoelectric point

The solubility of a protein varies with pH and is least near the isoelectric point. When working with large amounts of sample there may be a tendency for some proteins to be eluted slightly later than their normal elution pH. This will be due to the protein precipitating at its isoelectric point as the gradient migrates down the column, and redissolving as the pH reaches a suitably lower value. At this point it will be repelled from the matrix and will migrate down the column with the eluent, until it tends to precipitate again. The elution pH will thus be slightly lower than the pI of the protein. Precipitation can be prevented by increasing the salt concentration or Polybuffer concentration, with some loss of resolution.

4. Other factors

Any comparison of the pI obtained by chromatofocusing and other methods such as isoelectric focusing should be made at the same temperature. The same is also true of comparisons made for the same protein on different chromatofocusing runs.

It is also important to take into account the presence of different counter-ions. It is particularly important to take care to exclude CO_2 from the system by degassing buffers as dissolved CO_2 readily causes disturbances in the pH gradient.

9. Ampholyte displacement chromatography

Ampholyte displacement chromatography (ADC) was first reported by *Leaback* and *Robinson* (8). In ampholyte displacement chromatography a column of conventional ion exchanger is eluted using carrier ampholytes. The major differences between chromatofocusing and ADC is that in ADC conditions are not so well defined. Specifically, conditions are not always chosen to favour the formation of a pH gradient. We have reserved the term ampholyte displacement chromatography to describe techniques in which the gel and eluent are at the same pH, and so no pH gradient is formed.

The elution mechanism in ADC is not clear, but is thought to involve competition between bound proteins and ions in the eluent for charged sites.

The major disadvantage of ampholyte displacement chromatography is the high cost of carrier ampholytes (12). Concentrations as high as 60 g ampholyte/l have been used for elution. Concentrations required for chromatofocusing are usually much lower (in the region of 10 g/l), since in chromatofocusing the protein is eluted from the gel primarily by losing its charge (15), and not by a displacement process.

Due presumably to the lack of a pH gradient, proteins are not always eluted in order of their isoelectric points in ADC (14) and there is no focusing effect (15). Several workers have reported difficulties in achieving linear gradients with conventional ion exchangers (8, 15); this can be attributed to variations in capacity ot these gels with pH.

A summary of the major differences between chromatofocusing and ADC is given below

Feature	Chromatofocusing	Ampholyte displacement chromatography
Cost of eluent	Low	High
Proteins elute in order of pI	Yes	No
Focusing effects occur to give high resolution	Yes	No
Linear pH gradient forms automatically	Yes	No

Fault finding chart

Problem	Cause	Comment
Protein does not bind to column	Column incorrectly equilibrated	Check that pH at bottom of column matches that of start buffer
	Start pH too low	Choose a start buffer with a higher pH
	Ionic strength of eluent too high	Use buffer composition recommended in instructions
	Ionic strength of sample too high	Remove salt in sample by gel filtration in Polybuffer
	Column contaminated with protein	Wash thoroughly before reuse (p. 24)
Protein binds, but does not elute from the column	Eluent composition incorrect	Check pH of eluent
	pH of eluent too high	Try a lower pH range
Gradient does not reach desired pH	Incorrect eluent composition	Check eluent pH. Make up fresh eluent if necessary
	Eluent contains CO_2	Degas all buffers prior to use
Gradient fluctuates	Incorrect counterion	Use recommended counterions
	CO_2 present	Degas all buffers prior to use. In alkaline regions, degas water *before* making up eluent
Bands skewed	Blocked bed net	Remove bed net. Clean or replace with a new net
	Poorly packed column	Check packing with cytochrome *c*. Repack bed if necessary.
Several bands on column, fewer on recorder	Poor design of end piece	Use correctly designed end piece. Narrow columns (10 mm) give the best results

Ordering information

	Type	Code No.	Pack size
Polybuffer	Polybuffer 74	17-0713-01	250 ml
	Polybuffer 96	17-0714-01	250 ml
Pharmalyte	Pharmalyte pH 8-10.5	17-0455-01	25 ml
Polybuffer exchangers	PBE 118	17-0711-01	200 ml
	PBE 94	17-0712-01	200 ml
Sephadex	Sephadex G-25, Coarse	17-0034-01	100 g
		Code No.	
Columns	SR 10/50	19-2638-01	
	C 10/40	19-5003-01	
	C 16/40	19-5102-01	
	C 16/70	19-5103-01	
	XK 16/40	18-8774-01	
	XK 16/70	18-8775-01	

A full list of columns and accessories is available free on request.

Peristaltic Pump-1	18-1110-91

References

1. Chromatofocusing: isoelectric focusing on ion exchangers in the absence of an externally applied potential. In *Proc. Int. Symp. Electrofocusing and Isotachophoresis.* Radola, B.J., Graesslin, D., Eds., (1977). de Gruyter, Berlin, pp 463—466. Sluyterman, L.A.Æ., Wijdenes, J.
2. Chromatofocusing: isoelectric focusing on ion exchange columns. I. General principles. *J. Chromatogr. 150 (1978) 17—30,* Sluyterman, L.A.Æ., Elgersma, O.
3. Chromatofocusing: isoelectric focusing on ion exchange columns. II. Experimental verification. *J. Chromatogr. 150 (1978) 31—44,* Sluyterman, L.A.Æ., Wijdenes, J.
4. *Ion Exchange Chromatography — Principles and methods.* Pharmacia.
5. A simple method for estimating isoelectric points. *Anal. Biochem. 11 (1965) 374—377,* Lampson, G.P., Tytell, A.A.
6. Size and charge distribution of macromolecules in living systems. *J. Chromatogr. 193 (1980) 1—8,* Gianazza, E., Righetti, P.G.
7. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72 (1976) 248—254,* Bradford, M.M.
8. Ampholyte displacement chromatography. A new technique for the separation of proteins illustrated by the resolution of β-N-acetyl-D-hexosaminidase isoenzymes unresolvable by isoelectric focusing or conventional ion-exchange chromatography. *Biochem. Biophys. Res. Commun. 67 (1975) 248—254,* Leaback, D.H., Robinson, H.K.
9. Rat liver proteins capable of transferring phospatidylethanolamine. Purification and transfer activity for other phospholipids and cholesterol. *J. Biol. Chem. 252 (1977) 1613—1619,* Bloj, B., Zilversmit, D.B.
10. Purification of mouse α-foetoprotein by ampholyte displacement chromatography. *Can. J. Biochem. 56 (1978) 853—856,* Pagé, M., Belles-Isles, M.
11. Ampholyte displacement chromatography for the preparation of alphafoetoprotein. In "*9th International Symposium on Chromatography and Electrophoresis 1978*". Pagé, M., Belles-Isles, M.
12. Ampholyte displacement chromatography for the separation of serum proteins. *Biochem. Soc. Trans. 6 (1978) 1051—1054,* Young, J.L., Webb, B.A., Coutie, D.G. et al.
13. Two methods for the separation of human α-fetoprotein and albumin. (A) Affinity chromatography using Blue Sepharose CL-6B and (B) ampholyte displacement chromatography. *Anal. Biochem. 88 (1978) 619—623,* Young, J.L., Webb, B.A.
14. The separation of serum proteins by ampholyte displacement chromatography. *Science Tools 25 (1978) 54—56,* Young, J.L., Webb, B.A.
15. Ampholyte displacement chromatography: an investigation of some factors affecting the separations obtained and a comparison with chromatofocusing. In "*Protides of Biological Fluids*" Proceedings of the 27th Colloquium, Peeters, M., Ed., Bruges 1979 pp 739—742. Young, J.L., Webb, B.A.
16. Ampholyte displacement chromatography: application to the separation of hemoglobin variants. In "*Protides of Biological Fluids*" Proceedings of the 27th Colloquium, Peeters, H., Ed., Bruges 1979 pp 743—746. Chapuis-Cellier, C., Francina, A., Arnaud, P.
17. Ampholyte displacement chromatography by column liquid chromatography on ion exchangers. Its application to the separation of human haemoglobins. *13th International Symposium on Chromatography Cannes 1980* C 10-1, Francina, A., Chapuis-Cellier, C., Cloppet, H. et al.
18. Carrier-ampholyte displacement chromatography (chromatofocusing) on ion-exchange papers. Application to the separation of haemoglobin variants. *J. Chromatogr. 222 (1981) 116—119* Francina, A., Dorleac, E., Cloppet, H.

More references are listed in the "Chromatofocusing Reference List" which is available on request.

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