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Recycling isoelectric focusing: use of simple buffers☆

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ABSTRACT

Using the recycling free-flow focusing (RF3) apparatus, we have demonstrated that single ampholytes can be utilized to establish very stable pH regions, separating all proteins into three groups: a sharply resolved zone of proteins isoelectric at the prevailing pH, this "pH window" being bracketed by zones of more acidic and/or basic proteins. The ampholytes used are either amino acids or their dipeptides and other derivatives. Where necessary, because of lack of an ampholyte with the required pH, a binary mixture of ampholytes can be utilized. The closer their isoelectric points (p*I*), the narrower will be the pH window, *i.e.*, the sharper the resolution of the bracketed proteins. This method overcomes the necessity of using ill-defined commmercial carrier ampholytes, such as Ampholine, for preparative isoelectric focusing. It is recommended that the ampholytes be utilized at relatively high concentration, 100 m*M* or higher, this contributing to pH stability and minimizing protein precipitation.

INTRODUCTION

Borrowing from the vocabulary of another "high-tech" discipline, scaling up of electrophoresis for the processing of relatively large quantities of proteins presents both hardware and software problems. Our prior work on the hardware problems resulted in the design of several instruments embodying novel concepts for regulation of fluid flow. While for scale-up of liquid chromatography enlarging column diameters is a feasible option, enlarging gel-based analytical electrophoretic instruments is rather unproductive. Gels and other solid support matrices complicate dissipation of Joule heat as well as product recovery. Thus, it was found necessary to devise new types of instruments, operating in free solution without gels or other supports. This requires control of fluid flow and avoidance of unwanted convection. In our hands, three strategies for convection control were found to be particularly effective for the scaling of isoelectric focusing: (i) stabilization of fluid flow by means of screen elements, in the RIEF [1] apparatus; (ii) stabilization by screen elements and rotation in the Rotofor [2] apparatus (available from Bio-Rad Labs., Richmond, CA, USA); and (iii) stabilization by means of rapid flow through narrow gaps, in the recycling free-flow focusing (RF3) instrument [3] (available from Rainin Instrument Co., Woburn, MA, USA).

Software problems are concerned with the selection of buffers and other operational parameters for optimal resolution. In the past, we have addressed this problem through computer modeling [4]. In the present paper we wish to address the problem inherent in the use of commercial carrier ampholytes (CAs) for the establishment of pH gradients. These CAs are chemically ill-defined, which makes it difficult to document their complete removal, where necessary. In analytical work, pH gradients are indispensable, but in preparative applications, the isolation of a single protein is usually sufficient.

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☆ It is a special pleasure and privilege to pay homage to Professor Jerker Porath at the occasion of his seventieth birthday. His part-time embracing of Tucson these last few years has given to my wife and me a unique opportunity to get to appreciate the personal warmth, love for life, and enthusiasm of Jerker and Ann Mari. Their friendship has enriched immeasurably our lives and we both thank them for it.

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Thus, we are in the process of developing a novel strategy for preparative isoelectric focusing based on the concept of "pH windows" rather than pH gradients.

A single simple ampholyte, such as an amino acid or a dipeptide, suffices to cut a broad spectrum of proteins, isolating a narrow zone of proteins within the prevailing pH. Used at rather high concentration, the ampholyte produces an exceptionally stable pH profile, separating the proteins into three fractions: those with isoelectric points (p*I*) more basic than the ampholyte, those with p*I* values more acidic, and those with the same p*I* as the ampholyte. It is this third fraction which has the narrowest p*I* distribution of proteins and exhibits highest resolution. If a suitable single ampholyte with the required p*I* is not available, a mixture of two ampholytes bracketing the desired pH value can be substituted. It is an extension of the concept of the use of amino acids as discrete spacers in isotachophoresis, introduced a number of years ago [5]. Such ampholytes are chemically well defined and biologically acceptable. Equally important, because of the high concentration, they minimize the isoelectric precipitation often encountered otherwise. Many are also lower in cost than commercial CAs. Thus, their use opens the possibility of cost effective high resolution large scale focusing.

THE CONCEPT AND USE OF pH WINDOWS

In isoelectric focusing (IEF) the essential pH gradient is usually generated naturally, through the migration of buffer components to their isoelectric points, the proteins migrating within this gradient to their own steady state distribution. Realizing the need for a large number of ionizable species, Vesterberg [6] synthesized a random mixture of polyamino–polycarboxylic acids, commercialized under the tradename of Ampholine. Ampholine and other similar CA mixtures have found widespread acceptance for analytical focusing, even if chemically ill-defined.

In a seminal paper, Svensson (now Rilbe) [7] examined the possible use of amino acids and other simple ampholytes for establishment of a pH gradient. He has given formulas for calculation of degree of ionization, conductivity and buffering of individual compounds. Svensson also established the now classical distinction between good and poor ampholytes. The p*I* of a simple ampholyte is given by the algebraic mean of its two dissociation constants, $pI = (pK_1 + pK_2)/2$. For an ampholyte with more than two ionizable groups, only the dissociation constants proximal to its p*I* are of consequence. Due to the logarithmic definition of pH, buffering decreases by a factor of 10 for every pH unit away from the p*K*. Thus, the degree of ionization of the ampholyte and its buffering at or near its p*I* will depend of the quantity $pI - pK_1$. Svensson rejected as unsuitable all ampholytes where $(pI - pK_1) > 2.5$, defining them as poor ampholytes, *i.e.* exhibiting too low conductivity to act as good carriers of the electrical current. Table I reproduces Svensson's [7] listing of acceptable ampholytes. Albert and Serjeant [8] have given a concise review of the methods for the determination of ionization constants and list additional data.

The Svensson calculations of the ionization, conductance and buffering were cumbersome and are greatly simplified by our computer program [4]. In previous work [4,9] we have published computer predictions of pH gradients resulting from focusing of various binary and ternary mixtures of amino acids. For instance, the ternary mixture of an acidic, a neutral and a basic amino acid (glutamic acid, cycloserine, and lysine) [9] creates two broad pH windows, between the acidic and neutral, and neutral and basic amino acids, respectively. These windows capture proteins isoelectric at those pH values. We have often used such systems to demonstrate the performance of our instruments for the admittedly easy separation of hemoglobin and bromophenol blue stained albumin, taken as models because of their color.

We now wish to present results obtained with single ampholytes, used in high concentration. These suffice to establish very stable pH zones isolating proteins isoelectric at the prevailing pH from those more acidic or more basic. In practice, we have found that good ampholytes, such as histidine or cycloserine, give good results at 100 m*M* concentration, but that even poor ampholytes can be used, provided the concentration is increased. Thus, we have used glycine at 1.5 *M* concentration and ε-aminocaproic acid (EACA) or glycyl-glycine at 0.5 *M* concentration. The upper concentration is mainly limited by the solubility of the compound. A rea-

TABLE I

LISTING OF CARRIER AMPHOLYTES ACCORDING TO SVENSSON [7]

Ampholyte	pI	pI − pK_1
Aspartic acid	2.77	0.89
Glutathione	2.82	0.70
Aspartyl-tyrosine	2.85	0.72
o-Aminophenylarsonic acid	3.00 (?)	0.77 (?)
Aspartyl-aspartic acid	3.04	0.34
p-Aminophenylarsonic acid	3.15 (?)	0.92 (?)
Picolinic acid	3.16	2.15
L-Glutamic acid	3.22	1.03
β-Hydroxyglutamic acid	3.29	0.96
Aspartyl-glycine	3.31	1.21
Isonicotinic acid	3.35	1.51
Nicotinic acid	3.44	1.37
Anthranilic acid	3.51	1.47
p-Aminobenzoic acid	3.62	1.30
Glycyl-aspartic acid	3.63	0.82
m-Aminobenzoic acid	3.93	0.81
Diiodotyrosine	4.29	2.17
Cystinyl-diglycine	4.74	1.62
α-Hydroxyasparagine	4.74	2.43
α-Aspartyl-histidine	4.92	1.90
β-Aspartyl-histidine	4.94	2.00
Cysteinyl-cysteine	4.96	2.31
Pentaglycine	5.32	2.27
Tetraglycine	5.40	2.35
Triglycine	5.59	2.33
Tyrosyl-tyrosine	5.60	2.08
Isoglutamine	5.85	2.04
Lysyl-glutamic acid	6.10	1.65
Histidyl-glycine	6.81	1.00
Histidyl-histidine	7.30	0.50
Histidine	7.47	1.50
L-Methylhistidine	7.67	1.19
Carnosine	8.17	1.34
α, β-Diaminopropionic acid	8.20	1.40
Anserine	8.27	1.23
Tyrosyl-arginine	8.38 (?)	1.00 (?)
	8.68 (?)	1.13 (?)
L-Ornithine	9.70	1.05
Lysine	9.74	0.79
Lysyl-lysine	10.04	0.59
Arginine	10.76	1.72

sonable estimate of the conductivity and the pI can be obtained by direct measurement on solutions of the pure ampholytes in distilled water, at the desired concentration.

As most ampholytes have very low concentrations of anionic or cationic species at their pI, they migrate only imperceptibly toward cathode or anode, and remain distributed throughout the system. This was confirmed experimentally as well as by the use of our computer program, recently utilized to model the performance of single ampholytes, and of binary mixtures of ampholytes, with closely similar electrokinetic properties [10]. At this point, it will suffice to give some general conclusions applicable to all simple ampholytes at their isoelectric state.

(1) Near neutrality, the conductivity of the solution is a linear function of concentration. Below pH 5 and above pH 9, the conductivity increases somewhat less than proportionally to concentration because of the contribution of hydrogen and hydroxyl ions.

(2) Near neutrality, the conductivity of the solution is a linar function of the electrophoretic ion mobility, this linearity decreasing at low or high pH. Ion mobility is roughly an inverse function of ion molecular weight.

(3) At same ionic mobility and close to neutrality, the conductivity of the solution decreases by roughly a factor of 9, when the value (pI − pK_1) increases from 1 to 2. This factor decreases to about 6 at pH 4.

(4) At same ion mobility, conductivity is nearly the same between pH 5 and 9, increasing at more extreme pH values.

(5) If the desired pH window cannot be established using a single ampholyte, binary mixtures of compounds with very close pI values seem to be equivalent. The resulting pH is intermediary to their individual pI values. Thus, such close binary mixtures permit the establishment of any desired pH window. As the ionization at the intermediate pH is still very low, there is little migration of the component ampholytes and the pH remains stable. A good approximation of the conductivity is given by the algebraic mean of component conductivities.

Above guidelines may suffice to lead the experimenter in the selection of appropriate ampholytes for the desired pH window. Detailed computer modeling data will be published separately.

EXPERIMENTAL

RF3 Apparatus (RF3)

Fig. 1 shows a schematic presentation of the recycling free flow focusing (RF3) apparatus. The work was carried out in a commercial RF3 apparatus

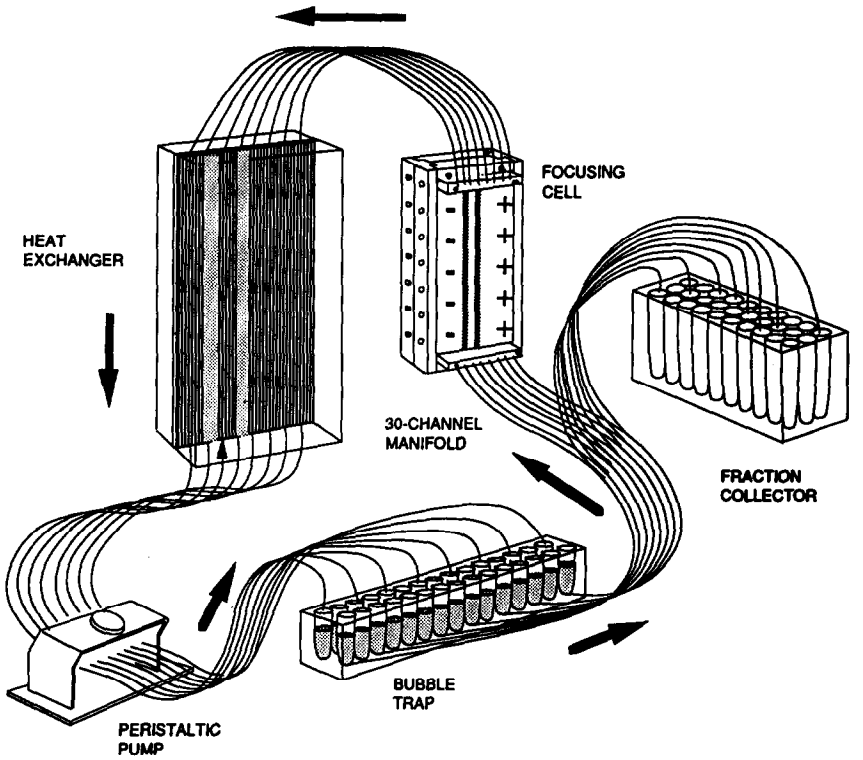


Fig. 1. A schematic presentation of the fluid flow pattern in the recycling free-flow focusing (RF3) apparatus. The main components are the focusing cell, the heat exchanger, a multichannel peristaltic pump and a bubble trap. See text for explanation.

(Protein Technologies, Tucson, AZ, USA) as well as in a home-made prototype. No difference in performance was noticed, though the commercial apparatus is simpler in usage.

The process fluid, containing the CAs and proteins to be fractionated, is continuously recycled through the focusing cell and a heat exchanger by means of a 30-channel peristaltic pump. The array of bubble traps inserted into the recycling loops are sealed by silicone septa, permitting addition of protein or withdrawal of small sample volumes during the focusing process. The key component of the apparatus is the focusing cell, illustrated schematically in Fig. 2. The cell is constituted by two parallel plexiglass plates defining a focusing cavity, 20 × 4 cm in size. Key to the stability of laminar flow through the cavity is its shallow depth, 0.75 mm, combined with the rapid flow of process fluid and the appropriately machined fluid inlet and outlet manifolds. Quick-connectors simplify the attachment of the 30 PTFE tubing loops to the focusing chamber. A multichannel peristaltic pump directs the flow of the focusing process fluid upward through the cell with a residence time of about 2–4 s. Thus, in each pass through the cell, only incremental migration of components toward their isoelectric points is achieved. Rapid flow is essential to avoid electrohydrodynamic distortion [3].

An external power supply can impose a d.c. voltage of up to 1500 V, 400 mA. The electrodes are lateral, separated from the focusing cavity by ion-permselective membranes. These can be utilized in two modes. In the electrodialysis mode, the cation-selective membrane will face the cathodic compartment, the anion-selective membrane facing the anodic compartment. This will result in transport of low-molecular-weight ions from the processing to the electrode compartments. Using commercial CAs, such an arrangement may cause loss of some buffering components. Thus, in focusing, we often

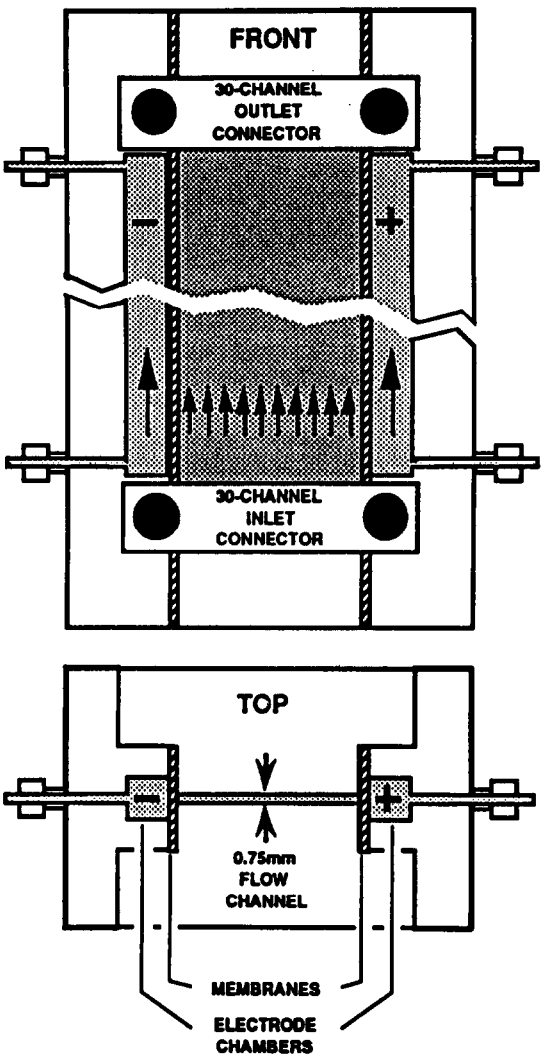


Fig. 2. A schematic presentation of the focusing cell of the RF3 apparatus. Of critical importance is the flow channel depth, indicated in the bottom panel.

use the reverse arrangement, cationic membrane facing the anode and vice versa. In such arrangement, no ions can escape from the processing compartment and are, instead, polarized proximally to the membranes. 0.1 *M* NaOH and phosphoric acid solutions are used typically as catholyte and anolyte, respectively.

Operating procedure

The cell volume is only about 6 ml, while total priming volume of the cell, the loops and the bubble trap is about 100 ml but can be increased up to 500 ml. The apparatus was primed with amino acid solutions of concentrations specified further on. Once recycling was started and current applied, the model protein solution to be fractionated was slowly injected into a central recycling loop by means of a Harvard apparatus syringe pump. The injection was carried out over a 30-min period, so as not to cause an excessive local protein concentration, which may alter to local pH value.

The focusing was carried out for 2 h at constant voltage of 1000V, the current varying as specified in figure legends. In some experiments, the field was decreased to 500 V during the last 30 min of focusing. With commercial CAs, current decreases significantly due to focusing of CA components. This is not the case in the present method, as the ampholyte is already loaded very close to or at its isoelectric pH. Thus, there is only a minor decrease of current density. At the end of the focusing, recycling and electrical power are stopped. The focusing chamber contents (6 ml) remix and are collected separately. The bulk of the fluid (94 ml) remains contained in the fluid loops and is collected simultaneously by shifting the bottom quick-connector from the cell to a test-tube array. Each of the 30 fractions contains approximately 3 ml of the fractionated fluid.

Amino acids

The EACA, histidyl-β-alanine, and lysyl-glutamine were obtained as gift from Vega Biotechnology (Tucson, AZ, USA). The other compounds were purchased from Sigma (St. Louis, MO, USA). No tests were made to ascertain the purity of the compounds.

Serum proteins

For most separations, a 10-ml aliquot (approx. 500 mg protein) of a frozen specimen of bovine serum was used as the model protein. In one experiment, a commercial sample of bovine immunoglobulin G (Sigma) was used. Both models were chosen as they represent rather broad distribution of proteins. To avoid overloading of the system with serum electrolytes, the serum was first equilibrated against 5 m*M* Tris buffer, pH 9.

TABLE II

PROPERTIES OF THE AMPHOLYTES USED

Ampholyte	p*I*	pK_a [a]	pK_b [a]	$\mu \cdot 10^4$ [b]	Exp. p*I* [c]
ε-Aminocaproic acid	7.6	4.4	10.8	?	N.A.
Cycloserine	5.9	4.4	7.4	3.42	5.9
Glycyl-glycine	5.7	3.15	8.25	3.08	5.9
Histidine	7.65	6	9.3	3.17	7.9
Histidyl-β-alanine	6.8	?	?	2.3	6.73
Lysyl-glutamic acid	6.1	4.47	8.45	1.96	6.15

[a] For histidine and other polyprotic ampholytes, only the two dissociation constants proximal to the p*I* are given.
[b] Electrophoretic mobility (cm^2/V s).
[c] Experimental p*I*, as obtained in the reported fractionations.

Analysis

The pH, ultraviolet adsorption and conductivity of all fractions were recorded. In addition, every second fraction was analyzed by conventional isoelectric focusing in polyacrylamide gels (PAG), using pH 3–10 Ampholine, the protein patterns being stained by Commassie Blue.

RESULTS

Of the many experiments carried out, we will report only a few illustrative examples. Serum proteins were used as a model, because of their polydispersity. In Table II are listed the p*I*, dissociation constants and electrophoretic mobility of the am-

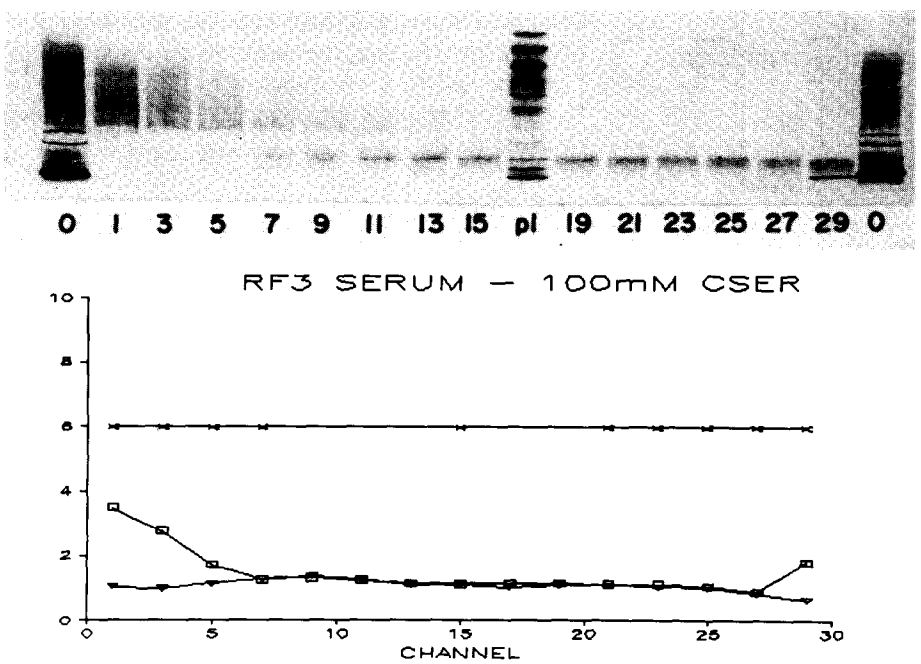


Fig. 3. RF3 separation of 10 ml of dialyzed serum using 90 ml of 100 m*M* cycloserine (CSER) as the sole buffering agent. Every second fraction was analyzed by focusing in PAG, 3–10 Ampholine (0 = original serum, p*I* = pH standard). Bottom plots present profiles of pH (x), absorbance at 280 nm (□) and conductivity in mS · 10 (▽). Initial conditions: 1000 V, 69 mA; final, after 2 h focusing: 1000 V, 42 mA. Throughout all the fractions, pH 5.9 was measured.

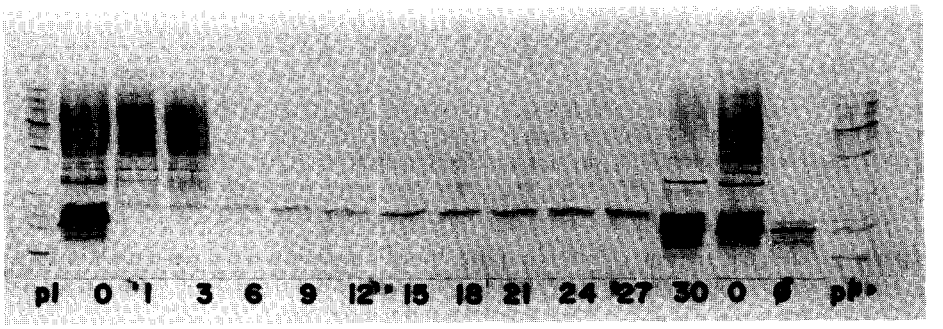
pholytes used. The last column in the table lists the pH of the plateaus seen in the reported experiments. There is in general a good agreement between the literature p*I* and the measured pH. Unfortunately, reliable literature data are not available for many potential ampholytes.

Fig. 3 illustrates the separation of the bovine serum proteins, using as the buffering agent a 100 m*M* solution of cycloserine, a "good" ampholyte. The upper part of the figure shows the IEF pattern of the separated fractions: basic proteins were found in the first 5 fractions, acidic in the last two. The original serum patterns are marked as 0. The bulk of the fractions, 11 to 27, contain essentially a single proteins, isoelectric at or near pH 6, coinciding with the band of the carbonic anhydrase, contained in the p*I* standard (marked p*I*). We have made no effort to identify this serum protein.

The bottom part of the figure shows the remarkable constancy of the pH across all the fractions. The pH 5.9 measured across all channels corresponds to the literature one, given in Table II. There was no polarization of low-molecular-weight ions in the terminal fractions, as the electrodialysis mode of perm-selective membrane arrangement was utilized. Absorbance readings at 280 nm, document the accumulation of proteins close to the two electrodes.

Fig. 4 shows quite similar results obtained using as the sole buffer a 500 m*M* solution of glycyl-glycine. This dipeptide is a rather "poor" ampholyte, therefore the current density was much lower, yet the resolution was comparable to that obtained with cycloserine. As the focusing mode of membrane arrangement was utilized, there was significant ion polarization, causing sharp increases of conductivity in the terminal fractions. These ions may have been contributed by the protein sample as well as by the buffering glycyl-glycine, used at high concentration.

Fig. 5 reproduces comparable data obtained with 100 m*M* lysyl-glutamic acid dipeptide. The polarization effect, noticed above for glycyl-glycine, is



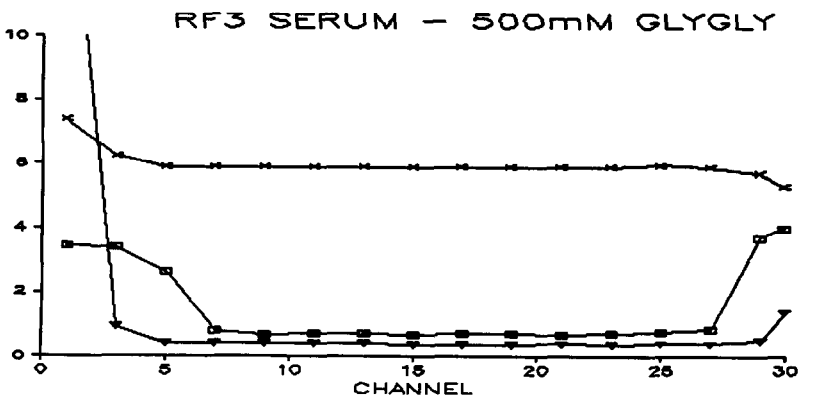


Fig. 4. RF3 separation in 500 m*M* glycyl-glycine (GLYGLY) buffer, a "poor" ampholyte. Initial: 1000 V, 19 mA; after 120 min: 1000 V, 16.4 mA; final 30 min: 500 V, 7.5 mA. Poor ampholytes result in lower current densities. A stable pH of 5.9 was measured between fractions 5 and 27. Symbols as in Fig. 3. ∅ = Sample irrelevant for example shown.

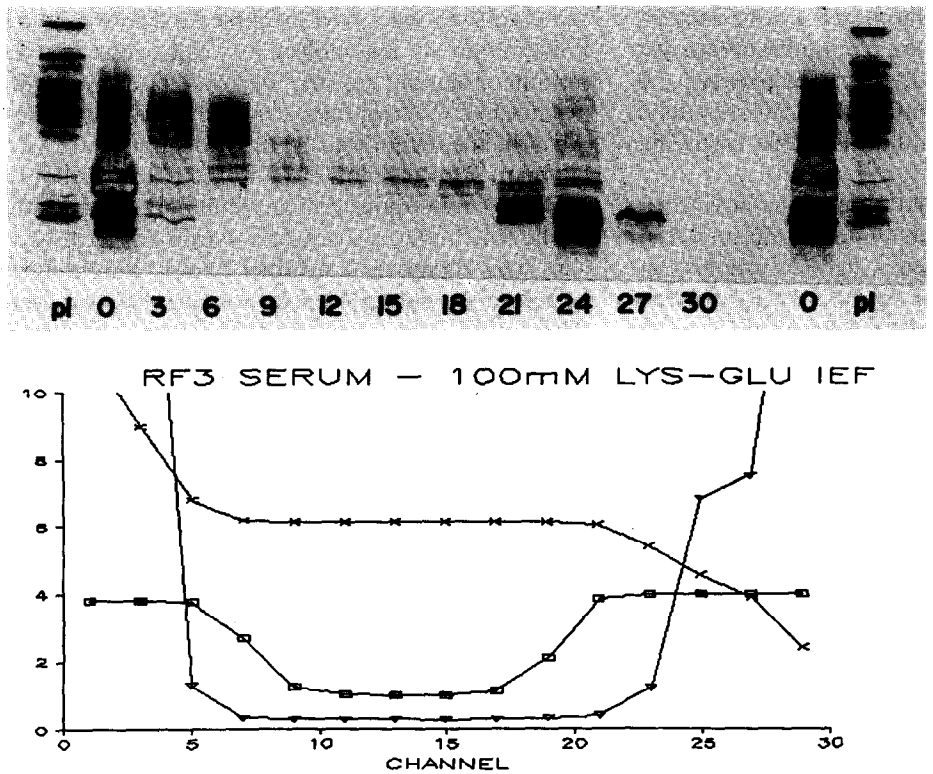


Fig. 5. Fractionation of 10 ml of serum in 100 m*M* lysyl-glutamic acid (LYS-GLU) dipeptide. Initial: 450 V, 200 mA; final: 500 V, 8.7 mA. The drastic decrease in current was due to the contamination of the lysine-glutamic acid by free glutamic acid, which focused at the anode, displacing all proteins. Nevertheless, a constant pH of 6.15 was measured in fractions 8 to 20. Symbols as in Fig. 3.

greatly accentuated, particularly in the acid region (fractions 25–30), where even the serum proteins were excluded. This effect was probably due to the presence of unreacted glutamic acid in the dipeptide used, which focused at the anode. This impurity notwithstanding, reasonable separation was obtained.

A separation of serum proteins within the histidyl-β-alanine pH window is presented in Fig. 6. The peptide concentration was lower, only 50 m*M*, and probably insufficient to properly buffer the apparatus contents during protein injection. Thus, fraction 30, which should comprise only acidic proteins, is heavily contaminated by basic proteins as well. Once proteins become trapped in regions close to the electrodes, they are slow to focus. The prevailing high conductivity results in a low local electric field and slow migration.

The results of separation using a binary mixture of two quite "poor" ampholytes is presented in Fig. 7. The combination of 500 m*M* each of EACA (p*I* 7.6) and glycyl-glycine (p*I* 5.7) resulted in an extended pH zone of pH 6.4–6.5, *i.e.*, close to the mean value. Being poor ampholytes, their ionization at this pH is still minimal, insufficient to bring about separation of the two amino acids even at the prolonged focusing time of 3 h. Thus, the mixture resulted in a very stable pH profile.

Finally, Fig. 8 shows the separation of 500 mg of bovine γ-globulin, using 100 m*M* histidine, a typical "good" ampholyte. The analysis of the fractions was carried out in a narrow range gel, pH 5–8 Ampholine. The data show that the spectrum of gamma globulins was resolved with high resolution.

DISCUSSION

Svensson [7] was the first to examine the use of

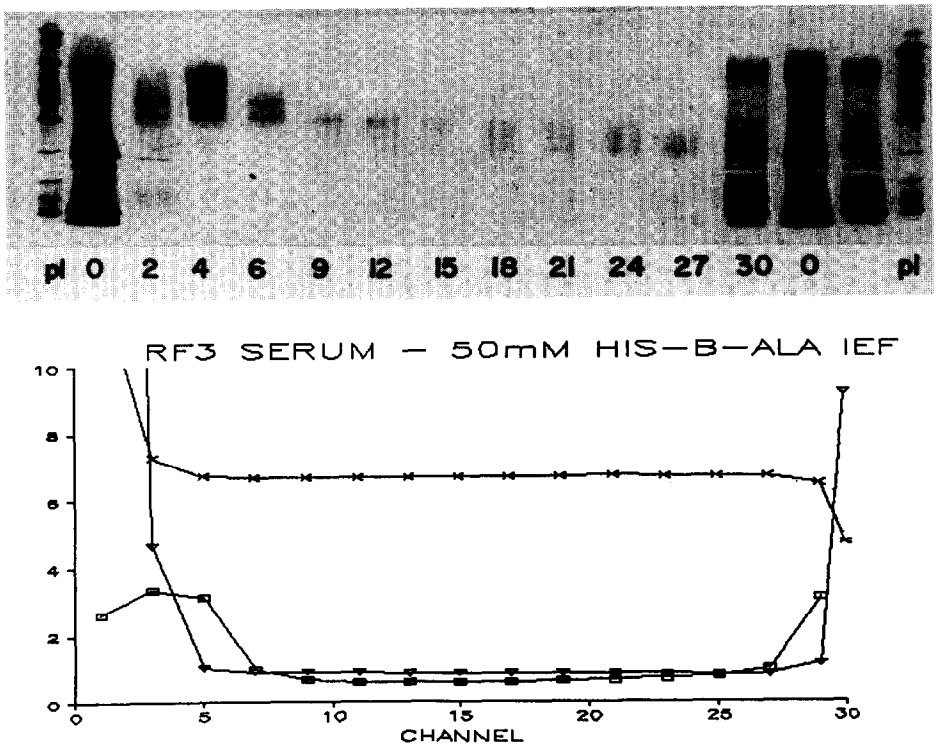


Fig. 6. Serum fractionation using 50 m*M* histydyl-β-analine (HIS-B-ALA) as the pH knife. Some basic protiens were trapped in the anodic compartment 30, possibly because of too low ampholyte concentration. Starting at 950 V, 110 mA, final 500 V, 24 mA. Channels 6 to 27 exhibited a constant pH of 6.73. Symbols as in Fig. 3.

amino acids and other simple ampholytes as current carrying buffers for isoelectric focusing. Nguyen and Chrambach [11] and others followed suit, while we have analyzed this possiblity by computer simulation [4,9]. The results, as a whole, were disappointing, because of the lack of sufficient ampholytes. More important, the gradients formed presented too many conductivity gaps, acting as protein traps.

We now propose a novel usage of amino acids and other similar simple ampholytes. Rather than trying to form pH gradients, we use individual amino acids to establish very stable regions of constant pH, into which the proteins to be separated are slowly injected. Due to the absence of any significant pH gradient, proteins do not focus in the conventional sense, but migrate by zone electrophoresis toward one or the other electrode. A third, intermediate zone is formed by the proteins isoelectric at the prevailing pH window imposed by the amino acid. These are sharply resolved from the more acidic or more basic ones. Where a single ampholyte of desired p*I* is not available, binary mixtures can be used. The closer their p*I* values, the sharper the resolution.

The proposed method is a software analogue to the hardware one developed by Righetti *et al.* [12]. Using the principle of recycling focusing [1], Righetti *et al.* recycle the protein to be purified though a compartment bounded on both sides by polyacrylamide gels, rendered isoelectric at the desired pH by incorporation of copolymerized buffering components (known as Immobiline). The advantage of our method is the avoidance of the need for controlled gel synthesis.

In is projected that for optimal use of our method, it would be advantageous to have available ampholytes covering the pH scale at 0.1 pH intervals. Table I lists the ampholytes proposed already by Svensson [7]. We are presently engaged in preparing

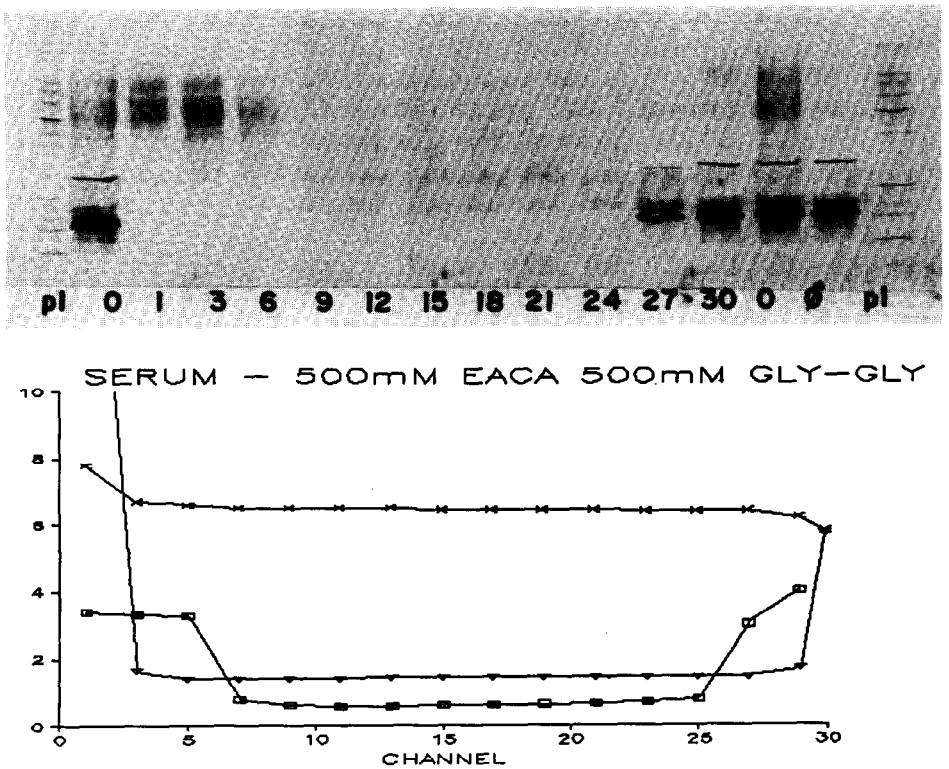


Fig. 7. Serum fractionation in a binary mixture of 500 m*M* ε-aminocaproic acid (EACA) and 500 m*M* of glycyl-glycine (GLY-GLY), two "poor" ampholytes. A shallow pH gradient, pH 6.5 in channel 6 decreasing to pH 6.4 in channel 27, was obtained. Few proteins were focused in this range. 1000 V were applied throughout the 3 h run, the current decreasing from 91 to 61 mA. Obviously, the two amino acids were poorly resolved, their ionization at this average pH value being minimal, but excellent resolution was obtained. Symbols as in Fig. 3.

an extended list of compounds, rendered possible through advances in amino acid chemistry and peptide synthesis. Moreover, we have demonstrated that intermediate pH regions can be established using binary mixtures of ampholytes. The closer their p*I* values, the narrower will be the pH window and the sharper the resolution. In addition, we are engaged in extensive computer modeling of such systems, to be published separately.

We feel that this method has overcome the main shortcoming of conventional preparative focusing by avoiding use of the chemically ill-defined commercial CAs. The amino acids are easily analyzed and, if necessary, easily removed by dialysis or gel sieving. In addition, protein precipitation is avoided or greatly decreased. Because of the low cost of many amino acids and other ampholytes, it opens the possibility of large scale focusing for production of therapeutic proteins. In work not yet reported [10], we were able to scale focusing to a rate of 500 ml/h, using a RIEF apparatus [1] with an effective cross-area of 100 cm^2.

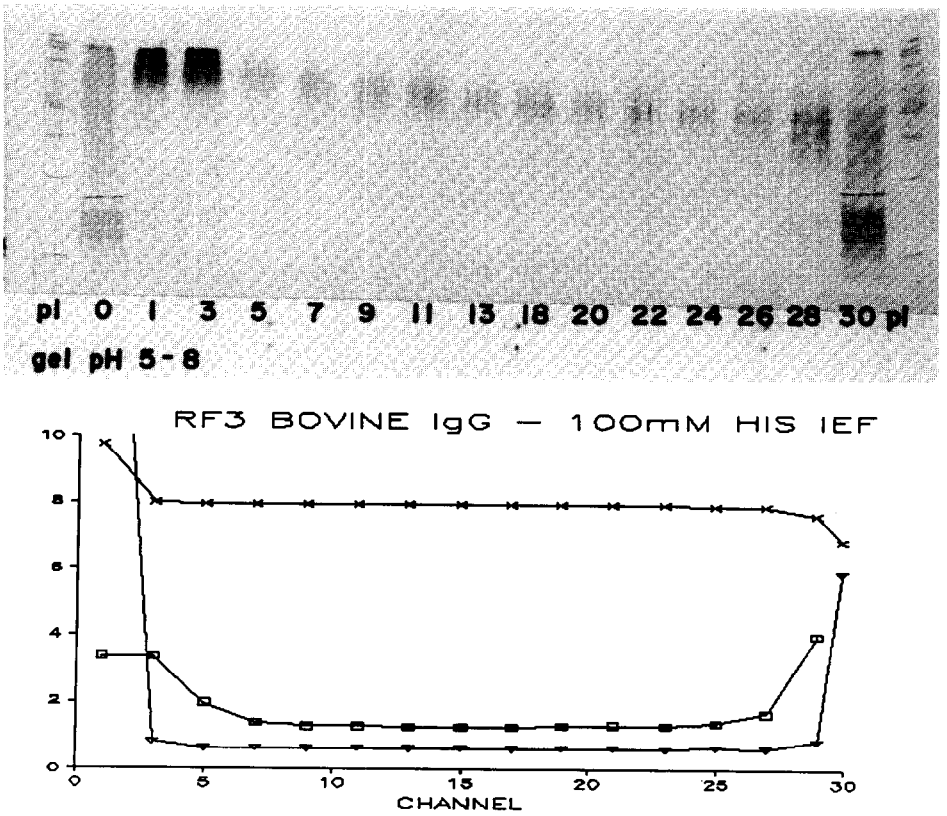


Fig. 8. Fractionation of 500 mg of bovine immunoglobulin G in 100 m*M* histidine (HIS) buffer. Clear subfractionation was obtained. Initial condition: 1000 V, 55 mA, final conditions: 500 V, 16.5 mA, pH 7.90 between channels 5 and 27. Symbols as in Fig. 3.

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