Journal of Chromatography, 205 (1981) 355–366
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,347

LIMITING FACTORS FOR THE RESOLVING POWER OF ISOELECTRIC FOCUSING IN NATURAL pH GRADIENTS

R. CHARLIONET*, C. MORCAMP, R. SESBOÜÉ and J. P. MARTIN
Unité U-78 de l'Inserm sur la Génétique des Protéines Humaines, 543 Chemin de la Bretèque, F-76230 Bois-Guillaume (France)

(First received May 22nd, 1980; revised manuscript received September 11th, 1980)

SUMMARY

The improvement of the resolving power of isoelectric focusing by the use of synthetic ampholytes is analysed. It is shown that (i) a high buffering capacity is an essential feature of suitable carrier ampholytes, and (ii) with the synthetic procedure utilised, *i.e.* the polycondensation of basic amphoteric structures, it is expected that the higher the molecular weight of the polyaminocarboxylic acid carrier ampholytes, the better the resolving power.

INTRODUCTION

The advent of isoelectric focusing in natural pH gradients, with the theoretical work of Svensson[1–3] and the practical developments of Vesterberg[4], represents a major advance in the field of electrophoretic separation of proteins. Its excellent resolving power and its handiness, especially when it is employed with polyacrylamide gel slab, make this technique an ideal tool for the study of genetic polymorphism of certain proteins.

The pH gradient is formed by the action of an electric current on a mixture of many ampholyte species with isoelectric points distributed evenly over the pH scale. We have recently[5] described the synthesis of highly diversified carrier ampholytes which, fractionated in a narrow pH range, allowed us to separate and to reveal new alleles of the Pi system (alpha-1-antitrypsin genetic polymorphism). A resolving power of the order of 0.005 pH units or less was obtained.

According to Rilbe[6], the smallest difference of isoelectric points that can be detected with certainty by the equilibrium-gradient separation method is:

$$\Delta(\mathrm{p}I) = 3 \sqrt{\frac{D \dfrac{\mathrm{d}(\mathrm{pH})}{\mathrm{d}x}}{\dfrac{-\mathrm{d}u}{\mathrm{d}(\mathrm{pH})} E}}$$

where D is the diffusion coefficient and $-\mathrm{d}u/\mathrm{d}(\mathrm{pH})$ the mobility slope of the proteins to be separated; E is the field strength and $\mathrm{d}(\mathrm{pH})/\mathrm{d}x$ the pH gradient.

0021-9673/81/0000–0000/$02.50

Giddings and Dahlgren[7], using another approach, derived a similar equation. They defined the resolution R_s for two peaks as $\Delta x/4\sigma$, where Δx is the distance between the centres of due peaks and σ is the average deviation in width of two peaks:

$$R_s = \frac{\Delta\text{pH}}{4} \sqrt{\frac{-FE\, dq/d\text{pH}}{RT\, d\text{pH}/dx}}$$

where ΔpH is the isoelectric pH increment between the two components, F the Faraday (96,500 coulombs), E the electric field strength, R the gas constant, T the absolute temperature, q the effective charge of the particle and x the distance moved. The two peaks are separated if $R_s \geqslant 1$.

It is thus found that a shallow pH gradient and a high field strength favour resolution. It should nevertheless be kept in mind that the quantities E and d(pH)/dx appear as the ratio $E/[d(\text{pH})/dx]$. If the conductivity is constant on the gel slab, this ratio equals $\Delta(V)/\Delta$pH, where $\Delta(V)$ is the potential difference and Δ(pH) the pH difference between the electrodes. Contrary to what has been asserted by several authors[8], the gel slab length does not influence the resolution of two proteins. The notion of resolving power should however be well differentiated from that of peak capacity[7]. The former refers to the smallest difference of isoelectric points between proteins which can be separated. The latter is defined as the theoretical maximum number of components resolvable under specified conditions. If component peaks of average width 4σ are distributed over path length L, the peak capacity is $n = L/4\sigma$. Giddings and Dahlgren gave the following expression for peak capacity in isoelectric focusing:

$$n = \sqrt{\frac{-FE\,(dq/d\text{pH})\,(d\text{pH}/dx)\,L^2}{16\,RT}}$$

In contrast to the resolving power, the peak capacity is favoured by high pH increment and is linearly dependent on the length of the gel slab. The conditions for optimum resolving power and for optimum peak capacity are thus not the same. In this paper we will consider only the resolving power of isoelectric focusing.

As a matter of fact, the pH gradient can not be reduced nor field strength increased indefinitely.

Considering a simplified system of carrier ampholytes in the presence of an electric field, Almgren[8] computed their concentration distribution and the resulting pH course at the steady state. From his results, we can deduce that above a certain value of the field strength, the concentration distributions are no longer Gaussian but tend to acquire a square form: the peaks flatten out, the slopes become steeper and the pH course tends towards a step function. This critical value of the field strength is dependent on the dissociation constants (therefore, the buffer capacities), the diffusion coefficients, the ion mobilities and the isoelectric points of the carrier ampholytes. Because under these conditions the pH gradient is increasing in a stepwise manner, the resolving power of the method is limited to the height of the pH step, *i.e.* to the difference of isoelectric points between successive focused ampholytes.

Our method[5] of decreasing the slope of the pH gradient is the electrophoretic fractionation of the carrier ampholytes in a narrow pH range. We have experimentally

found that the minimum pH range which can be used is, in our system of ampholytes, *ca.* 0.3 pH unit. Owing to overlapping in the ampholyte distribution, fractionation over a narrower pH range leads to a steady state with a new concentration distribution of the carrier ampholytes, covering a pH zone of *ca.* 0.3 pH unit. In this case, the width of the concentrations of each focused ampholyte species is the limiting factor for decreasing the pH gradient. According to Almgren[9] and Rilbe[3], it is a function of the buffer capacity (dissociation constants), the diffusion coefficients and the ion mobilities of the ampholytes.

The limiting factors for the resolving power of isoelectric focusing in natural pH gradients thus probably lie in the physico-chemical nature of the carrier emopholytes.

We have investigated the qualities of carrier ampholytes particularly with a view to improving the resolving power of isoelectric focusing. Two methods have been used for this purpose:

(1) The further physical characterization of our synthetic ampholytes, by the determination of their molecular weight distribution. In this context, Gelsema *et al.*[10] have recently shown that the buffer capacity and the conductivity of ampholytes are linked by the mean molecular weight; furthermore, the diffusion coefficients and ion mobilities of ampholytes are also dependent on the molecular weight.

(2) An assessment of the value of succinylated albumin as carrier ampholytes. This system of ampholytes can be physicochemically characterized and its behaviour in isoelectric focusing experiments analysed.

EXPERIMENTAL

Materials

The chemical reagents and their sources were: ammonium persulphate, riboflavin, sucrose, hydrindantin and ninhydrin from Merck (Darmstadt, G.F.R.); acrylamide, N,N'-methylenebisacrylamide and N,N,N',N'-tetramethylethylenediamine (TEMED) from O.S.I. (Paris, France); trichloroacetic acid from Prolabo (Paris, France); bovine albumin 30% solution from S.P.C.I. (Laplaine-Saint-Denis, France); ethylene glycol monomethylether, succinic anhydride, acetic acid sodium salt from Carlo Erba (Milan, Italy); Bio-Gel P-6 Bio-Rad Laboratories (Richmond, CA, U.S.A.); Sephadex G-15 from Pharmacia (Uppsala, Sweden); glutathione reduced, cytochrome *c* from Aldrich (Paris, France); insulin, glucagon from Fluka (Paris, France); Coomassie Brillant Blue R-250 from Serlabo (Paris, France); cyanocobalamine from Laboratoire Roussel (Paris, France); agarose, indubiose from I.B.F. (Clichy, France); rabbit anti-alpha-1-antitrypsin serum from Blood Transfusion Centre (Bois-Guillaume, France).

The ampholyte pH ranges (4.3–4.9), (3.7–4.3), (5–6) and (6–8) were synthesized and fractionated as previously described[5].

Methods

Ampholyte molecular weight determination. All analyses were performed on a Technicon amino acid analyser. Approximately 1 mg of ampholytes (calculated as dry weight) in 1 ml of 0.35 *M* acetate buffer pH 5 was placed on a 150 × 1 cm I.D. column of Bio-Gel P-6 (200–400 mesh) or Sephadex G-15 previously equilibrated with

the same buffer. A proportioning pump was used to collect effluent from the column at a flow-rate of *ca.* 25 ml/h and to mix it continuously with 2.9 ml of ninhydrin reagent (20 g of ninhydrin, 1.5 g of hydrindantin, 2.150 l of ethylene glycol, 0.350 l of 4 *M* acetate buffer pH 5.5 and 1.5 l of water). The mixture was passed through a heating bath (95°C) for *ca.* 12 min and a jacketed cooling coil. The absorbance was then measured in a colorimeter at 570 nm and recorded.

The columns were calibrated with reduced glutathione (mol. wt. 307), cyanocobalamine (mol. wt. 1300) and cytochrome *c* (mol. wt. *ca.* 13,000) for the Sephadex G-15 gel, and cyanocobalamine, glucagon (mol. wt. 3500), insulin (mol. wt. 6000) and cytochrome *c* for the Bio-Gel P-6 gel.

Succinylation of bovine albumin. Bovine albumin was chemically modified[1] by the action of succinic anhydride on the amino group of basic residues, especially of lysines.

Succinic anhydride (1 g) is added slowly to a water solution of bovine albumin (60 ml, 2 g) under continuous stirring. The pH of the mixture is maintained at 5.2 by addition of 2 *M* sodium acetate. The reaction continues for 3 h. The products are then dialysed against deionized water and concentrated to 30 ml (*i.e.* 6.7%, w/v).

The effect of succinylation on the isoelectric points of the produced proteins is followed by isoelectric focusing on a polyacrylamide gel slab with an LKB Multiphor apparatus. Our synthetic ampholytes in the pH range 4.3–4.9 are employed and the technique is carried out as described previously[5].

Use of succinylated bovine albumin as carrier ampholytes. The isoelectric focusing experiments with succinylated bovine albumin as carrier ampholytes are performed on polyacrylamide gel plates with a Pharmacia Multiphor apparatus. The gels are made to a final concentration of acrylamide 5% (w/v), sucrose 12% (w/v), synthetic carrier ampholytes from 0 to 1% (w/v) and succinylated bovine albumin from 0.5 to 2% (w/v).

Polymerization is accomplished with riboflavin (0.04%, v/v) and UV light for 1 h when synthetic carrier ampholytes are present; otherwise, with TEMED 0.1% (v/v) and ammonium persulphate 0.05% (w/v). A pre-run is carried out for 1 h, and after an eventual application of the serum sample isoelectric focusing is performed for 2 h under the following maximum electrical conditions: 10 W, 10 mA and 1600 V. The distance between electrodes is generally set at 10 cm. Some experiments have been performed with electrodes separated by 22 cm during the pre-run and by 8 cm during the run.

The pH gradients in the gel slabs are measured with a contact electrode (Ingold, Sofranie, France) and read on a digital pH meter.

Immunofixation. The pattern of the alpha-1-antitrypsin phenotypes after isoelectric focusing with succinylated bovine albumin as carrier ampholytes, cannot be revealed by fixation with trichloroacetic acid and staining with Coomassie blue, as the whole plate would be stained. We had to use immunofixation[12]: 20 ml of agarose containing 4 ml of anti-alpha-antitrypsin serum was poured on the polyacrylamide gel and incubated in a humid atmosphere for 12 h.

RESULTS

Molecular weight determination

Figs. 1 and 2 show the weight distribution of synthetic carrier ampholytes

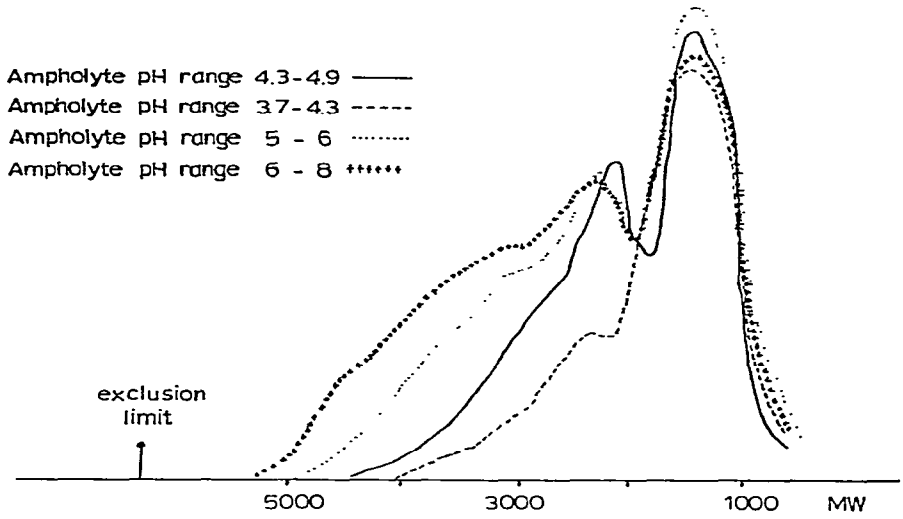

Fig. 1. Molecular weight distribution of carrier ampholytes fractionated in pH ranges 3.7–4.3, 4.3–4.9, 5–6 and 6–8. The gel filtration was performed with Bio-Gel R-6 (200–400 mesh) on a 150 × 1 cm I.D. column, equilibrated with 0.35 *M* acetate buffer, pH 5. The elution was analysed on a Technicon amino acid analyser. The column was calibrated with cyanocobalamine (mol.wt. 1300), glucagon (mol.wt. 3500), insulin (mol.wt. 6000) and cytochrome *c* (mol.wt. 13,000).

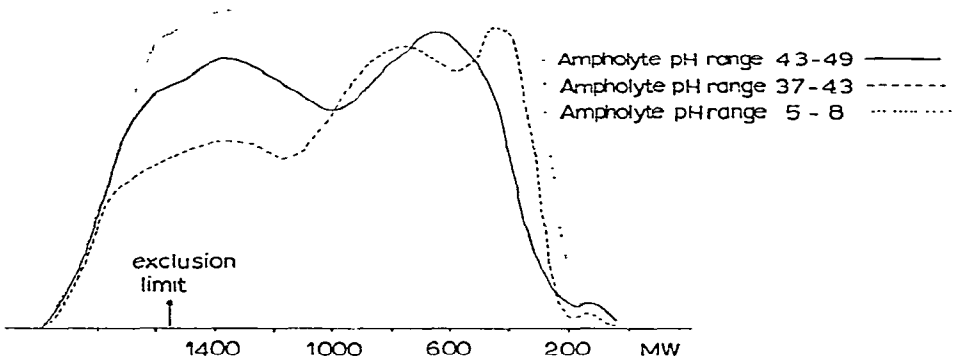

Fig. 2. Molecular weight distribution of carrier ampholytes fractionated in pH ranges 3.7–4.3, 4.3–4.9 and 5–8. The gel filtration was performed with Sephadex G-15 on a 150 × 1 cm I.D. column, equilibrated with 0.35 *M* acetate buffer, pH 5. The elution was analysed on a Technicon amino aicd analyser. The column was calibrated with reduced glutathione (mol.wt. 307), cyanocobalamine (mol.wt. 1300) and cytochrome *c* (mol.wt. 13,000).

fractionated in the pH ranges 3.7–4.3, 4.3–4.9, 5–6 and 6–8. The gel filtrations were performed with Bio-Gel P-6 (200–400 mesh) (Fig. 1) and Sephadex G-15 (Fig. 2), the separation range (daltons) being respectively 1000–6000 and 0–1500.

Differences in the molecular weight distributions are particularly evident between the various pH ranges analysed. It appears that polymerized ampholytes have a COOH/NH ratio smaller than that of the monomers (Figs. 1 and 2). Amongst the monomeric ampholyte species the acidic monomers, which are able to bind more acrylic acid molecules, have the highest molecular weights. Amongst the polymerized

ampholytes, where molecules with molecular weights up to 5200 daltons are found, the alkaline species have the largest molecular sizes. The observations are, in essence, similar to those of Radola *et al.*[13].

Concerning the pH range 4.3–4.9 ampholytes, three distinct peaks are observed. The first peak includes ampholytes of molecular weight between 550 and 750 daltons. These could correspond to the following basic molecules of ampholytes:

	1 TEPA and 5 acrylic acid	mol. wt. 550
	1 TEPA, 5 acrylic acid and 1 epoxypropanol	mol. wt. 624
or	1 TEPA, 5 acrylic acid and 2 epoxypropanol	mol. wt. 700

(TEPA is tetraethylenepentamine)

The second peak is broader and contains ampholytes of average size near 1400 daltons. It could be explained by two basic molecules of ampholytes linked by one bridge of diepoxyoctane (mol. wt. 142). The third peak comprises ampholytes of molecular weight 2000–2700. These could be trimers or tetramers of basic molecules of ampholytes linked by two or three diepoxyoctane bridges.

Concerning the other pH range ampholytes, peaks are seen as expected, corresponding to the basic molecules of ampholytes: for pH range 3.7–4.3, 1 TEPA, 6 acrylic acid and 0, 1 or 2 epoxypropanol; for pH range 5–8, 1 TEPA, 2 or 3 acrylic acid and 0, 1 or 2 epoxypropanol.

In addition to these major peaks there are also minor peaks corresponding to ampholytes of lower molecular weight. They should be the products of acrylic acid with small polyamines, such as triethylenetetramine (TETA) and diethylenetriamine (DiETriA). It is worth noting that such a peak does not appear with ampholytes of pH range 4.3–4.9. Condensation of TETA or DiEtriA with acrylic acid does not lead to ampholytes of that pH range. This correlates well with the findings of Vesterberg[4] that DiETriA and TETA ampholytes give rise to a pH course with two plateaus at pH 4 and 5, *i.e.*, there is a deficiency of TETA and DiETriA ampholytes with p*I* between these two pH values.

The amounts of ampholytes recovered from Bio-Gel P-6 and Sephadex G-15 columns have not been quantified. The ninhydrin reagent is extremely sensitive to all compounds with free amino groups, yielding a coloured product (Ruhemann's purple) which absorbs 570-nm light. Integration of the curves from the chromatograms cannot give, in this case, the precise amount of ampholytes, as the different ampholyte species do not all yield the same amount of colour per mole. We can only ascertain that in synthesizing highly diversified carrier ampholytes as previously described, we succeeded in producing an important fraction of di-, tri-, tetra- and even higher polymers. An important proportion of ampholytes pH range 4.3–4.9 have a molecular weight well over 1000 daltons

Use of succinylated bovine albumin as carrier ampholytes

Fig. 3 shows the distribution of the isoelectric points of the modified bovine albumins; *ca.* 40 μg of these proteins were analysed. It is clear that succinylation of bovine albumin yields a very large number of modified proteins with p*I* values well distributed over the zone of pH 4.3–4.7. We can assume that three or four molecules of succinic anhydride are needed to lower the p*I* of native bovine albumin from 5.2 to *ca.* 4.5 (ref. 14). As there are 59 residues of lysine which can react with the anhy-

Fig. 3. Distribution of the isoelectric points of modified bovine albumin. On the left is the isoelectrofocusing pattern of several alpha-1-antitrypsin phenotypes (namely M_1M_2, M_1S and M_1). On the right is the isoelectrofocusing pattern of succinylated bovine albumins; 40 μg of these proteins are analysed. Isoelectric focusing was performed on polyacrylamide gels labs on a LKB Multiphor apparatus, with carrier ampholytes pH range 4.3–4.9, as described under *Methods*.

dride, the number of different modified proteins obtained would be between 32,506 and 455,126. These numbers have been calculated from $C3^{59}$ and $C4^{59}$, which represent the possible sets of three or four things selected from 59, irrespective of arrangement within the sets. All these slightly different proteins will focus between pH 4.3 and 4.7, as can be seen in Fig. 3.

Although bovine albumin succinylated in these conditions constitutes a really highly diversified ampholyte system, these proteins do not possess the necessary qualities to be carrier ampholytes, as illustrated by Figs. 4 and 5.

Fig. 4 shows the pH gradients created in polyacrylamide gel slabs containing:

(1) Only modified bovine albumin as ampholytes (final concentration 2%);

(2) Modified bovine albumin (1%) and synthetic carrier ampholytes pH range 4.3–4.9 (0.5%);

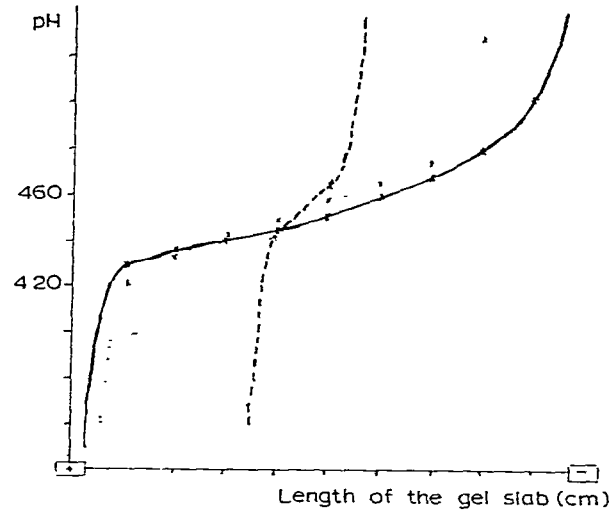

Fig. 4. Comparison of the pH gradients created in polyacrylamide gel slabs containing the following ampholytes: broken line, only modified bovine albumin (final concentration 2%); dotted line, modified bovine albumin (1%) and synthetic carrier ampholytes pH range 4.3–4.9 (0.5%); solid line, modified bovine albumin (0.5%) and synthetic carrier ampholytes pH range 4.3–4.9 (1%). Conditions for isoelectric focusing are described under *Methods*.

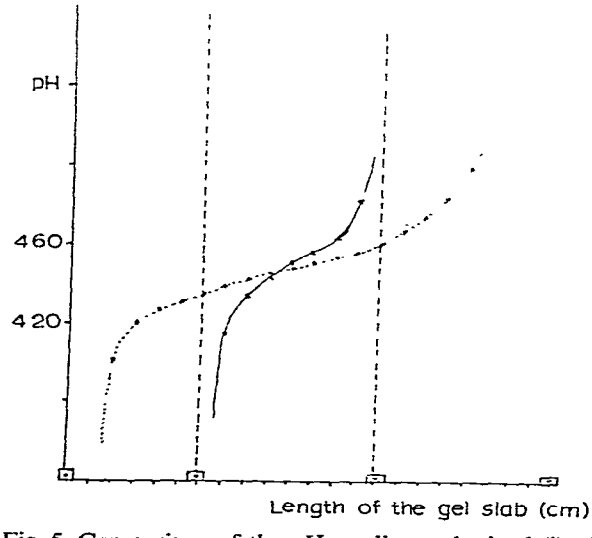

Fig. 5. Comparison of the pH gradients obtained (i) after a pre-run of 2 h, in a gel slab of 22 cm containing synthetic carrier ampholytes (1%) and modified bovine albumin (1%) (dotted line); (ii) after a further run of 2 h, in the same gel slab which has been shortened to 8 cm encompassing the zone of pH 4.35–4.60 of the previous pH gradient (solid line). Isoelectric focusing was performed on a Pharmacia Multiphor apparatus as described under *Methods*.

(3) Modified bovine albumin (0.5%) and synthetic carrier ampholytes pH range 4.3–4.9 (1%).

A flat pH gradient is created only if synthetic ampholytes are present in sufficient concentration (1%). When modified bovine albumins are the only ampho-

lytes, nearly half of the 10-cm wide polyacrylamide gel slab, anodically, has a pH less than 2. On the other half of the slab, the cathodic side, the pH is above 11. Near the middle of the slab, the pH alters very quickly from 2 to 11 with a slight "plateau" of *ca.* 1 cm, pH 4.2–4.7, where all the modified albumins are focused. Concentrations of bovine albumin greater than 2% cannot be employed owing to the insolubility of these proteins in the vicinity of their pI.

Fig. 5 illustrates the pH course obtained:

(1) In a gel slab of 22 cm containing synthetic carrier ampholytes 1% and modified bovine albumin 1% after a pre-run of 2 h;

(2) In the same gel slab which has been shortened to 8 cm encompassing originally a zone of pH 4.35–4.60, after another run of 2 h.

The new pH gradient is steeper than the first one and extends between 4.2 and 4.8. This phenomenon, which can be explained by the overlapping of the ampholyte distribution, indicates that the presence of modified bovine albumin did not stabilize the pH gradient.

Fig. 6 shows the pattern of isoelectric focusing of Pi M_1M_2 and Pi M_1S in polyacrylamide gel slab containing modified bovine albumin (1%) and carrier ampholytes (1%) revealed by immunofixation. It is in all respects similar to the pattern seen after electrofocusing in the absence of albumin, and indicates that although the presence of the albumin does not improve the resolving power, it allows the steady state to be established.

DISCUSSION

With the exception of ampholytes containing inorganic acid groups such as phosphoric or sulphuric acids, which are used only for extending the pH range down to 2, all manufactured or "home-made" ampholytes have the same fundamental polyamine–polycarboxylic acid structure. The only differences are in the synthetic procedure:

(1) Vesterberg[4] used various aliphatic polyamines with the general formula $R_2N(CH_2)_nNR_2$, where R is H or $(CH_2)_nNR_2$ and n is between 2 and 4. The main polyamines employed are PEHA (pentaethylenehexamine) and TEPA. Unsaturated carboxylic acids such as acrylic or itaconic acid are added. Vinogradov[15], Righetti[16,17], Just[18] and the authors[5] used the principle of this synthetic process.

(2) Grubhofer[19] started his synthesis by preparing aliphatic amines with a large number of isomers. It was conducted by condensing ethyleneimine with propylenediamine under conditions based on the work of Könnecke[20].

(3) Carrier ampholytes called "pharmalytes" are presented in a brochure from the manufacturer as being synthesized by copolymerisation of amino acid such as glycine and glycylglycine, amine, and epichlorhydrin.

All these polyamine–polycarboxylic acid ampholytes could differ by the density of charged groups and by the number of amphoteric species with various isoelectric points and dissociation constants.

When the conductivity and buffer capacity of different commercially available ampholytes are compared[10,21] they are found to be similar but not quite identical over the whole pH range. Furthermore, the differences that exist between manufacturers also occur to some extent between different batches[10]. Despite some variation the

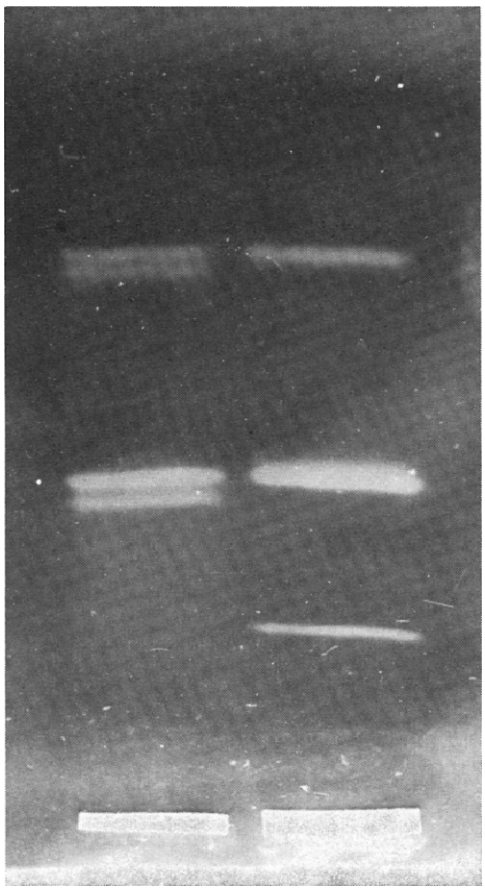

Fig. 6. Immunofixation revealing the pattern of isoelectric focusing of Pi M_1M_2 and Pi M_1S in a polyacrylamide gel slab containing modified bovine albumin (1 %) and synthetic carrier ampholytes pH range 4.3–4.9 (1 %).

commercial ampholytes have nevertheless "minimal" buffer capacity, sufficiently good distribution of isoelectric points and evenly distributed conductivity, to be suitable carrier ampholytes, and indeed some, under good operating conditions, separate proteins whose p*I*'s differ by only 0.02 or even 0.01 pH units[22–24]. However, as we have previously shown[5] in parallel experiments, the commercial ampholytes even when used in a narrow pH range do not give a level of resolution as good as that achieved using the synthetic ampholytes described in the present paper.

If a superior resolving power is needed, the commercial ampholytes are of little use. Our synthetic ampholytes can be used to separate proteins whose p*I*'s are less than 0.005 pH unit apart and allowed us to detect at least six new variants of alpha-1-antitrypsin[25]. The success of our synthetic ampholytes in the separation of genetic variants of alpha-1-antitrypsin has already been explained by the increased diversity of amphoteric components due to the use of condensing reagents such as epoxypropanol and diepoxyoctane. We had calculated that, in the zone of pH 4.3–5.0, more than 4000 ampholyte species with different isoelectric points and dissociation constants could have been created.

Another explanation may be put forward to explain this improved resolving power. We have demonstrated in this study that our ampholytes are generally heavier than the manufactured ones. An important fraction of ours are dimers, trimers and tetramers of the basic ampholytes (550–700 daltons). As a consequence the buffer capacity of our ampholytes should be very good in their focused state because they exhibit a high conductivity[5], and the ratio of buffer capacity to conductivity is proportional to the cube root of the molecular weight[10]. Further, the width of the concentration distribution peak of our ampholytes should be very small because the relatively high molecular weights affect both the buffer capacity and the diffusion coefficient[26].

We have employed succinylated bovine albumins as carrier ampholytes, in order to study the behaviour of a system with the following characteristics: very high molecular weight (*ca.* 67,000); very sharp zone of focusing for each species of modified albumins as is the case for nearly all proteins; good repartition, over the pH scale 4.3–4.7, of the isoelectric points of the highly diversified albumins; but a buffer capacity much lower than that of the synthetic ampholytes. This last feature can be illustrated by comparing the density of protolytic group involved in buffering activity near pH 4.5 for bovine albumin and for synthetic ampholytes. Only the aspartic and glutamic acid residues are concerned by buffering activities at pH 4.5 for bovine albumin, *i.e.*, 100 protolytic groups for proteins of mol. wt. 66,200 (ref. 27). For an equivalent weight, a synthetic ampholyte would have *ca.* 500 carboxylic groups and several hundred amine groups whose dissociation constants are near $pK = 4.5$ (pK_4 of TEPA[4]). Such a system has been proved in this study to be absolutely inoperative as carrier ampholytes. This reveals the importance of the buffer capacity for carrier ampholytes.

High buffer capacity is thus, as has already been shown by Svensson, desirable for carrier ampholytes to enable a small amount of them to dictate the pH course in the steady state even in the presence of proteins, and to repel strong electrolytes in the vicinity of their respective electrodes. Increasing the amount of carrier ampholytes, as a palliative for an insufficiency of buffer capacity, must be done cautiously: as the amount of ampholytes that is added increases, the approach to steady state becomes slower and more difficult[9].

The method of decreasing the slope of pH gradients that we have chosen is the isoelectric focusing fractionation of the carrier ampholytes in a narrow pH range. The limiting factor for a low pH gradient is then the width of the focused zones of each ampholyte species. Narrow focused bands are promoted by high buffer capacity or by high molecular weight of the ampholytes. It seems very difficult to increase the number of charged groups in the actual polyamine–polycarboxylic acid structure of ampholytes. However, it seems reasonable to think that more attempts could be made to increase the molecular weight of carrier ampholytes. A sensible control of an equilibrated weight distribution in the mixture of ampholytes would lead to a gradient of molecular weight. Part of the ampholyte mixture should contain species of low molecular weight and minimal buffer capacity with a view to ensuring an even conductivity on the whole chosen pH scale. Ampholytes with increasing molecular weight and increasing buffer capacities should also be included so that they can be fractionated in narrower and narrower pH ranges. Molecular weight cannot, however, be increased indefinitely as carrier ampholytes must be soluble in their isoelectric state.

It must be remembered that considerable diversification of isoelectric points of the ampholytes is necessary to compensate for the reduced width of ampholyte

focusing zone, in order to obtain a linear pH course even for high electric field strength. We have seen that this diversification is obtained in our synthetic procedure by poly-condensing basic ampholyte molecules.

For all these reasons, and keeping in mind that ampholytes must possess a minimal buffer capacity to be suitable carrier ampholytes, we can state that the limiting factor for the resolving power of isoelectric focusing in a natural pH gradient formed by polyamine–polycarboxylic acid, appears to be the molecular weight of ampholytes: the higher the molecular weight of the polyaminocarboxylic acid carrier ampholytes, the better the resolving power expected. It must be clear that these ampholytes of high molecular weight should not be employed for quantitive purposes as it could be difficult to separate them from proteins and peptides. They should not be recommended either where a maximum peak capacity is needed (*e.g.*, for fractionating membrane components). They are particularly well adapted for analytical purposes where a very good resolving power is needed to separate two identified proteins, as is the case for the study of genetic polymorphisms.

ACKNOWLEDGEMENTS

This work was supported in part by Inserm, Grant No. ATP 55-77-87, and the University of Rouen.

REFERENCES

1 H. Svensson, *Acta Chem. Scand.*, 15 (1961) 325.
2 H. Svensson, *Acta Chem. Scand.*, 16 (1962) 456.
3 H. Rilbe, in N. Catsimpoolas (Editor), *Isoelectric Focusing*, Academic Press, London, 1976, p. 14.
4 O. Vesterberg, *Acta Chem. Scand.*, 23 (1969) 2653.
5 R. Charlionet, J. P. Martin, R. Sesboüé, P. J. Madec and F. Lefebvre, *J. Chromatogr.*, 176 (1979) 89.
6 H. Rilbe, *Ann. N.Y. Acad. Sci.*, 209 (1973) 11.
7 J. C. Giddings and H. Dahlgren, *Separ. Sci.*, 6 (1971) 345.
8 R. C. Allen, R. A. Harley and R. C. Talamo, *Amer. J. Clin. Path.*, 62 (1974) 732.
9 M. Almgren, *Chem. Scripta*, 1 (1971) 69.
10 W. J. Gelsema, C. L. de Ligny and N. G. van der Veen, *J. Chromatogr.*, 173 (1979) 33.
11 K. H. Gustavson, *Arkiv. Kemi.*, 17 (1961) 541.
12 C. A. Alper and A. M. Johnson, *Vox Sang.*, 17 (1969) 445.
13 B. J. Radola, H. Tschesche and H. Schuricht, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, New York, 1977, p. 97–110.
14 P. G. Righetti, *J. Chromatogr.*, 173 (1979) 1.
15 S. N. Vinogradov, S. Lowenkron, M. R. Andonian, J. Bagshaw, K. Felgen Hauer and S. J. Pak, *Biochem. Biophys. Res. Commun.*, 54 (1973) 501.
16 P. G. Righetti, M. Pagani and E. Gianazza, *J. Chromatogr.*, 109 (1975) 341.
17 P. G. Righetti, L. Balzarini, E. Gianazza and O. Brenna, *J. Chromatogr.*, 134 (1977) 279.
18 W. W. Just, *Anal. Biochem.*, 102 (1980) 134.
19 N. Grubhofer and C. Borja, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, New York, 1977, pp. 111–120.
20 H. G. Könnecke and M. Heisse, *J. Prakt. Chem.*, 9 (1959) 232.
21 S. Fredriksson, *J. Chromatogr.*, 135 (1977) 441.
22 T. Genz, J. P. Martin and H. Cleve, *Hum. Genet.*, 38 (1977) 325.
23 R. R. Frants, G. T. Noordhoek and A. W. Eriksson, *Scand. J. Clin. Lab. Invest.*, 38 (1978) 457.
24 O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, 20 (1966) 820.
25 J. P. Martin, R. Charlionet and R. Sesboüé, in preparation.
26 P. G. Righetti, *J. Chromatogr.*, 138 (1977) 213.
27 J. R. Brown, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 34 (1975) 591.