Journal of Chromatography, 320 (1985) 15–22
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,236

AUTOFOCUSING — A METHOD FOR ISOELECTRIC FOCUSING WITHOUT CARRIER AMPHOLYTES

O. SOVA
Institute of Animal Physiology of the Slovak Academy of Sciences, Palackého 12, 040 01 Košice (Czechoslovakia)

SUMMARY

Autofocusing* is defined as a modification of isoelectric focusing in which the separation is run with only one class of ampholytes, of high molecular weight (*e.g.*, proteins), without carrier ampholytes. The proteins act as the working ampholyte solution and the pH gradient and at the same time they are isoelectrically separated as a sample. The method has two criteria, the conductivity of the working solution and the concentration of the material to be separated. The advantage of autofocusing lies in its very high capacity, suitable for large-scale separations.

INTRODUCTION

Isoelectric focusing in its modern form was adapted for widespread use by Vesterberg's[1] synthesis of suitable carrier ampholytes. The success of this method has been remarkable, but the expense of carrier ampholytes certainly limits the use of isoelectric focusing in many laboratories and in industry. Even the authors had great problems with the development of electrofocusing equipment for the preparation of various materials on a large scale[2].

Hence it is desirable to have buffer systems that will allow inexpensive isoelectric focusing using an arbitrary ionic strength in a well defined electrolytic environment with an easily controlled conductivity course. Attempts to create stable pH gradients without the use of carrier ampholytes have already been reported by Luner and Kolin[3], Lundahl and Hjertén[4], Troitsky *et al.*[5], Nguyen and Chrambach[6] and, in steady-state rheoelectrolysis, by Rilbe[2] and others[7,8].

The pH gradient during isoelectric focusing 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. According to Rilbe[9], the smallest difference in isoelectric points that can be detected with certainly by the equilibrium-gradient separation method is

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

* Czechoslovak Patent No. 211,856, 1979.

0021-9673/85/$03.30

where D is the diffusion coefficient, $-du/d(pH)$ is the mobility slope of the protein to be separated, E is the field strength and $d(pH)/dx$ is the pH gradient. Several workers have reported that the slab length did not influence the resolution of two proteins[10]. From the results of Almgren[11] we can deduce that above a certain 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, the diffusion coefficients, the ion mobilities and the isoelectric points of the carrier ampholytes. According to these conditions, the resolving power of the method is limited to the height of the pH step, *i.e.*, to the difference in isoelectric points between focused 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 ampholytes.

The invention of isoelectric focusing can be looked upon as the idea of simultaneous focusing of two classes of ampholytes that can easily be separated by other means: one low-molecular-weight class (carrier ampholytes) and one high-molecular-weight class (*e.g.*, proteins). In principle, then, it is possible to obtain ampholytes within each class that are contaminated only by members of the other class.

These definitions were the starting point for the development of a new technique for isoelectric focusing without carrier ampholytes, presented in this paper.

The usual biochemical analytical and preparative methods work under a classical arrangement, in which a working solution (buffer, carrier ampholytes), some medium (free, paper, gel) and a sample to be separated are present in an apparatus. The ratios of the amounts of the sample to that of the other components are very interesting. In electrophoresis, the ratio of the amount of sample to that of the working solution (buffer) is about 1:2000 (v/v). In chromatography it is higher, about 1:200, and in free isoelectric focusing it is about 1:100.

When the simultaneous separation of two classes of ampholytes in isoelectric focusing, as mentioned above, is reduced to the separation of only one class, the ratio of the amount of sample, now the only class to be focused, to that of the working solution can be adjusted to 1:1. For example, when the carrier ampholytes were separated by isoelectric focusing for the characterization of their capacity or function[12], the low-molecular-weight ampholytes were acting as the working solution and pH gradient and simultaneously were separated as a sample.

On the other hand, when we try to adapt this system so that the separation will run with only the other class of ampholytes, the high-molecular-weight ampholytes (*e.g.*, proteins), as a result of the pH gradient the proteins could be separated according to their isoelectric points and the same process be developed as before. The proteins would act as the working ampholyte solution and at the same time they could be isoelectrically analysed as a sample.

This notion sounds very interesting and simple —proteins in isoelectric focusing will be diluted in pure distilled water and focused by usual way. However, according to the theory and practice of isoelectric focusing, there are some contradictions. It is considered that the proteins are surrounded by an unknown number of ampholytes, each with an unknown structure, conformaation, molecular weight and concentration. Another problem is the insufficient knowledge of the medium in which each individual protein or other material is brought to rest in its isoelectric state.

Some from the limiting factors in isoelectric focusing are the diffusion coefficient and the concentration of ampholytes and their conductivity. On the other hand, the "hot spots" in the forming pH gradient, occurring at a very low conductivity in the isoprotic state, are characteristic of natural amino acids and proteins. For the development of new methods these difficulties must be resolved.

EXPERIMENTAL

In all experiments a distilled water with a conductivity of 3 μS was used. No other chemicals were used except sucrose of analytical-reagent grade for gradient formation.

Freeze-dried bovine serum albumin (Sevac Works, Czechoslovakia) RMS 151 plasmid DNA isolated according to Mayers *et al.*[13] and crystalline chloramphenicol (SPOFA Works, Czechoslovakia) were used as samples. These materials were subjected to autofocusing treatment at various concentrations by means of gradient mixer so that the conductivity of the solution obtained varied from 50 to 100 μS.

For autofocusing we used a 440-ml LKB column stabilized with a 5–45% gradient of sucrose applied with a gradient mixer together with the sample. The central electrode was connected as the cathode and the whole apparatusdd was cooled with water. No anolyte or catholyte was used. Focusing was applied at a power of 3 W until the current decreased to zero and the field strength increased to 1000 V. The column was then emptied with a peristaltic pump at flow-rate 4 ml/min and 10-ml fractions were collected with an automatic fraction collector. In each fraction the absorbance and the pH were determined.

RESULTS

During autofocusing of three samples the pH gradient was automatically formed without carrier ampholytes when the concentration of the sample compounds

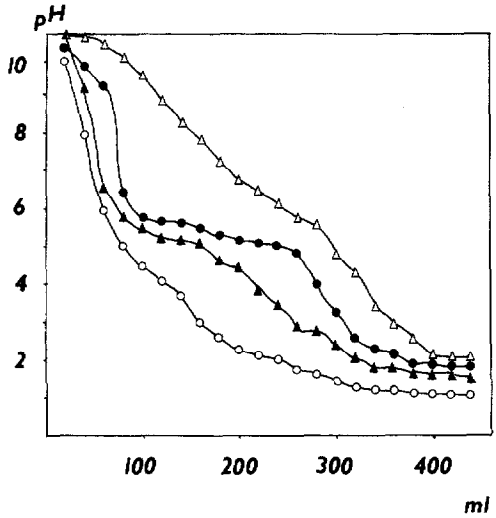

Fig. 1. Formation of a pH gradient by isoelectric focusing of bovine serum albumin without carrier ampholytes at various concentrations corresponding to conductivities of 100 (△), 200 (●), 400 (▲) and 800 (○) μS.

in solution was sufficient. Fig. 1 shows the pH gradient formed by the focused solution of bovine serum albumin at various concentrations corresponding to conductivities of 100, 200, 400 and 800 μS. The pH gradient was linear and smooth enough at 100 μS, but as the conductivity was increased the pH gradient became more and more undulating. The results obtained with DNA and chloramphenicol were similar (see Figs. 3 and 4).

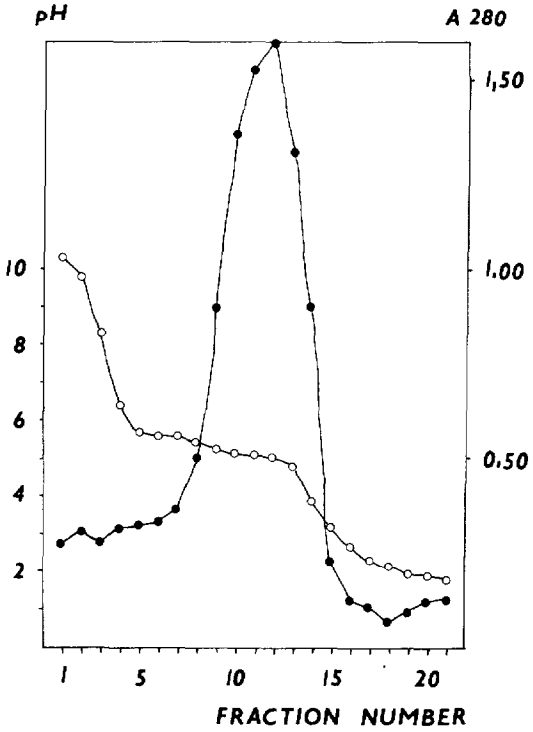

Fig. 2. Autofocusing of bovine serum albumin. ○, pH gradient; ●, absorbance at 280 nm.

Fig. 2 shows the autofocusing results for bovine serum albumin. The pH position of the main peak approximated its p*I* of 4.8. Fig. 3 illustrates the separation of plasmid DNA by autofocusing, with two peaks at pH 3.77 and 2.02, and Fig. 4 shows the results for chloramphenicol, with two peaks at pH 10.08 and 3.32.

The dependence of the duration of autofocusing on the conductivity of the focused solution is shown in Fig. 5. Fig. 6 illustrates the relationship between the conductivity and the concentration of the three compounds examined.

DISCUSSION

The automatic creation of a natural pH gradient by the compounds studied, diluted in distilled water only, during focusing has not been reported prior to this study. The fact that various substrates form pH gradients on focusing themselves could be explained by the existence of impurities in the "pure" isolates or by the

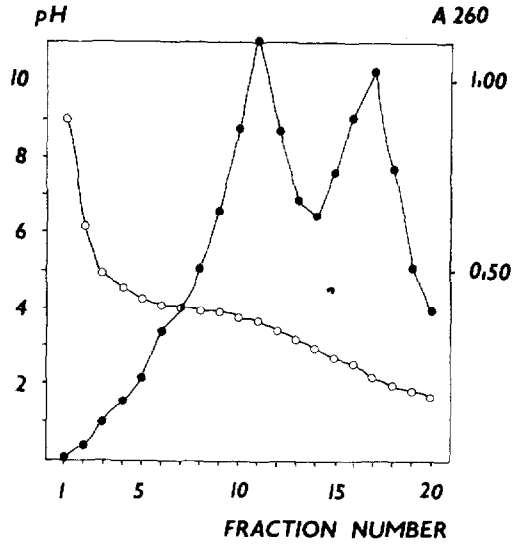

Fig. 3. Measurement of RMS 151 plasmid DNA at 260 nm (●) and the pH gradient (○) after autofocusing.

existence of surrounding ampholytes of unknown structure and molecular weight around all these materials[2]. The success of autofocusing therefore depends on the suitable ratio of the concentration of these surrounding ampholytes to that of the protein or other material to be separated.

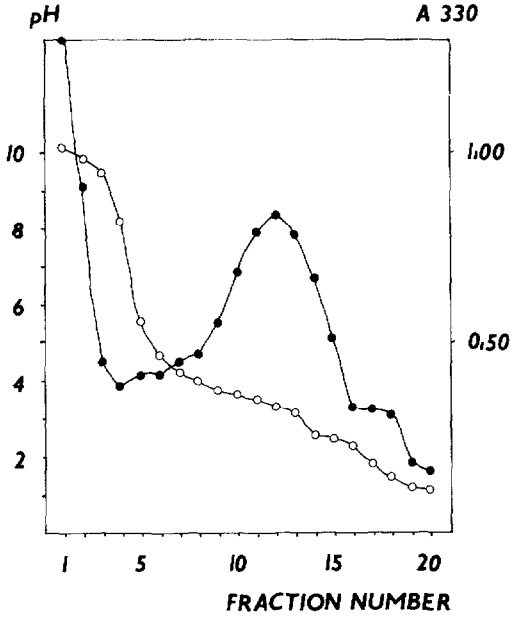

Fig. 4. Autofocusing of chloramphenicol. ○, pH gradient; ●, absorbance at 330 nm.

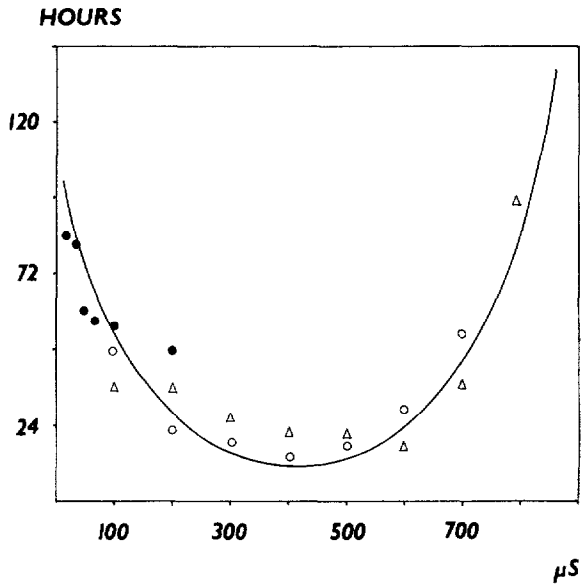

Fig. 5. Dependence of the duration of autofocusing on the conductivity of the focused solution. ●, DNA; ○, bovine serum albumin; △, chloramphenicol.

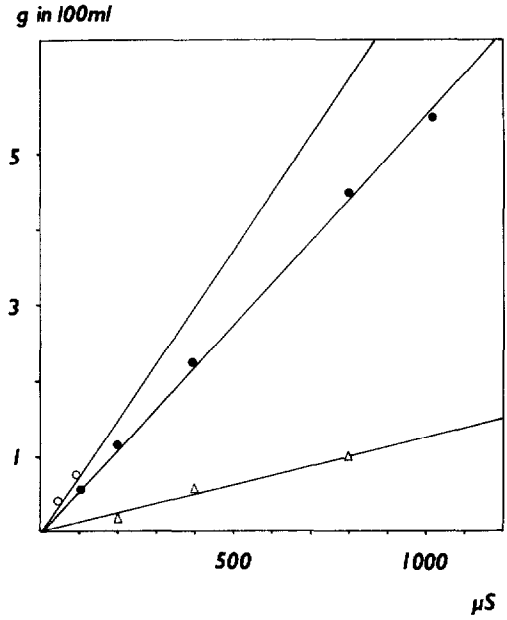

Fig. 6. Relationship between conductivity and concentration: ○, DNA; ●, bovine serum albumin; △, chloramphenicol.

It was found that the separations of the studied compounds by autofocusing were comparable to those obtained by analytical isoelectric focusing. Thus the separation of bovine serum albumin was comparable to the results of Caspers and Chrambach[14], the separation of plasmid DNA corresponds to the results reported elsewhere[15], as also did the separation of chloramphenicol[16].

It seems that the compounds separated in autofocusing reach an appropriate p*I* along the forming pH gradient as in classical isoelectric focusing. Irrespective of the undulations or steps in the pH gradient, the peaks are sufficiently far apart from each other for their later analysis. However, conceivably, large p*I*–pH differences in the pH gradient steps may retard the migration near the p*I* of single peaks, so that they are broader than the corresponding peaks obtained by the usual electrodocusing with carrier asmpholytes. The separation of proteins or other materials by autofocusing at their p*I* values occurs after a sufficient time period for the current to have droppped to zero. This time is dependent on the conductivity and/or concentration of the separated compounds. Fig. 5 shows that either a low or a high conductivity prolongs the focusing time. The best results were obtained with conductivity in the range 200–600 μS. However, it is very difficult to state the dependence of the duration of autofocusing on the concentration of the material to be separated, because as is shown in Fig. 6, the ratio between conductivity and concentration differs for the various compounds. With DNA its viscosity is a limiting factor but with chloramphenicol only its conductivity is a limiting factor. Therefore, two criteria are important for the realization of autofocusing. The first is the conductivity, which is optimal between 200 and 600 μS (with a maximum range of 100–1000 μS). The second is concentration, for which we can only tentatively recommend that conditions should be used such that precipitation should not occur and the viscosity should not retard the action of focusing.

The above-mentioned "hot spots" were measured along the pH gradient more frequently than in classical isoelectric focusing, but only when a power was applied near or higher than the recommended 3 W. However, the temperature in such spots must be regulated by decreasing the field strength or cooling.

The main advantage of autofocusing over isoelectric focusing lies in its possible using in large-scale preparations. As a first step the usual columns for free electrofocusing may be used. The capacity of the method depends on the quality of material to be separated. In the experiments reported here, amounts of 5 g of proteins, about 300 mg of DNA and 1 g of antibiotic per 100 ml were used. The total capacity of the 440-ml column is about 20 g of protein or 5 g of drugs. The limits of time and the dimensions of the apparatus set a practical limit to the load capacity.

REFERENCES

1 O. Vesterberg, *Acta Chem. Scand.*, 23 (1969) 2653.
2 H. Rilbe, *Electrophoresis*, 2 (1981) 261.
3 S. J. Luner and A. Kolin, *Proc. Nat. Acad. Sci. U.S.*, 66 (1970) 898.
4 P. Lundahl and S. Hjertén, *Ann. N.Y. Acad. Sci.*, 209 (1973) 94.
5 G. V. Troitsky, I. F. Zav'yalov, I. F. Kirjukhin, V. M. Abramov and G. J. Agitsky, *Biochim. Biophys. Acta*, 400 (1975) 24.
6 N. Y. Nguyen and A. Chrambach, *Anal. Biochem.*, 87 (1978) 576.
7 M. Bier, *Electrophoresis*, 83 (1984) 99.

8 K. Shimao, *Electrophoresis*, 83 (1984) 60.
9 H. Rilbe, *Ann. N.Y. Acad. Sci.*, 209 (1973) 11.
10 R. Charlionet, C. Morcamp, R. Sesboüé and J. P. Martin, *J. Chromatogr.*, 205 (1981) 355.
11 M. Almgren, *Chem. Scr.*, 1 (1971) 69.
12 W. J. Gelsema and C. L. deLigny, *J. Chromatogr.*, 130 (1977) 41.
13 J. A. Mayers, D. Sanchez, L. E. Elwell and S. Falkow, *J. Bacteriol.*, 127 (1976) 1529.
14 M. L. Caspers and A. Chrambach, *Anal. Biochem.*, 81 (1977) 28.
15 O. Sova, A. Sokol and V. Kmet', in S. Mitsuhashi, L. Rosival and V. Krčméry (Editors), *Antibiotic Resistance*, Springer Verlag, Berlin, Heidelberg, New York, 1980, p. 147.
16 O. Sova, *Proceedings of the Czechoslovak Congress on Chemotherapy with International Participation, Prague, April 15–19, 1984*, Abstracts, p. 186.