Journal of Chromatography, 470 (1989) 407–414
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1597

ISOTACHOPHORESIS OF PROTEINS

FERNANDO ACEVEDO

Department of Dermatology, Karolinska Hospital, Karolinska Institute, 104 01 Stockholm (Sweden)

SUMMARY

The analytical separation of proteins by isotachophoresis (ITP) was achieved in a short electrophoretic path and with a resolution comparable to that of isoelectric focusing by the appropriate selection of (1) a mixture of ampholytes as spacers to generate linear gradients of electrophoretic mobility and (2) the counter ions chosen to buffer the complete pH gradient generated. This ITP technique is exemplified by the analysis of plasma proteins in agarose gels. Up to 46 samples in the same gel plate were analysed. The resolution was such that at least 30 clear and discrete bands per sample could be observed after staining with Coomassie Brilliant Blue. The resolving power of ITP could be further increased for the study of a particular protein or zone by the selection of suitable spacers and counter ions.

INTRODUCTION

Isotachophoresis (ITP) has the resolving capacity inherent in isoelectric focusing (IEF) and the advantage of flexibility of pH and ionic strength, which confers great potential for analytical and preparative separations of proteins. However, the use of ITP for the separation of proteins has been scant. Two major problems that limit this application have been pointed out by Nguyen and Chrambach[1]: first, the lack of spacers to generate a continuous gradient of electrophoretic mobility, and second, the length of the electrophoretic path required to obtain a steady state. In this paper, we present an ITP technique for the analytical separation of proteins in which mixtures of commercially available ampholytes are used as spacers. The analytical separation of plasma proteins in agarose gels is used as a model system to illustrate the technique.

THEORETICAL

Theoretically, in ITP the components of the mixture should migrate at the same velocity and stack according to their electrophoretic mobilities (U). The Kohlrausch function, defined as

$$\omega_i = \sum_j C_{ij}/U_{ij},$$

where C_{ij} is the concentration of the jth component in the ith phase, should have the

 © 1989 Elsevier Science Publishers B.V.

same value in all phases. The concentration of each component in the stack is given by the concentration and electrophoretic mobility of the components in the leading zone, and the system is self-regulated against diffusion[2]. The leading ion should have (1) an electrophoretic mobility slightly higher than that of any component in the mixture, in order to avoid conductivity jumps, and (2) a concentration such that all the components in the mixture stack in an adequate length.

Two aspects require attention in the design of an isotachophoretic system for the separation of proteins: the counter ions and the spacers.

Counter ions

Electroneutrality requires that the concentration of ions of different sign should be equal in any phase. Therefore, the charge provided by the counter ions is an important factor in the stablishment of an ITP system. To illustrate this, let us consider IEF as a special case of ITP where the final velocities of the components are close to zero (Fig. 1A).

The addition of a strong ion, for instance Na^+, to ampholytes which are focused in an electric field causes the migration of the stack of ampholytes and the pH gradient towards the anode. As the counter ion is strong, it will cause the protonation of weakly ionized groups of the ampholytes. Consequently, the charges of all stacked ampholytes

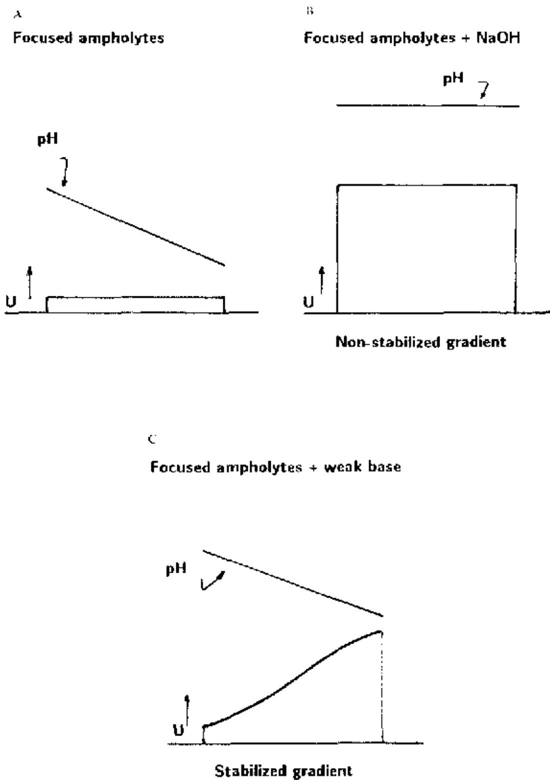

Fig. 1. Electrophoretic mobility *vs.* distance. (A) Focused ampholytes; (B) focused ampholytes after addition of a strong base; (C) focused ampholytes after the addition of a weak base.

will be similar and they will mix, with the consequence that the pH gradient will vanish during the migration (Fig. 1B). On the other hand, the addition of a weak ion, *i.e.*, with a pK_b value in the p*I* zone of the focused ampholytes, will stabilize the pH gradient; the ampholytes on the acidic side of the pK_b will migrate faster than those on the basic side towards the anode, owing to the higher charge conferred upon the former by the weak base used as the counter ion (Fig. 1C).

Spacers

The separation of proteins by ITP is based on their differences in electrophoretic mobility. Therefore, suitable spacers for analytical ITP should generate a continuous linear gradient of electrophoretic mobility. According to the Kohlrausch function for a moving boundary, the concentration of the ions in the different zones obtained by ITP is determined by their electrophoretic mobility. Consequently, in order to develop a linear gradient of electrophoretic mobility, the amount of any particular spacer should be proportional to its electrophoretic mobility. Commercially available ampholyte mixtures are designed for IEF where an equal concentration of all the different p*I* ampholytes is desired. Therefore, a non-linear gradient of electrophoretic mobility will be generated when these ampholyte mixtures are used in ITP. However, IEF ampholytes can be used as spacers for ITP if the concentration of each component is adjusted to its electrophoretic mobility. This can be achieved by fractionating commercially available ampholytes according to their p*I* and then preparing a mixture with concentrations inversely proportional to their p*I* values. Commercially available ampholytes of narrow pH range can be mixed to generate nearly linear gradients of electrophoretic mobility and produce good results, as illustrated in this paper. The addition of a terminating ion with a lower mobility will, together with the leading ion, set the limits of the migration velocities and complete the isotachophoretic system.

EXPERIMENTAL

Human plasma samples were supplied by the blood centre at the Karolinska Hospital. Ampholytes and paper electrode strips were obtained from LKB (Bromma, Sweden), agarose, electrode strips and paper sample applicators from Pharmacia (Uppsala, Sweden), rabbit immunoglobulins (Igs) against human antigens and horseradish peroxidase-conjugated swine antibodies against rabbit Igs from Dakopatts (Copenhagen, Denmark), Gelbond from FMC Bioproducts (Rockland, ME, U.S.A.), swine serum from Flow Laboratories (Uxbridge, Middlesex, U.K.) and dry defatted milk from Semper (Stockholm, Sweden). All chemicals were of analytical-reagent grade.

Isotachophoresis in agarose gels

The procedure was basically as described by Acevedo[3]. The plasma proteins were electrophoresed towards the anode in 0.1 × 12 × 24 cm 1% agarose IEF, 12% sorbitol gels, at 10°C, on a flat-bed electrophoresis apparatus (Pharmacia FBE3000).

The gels were cast in buffers formed by the leading ion and the counter ion or counter ions. The spacers were either included in the gel, in the electrode solution, or in a small portion of the gel behind the samples. The electrode solutions, 10 or 20 ml, which contained the leading and counter ions (anode) or the terminating ion (cathode),

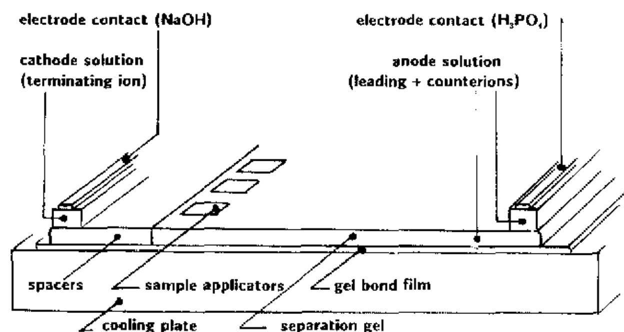

Fig. 2. Assembly for agarose gel ITP. The use of electrode vessels for the electrode solutions connected to the gel by means of paper wicks produces the same results.

were absorbed in electrode strips, placed on the edge of the gel. The use of 500-ml electrode vessels for the electrode solutions, connected to the gel by means of paper wicks, produced the same results. In the former instance, the contact between the electrode wires and the strips containing the electrode solutions was mediated by either 1 *M* phosphoric acid or sodium hydroxide, respectively (see Fig. 2).

The gels were pre-run for about 1 h at 5–10 W until the isotachophoretic front reached 1.5–2 cm from the cathode. Then the samples (8 μl of 20% plasma in 5% Ampholytes 5–7) were applied on paper sample applicators, cut to half the width (1 × 0.25 cm) and placed behind the front and perpendicular to it. Electrophoresis was carried at 5–20 W (300–1200 V) until the steady state was reached. This was observed by the formation of sharp lines at the front and rear ends, which migrated at the same velocity, or until the leading front reached the anode, after *ca.* 3–5 h at 10°C. In some instances, the gel shrank at the cathodic side. This was observed as a decrease in the current and a flattening of the gel surface immediately at the electrode contact. In such an event, to avoid burning of the gel, the electrode contact was advanced a few millimetres (2–10) and electrophoresis was continued at a lower power setting. The protein bands were revealed with Coomassie Brilliant Blue (CBB) or by immuno techniques and scanned in a LKB laser gel scanner.

Conductivity and pH measurements

Pieces of gel of 1 × 0.5 cm were cut and left overnight at room temperature in 1.5 ml of distilled water for conductivity measurements or in 1 ml of 10 m*M* potassium chloride solution for pH measurements.

Immunodetection

The proteins were transferred to a nitrocellulose (NC) filter by overlaying the gel with a stack, consisting of a wet NC filter, a layer of wet blotting paper, eight layers of dry blotting paper (Munktell, Stora, Sweden), a glass plate and, on the top, a 5-kg weight for 20 min at room temperature. The immunological detection of proteins on NC filters was performed essentially as described by Andrews[4], except that 5% dry defatted milk in 0.9% NaCl–10 m*M* Tris–HCl buffer (pH 7.4) (TBS) was used instead of bovine serum albumin. The Igs were diluted 1:1000 in 5% dry defatted milk–10% swine serum in TBS. Peroxidase-labelled antibodies were detected with 4-chloro-1-naphthol.

RESULTS

The result of an isotachophoretic separation of plasma proteins is illustrated in Fig. 3A. The protein bands, stained after ITP, are as sharp as expected for IEF, in spite of the 1-cm-long application zone, and more than 30 clear and discrete bands, stained with Coomassie Brilliant Blue, as illustrated by the scanning result shown in Fig. 4. The result of conductivity and pH measurements on a gel similar to that shown in Fig. 3A is illustrated in Fig. 5 and shows a linear pH gradient, from 5 at the front to 9 at the terminating zone, and a non-linear conductivity gradient, which are conditions expected for an ITP stack. Other characteristics of the ITP system described here were that the migration velocities of the leading and the terminating fronts were the same, and consequently the length of the ITP train was constant (this length was dependent on the type and amount of ampholytes used and the type and concentration of the leading ion), and the pH value for any protein zone in the stack was higher than the protein's isoelectric point.

The resolution can be increased for a particular protein or pH zone by the selection of ampholyte mixtures with a narrow p*I* interval as spacers and counter ions with pK_b in this interval, as illustrated in Fig. 3. Two different transferrin forms,

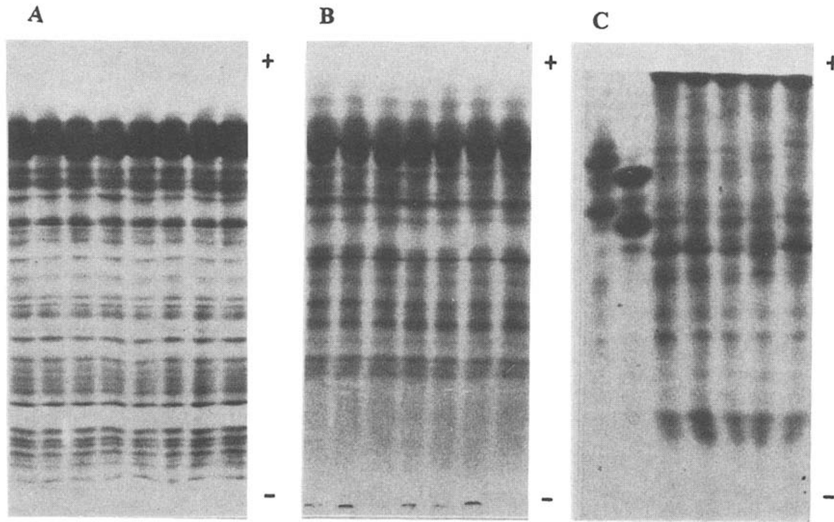

Fig. 3. Agarose gel ITP of plasma proteins. CBB staining. (A) The spacers (pH 3.5–10) were placed behind the separation gel. Separation gel (24 × 10 × 0.1 cm): 120 m*M* glutamic acid–40 m*M* Tris-base–20 m*M* imidazole–20 m*M* Bis-Tris–40 m*M* pyridine. Spacer-containing gel (24 × 2 × 0.1 cm): 565 μl of Ampholytes 3.5–10, 175 μl of 7–9, 175 μl of 6–8, 350 μl of 5–7, 350 μl of 4–6, 400 μl of 3.5–5 and 120 m*M* lysine. Terminating ion: lysine. Anode: 20 ml of 400 m*M* Tris–200 m*M* imidazole–200 m*M* Bis-Tris–400 m*M* pyridine–400 m*M* glutamic acid. Cathode: 10 ml of 120 m*M* lysine. Power setting: 10 W. (B) The spacers (pH 3.5–7) were included in the separation gel (24 × 12 × 0.07 cm): 120 m*M* glutamic acid–30 m*M* imidazole–90 m*M* histidine. 600 μl of Ampholytes 3.5–5, 400 μl of 4–6 and 400 μl of 5–7. Terminating ion: β-alanine. Anode: 20 ml of 150 m*M* imidazole–450 m*M* histidine–300 m*M* glutamic acid. Cathode: 10 ml of 1 *M* β-alanine–250 m*M* histidine. Power setting: 10 W. (C) The spacers (pH 5–7) were included in the separation gel. Separation gel (24 × 12 × 0.07 cm): 20 m*M* glutamic acid–60 m*M* histidine. 500 μl of Ampholytes 5–7 and 500 μl of Pharmalytes 5–6. Terminating ion: histidine. Anode: 20 ml of 250 m*M* histidine–20 m*M* glutamic acid. Cathode: 10 ml of 250 m*M* histidine. Power setting: 5 W. Two different transferrin forms are included as markers.

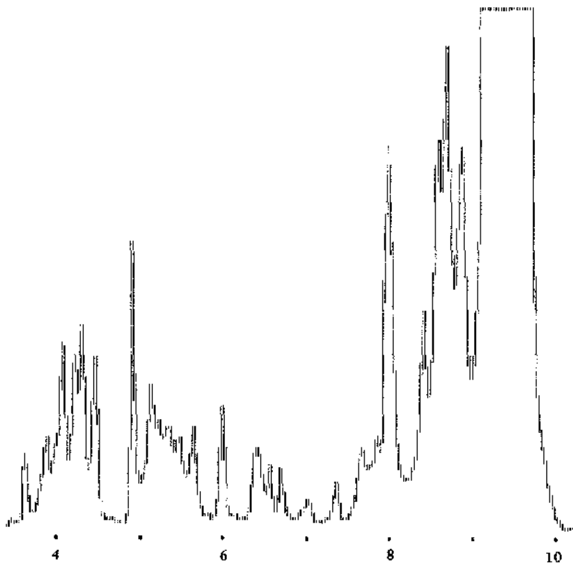

Fig. 4. Scanning of one track of the gel illustrated in Fig. 3A (LKB laser scanner). Abscissa, distance in cm; ordinate, absorbance.

prepared in our laboratory, are included as markers in Fig. 3C. The application of immuno techniques after ITP is illustrated in Fig. 6.

Results obtained with T_5C_3 polyacrylamide gel slabs were similar to those on agarose gels (not shown), but the latter are easier to handle and more suitable for immunological and preparative purposes.

DISCUSSION

Two aspects demand special attention in the design of an isotachophoretic system for the analytical separation of proteins: first, commercially available mixtures of ampholytes, designed for IEF, generate non-linear gradients of electrophoretic

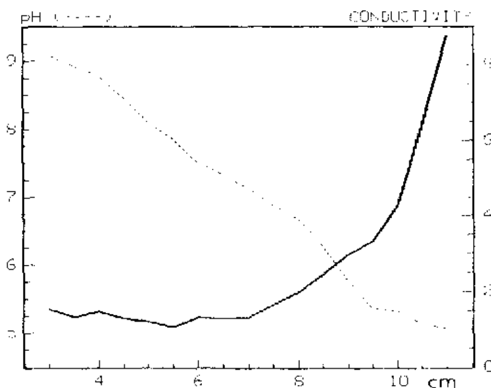

Fig. 5. Conductivity and pH measurements after agarose gel ITP, as illustrated in Fig. 3A.

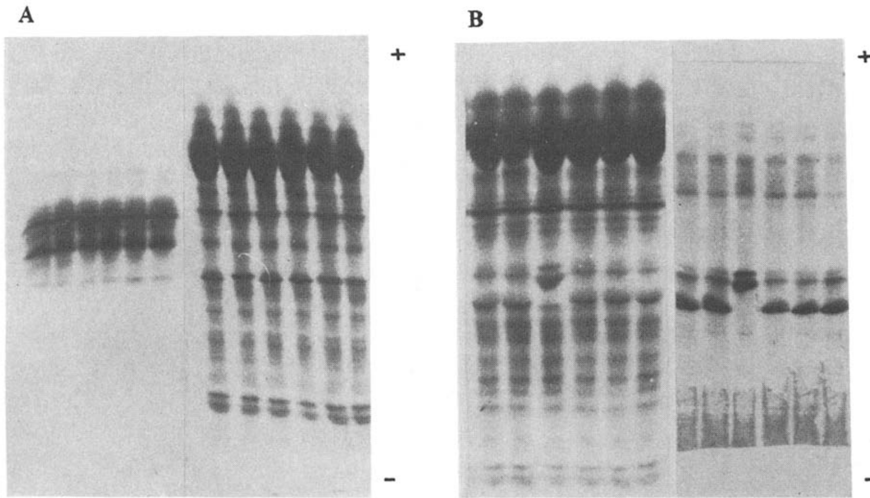

Fig. 6. Agarose gel ITP of plasma proteins, followed by immunoblot. (A) Transferrin and CBB stain. The spacers (pH 4–7) were included in the separation gel (24 × 12 × 0.1 cm): 80 m*M* glutamic acid–20 m*M* imidazole–60 m*M* histidine. 1.4 ml of Ampholytes 4–6, 700 μl of 5–7. Terminating ion: histidine. Anode: 20 ml of 400 m*M* glutamic acid–100 m*M* imidazole–300 m*M* histidine. Cathode: 10 ml of 250 m*M* histidine–80 m*M* imidazole. Power setting: 20 W. (B) CBB stain and C_3 complement. The spacers (pH 3.5–7) were included in the separation gel. Separation gel (24 × 12 × 0.07 cm): 40 m*M* glutamic acid–40 m*M* histidine. 600 μl of Ampholytes 3.5–5, 400 μl of 4–6, 400 μl of 5–7. Terminating ion: β-alanine. Anode: 20 ml of 250 m*M* histidine–20 m*M* glutamic acid. Cathode: 10 ml 1 *M* β-alanine–250 m*M* histidine. Power setting: 10 W.

mobility on ITP and therefore for this application they should be remixed inversely proportional to their p*I* value, as outlined above, and second, the buffer capacity of the counter ion(s) should cover the complete pH zone used for the separation.

For the design of the ITP systems illustrated in this paper, the electrophoretic mobilities of the leading, spacer and terminating ions were considered to be proportional to their charge. When the ITP condition is established, the total charge of the spacer in a particular pH zone in the system is defined by the charge of the counter ions in the zone, as the condition of electroneutrality must be satisfied. Therefore, the relative proportions between the leading ion and the counter ion(s) at the start, which gives the pH in the separation gel, regulate the charge of the spacers in the zones. Then, according to the Kohlrausch function, the starting pH regulates the concentration of spacers in the zones and thereby the length of the stack. The distance required to reach the steady state will be shorter for starting pH values closer to the p*I* of the fastest spacer ampholyte used, where its mobility is low. The starting pH is determined by all ions present in the separation gel: the leading ion, the counter ion(s) and the spacers, if they are included in the separation gel. In the latter instance, the spacers can also initially act as counter ions.

CONCLUSIONS

The separation of proteins by ITP offers, first, a high resolving capacity, which is adjustable for the study of any particular protein and, second, flexibility in pH and

ionic strength for the separation. The use of mixtures of ampholytes that generate linear gradients of electrophoretic mobility as spacers and the selection of counter ions to buffer the complete pH gradient generated are critical factors for the analytical separation of proteins by ITP. The analytical application of ITP illustrated in this paper is simple and rapid, comparable to gel electrophoresis or IEF, and it does not require special equipment or training.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Medical Research Council (12-5665), the Karolinska Institute, the Swedish Medical Association, the Swedish Psoriasis Association and the Foundations of Magnus Bergvall, Finsen and Edvard Wellander.

REFERENCES

1 N. Y. Nguyen and A. Chrambach, in B. D. Hames and D. Rickwood (Editors), *Gel Electrophoresis of Proteins: a practical approach*, IRL Press, London, 1981, Ch. 3, p. 145.
2 P. Boček, M. Deml, P. Gebauer and V. Dolník, in B. J. Radola (Editor), *Analytical Isotachophoresis*, VCH, Weinheim, 1988, pp. 25, 43.
3 F. Acevedo, in C. Schafer-Nielsen (Editor), *Electrophoresis '88*, VCH, Weinheim, 1988, p. 112.
4 A. T. Andrews, *Electrophoresis —Theory, Techniques, and Biochemical and Clinical Applications*, Oxford Science Publications, Oxford, 1986, p. 67.