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High-performance displacement electrophoresis in 0.025- to 0.050-mm capillaries coated with a polymer to suppress adsorption and electroendosmosis

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ABSTRACT

The risk of adsorption onto the walls of an electrophoresis tube increases with decreases in the bore of the tube and in the ionic strength (conductivity) of the buffers. It is therefore important to suppress adsorption in high-performance displacement electrophoresis where the diameter of the tube is often as narrow as 0.025 mm and the electrical conductivities are low (as they are in most displacement electrophoresis experiments at the steady state), for instance $10^{-4}\ \Omega^{-1}\ cm^{-1}$. This can be accomplished by coating the electrophoresis tube with a thin (preferably monomolecular) layer of a hydrophilic non-ionic polymer, which also eliminates electroendosmosis.

The narrow-bore tubes permit high field strengths without serious thermal zone broadening and therefore have the advantage of affording fast runs with high resolution (displacement electrophoresis often gives lower thermal zone broadening at a given field strength than does zone electrophoresis, because the average conductivity in the former method is usually lower than the conductivity in the latter). An analysis of serum proteins at a field strength of 860 V/cm took about 10 min. In the presence of continuous spacers the resolution increased dramatically: human serum could be resolved into about 30 components. Twelve nucleotides were separated within 10 min.

Displacement electrophoresis in coated tubes gives reproducible electropherograms, which makes the method suitable for clinical analyses, exemplified by a study of serum from a patient with multiple myeloma.

The zone broadening is considered with regard to diffusion, Joule heat and adsorption. The terms displacement electrophoresis and isotachophoresis are discussed.

INTRODUCTION

A common diameter of the electrophoresis tube in displacement electrophoresis (isotachophoresis) experiments is 0.2 mm. Theoretically, it is an advantage to use even smaller diameters, since the thermal zone deformation is proportional to $(dF)^2$, where d is the diameter of the tube and F is the field strength [1] (if d is decreased the field strength can be increased proportionally to get the same zone deformation). Another advantage of high field strengths is that the automatic zone-sharpening characteristic of displacement electrophoresis is more pronounced the higher is the field strength (broadening caused by diffusion is thus of less importance). Displace-

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ment electrophoresis experiments should therefore be conducted at high field strengths to give high resolution and short analysis times, but not so high that the thermal zone deformation becomes the dominating cause of zone broadening.

There are, consequently, theoretical reasons why one should try to decrease the diameter of the electrophoresis tubes. However, a reduction in tube diameter means, in practice an increased risk of adsorption onto the tube wall of the solutes to be separated. Only if the adsorption can be virtually eliminated can one take advantage of diameters as small as 0.025–0.050 mm. The purpose of this paper is to investigate whether the method successfully used to suppress adsorption in zone electrophoresis (*i.e.* coating the inside of the electrophoresis tube with a hydrophilic polymer, which also eliminates electroendosmosis) [2] is applicable in displacement electrophoresis experiments in tubes with these small bores.

Most of the experiments described here in have been presented at two recent symposia [3,4].

MATERIALS AND METHODS

Normal human serum was from the University Hospital in Uppsala, Sweden, and serum of a multiple myeloma patient from the Department of Neurology, University of Pécs, Hungary. Acrylamide, potassium persulphate, N,N,N′,N′-tetramethylethylenediamine (TEMED), agarose (zero = m_r), tris(hydroxymethyl)aminomethane (Tris) and the carrier ampholytes Bio-Lyte were obtained from Bio-Rad Laboratories, Richmond, CA, USA. γ-Methacryloxypropyltrimethoxysilane (Bind-Silane) was purchased from Pharmacia, Uppsala, Sweden, and the fused-silica tubing (inside diameters, 0.025–0.05 mm; lengths, 290–360 mm) from Scientic Glass Engineering, Ringwood, Australia.

The coating of the tubes was performed as described in ref. 2 with minor modifications. A 15-μl sample of Bind-Silane was mixed with 0.5 ml of 50% ethanol (pH 3.6). The 4% (w/v) acrylamide solution contained 0.8 μl of TEMED and 2 mg of ammonium persulphate per ml solution. The tubes were treated twice with Bind-Silane and once with the acrylamide solution. Excess polymerized non-bound acrylamide was displaced from the capillary tube with the aid of a high-performance liquid chromatography (HPLC) pump. The coupling between the tube and the pump was accomplished with standard HPLC connectors.

In all displacement electrophoresis experiments for the separation of serum proteins we employed the following buffer system: leading buffer, 0.01 *M* HCl adjusted to pH 8.3 with solid Tris [5]; terminating buffer, 0.1 *M* β-alanine adjusted to pH 9.2 with solid barium hydroxide. Barium ions precipitate bicarbonate formed by uptake of atmospheric carbon dioxide [5,6]. A 0.01 *M* HCl solution adjusted to pH 3.9 with solid β-alanine served as leading buffer for the separation of nucleotides and 0.01 *M* caproic acid (pH about 3.7) as terminator. In all experiments the anode vessel contained the leading and the cathode vessel the terminating solution. Coated electrophoresis tubes were filled (with the aid of water suction) with leading and non-coated tubes with terminating buffer. A 30-mm length of buffer was sucked off from one end of the tube. The opposite end was dipped into the sample solution, which by capillary forces entered the tube within about 20 s. The starting zone thus had a length of 30 mm.

The sample end of the tube was pressed into a 3% agarose gel prepared in the terminator. A 1 to 2-mm gel plug thus closed the tube and prevented hydrodynamic flow in the tube during the run.

The on-tube detector was a modified Zeiss spectrophotometer (Model M4 QIII). The proteins were recorded at 280 and the nucleotides at 254 nm. The 30-kV power supply was purchased from Glassman, Whitehouse Station, NY, USA. The electrophoretic migration distance up to the UV detector was 30 mm shorter than the length of the tube.

EXPERIMENTS AND RESULTS

Displacement electrophoresis in polymer-coated and non-coated capillaries

The experiment was performed at a voltage of 10 kV in a 340 × 0.025 mm I.D. fused-silica tube coated with linear polyacrylamide. As sample we used normal human serum diluted 6:1 with 0.7 *M* β-alanine titrated with solid barium hydroxide to pH 9.2. The electropherogram is presented in Fig. 1a. To test the stability of the coating the experiment was repeated every fourth day during a period of twenty days. Between the runs the tube was filled with the terminator (pH 9.2). No differences in the appearance of the electropherograms were observed.

The experiment was then repeated in a non-coated tube. Since non-coated tubes have a cathodic electroendosmotic velocity which is larger than the anodic electrophoretic velocity of the solutes, these will be transported toward the cathode. The non-coated tubes were therefore filled with terminator. The tube end with the 30-mm sample was in contact with the leading solution in the anode vessel. The cathodic vessel contained the terminating solution. The protein pattern obtained in a non-coated tube is shown in Fig. 1b. The zones in this figure are broader and less concentrated than those in Fig. 1a, indicating that a polymer coating suppresses disturbances caused by adsorption or/and electroendosmosis.

Displacement electrophoresis in polymer-coated capillaries of different diameters

The experiment presented in Fig. 1a was repeated with the difference that the diameter of the capillary tube was 0.05 mm instead of 0.025 mm. The electropherogram (Fig. 2) was similar to that shown in Fig. 1a.

Displacement electrophoresis in polymer-coated capillaries at different field strengths

The experimental conditions were the same as those in the experiment shown in Fig. 1a, but the voltage was increased to 30 kV (= 860 V/cm). The serum pattern (Fig. 3) resembled that in Fig. 1a, *i.e.* a field strength of 860 V/cm can be used for tubes with an inner diameter of 0.025 mm without loss of resolution.

Displacement electrophoresis in polymer-coated capillaries in the presence of discrete spacers

The sample was prepared by mixing 80 μl of normal human serum with 10 μl of 0.7 *M* β-alanine (titrated to pH 9.2 with solid barium hydroxide) and 10 μl of 0.1 *M* β-alanine (pH 9.2) containing 0.119 mg of glycylglycine, 0.026 mg of asparagine, 0.024 mg of threonine and 0.015 mg of glycine [7]. The peptide and the amino acids served as discrete spacers; their final concentrations in the applied sample were 9, 2, 2 and 2 m*M*, respectively. The protein pattern is shown in Fig. 4.

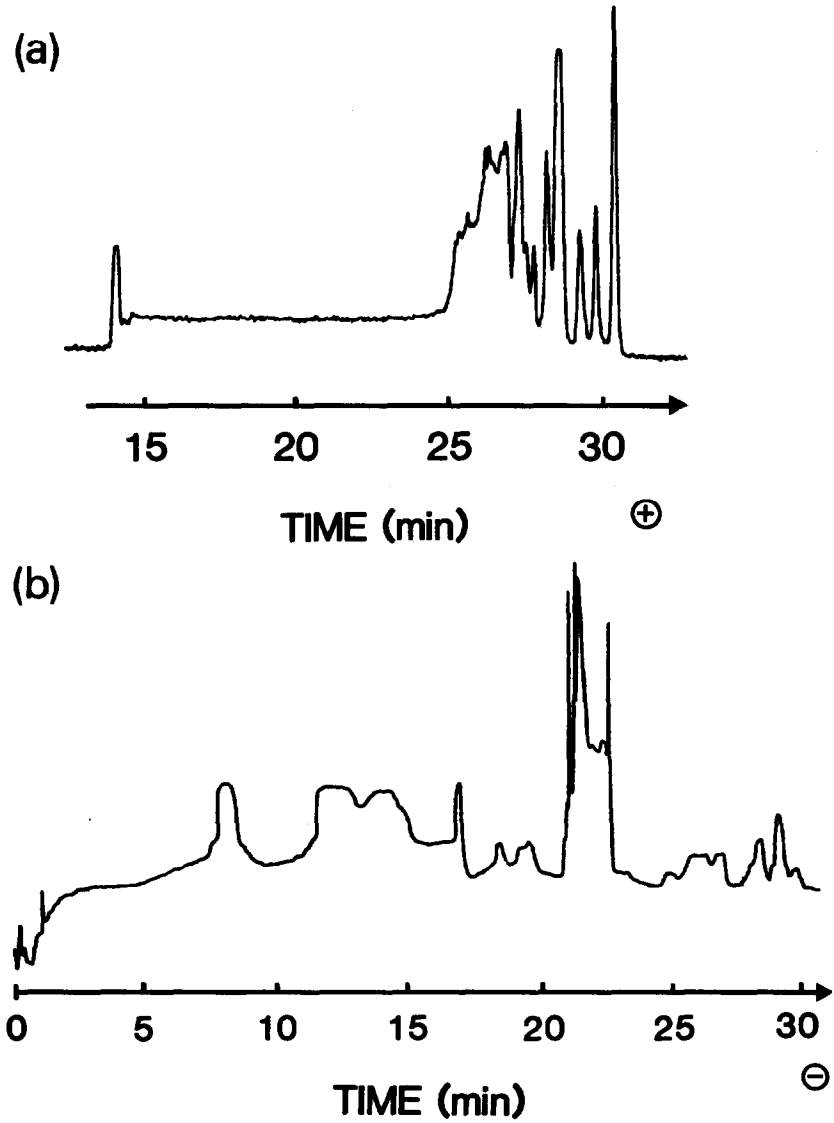

Fig. 1. High-performance displacement electrophoresis of human serum in polymer-coated (a) and non-coated (b) capillaries. Sample volume: 15 nl (= 30 mm). 0.01 *M* HCl adjusted to pH 8.3 with Tris served as anolyte (leading solution) and 0.1 *M* β-alanine titrated to pH 9.2 with barium hydroxide as catholyte (terminator). The electrophoresis tube was filled with leading solution in (a) and terminator in (b). Dimensions of the electrophoresis tubes: 340 × 0.025 mm I.D. Voltage: 10 kV. Detection wavelength: 280 nm. Anodic migration in (a), cathodic in (b) caused by electroendosmosis. The zones are narrower in (a) than in (b) because of suppression of adsorption or/and the absence of electroendosmosis.

Displacement electrophoresis in polymer-coated capillaries in the presence of continuous spacers

A 168-μl portion of 0.7 *M* β-alanine, pH 9.2, was mixed with 60 μl of Bio-Lyte 3/10. The pH was adjusted to 9 by the addition of 7 μl of 1 *M* NaOH. A 15-μl aliquot

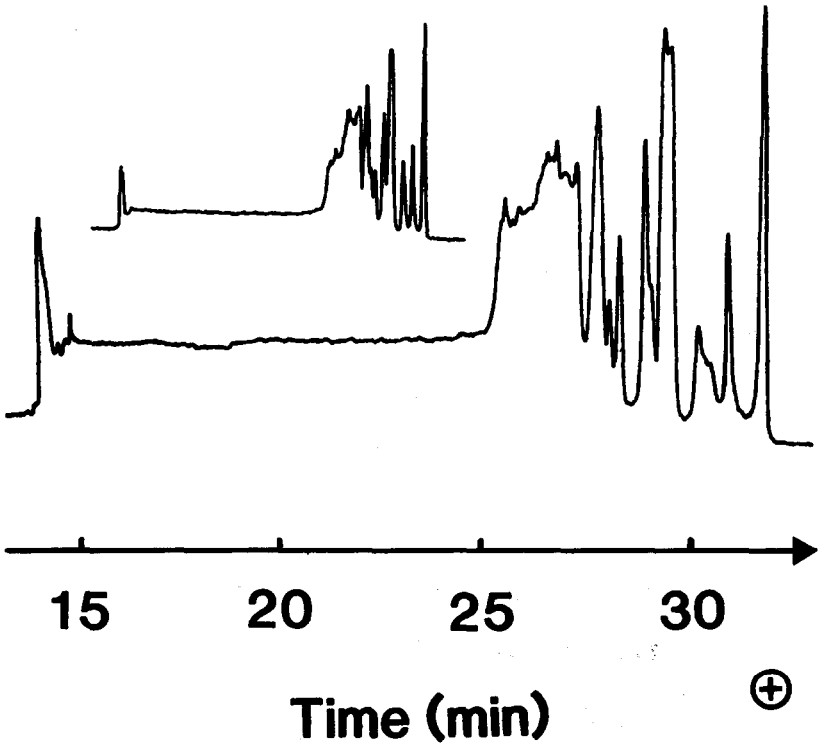

Fig. 2. High-performance displacement electrophoresis of human serum in polymer-coated capillaries of different diameters. Sample volume: 59 nl (= 30 mm). Anolyte, catholyte, voltage and wavelength are similar to those in the experiment shown in Fig. 1a. Dimensions of the electrophoresis tube: 350 × 0.05 mm I.D. A comparison with Fig. 1a (inserted) indicates that one can decrease the diameter of the capillary down to at least 0.025 mm without loss of resolution.

of this solution was mixed with 80 μl of normal human serum. A displacement electrophoresis of this sample gave the pattern in Fig. 5. A comparison with Fig. 2 shows the increased resolution achieved in the presence of spacers, Bio-Lyte (Svendsen and Rose [8] were the first to use carrier ampholytes for isoelectric focusing as spacers in displacement electrophoresis). Alternative conditions for displacement electrophoresis of serum proteins in the presence of such spacers are given in an early publication on free capillary electrophoresis [9]; see also ref. 10.

Displacement electrophoresis in polymer-coated capillaries of serum from a patient with multiple myeloma

The run was performed essentially as described for the experiment in Fig. 1a. The electropherogram (Fig. 6) differs chiefly from that in Fig. 2 in that it has a larger slowly migrating zone, corresponding to an increased concentration of immunoglobulins [11].

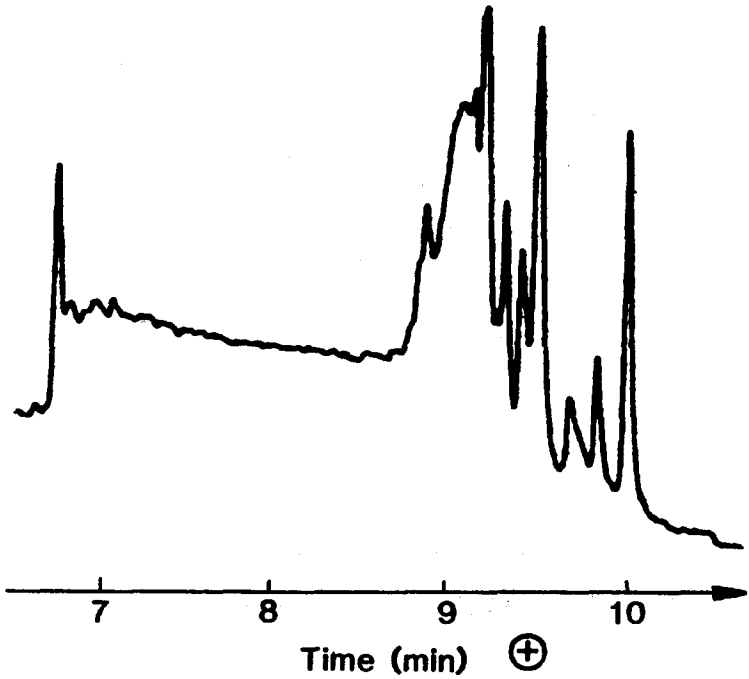

Fig. 3. High-performance displacement electrophoresis of human serum on polymer-coated capillaries at increased field strength. Sample volume: 15 nl (= 30 mm). Anolyte, catholyte and wavelength are similar to those in Fig. 1a. Voltage: 30 kV. Dimensions of the electrophoresis tube: 350 × 0.025 mm I.D. A comparison between Figs. 1a and 3 shows that one can use at least 30 kV (maximum voltage of the power supply) without loss of resolution and that there is a good correlation between field strength and run time.

Displacement electrophoresis of nucleotides in a polymer-coated capillary

Sample of 1 mg of each of the nucleotides AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, UMP, UDP and UTP were dissolved in 200 μl of water, and 30 μl of 0.07 *M* caproic acid was added [11]. The starting zone had a length of 2 mm. The separation pattern is shown in Fig. 7.

Thermal zone broadening

The Joule heat generated in an electrophoresis experiment gives rise to a temperature difference between the axis of the electrophoresis tube and its inner wall. The solute molecules therefore migrate faster at the axis than at the wall. Eqn. 73 in ref. 1 was used to calculate the percentage difference in these migration velocities as a function of the square of the field strength for different radii of the electrophoresis tube (Fig. 8). This equation is only approximately valid for the displacement electrophoresis experiments described in the present paper (for instance, the capillaries have an outer polyimide coating and are not liquid-cooled), but still gives an idea as to how the field strength and the capillary radius affect the zone broadening.

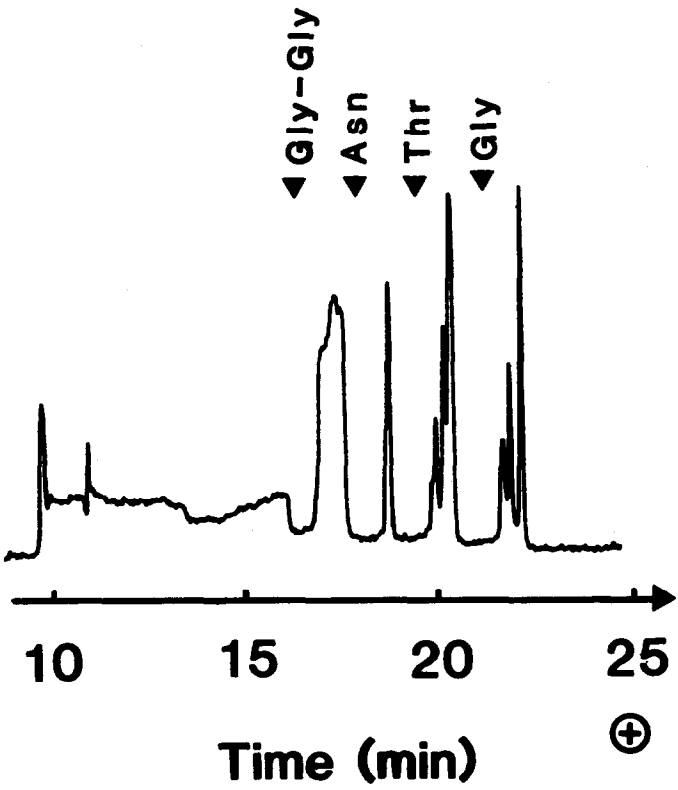

Fig. 4. High-performance displacement electrophoresis of human serum in a polymer-coated capillary in the presence of discrete spacers. Sample volume: 15 nl (= 30 mm). Anolyte, catholyte, voltage and wavelength are similar to those in Fig. 1a. Dimensions of the electrophoresis tube: 320 × 0.025 mm I.D. The spacers have negligible absorption at 280 nm, the wavelength used for detection.

DISCUSSION

The importance of correct nomenclature

Since the following equation applies to all methods based on electrophoresis, chromatography and centrifugation, any separation method within one of these three techniques has a counterpart within the other two [12,13].

$$c_j^{\alpha} v_j^{\alpha} - c_j^{\beta} v_j^{\beta} = v^{\alpha\beta} (c_j^{\alpha} - c_j^{\beta}) \quad (1)$$

where α and β are two phases separated by a moving boundary, which migrates with the velocity $v^{\alpha\beta}$; c_j^{α} and v_j^{α} are the concentration and the velocity, respectively, of the ion j in the α phase (c_j^{β} and v_j^{β} refer to the β phase). For instance, there must accordingly exist a method which is the electrophoretic counterpart of displacement chromatography and it is natural to give this method an analogous name, *i.e.* displacement electrophoresis —a term preferred and used by one of the pioneers in this field, A. J.

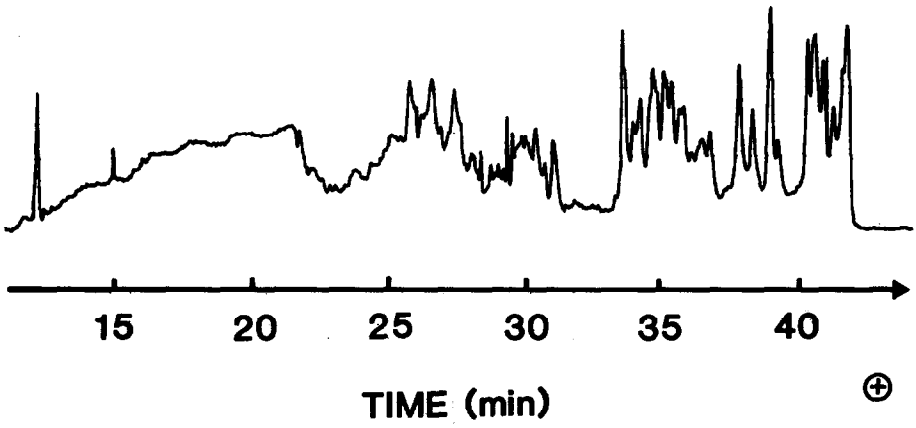

Fig. 5. High-performance displacement electrophoresis of human serum in polymer-coated capillaries in the presence of continuous spacers. Spacers: Bio-Lyte 3/10. Sample volume: 59 nl (= 30 mm). Anolyte, catholyte, voltage and wavelength are similar to those in Fig. 1a. Dimensions of the electrophoresis tube: 350 × 0.05 mm I.D. A comparison with Fig. 2 shows that the presence of continuous spacers increases the resolution considerably.

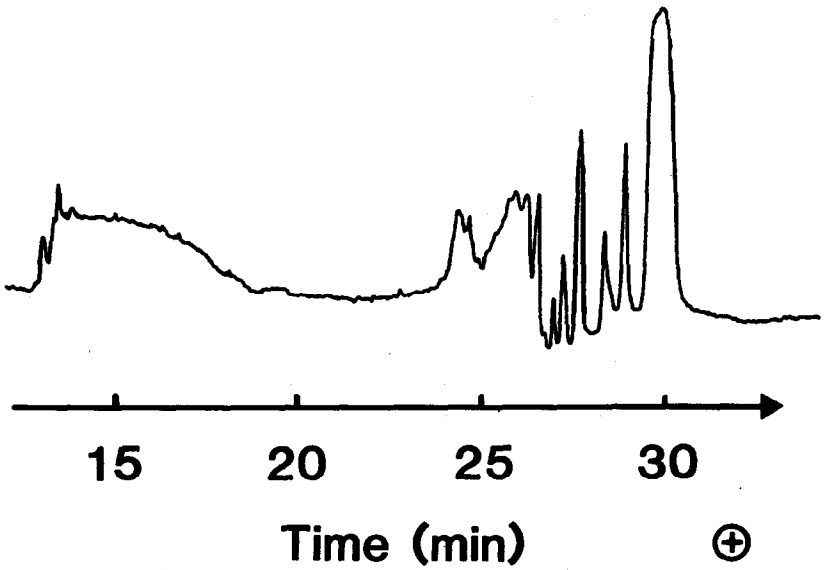

Fig. 6. High-performance displacement electrophoresis of a multiple myeloma serum in a polymer-coated capillary. Sample volume: 59 nl (= 30 mm). Anolyte, catholyte, voltage and wavelength are similar to those in Fig. 1a. Dimensions of the electrophoresis tube: 340 × 0.05 mm I.D. A comparison with Fig. 2, where serum from a healthy person was used, shows that multiple myeloma patients have an elevated level of immunoglobulins (the last zone).

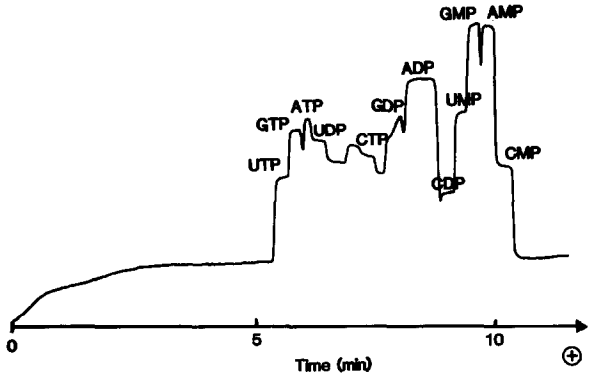

Fig. 7. High-performance displacement electrophoresis of nucleotides in a polymer-coated capillary. Sample volume: 4 nl (= 2 mm). Anolyte: 0.01 *M* HCl adjusted to pH 3.9 with solid β-alanine. Catholyte: 0.01 *M* caproic acid. Dimensions of the electrophoresis tube: 290 × 0.05 mm I.D. Voltage: 10 kV. Detection wavelength: 254 nm. The analysis time is about fourfold shorter than that obtained in commercial equipment with a non-coated 0.2-mm capillary.

P. Martin [14,15] and employed by Everaerts in his thesis [16]. One can expect these analogous methods to have analogous separation mechanisms and to be described by analogous equations. This is, in fact, true: in both methods the width of a zone is proportional to the amount of the solute in the zone; and the solute concentration in the zone is independent of its concentration in the applied sample but is characteristic of the solute and can be used to identify it. Furthermore, the leading and terminating solutions in displacement electrophoresis correspond to the equilibration and displacing buffers, respectively, in displacement chromatography. The two methods also give similar separation patterns: a step-formed diagram when the solute concentrations are measured in suitable units.

From the above considerations it might be evident that all analogous separation methods should be given analogous notations, since this stresses the analogy and facilitates theoretical understanding of the separation mechanisms, and thereby rational utilization of the methods. For this reason we prefer the term displacement electrophoresis to isotachophoresis.

It is very likely that progress in separation science has been delayed —and therefore also that in life sciences— because the existence of a common equation for electrophoresis, chromatography and centrifugation —and the consequences of it— have not until recently been emphasized [12,13]. For instance, isoelectric focusing in natural pH gradients was described in 1961 [17], but the first paper on the chromatographic counterpart, chromatofocusing, was not published until 1978 [18]. Seventeen years is too long for the introduction of the analogous chromatographic method. Another example of a consequence of ignorance of eqn. 1 is that indirect detection in chromatography was not utilized [19] until many years following introduction of this detection method in electrophoresis [1].

Factors causing zone broadening

In zone electrophoresis the following factors affect the width of a zone: diffusion; Joule heat; adsorption; and pH and conductivity differences between the solute

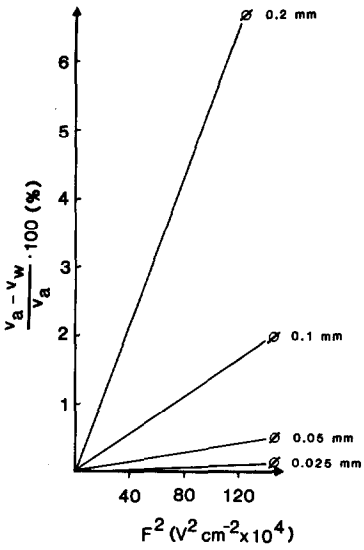

Fig. 8. Rough estimation of thermal zone broadening as a function of the square of the field strength, F (V/cm), for different tube diameters (Ø). v_a and v_w are the electrophoretic migration velocities at the axis and the wall of the electrophoresis tube, respectively. Eqn. 73 in ref. 1 was used with obvious modifications for the calculation of the zone braodening. The following parameter values were employed: a constant, B = 2400 K, electrical conductivity, $\kappa = 4.8 \cdot 10^{-4}\ \Omega^{-1}\ cm^{-1}$, thermal condctivity, $\lambda = 6.0 \cdot 10^{-3}\ J\ s^{-1}\ cm^{-1}\ K^{-1}$, T = 295 K. The figure, illustrating the importance of using narrow-bore tubes to suppress thermal zone broadening, permits an estimation of the zone broadening at different field strengths and for varying diameters of the electrophoresis tube.

zone and the surrounding buffer (for a detailed treatment, see ref. 13). In displacement electrophoresis the conductivity difference causes not broadening but rather zone sharpening, which decreases the disturbing influence of diffusion. For proteins, with their low diffusion constants, the diffusional zone broadening in displacement electrophoresis is therefore often negligible at high field strength. The adsorption, which may be very disturbing in uncoated tubes, particularly for proteins with their multipoint attachment to the tube wall [20], is often more pronounced in displacement electrophoresis than in zone electrophoresis. One reason is that a displacement electrophoresis must be conducted in the absence of a carrier buffer and therefore the conductivity (ionic strength) is lower, with attendant increase in the risk of adsorption (electrostatic interaction). That the adsorption is very disturbing in uncoated tubes of the small diameters used in this study is evident not only from a comparison of Fig. 1a and b, but also from our observation that the electropherogram in Fig. 1b was far from reproducible (often no proteins passed the detector). The difficulty in avoiding adsorption of proteins onto uncoated tubes in zone electrophoresis also is obvious from investigations by Lauer and McManigill [21] and many others.

Since the ionic strength is low in displacement electrophoresis, one can use very high field strengths without significant thermal zone broadening. However, zone broadening induced by reversible adsorption increases with the field strength [13,22], so one cannot expect in displacement electrophoresis high resolution at high field

strengths in uncoated tubes, although the automatic zone sharpening in displacement electrophoresis decreases the adsorptive zone broadening.

In uncoated quartz tubes the solutes are transported much faster by electroendosmosis than by electrophoresis. Uncoated tubes must, accordingly, be longer than coated tubes (for a given electrophoretic migration distance). Uncoated tubes therefore have the drawback that they require a higher voltage for a given field strength, with attendant risk of increased noise caused by electrical shock (current leakage). One should also recall that the longer the transport distance, the greater is the zone broadening caused by adorption.

Electroendosmosis can also be suppressed by addition of a hydrophilic polymer to the buffer [23]. This technique, which is often used in displacement electrophoresis, will also suppress adsorption. However, a thin coating on the tube itself is more efficient and does not increase the viscosity of the bulk of the buffer (an increase in viscosity decreases the migration velocities of the solutes, *i.e.* increases the analysis time).

Some proteins are not soluble at low ionic strengths and therefore have a tendency to precipitate in displacement electrophoresis [24], which may explain the small variations observed among some of the electropherograms presented herein. The precipitation may be suppressed or eliminated if the experiments are performed in the presence of ethylene glycol or a neutral detergent [24].

The conductivity used for the plot in Fig. 8 corresponds roughly to the average value of that in the experiment shown in Fig. 3. It is evident from Fig. 8 that the field strength used in this experiment (860 V/cm) gives a thermal zone broadening of only 0.06% (capillary diameter, 0.025 mm). For the experiment illustrated in Fig. 2 (field strength, 285 V/cm; capillary diameter, 0.050 mm) the corresponding figure is 0.03%. The electrophoretic migration distance was in both experiments about 320 mm. The thermal zone broadening was, accordingly, $320 \times 0.06/100 = 0.19$ mm and $320 \times 0.03/100 = 0.10$ mm, respectively, *i.e.*, almost negligible, which is in agreement with the observation that the resolution in Figs. 2 and 3 is about the same. Fig. 8 also shows that a capillary diameter of 0.2 mm —a common diameter in most equipment for displacement electrophoresis— gives a thermal zone broadening of 4 and 0.5% at the above field strengths, respectively, corresponding to 13 and 1.6 mm broadening for a 320-mm migration distance. This comparison illustrates the importance of reducing the diameter of the electrophoresis tube, provided that the adsorption and electroendosmosis can be eliminated, for instance, with a polymer coating as described herein (see also refs. 2, 13 and 25).

ACKNOWLEDGEMENTS

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