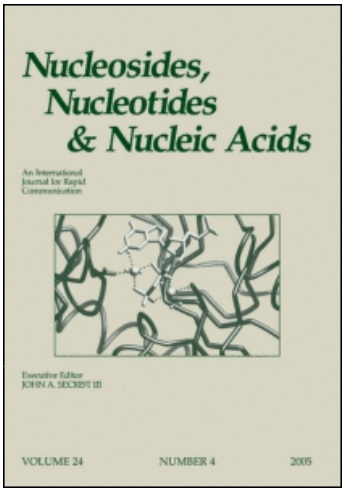

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High-Performance Electrophoresis Including Separation of Nucleic Acids and their Degradation Products. Interplay Between Theory and Practical Experiments

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HIGH-PERFORMANCE ELECTROPHORESIS INCLUDING SEPARATION OF NUCLEIC ACIDS AND THEIR DEGRADATION PRODUCTS. INTERPLAY BETWEEN THEORY AND PRACTICAL EXPERIMENTS.

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Abstract: The analogy between electrophoresis, centrifugation and chromatography is stressed by consideration of a formula which is valid for all three methods. An equation for electrophoretic plate heights forms the basis for a discussion of the experimental conditions for maximum resolution. The difference between true and apparent plate numbers and plate heights is underlined. Separations of nucleotides and DNA fragments are presented.

Introduction

In both electrophoresis, centrifugation and chromatography the separations obtained are based on differences in transport velocities among the substances to be fractionated. The transport is achieved by an electrical field in electrophoresis, a centrifugal field in centrifugation and a hydrodynamic force (flow) in chromatography (the retarding forces originate from frictional resistance in electrophoresis and centrifugation and from adsorption to the support in chromatography). These qualitative considerations reveal many analogies between electrophoresis, centrifugation and chromatography (1, 2). In fact, using the law of conservation of mass one can, by proper choice of parameters, derive the same equation for all three methods (3, 4):

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

, where α and β are two phases, separated by a moving boundary; c_j^{α} and c_j^{β} are the concentrations of the ion j in the α and β phase, respec-

tively; v_j^{α} and v_j^{β} are the velocities of the ion j in the α and ß phase, respectively. $V^{\alpha\beta}$ is the velocity of the moving boundary.

With the aid of eqn. (1) one can show that the function $H = \Sigma c_j \cdot v_j$ has the same value in all phases separated by a moving boundary, i.e. $H^{\alpha} = H^{\beta} = H^{\gamma} = \ldots$

Eqn. (1) can, of course, be used for theoretical calculations, but it also shows, for example, that for any chromatographic method there is an analogous method in electrophoresis and centrifugation. There exists therefore an electrophoresis method which is equivalent to high-performance liquid chromatography (HPLC). This paper will briefly deal with this method, called high-performance electrophoresis (HPE) (1, 2, 5, 6). The method is also called high-performance capillary electrophoresis (HPCE) or capillary electrophoresis (CE).

The main purpose of this paper is to treat certain aspects of high-performance electrophoresis which albeit of practical importance are not so often discussed in the literature. Not much attention will be devoted to applications for which there is already copious literature. The discussions herein are of a general character and thus refer to both low-molecular-weight compounds and macromolecules, for instance nucleosides, nucleotides and DNA fragments - substances of interest at this symposium.

The Principle of the Method.

The characteristic features of any high-performance separation method are high resolution and short run times. In the case of electrophoresis these requirements can be fulfilled only if the zone--distorting Joule heat is rapidly dissipated, which can be accomplished if the experiments are performed in an electrophoresis chamber of a small cross-sectional area, for instance, in fused silica tubes with diameters below 100 μm. However, when the radius (r) decreases, the ratio (2/r) between the surface area of the inner wall of the tube and its volume increases, and thereby also the risk of adsorption of the analyte to the tube wall. To suppress this adsorption the wall should be coated with a polymer (7). This coating has also the advantage to eliminate the electroendosmosis, i.e., the liquid flow that attends the electrophoresis and is caused by charges on the tube wall (7).

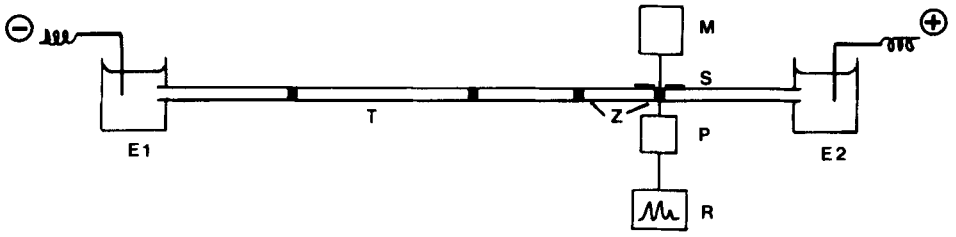

FIG. 1. The analytical HPE apparatus (on-tube detection). T = electrophoresis tube, Z = analyte zones, M = monochromator, S = slit, P = photo multipler, R = recorder and E1, E2 = electrode vessels.

A sketch of the apparatus used in HPE is shown in Fig. 1. The analyte zones Z are monitored as they pass the detector (M, S, P, R), which often is based on UV-absorption or fluorescence. This on-line detection method is particularly useful for analytical purposes.

For micro-preparative runs we have designed an apparatus which is depicted in Fig. 2 (8-10). As the solute zones Z leave the capillary tube T electrophoretically a stream of buffer from the pump P transfers them to the standard flow-through cuvette of an ordinary HPLC detector D and then to a fraction collector. The volume of the flow cuvette is considerably larger than the volume of a solute zone, but this has little or no effect on the resolution (8, 9). The same detection system can be used with advantage in micro-bore chromatography without using micro flow cells. The sensitivity is as high as in on-tube detection (8, 9), although the method does dilute the sample zones. An obvious advantage of this off-tube detection method is that it permits the use of any ordinary HPLC monitor.

Plate Height as a Function of Initial Zone Width and Zone Broadening Caused by Diffusion, Joule Heat and Conductivity Difference between Solute Zone and Buffer.

The following factors cause broadening of the applied zone: (1) diffusion; (2) Joule heat; (3) differences in electrical conductivity between a solute zone and the surrounding buffer; (4) convection; (5) adsorption of solutes to the inner wall of the electrophoresis chamber and (6) electroendosmosis.

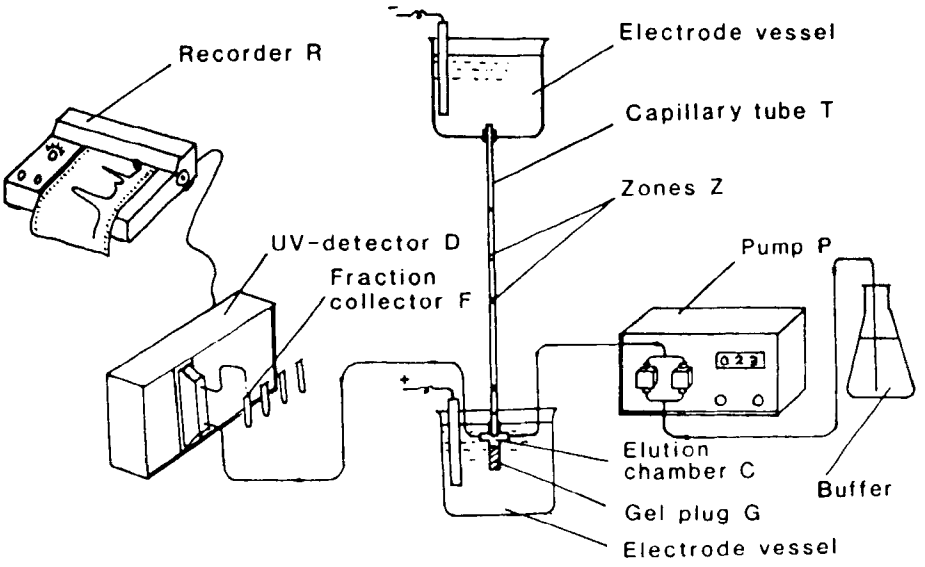

FIG. 2. The micro-preparative HPE apparatus (off-tube detection). Reproduced from ref. 9 with permission of the authors and the publisher.

The last two factors become negligible if the electrophoresis experiments are performed in polymer-coated capillaries (see above). Convection is virtually eliminated when the inside diameters are small (for instance 0.025-0.1 mm). Under these conditions the plate height H is determined only by the width ΔX_{inj} of the starting zone and the first three factors:

$$H = h_{inj} + h_{diff+cond} + h_{Joule} \qquad (2)$$

It is known (11, 12) that

$$h_{inj} = \frac{\Delta X^2_{inj}}{12L} \qquad (3)$$

In previous reports (3, 13-15) we have shown that

$$h_{Joule} = \frac{L}{16} \cdot \left(\frac{B\kappa}{\lambda}\right)^2 \cdot \left(\frac{RF}{2T}\right)^4 \qquad (4)$$

and

$$h_{diff+cond} = \frac{2D}{uF} \qquad (5)$$

, where L = the electrophoretic migration distance (cm), B = 2 400 °K, κ = the electrical conductivity (ohm^{-1} cm^{-1}), λ = the thermal conductivity of the buffer (Joule sec^{-1} cm^{-1} $degree^{-1}$), R = the radius of the electrophoresis tube (cm), T = the temperature (°K), F = the field strength (volt cm^{-1}), D = the diffusion coefficient (cm^2 sec^{-1}) and u = the mobility (cm^2 sec^{-1} $volt^{-1}$).

Insertion of eqns. (3)-(5) into eqn. (2) gives

$$H = \frac{\Delta X^2_{inj}}{12L} + \frac{2D}{uF} + \frac{L}{16} \cdot \left(\frac{B\kappa}{\lambda}\right)^2 \cdot \left(\frac{RF}{2T}\right)^4 \qquad (6)$$

We have determined experimentally the plate heights at different field strengths and compared them with plate heights calculated according to eqn. (6). The agreement has been satisfactory considering the approximate nature of this equation. One should also remember that asymmetrical peaks give incorrect plate heights when calculated from formulas which are derived on the assumption that the peaks have the form of symmetrical Gaussian curves. Perhaps more interesting than the values of the plate heights is the field strength that gives a minimum in plate height. This optimal field strength, F_{opt}, can be calculated with the aid of eqn. (7):

$$F_{opt} = 1.44 \left(\frac{D}{Lu}\right)^{1/5} \cdot \left(\frac{1}{\kappa}\right)^{2/5} \cdot \left(\frac{1}{R}\right)^{4/5} \qquad (7)$$

The constant 1.44 applies for the case when the temperature in the tube is about 25°C.

Field strengths calculated with this equation have in all verification experiments shown good agreement with the field strengths that correspond to a minimum in plate height in a plot of experimentally determined plate heights against the field strength. One should also observe that the plate height, at its minimum, varies relatively

little with the field strength. Therefore, without significant loss in resolution one can, in practice, use field strengths which deviate relatively much from the optimal one. Interestingly, F_{opt} is not strongly dependent on the values of the diffusion coefficient (D) and the mobility (u), since these parameters in eqn. (7) are raised to 1/5. Accordingly, it is sufficient to determine D and u only roughly. In a forthcoming paper we will present fast methods for such estimations.

Eqn. (6) takes into account peak asymmetry caused by differences in conductivity between the solute zone and the buffer. However, the equation must be modified when the asymmetry is so pronounced that the diffusion at one boundary of the zone is negligible due to the zone-sharpening effect of the conductivity difference. This modification utilizes the finding that the zone broadening at this boundary is $L \cdot \frac{\Delta\kappa}{\kappa}$.

The above treatment of zone broadening might be the first one in which the asymmetry of a peak caused by conductivity differences is considered. Among those who have previously treated electrophoretic zone broadening are Hjertén (16); Cox, Hessels and Teven (17); Virtanen (11); Brown and Hinckley (18); Jorgensen and Lukacs (19); Foret, Deml and Boček (20); Grushka, McCormick and Kirkland (21); Jones and Grushka (22); Knox (23); Terabe, Otsuka and Ando (24).

Correction of Plate Numbers Measured in the Presence of Electroendosmosis.

Assume that two electrophoresis experiments are performed under identical conditions, except that the first experiment is done in the absence of electroendosmosis and the second in the presence of electroendosmosis. The field strengths and the run times are thus the same in both experiments, which means that the electrophoretic migration distances and zone widths in the tubes are the same (but not the peak widths on the recorder chart and the actual lengths of the capillary tubing (5)). The relation between the true plate number (N_{true}) in the absence of electroendosmosis and the apparent plate number (N_{app}) in the presence of electroendosmosis is

$$N_{true} = \left(\frac{L'}{L''}\right)^2 \cdot N_{app} \qquad (8)$$

, where L' and L" are the migration distances in the absence and presence of electroendosmosis, respectively. Eqn. (8) can also be written

$$N_{true} = \left(\frac{v}{v_{eo} \pm v}\right)^2 \cdot N_{app} \qquad (9)$$

The plus and minus signs refer to the cases when the electroendosmotic and electrophoretic velocities (v_{eo} and v) have the same and opposite directions, respectively.

We have assumed here that the plate numbers N_{true} and N_{app} are calculated directly from retention times t and peak widths t_w on the recorder chart without any corrections ($N = C\,(\frac{t}{t_w})^2$).

The electroendosmotic flow velocity is often 3- to 10-fold that of the electrophoretic velocity, i.e. $N_{true} = (\frac{1}{(3 \text{ to } 10) \pm 1})^2 N_{app} = (\frac{1}{2} \text{ to } \frac{1}{11})^2 N_{app}$, which means that one should divide the apparent plate number with a factor in the range of 4 to 120 to get the true plate number. All plate numbers determined in the presence of electroendosmosis and reported in the literature might only be the apparent ones and should therefore be corrected as indicated.

Since $\frac{L'}{L''} = \frac{v}{v_{eo} \pm v}$, one can conclude that an electroendosmosis-free tube can be about 2- to 10-fold shorter than a tube with electroendosmosis and yet give the same resolution.

The relation between true and apparent plate heights is

$$H_{true} = \frac{L''}{L'} \cdot H_{app} = \frac{v_{eo} \pm v}{v} \cdot H_{app} \qquad (10)$$

Some Aspects of the Design of an Experiment and Applications.

There are many details which are of importance for the attainment of successful and reproducible results. Some of these will be discussed in this section.

If the run is to be performed in free solution, i.e. in buffer alone without a supporting medium, it is essential to coat the tube with a polymer, for instance linear polyacrylamide, in order to eliminate adsorption and electroendosmosis. The coating procedure is described in ref. 7. In the absence of electroendosmosis one can use a

short electrophoresis tube, as pointed out above: 15-20 cm is a common length. A non-coated tube has often a length around 80 cm. Such a long tube requires a four-fold higher voltage than does a 20-cm coated tube to achieve the same field strength. A short tube is also easy to handle and prepare for a run.

Experimentally (24) and theoretically (from eqn. (6)) one can show that high resolution often requires the widths of the starting zones to be smaller than one mm. With the conventional application methods based on electrophoresis/electroendosmosis or a pressure difference between the ends of the electrophoresis tube, it can be difficult to apply such narrow starting zones reproducibly. Therefore, I have developed a novel thermal loading technique: When the temperature of the electrophoresis tube is lowered the buffer will contract and the sample, placed at one end of the tube, is sucked in. The accuracy and reproducibility approach that of a thermometer. The length of the starting zone can be calculated easily and exactly from the temperature drop.

Liquid cooling of the electrophoresis tube (2) increases the reproducibility and decreases the drift in the base line of the electropherograms. The liquid cooling has also the advantage to diminish the risk of formation of air bubbles in the tube which is particularly great when the tube diameter exceeds 75 μm. This risk can be suppressed by deaeration of the buffers.

The reproducibility of electrophoresis experiments in tubes filled with polyacrylamide gels increases if the tube diameter exceeds 0.1 mm. In such experiments it is particularly important to deaerate the monomer solutions thoroughly and to actively cool the tube. When on-tube UV-monitoring of proteins is done at very short wavelengths (190-225 nm) it may be appropriate to have the section of the tube where the detection takes place filled with buffer alone, since polyacrylamide gels have a strong absorption at these wave lengths. The absorption is lower if the tubes are used the day after the polymerization was done.

HPE has a very broad range of applications: it permits studies of inorganic and organic ions, peptides, nucleosides, nucleotides, proteins, nucleic acids, virus and whole cells (for instance bacteria),

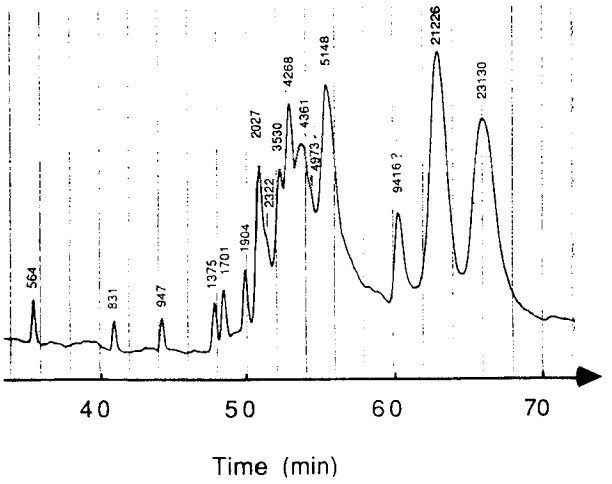

FIG. 3. High-performance field-inversion electrophoresis of DNA fragments in a HydroLink™ gel. Sample: a mixture of digested λ (Hind III) and λ (Hind III + EcoR I). Tube dimensions: 0.075 (i.d.) x 110 mm. Buffer: 0.089 M Tris-borate + 0.002 M EDTA (pH 8.0). Medium: HydroLink™; T = 4%, C = 3% (these parameters are defined in ref. 28). Voltage: 750 volts. The switching cycle comprised 160 msec of forward migration and 40 msec of reverse migration. This periodic inversion of the electrical field gave higher resolution of the larger, slowly migrating DNA fragments than did ordinary electrophoresis without reversal of the electrical field. Detection wave length: 260 nm. Similar electropherograms were obtained by field-inversion polyacrylamide gel electrophoresis. The experiments were performed by Dr. Tasanee Srichaiyo in the author's laboratory.

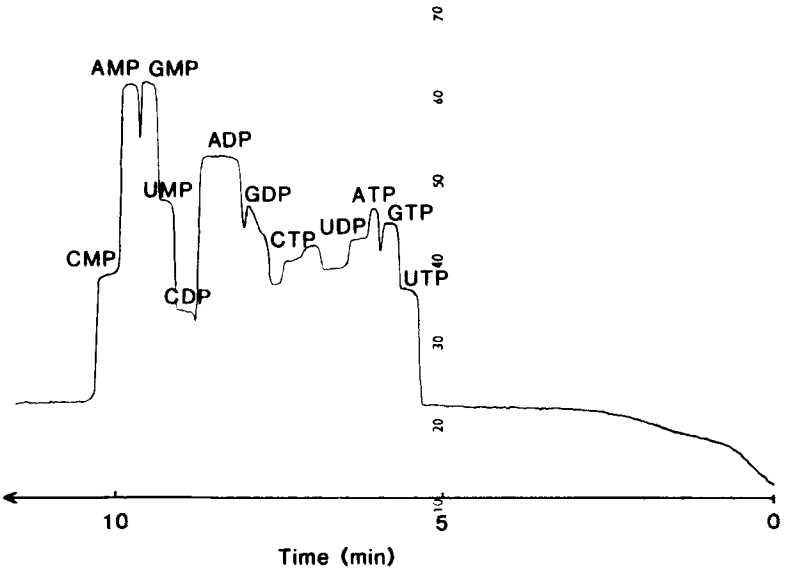

FIG. 4. High-performance displacement electrophoresis of nucleotides. Sample: an artificial mixture of nucleotides. Tube dimensions: 0.05 mm (i.d.) x 290 mm. Voltage: 10,000 volts. Leading buffer: 0.01 M HCl, titrated to pH 3.9 with ß-alanine. Terminating buffer: 0.01 M caproic acid (pH ≈ 3.7). Detection wave length: 254 nm. The experiment was performed by Dr. Marianne Kiessling-Johansson in the author's laboratory.

both by zone electrophoresis, isoelectric focusing and displacement electrophoresis. The applications presented herein must necessarily be few and are therefore limited to substances whithin the scope of this symposium. Fig. 3 thus shows an electrophoresis of DNA fragments in a gel of HydroLink™ (25, 26). The direction of the electric field was reversed periodically as described by Carle et al. for DNA separations in agarose gels (27).

Fig. 4 shows a displacement electrophoresis of nucleotides. Since the tube was only 0.05 mm in diameter and was coated with a layer of polyacrylamide to eliminate electroendosmosis and adsorption the analysis time was four-fold shorter than that reported in the literature for the same sample mixture (29).

For further information on DNA, nucleoside and nucleotide separations by HPE, see papers by Zhu and Hjertén (8); Cohen et al. (30, 31).

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