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Review

Theoretical background for clinical and biomedical applications of electromigration techniques

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ABSTRACT

This review presents the theoretical principles of analytical electrophoresis. The basic rules which control the movement of ionic species in electrostatic fields, together with the phenomenological theory of the resulting mass transport are analysed. The separation principles and capabilities of zone electrophoresis, isotachophoresis, isoelectric focusing, micellar electrokinetic chromatography and gel electrophoresis are evaluated. The most important effects accompanying electrophoresis, such as the production of Joule heat, electroosmosis and diffusion are discussed.

CONTENTS

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1. INTRODUCTION

There is no doubt that electrophoresis is one of the most important and widely used separation tools in biochemistry and clinical chemistry. Its main features are an extremely high separation efficiency, a short analysis time and the potential to separate native species under such conditions that the risk of denaturation is minimized. However, electrophoresis is still far from being a black box with an inlet for samples and an outlet providing complete and unambiguous information on the qualitative and quantitative composition of a sample. For a correct interpretation of the data provided by electrophoretic analyses and to solve new separation and identification problems, a theoretical background of electromigration techniques is essential.

The goal of this paper is to provide an introduction to the theoretical background of the methodology of electrophoresis and to serve as a reference source and practical guide for those already working in the field. It is worthwhile to mention at this point publications discussing the present state-of-the-art of electrophoretic methods [1–6], surveys of the theory and comprehensive articles [7–20], an exhaustive survey of the literature [21], as well as reviews of applications where some fundamental phenomena are discussed [22–31].

2. BASIC CONCEPTS

2.1. Electrophoretic mobility

An ion or any other charged particle i which carries z_i elementary charges of an electron e (elementary charge of an electron is $1.602 \cdot 10^{-19}$ C) when placed in a homogeneous electric field, the intensity of which is E, is accelerated by the force

$$F_e = z_i e E \tag{1}$$

Here, the electric field intensity E (V m^{-1}) is defined as the gradient of the electric potential ϕ (V) in the form

$$E = -\frac{d\phi}{dx} \tag{2}$$

In the present forms of electrophoresis, the electric field intensities used range from 1 kVm^{-1} in gels up to 50 kV m^{-1} in capillaries.

In a viscous hydrodynamic medium, the drag force F_d is exerted on a moving particle which is, in general, proportional to its migration velocity v and to the Newtonian viscosity η of the medium

$$F_d = k\eta v \tag{3}$$

Hence, the acceleration of a particle proceeds until the force F_e is compensated by the drag force F_d and then the particle i moves with a constant velocity v_i which is given by

$$v_i = \frac{z_i e}{k\eta} E \tag{4}$$

This is illustrated in Fig. 1.

The proportionality factor between the migration velocity v_i and the electric field intensity E is called the electrophoretic mobility, u_i. It follows from eqn. 4 that the electrophoretic mobility u_i is a positive or negative quantity according to the sign of the charge z_i. Hence, cations possess positive mobilities and anions negative mobilities. The migration of cations to the cathode (cathodic migration) is usually considered to have a positive sense.

For a spherical particle under laminar flow conditions in a continuum, the drag force is given by Stokes' law [32]

$$F_d = 6\pi\eta r_i v_i \tag{5}$$

where r_i is the sphere radius. (In many electrophoretic experiments in free aqueous solutions, the viscosity of the medium may be substituted by the viscosity of water which is $\eta = 1.002$ mPa s, at 293 K.) Equating forces F_e (eqn. 1) and F_d (eqn. 5) we have for spherical charged particles

$$v_i = u_i E = \frac{z_i e}{6\pi\eta r_i} E \tag{6}$$

The electrophoretic mobility u_i is given by

$$u_i = \frac{z_i e}{6\pi\eta r_i} \tag{7}$$

The numerical parameter 6 in eqns. 5–7 holds true for spherical particles under the assumption that the velocity of a liquid, relative to a particle, vanishes at the particle surface [33]. For real molecules which are not spherical and/or for small ions the size of which is comparable to that of the solvent molecules, the numerical parameter differs from 6 [34]. The ion radius r_i is a radius of the hydrodynamic equivalent sphere which has the same transport properties as the ion in question [35]. This quantity is determined not only by the size and shape of the molecule, but also by the attached solvation shell.

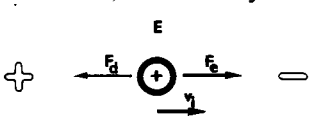

Fig. 1. Forces acting on a migrating charged particle in an electric field.

The variation of electrophoretic mobility with temperature, seen from eqn. 8, is almost wholly accounted for by the variation of solvent viscosity with temperature. The temperature dependence of the electrophoretic mobility is only to a minor extent due to the change in solvation. The increase in electrophoretic mobility with temperature is usually expressed by

$$u_{T_2} = u_{T_1}(1 + \alpha \Delta T) \tag{8}$$

where $\Delta T = T_2 - T_1$, and the coefficient α has an average value of 0.02 K^{-1} for aqueous solutions. This equation shows that in a water solution of electrolytes, the mobilities of ions increase by 2% for each degree Kelvin temperature rise.

An ion in a solution is always surrounded by an atmosphere of ions of the opposite sign. The electrostatic interaction between such an ion and the ion atmosphere, which migrates in the opposite direction, will affect the mobility of the former. Thus the mobility decreases with increasing ionic strength. The ionic strength (υ) of the solution is defined as

$$\upsilon = \frac{1}{2} \sum_{i=1}^{n} c_i z_i^2$$

The dependence of the electrophoretic mobility on the ionic strength is expressed by the Onsager equation [5]

$$|u_i| = |u_i^0| - (0.23\,|u^0 z_i z_R| + 31.43 \cdot 10^{-9}|z_i|)\frac{\upsilon^{1/2}}{1 + \upsilon^{1/2}} \tag{9}$$

where u_i^0 is the limiting electrophoretic mobility at zero concentration and z_R is the charge number of the counterion. The measured absolute values of the limiting ionic mobilities of different species are given in Table 1.

In practice, the dependence $u_i = f(\upsilon)$ is advantageously expressed by a series applied for a certain ion serving as a reference standard, and, for other ions, relative mobilities are used which can be considered as independent of υ. The dependence of $u_{Na} = f(\upsilon)$ for 25°C is given here as an example [36]

$$u_{Na} = 51.92 - 42.88\upsilon^{1/2} + 52.25\upsilon - 30.24\upsilon^{3/2} \tag{10}$$

The plot of this function is shown in Fig. 2.

The electrophoretic movement of ions in a solution manifests itself macroscopically as a conduction of electric current by the solutions of electrolytes. The Kohlrausch law for the independent motion of ions says that both cations and anions contribute independently to the whole rate of charge transfer, *i.e.* to the actual electric current which can be measured by an ammeter. This is shown schematically in Fig. 3; it holds for the current density i (Am^{-2})

TABLE 1

ABSOLUTE VALUES OF LIMITING IONIC MOBILITIES AT 298 K

Abbreviations: Tris = Tris(hydroxymethyl)aminomethane; HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; MOPS = 4-morpholinepropanesulphonic acid; MES = 4-morpholinethanesulphonic acid; AMP = adenosine monophosphate.

Cation	$\lvert u^\circ \rvert \cdot 10^9$ ($m^2\ V^{-1}\ s^{-1}$)	Anion	$\lvert u^\circ \rvert \cdot 10^9$ ($m^2\ V^{-1}\ s^{-1}$)
H_3O^+	362.5	OH^-	205.5
Li^+	40.1	F^-	57.4
Na^+	51.9	Cl^-	79.1
K^+	76.2	NO_3^-	74.1
Ammediol	29.5	SO_4^{2-}	82.9
Tris	29.5	HEPES	21.8
β-Alanine	36.7	MOPS	24.4
Ethanolamine	44.3	MES	26.8
Imidazole	52.0	AMP	22.6

$$i = FE \sum_{j=1}^{n} c_j z_j u_j \tag{11}$$

where c_j is the concentration of the ionic species j in kmol m^{-3} (numerically equal to mol l^{-1}) and F is the Faraday constant (96 487 C mol^{-1}). This holds for the specific conductivity

$$\varkappa = \frac{i}{E} = F \sum_{j=1}^{n} c_j z_j u_j \tag{12}$$

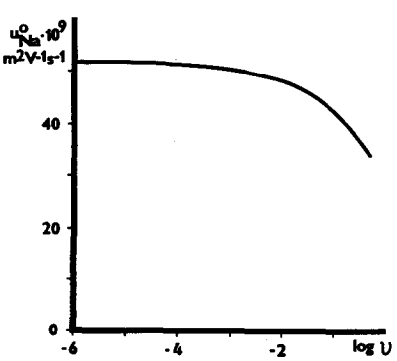

Fig. 2. Dependence of the electrophoretic mobility of the sodium cation on the ionic strength (eqn. 10).

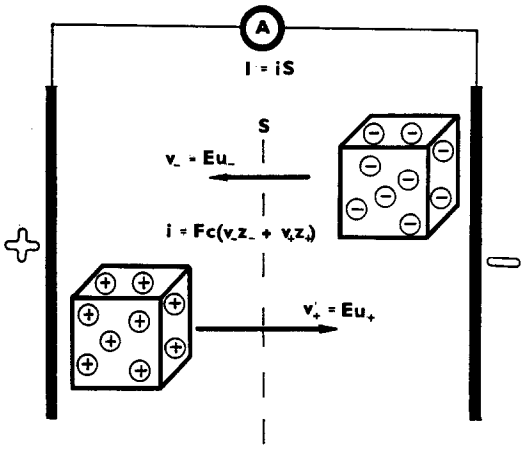

Fig. 3. Schematic representation of the independent motion of ions in the electric field.

These considerations are all connected with solutions of strong electrolytes. In practice, however, weak electrolytes are more common in the solutions. In such solutions, the concept of effective electrophoretic mobility (further referred to as effective mobility) $\bar{u}$ is very important. The well known definition by Tiselius [7] may be cited here. Substance A, present in solution in forms (A_1, A_2, ... A_n), whose molar fractions are x_1. x_2, ... x_n, and mobilities are u_1, u_2, ... u_n, with the individual forms in rapid dynamic equilibrium with one another, migrates in an electric field as a single substance with an effective mobility

$$\bar{u}_A = \frac{1}{\bar{c}_A} \sum_{i=1}^{n} c_i u_i = \sum_{i=1}^{n} x_i u_i \tag{13}$$

where $\bar{c}_A = \sum_{i=1}^{n} c_i$ is the total, or analytical, concentration of A.

A practical example of the effective mobility in solution is provided by the effective mobility of oxalate ions

$$\bar{u}_{(COOH)_2} = x_{\substack{COO^- \\ COOH}} \cdot u_{\substack{COO^- \\ COOH}} + x_{\substack{COO^- \\ COO^-}} \cdot u_{\substack{COO^- \\ COO^-}}$$

The undissociated part of oxalic acid with zero mobility does not contribute to the effective mobility. Obviously, the actual magnitude of the molar fractions of the ionic components can be affected by a change in pH of the background electrolyte, which results in a shift of the equilibrium reactions. Hence, by controlling the pH, the effective mobilities and thus the migration time of analytes can also be controlled. The dependence of the effective mobility $\bar{u}$ of weak acids and weak bases on pH has a characteristic shape corresponding to the dissociation curve of weak acids and the protonation curves of weak bases.

This holds for the effective mobility $\bar{u}_B$ of base B with ionic mobility u_{BH} in the protonated form BH^+

$$\bar{u}_B = \frac{[H^+]}{K_{BH} + [H^+]} u_{BH} \tag{14}$$

where $K_{BH} = [H^+] \cdot [B]/[BH^+]$ is the constant of the acid–base equilibrium involved. Fig. 4 (curve 1) depicts the function $\bar{u}_B = f(pH)$, the mobility curve for tris(hydroxymethyl)aminomethane (Tris), with an ionic mobility of $u_{Tris} = 28 \cdot 10^{-9}\ m^2\ V^{-1}s^{-1}$ and a dissociation constant of the protonated (acid) form of $pK_{TRIS} = 8.0$.

This holds for a weak acid HA

$$\bar{u}_A = \frac{K_{HA}}{K_{HA} + [H^+]} u_A \tag{15}$$

where $K_{HA} = [H^+][A^-]/[HA]$ is the dissociation constant of the acid HA. Fig. 4 (curve 2) depicts the mobility curve for acetate with an ionic mobility $u_{Ac} = -40 \cdot 10^{-9}\ m^2\ v^{-1}\ s^{-1}$ and $pK_{HAc} = 4.76$.

Ampholytes have special properties with respect to electrophoretic separations. These ampholytes are very important in medicine, biology and biochemistry and include amino acids, nucleotides, proteins and other compounds which exist as cations or anions according to the acidity of their environment. Amino acids serve as examples of zwitterionic behaviour, *e.g.* the zwitterionic form of α-alanine is

$$\begin{array}{l} CH_2 - CH_2 - COO^- \\ | \\ NH_3^+ \end{array}$$

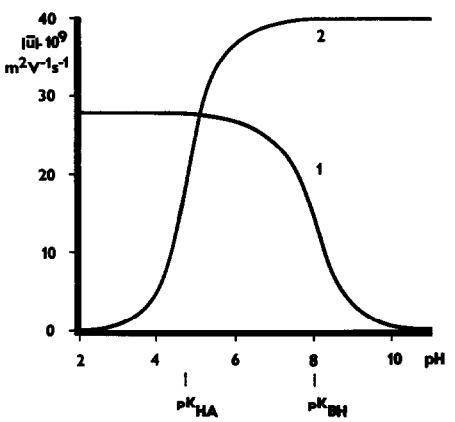

Fig. 4. Dependence of the absolute value of the effective mobility on pH. 1 = TRIS; $pK_{TRIS} = 8.0$; $u_{TRIS} = 28 \cdot 10^{-9}\ m^2\ V^{-1}\ s^{-1}$; 2 = acetic acid; $pK_{HAc} = 4.76$; $u_{HAc} = -40 \cdot 10^{-9}\ m^2\ V^{-1}\ s^{-1}$.

The ampholytic character of amino acids is characterized by their dissociation constants, numbered from the acidic to the basic region. It should be realized that the pK_1 values characterize the protonation of the carboxyl groups in the acidic region, *i.e.* the process in which the zwitterion constitutes the cation

$$K_1 = \frac{[H^+][NH_3^+RCOO^-]}{[NH_3^+RCOOH]} \tag{16}$$

The second dissociation constant characterizes the dissociation of the amino group when the zwitterion constitutes the anion

$$K_2 = \frac{[H^+][NH_2RCOO^-]}{[NH_3^+RCOO^-]} \tag{17}$$

For β-alanine, $pK_1 = 3.8$ and $pK_2 = 9.6$, the ionic mobility of the cation equals $36 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} and that of the anion is $-31 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}. Fig. 5 depicts the dependence of the effective mobility on pH. It can be seen that amino acids of the mono-amino–mono-carboxy type migrate cathodically (as cations) in an acidic medium and anodically in an alkaline medium. It should be noted that the mobility is practically zero in a certain pH region, the isoelectric region. Here the substance can be considered as neutral, at least from a macroscopic point of view.

The typical effective mobility dependence on the pH for proteins (Fig. 6) shows that zero mobility occurs at a single pH value, called isoelectric point p*I*. This is due to the presence of a large number of free acidic and basic groups with similar values.

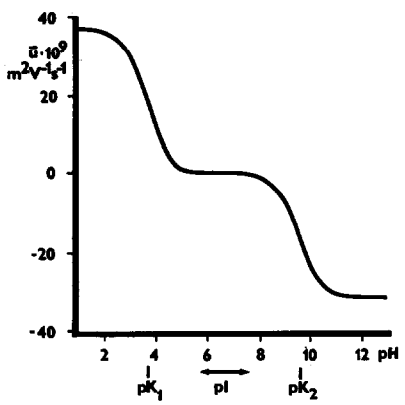

Fig. 5. Dependence of the effective mobility of β-alanine on pH. $pK_1 = 3.8$; $pK_2 = 9.6$; ionic mobilities of the cationic and anionic forms are $36 \cdot 10^{-9}$ and $-31 \cdot 10^{-9}$, respectively.

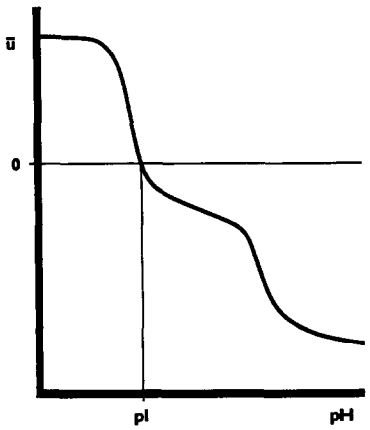

Fig. 6. Schematic dependence of the effective mobility of a protein on pH.

2.2. Electric current and mass transfer

The basic principle of the separation methods is a selective mass transport of components of an analysed mixture. The mass transport of component i can be described as the mass current density, or mass flux, J_i. If the movement of particles i can be described by a single macroscopic velocity v_i, the corresponding flux is [32]

$$J_i = c_i v_i \tag{18}$$

When the velocity is a random quantity, the phenomenological definition as for diffusional flux (Section 2.3) must be used.

Summing all the possible important contributions to the net flux in electrophoretic experiments gives, for the one-dimensional case,

$$J_i = c_i(u_i E + v_{eo} + v_f) - D_i(\mathrm{d}c_i/\mathrm{d}x) \tag{19}$$

where the velocities in parentheses at the first term on the right-hand side are, respectively, the electromigration velocity (see eqn. 6), the local bulk velocity of the fluid due to electroosmosis (see Section 2.3) and the local bulk velocity due to the hydrodynamic flow of the fluid. The last term on the right-hand side of eqn. 19 is the diffusional mass flux (see eqn. 63, Section 2.3).

The continuity equation for particles i, *e.g.* ion i, can be written in the form [32]

$$\frac{\partial c_i}{\partial t} = -\frac{\partial}{\partial x} J_i + R_i \tag{20}$$

where t is the time, J_i is the mass flux defined by eqn. 18 and $R_i = f(x,t)$ is the production of species i by a chemical reaction per unit volume and unit time. It should be noted that the mass of a reacting system is always conserved; however, the number of moles may change considerably.

A general solution to eqn. 20 is not known; the known solutions are valid for special cases only. Systems of strong electrolytes can be regarded as systems where chemical reactions do not take place. Neglecting any convection and assuming that mass transport is only due to electromigration and diffusion, eqn. 19 gives

$$J_i = Ec_iu_i - D_i \frac{dc_i}{dx} \tag{21}$$

The description of the migration of weak electrolytes is important from the practical point of view; however, explicit solutions are known only for the systems of uni–univalent weak electrolytes if diffusion is neglected [37]. Then the continuity eqn. 20 is in the form

$$\frac{\partial c_i}{\partial t} = -\frac{\partial}{\partial x}(Ec_iu_i) + R_i \tag{22}$$

By summing up such equations for all particles i of a given substance A the reaction term is eliminated because

$$\sum_{i=1}^{n} R_i = R_A = 0 \tag{23}$$

This means that the total amount of substance A, in moles, does not change. The electromigration transport equation for substance A can now be written as

$$\frac{\partial \bar{c}_A}{\partial t} = -\frac{\partial}{\partial x}(E\bar{c}_A\bar{u}_A) \tag{24}$$

Assuming a constant current density i passing through the whole length of a column with a constant cross-section S, the intensity of the electric field can be substituted with the help of eqn. 12

$$\frac{\partial \bar{c}_A}{\partial t} = -\frac{I}{S}\frac{\partial}{\partial x}\left(\frac{\bar{c}_A\bar{u}_A}{\varkappa}\right) \tag{25}$$

where I is the electric current. To describe the transport in an electrolyte system,

the number of transport equations is equal to the number of substances A, B, C, ... present in the system.

Of great importance for a more precise understanding of electromigration are the first integrals of the simplified transport equations. These first integrals can be derived easily in an illustrative way for systems of uni-univalent strong electrolytes, assuming constant temperature, constant ionic mobilities (independent of ionic strength) and with diffusion neglected. Their differences stem from the starting conditions and the type of electrolyte system.

2.2.1. Regulating function

The regulating function is not an independent equation describing the electromigration. It is one of the forms of the first integrals of the transport equation system where the diffusion is neglected and electrophoretic mobilities are assumed to be constant. This function was derived by Kohlrausch in 1897 for a system of uni–univalent strong electrolytes [38]. Later, the regulating function was also derived for uni–univalent weak electrolytes [39].

The importance of the regulating function stems from the fact that it enables the characterization of the system in an informative way [40]. It is useful for systems of strong electrolytes. However, in contrast to the moving boundary equation (which is also the first integral of the transport equation), the regulating function is also valid if the composition of the zones along the migration path in time is not constant. It is useful to introduce briefly one of the derivations of the Kohlrausch regulating function (ω) to illustrate its importance and to make clear the assumptions needed.

Consider that the transport equation of the type eqn. 25 is valid for strong or weak uni–univalent electrolytes, where $\bar{c}_i\bar{u}_i = c_iu_i$. In this case the subscript i for ionic form is interchangable with the subscripts A, B, C, ..., for substances. After multiplication of eqn. 25 by the factor z_i/u_i, the following equation is obtained

$$\frac{\partial}{\partial t}\left(\frac{c_iz_i}{u_i}\right) + \frac{I}{S}\frac{\partial}{\partial x}\left(\frac{c_iz_i}{\varkappa}\right) = 0 \tag{26}$$

By summing all equations for all ions of all substances present in the solution, $i = 1, 2, ...$, and assuming electroneutrality expressed as

$$\sum_{i=1}^{n} c_iz_i = 0 \tag{27}$$

we obtain

$$\frac{\partial}{\partial t}\sum_{i=1}^{n}\frac{c_iz_i}{u_i} = 0 \tag{28}$$

Hence it follows

$$\omega(x) = \sum_{i=1}^{n} \frac{c_i z_i}{u_i} \tag{29}$$

i.e. $\omega(x)$ is a function of x only and is independent of time.

The physical meaning of the regulating function is as follows. If the electric current is passing through the column filled originally with the electrolytes A^+R^- and B^+R^- which form two zones 1 and 3 separated by a sharp boundary (Fig. 7a) then, as a result of electromigration, the third zone 2 appears (Fig. 7b). The concentration of the electrolyte B^+R^- in zone 2 is not deliberate but is fixed to a value satisfying the condition (Eqn. 29) $\omega_1 = \omega_2$. Zone 2 is said to be adjusted to zone 1. This is a general mechanism which says that any of the migrating zones along the column follow the concentration profile which was formed along the column before the electromigration started. If the column was filled with a discontinuous electrolyte system before the experiment started, the regulating function along the column has as many different values as there are electrolyte phases and these values are independent of time. When the electromigration starts, the migrating zones copy all discontinuities and concentration levels at places where they were created prior to the switching on of the voltage across the column.

It is important to mention another form of regulating function. Multiplying eqn. 29 by the constant u_R/z_R, the following regulating function is obtained [41]

$$\omega' = \omega \frac{u_R}{z_R} \tag{30}$$

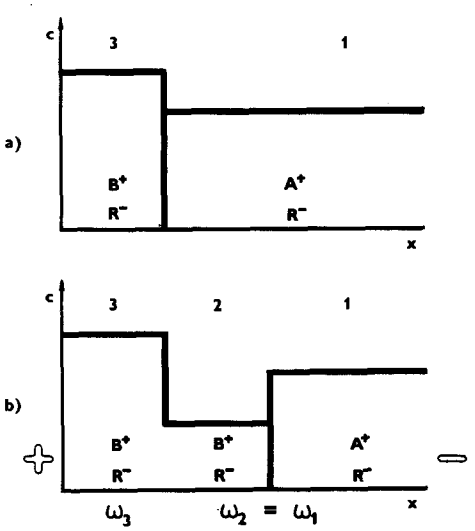

Fig. 7. Concentration profiles of electrolytes A^+R^- and B^+R^- during electrophoretic migration in the separation tube ($u_B < u_A$). (a) The starting situation. (b) As a result of electromigration, the adjusted zone 2 appears. For explanation of the regulating function ω, see text.

which has the dimension of concentration. The subscript R denotes the counterion. This form of regulating function is very useful for the description of unsteady-state migration.

2.2.2. Steady-state migration

The solution to the electrophoretic transport equation which corresponds to the steady-state migration is now described. Consider, once again, two strong electrolytes with a common counterion, A^+R^-, B^+R^-, which are separated from one another by the sharp zone boundary prior to electromigration, as seen in Fig. 7a. It is known from experiment that if zone A is in front of zone B in the direction of their electrophoretic migration and if it holds everywhere in the system that $u_B < u_A$ then, when the electric field is applied, the result is steady-state migration. This can be characterized by the formation of a new zone 2 and a moving boundary $2 \rightarrow 1$, where the concentrations of the species A and B in their zones are constant (Fig. 8). Considering a constant electric current density, the concentration profiles of the ionic components A, B and the zone boundary $2 \rightarrow 1$ move without changing the shape at a constant speed $v_{1,2}$. At the point of the original boundary between zones 3 and 1 (Fig. 7a), a certain discontinuity survives which does not move and is called the stationary boundary, denoted as 3,2.

The changes with time of the concentration of ionic component i at a point fixed with respect to the external coordinate system can be described by eqn. 20 where the mass flux J_i is substituted by eqn. 18 and v is assumed constant for this steady-state situation

$$\frac{\partial c_i}{\partial t} = -v_{1,2} \frac{\partial c_i}{\partial x} \tag{31}$$

As already shown by Kohlrausch [38] and Weber [41], the particular solution of this equation is

$$c_i = f_i(x - v_{1,2}t) \tag{32}$$

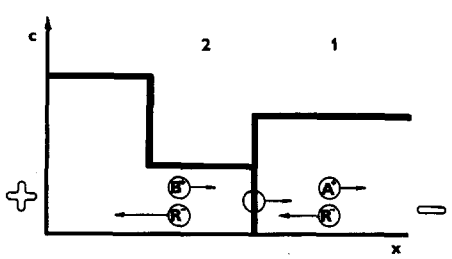

Fig. 8. Concentration profiles of electrolytes A^+R^- and B^+R^- ($u_B < u_A$) and velocities of ionic components and moving boundary 2–1 during steady-state electromigration (eqns. 35–39).

where f_i is an arbitrary function which is given by the concentration distribution after the steady state is reached and does not change with time.

To find the concentration distribution of component i and velocity $v_{1,2}$, the right-hand side of the continuity equation (eqn. 25) (the subscript i is interchangeable with the subscripts A, B, C, ... for strong electrolytes) is made equal to the right-hand side of the continuity equation for steady migration (eqn. 31)

$$\frac{I}{S}\frac{\mathrm{d}}{\mathrm{d}x}\left(\frac{c_i u_i}{\varkappa}\right) = v_{1,2}\frac{\mathrm{d}c_i}{\mathrm{d}x} \tag{33}$$

Integration of this equation gives the moving boundary equation in the form

$$I/S\left(\frac{c_{i,1}u_{i,1}}{\varkappa_1} - \frac{c_{i,2}u_{i,2}}{\varkappa_2}\right) = v_{1,2}(c_{i,1} - c_{i,2}) \tag{34}$$

The numerical subscripts 1 and 2 relate to the neighboring zones 1 and 2.

The following scheme gives the conditions for the steady-state electrophoretic migration together with the relationships for calculations of the concentrations and velocities of the components.

Zone 2 Boundary 1,2 Zone 1

Given conditions

$$\begin{aligned} u_{\mathrm{B},2}\ (\mathrm{const}) &< u_{\mathrm{A},1}\ (\mathrm{const}) \\ u_{i,2} &\neq u_{i,1}\ (\mathrm{generally}) \\ i_2 &= i_1 \\ \varkappa_2 &< \varkappa_1 \\ E_2 &> E_1 \end{aligned}$$

Velocities

$$v_{1,2} = \frac{I/S\left(\frac{c_{i,1}u_{i,1}}{\varkappa_1} - \frac{c_{i,2}u_{i,2}}{\varkappa_2}\right)}{c_{i,1} - c_{i,2}} \tag{35}$$

$$v_{\mathrm{R},2} = E_2 u_{\mathrm{R},2} = \frac{I}{S}\frac{u_{\mathrm{R},2}}{\varkappa_2} \neq v_{\mathrm{R},1} = E_1 u_{\mathrm{R},1} = \frac{I}{S}\frac{u_{\mathrm{R},1}}{\varkappa_1} \tag{36}$$

$$v_{1,2} = \frac{c_{\mathrm{R},1}v_{\mathrm{R},1} - c_{\mathrm{R},2}v_{\mathrm{R},2}}{c_{\mathrm{R},1} - c_{\mathrm{R},2}} \tag{37}$$

$$v_{\mathrm{B},2} = E_2 u_{\mathrm{B},2} = \frac{I}{S}\frac{u_{\mathrm{B},2}}{\varkappa_2} = v_{1,2} = v_{\mathrm{A},1} = E_1 u_{\mathrm{A},1} = \frac{I}{S}\frac{u_{\mathrm{A},1}}{\varkappa_1} \tag{38}$$

Concentrations

$$c_{B,2} = \frac{u_{B,2}}{u_{B,2} + |u_{R,2}|} \cdot \frac{u_{A,1} + |u_{R,1}|}{u_{A,1}} \cdot c_{A,1} \qquad 39$$

$c_{A,2} = 0$ $\qquad$ $c_{B,1} = 0$

Relationships for calculating the moving boundary velocity $v_{1,2}$ (eqn. 35) are derived from the moving boundary equation (eqn. 34). The migrating velocity of components A, B, R (eqns. 36, 38) are derived from the electrophoretic mobility definition (eqn. 6), with the help of eqn. 12. However, eqns. 38 can also be obtained from eqn. 35 written for ions A or B. It follows that the component which forms the steady-state zone on one side of the moving boundary migrates at the speed $v_{1,2}$ of this boundary. Thus eqns. 38 are the definitions of electrophoretic mobilities which also indicate the method of their measurement.

Component R, which forms steady-state concentration profiles in both zones 1 and 2, does not move at the speed of the boundary and $v_{R,1}$ differs from $v_{R,2}$. After substitution of eqns. 36 into eqn. 35, the relationship between the boundary velocity $v_{1,2}$ and the velocities and concentrations of component R in zones 1 and 2 is obtained (eqn. 37).

The concentrations $c_{B,2}$ and $c_{A,1}$ are not arbitrary but are adjusted to each other by the mobilities of all three components, as seen from eqn. 39. This equation was obtained from eqns. 38 by substitution of the specific conductivity definition (eqn. 12) where the electroneutrality condition $c_{A,1} = c_{R,1}$ and $c_{B,2} = c_{R,2}$ was used. With the help of this electroneutrality condition, eqn. 39 can be rewritten for concentrations $c_{R,1}$ and $c_{R,2}$.

The moving boundary 2 → 1 exhibits a characteristic property, known as a self-sharpening effect. It is supposed that $\varkappa_2 < \varkappa_1$. Hence, it follows from eqn. 12 that $E_2 > E_1$. Thus, if B^+ is pushed from zone 2 to zone 1 by diffusion, its speed decreases due to the lower electric field strength in this zone and the ion is quickly returned to its own zone 2. If, on the other hand, A^+ penetrates into zone 2, the higher electric field strength in this zone will push it back into zone 1. Hence, this self-sharpening effect balances dispersion processes [42].

2.2.3. Unsteady-state migration

The unsteady-state migration of a zone is characteristic phenomenon which always takes place when at least one zone boundary does not show the self-sharpening effect. During the unsteady-state migration, the initially sharp boundaries of the zones are dispersed not only by the diffusion but also by the electromigration itself. The latter effect is known as the electromigration dispersion and it manifests itself by the broadening of zones more quickly than would occur as a result of simple diffusion.

Such a situation can be illustrated with the system of strong electrolytes A^+R^- and B^+R^- already discussed. However, in contrast to Fig. 8, zone 3 containing the ions A^+ with a higher mobility is now behind the less mobile ions B^+ in zone 1 (Fig. 9a). As shown in Fig. 9b, when the electric field is applied, ion A^+ penetrates the rear boundary of zone 1 containing ion B^+ and is further accelerated during electromigration. Thus in addition to the adjusted zone of pure electrolyte A^+R^- (zone 2), a time-variable mixed zone (1–2) containing both A^+ and B^+ is formed.

The solution to partial differential equations of the type eqn. 25, where the diffusion is neglected, is known [41]. The regulating function in the form of eqn. 30 is very important if the concentration distributions in both zones 2 and 1–2 are to be found. For the starting conditions the regulating functions of the respective zones can be written in the forms

for $t = 0$, $x>0$:

$$\omega'_1 = \left(\frac{c_{B,1}}{u_B} + \frac{c_{R,1}}{|u_R|}\right)|u_R| = \frac{u_B + |u_R|}{u_B}\, c_{B,1} \tag{40}$$

and analogously for $t>0$, $x>0$:

$$\omega'_{12} = \frac{u_A + |u_R|}{u_A}\, c_{A,12} + \frac{u_B + |u_R|}{u_B}\, c_{B,12} \tag{41}$$

$$\omega'_2 = \frac{u_A + |u_R|}{u_A}\, c_{A,2} \tag{42}$$

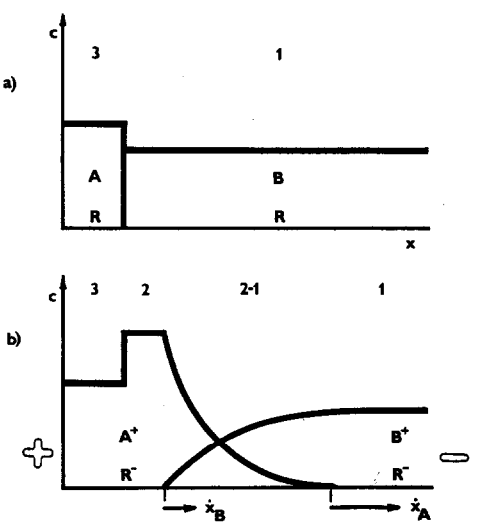

Fig. 9. Evolution of concentration profiles of electrolytes A^+R^- and B^+R^- ($u_A > u_B$) during the unsteady-state electromigration. For explanation, see text.

At the position of this system where zones 1, 1–2, 2 have consecutively taken place during the course of an analysis, the value of the regulating function does not change

$$\omega'_1 = \omega'_{12} = \omega'_2 \tag{43}$$

Combining eqns. 40, 42 and 43, the concentration in zone 2 can be evaluated

$$c_{A,2} = \frac{u_A}{u_A + |u_R|} \cdot \frac{u_B + |u_R|}{u_B} c_{B,1} \tag{44}$$

Compare this with eqn. 39.

The speed of the rear boundary of ion B^+, $\dot{x}_B$, is equal to the speed of the isolated ion B^+ in zone 2

$$\dot{x}_B = u_B E_2 = u_B \frac{I}{S \varkappa_2} \tag{45}$$

and in combination with eqn. 44 it follows

$$\dot{x}_B = \frac{u_B^2 I}{SFu_A(u_B + |u_R|)c_{B,1}} \tag{46}$$

In analogy, it holds for the cation A^+

$$\dot{x}_A = \frac{u_A I}{SF(u_B + |u_R|)c_{B,1}} \tag{47}$$

where $\dot{x}_A$ is equal to the speed of the isolated ion A^+ in zone 1. By combining eqns. 46 and 47 it holds

$$\dot{x}_A = \frac{u_A^2}{u_B^2} \dot{x}_B \tag{48}$$

i.e., the speeds of both boundaries are in the ratio of the squares of their mobilities.

To calculate the composition of the mixed zone 1–2, in addition to the condition $\omega'_{12} = \omega'_1$ still another equation must be solved. By combination of the transport equations of the type eqn. 20, written for ions A^+ and B^+, a partial differential equation with the new variable

$$Z = \omega'/\varkappa \tag{49}$$

can be obtained. The solution of this equation can be found in the form

$$Z = (a/b)^{1/2} \tag{50}$$

where

$$a(x) = \frac{1}{Fu_A u_B} \int_0^x \omega'(x)\mathrm{d}x \tag{51}$$

and

$$b(x) = \frac{1}{S} \int_0^t I\mathrm{d}t \tag{52}$$

In this case, where for $x > 0$ and $t > 0$ it holds that ω = constant and I = constant, the following simple formula is obtained

$$Z(x,t) = \left(\frac{S\omega' x}{Fu_A u_B It}\right)^{1/2} \tag{53}$$

By explicitly expressing the variables ω' and $\varkappa$ in eqns. 43 and 49 and by combining these with eqn. 53, the concentrations of ions A^+ and B^+ in their mixed zone 1–2 are given by

$$c_{A,12}(x,t) = c_{B,1} \frac{u_A}{u_A - u_B} \frac{u_B + |u_R|}{u_A + |u_R|} \left(\frac{1}{Fu_B Z} - 1\right) \tag{54}$$

$$c_{B,12}(x,t) = c_{B,1} \frac{1}{u_A - u_B} \left(u_A - \frac{1}{FZ}\right) \tag{55}$$

The concentration distributions of ions A and B during unsteady-state migration is shown in Fig. 9b [15].

2.3. Effects accompanying electrophoresis

Electrophoresis is always accompanied by several phenomena which can influence the separation of the detector signal. The most important accompanying phenomena are the production of Joule heat, electroosmosis, diffusion and interactions with the boundary walls. The dispersive effects of these phenomena decrease the resolving power of the technique and, therefore, their minimization is of great importance [43].

2.3.1. Joule heat

During the passage of an electric current I through a separation column of cross-section S and length l filled with an electrolyte the conductivity of which is $\varkappa$, the Joule heat produced per unit volume per second is

$$P_v = \frac{EI}{S} = \frac{I^2}{\varkappa S^2} \tag{56}$$

Eqn. 12 is used for the substitution of E.

Owing to the Joule heat produced in the electrolyte, its mean temperature increases, which results in a change in the effective mobilities. Moreover, an excessive elevation of the temperature can cause the destruction of thermolabile compounds, or can give rise to bubbles breaking the current circuit.

For a description of the increase of the steady-state mean temperature of an electrolyte, a parameter $\bar{Q}$ has been introduced [44], defined as

$$\bar{Q} = (\bar{T} - T_0)/P_l \tag{57}$$

where $\bar{T}$ is the steady-state mean temperature of an electrolyte, T_0 is the temperature of a thermostatic medium and P_l is the Joule heat produced in a unit length of a column. The parameter $\bar{Q}$ is easily accessible by experiment where a variable voltage is imposed across an electrophoresis column and the resulting electric current is measured. The deviation from Ohm's law is caused by the heating, *i.e.* by ΔT. Parameter $\bar{Q}$ is useful for a comparison of various column types from the point of view of the column thermostat efficiency.

The Joule heat brings about not only a temperature increase but also the occurrence of temperature gradients [45–50]. Assuming the constant temperature conductivity of the electrolyte λ (J s^{-1} m^{-1} K^{-}), the solution of the equation of the steady-state heat transfer inside the column of circular cross-section is obtained in the form [32]

$$T(r) = \frac{P_v}{4\lambda}(R^2 - r^2) + T_0 \tag{58}$$

where r is a distance from the longitudinal axis of the tube and R its inner radius. Fig. 10 shows this steady-state radial profile of temperature [51].

2.3.2. Electroosmosis

The bulk movement of the liquid towards one or the other of the electrodes due to the electric potential applied is called the electroosmotic flow [52]. When the separation column is filled with an electrolyte, an electric double layer is formed on the inner wall surface due to ionizable groups of the wall material and/or ions of positive or negative charge preferably adsorbed onto the wall.

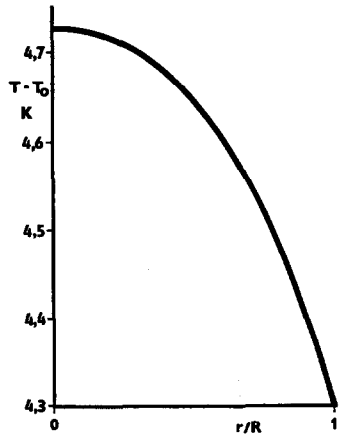

Fig. 10. Radial distribution of temperature increase $T - T_0$ due to Joule heat production in the capillary with inner radius $R = 0.4$ mm (eqn. 58). $P_l = 3.3\ \mathrm{J\ s^{-1}\ m^{-1}}$; $\bar{Q} = 1.36\ \mathrm{K\ m\ s\ J^{-1}}$ [51].

For example, in quartz capillaries, the silanol groups present at the surface form the fixed negative part of the electric double layer [53,54]. Positive ions are then attracted towards the negative surface and negative ions are repelled (Fig. 11). A fraction of the ions forming the electrolyte part of the electric double layer is always fixed by electrostatic forces near the capillary wall and forms the so-called Stern layer. The rest of these ions form the space charge of the mobile diffusive layer. As the condition of electroneutrality is not fulfilled, the space charge of the mobile layer is affected by the electric field. The cylindrical shell of

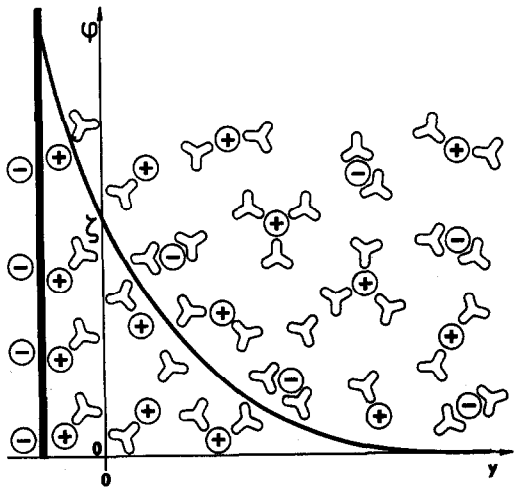

Fig. 11. Schematic representation of the electric double layer, double layer potential ϕ, and ζ potential. Three-pronged symbols are solvent molecules; (■) surface; (|) slipping plane.

the space charge, separated from the stationary Stern layer by a slipping plane ($y = 0$), will move to the respective electrode together with the ion solvation atmosphere. Owing to frictional forces among the solvent molecules, this movement is immediately spread over the whole liquid [40].

At the steady-state the electrostatic force and the frictional force can be equated. For a two-dimensional model with a plane wall

$$\frac{\partial \phi}{\partial x} \rho = \eta \frac{\partial^2 v}{\partial y^2} \tag{59}$$

where ρ is the charge density and x, y are Cartesian coordinates. With the help of the Poisson equation $d^2\phi/dy^2 = -\rho/\varepsilon$ [55], where ε is the electrolyte permitivity and ϕ is the potential of the double layer shown in Fig. 11, the charge density can be substituted in eqn. 59

$$\frac{\partial \phi}{\partial x} \frac{\partial^2 \phi}{\partial y^2} = -\frac{\eta}{\varepsilon} \frac{\partial^2 v}{\partial^2 y} \tag{60}$$

The solution to this equation gives the velocity profile of the electroosmotic flow, with the following boundary conditions: the electrostatic potential of the double layer and the derivative of electroosmotic flow velocity vanish as y tends to infinity; at the slipping plane, where $y = 0$, the velocity is zero and the electrostatic potential has the value ζ (Fig. 1,1), the velocity profile is

$$v(y) = \frac{E\varepsilon}{\eta} [\zeta - \phi(y)] \tag{61}$$

Eqn. 2 was used for rearranging. This equation indicates that the shape of this velocity profile is determined by the double layer potential curve. The characteristic thickness of this is several tens of nanometres. Thus the resulting profile is almost flat [56,57] (Fig. 12). The linear velocity of this plug profile, where $\phi(y) = 0$, is

$$v_{eo} = \frac{\varepsilon \zeta}{\eta} E \tag{62}$$

The term $\varepsilon\zeta/\eta$ is frequently denoted as u_{eo} and named the electroosmotic mobility.

The value of the zeta potential is dependent not only on the surface charge but also on the electrolyte concentration. The higher the electrolyte concentration, the faster the potential drop, and, consequently, the lower the zeta potential. For quartz capillaries the value -100 mV with a 0.001 M water solution of potassium chloride can be assumed. The resulting value of u_{eo} under such conditions is about $71 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} [54].

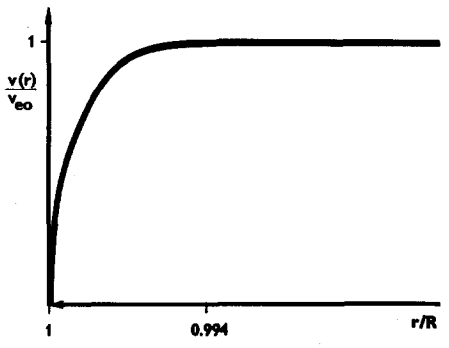

Fig. 12. Dependence of relative electroosmotic velocity $v(r)/v_{eo}$ on relative radial position r/R in a tube.

2.3.3. Diffusion

Diffusion is the motion of a substance under the action of a gradient of chemical potential or, in a simplified way, under the action of a gradient of the concentration of this substance. This movement can be observed macroscopically as a flow of mass due to the existence of its concentration gradient. This can be described phenomenologically as a proportionality of the concentration gradient of component i (dc_i/dx) to its mass flux in a given solvent [58]

$$J_i = -D_i(dc_i/dx) \tag{63}$$

where the diffusion coefficient D_i is the proportionality coefficient which characterizes the ability of the particle to move, or diffuse, in a medium due to chaotic Brownian movement.

This diffusional flux can be compared to the electromigrational flux ($c_i u_i E$), induced by an external electric field, under steady-state conditions when these two fluxes are exactly counterbalanced.

$$c_i u_i E = -D_i(dc_i/dx) \tag{64}$$

Solving this equation under the assumption that E, u_i and D_i are constants, the steady-state concentration distribution is obtained

$$c_i = c_0 \exp(-u_i E x/D_i) \tag{65}$$

where the concentration c_0 (at $x = 0$) is taken as an integration constant.

A very important feature of the diffusion is that there exists a close relationship between the binary diffusion coefficient and the electrophoretic mobility which enables the estimation of data on u_i using data on D_i and *vice versa*. Comparing eqn. 65 with the Boltzmann distribution [55] $c_i = c_o \exp(-U_i/kT)$, where k is the

Boltzman constant, $U_i = z_i e\phi$ is the potential energy and T is the absolute temperature, the Nernst–Einstein equation is obtained with the help of eqn. 2

$$D_i = u_i kT/(z_i e) \tag{66}$$

If the electrophoretic mobility is substituted by eqn. 7, the well known Stokes–Einstein equation is obtained for a sphere of radius r_i

$$D_i = kT/(6\pi\eta r_i) \tag{67}$$

There is an empirical formula for estimating D_i from mobility

$$D_i = 0.025u_i \tag{68}$$

In electrophoresis, diffusion causes band broadening which counteracts the separation. Provided that the sample zone is initially formed as a sharp pulse of analysed substances, then after a certain time t, due to diffusion, the result is a bell-like, Gaussian concentration profile. This profile can be characterized by its variance σ_i^2, which is related to the diffusion coefficient D_i of a substance by the Einstein equation [59]

$$\sigma_i^2 = 2D_i t \tag{69}$$

The change of the variance of the concentration distribution with time can be seen in Fig. 13.

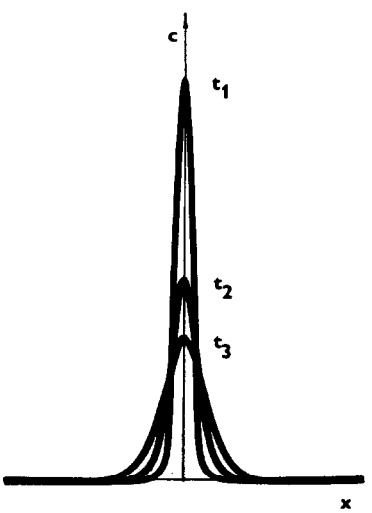

Fig. 13. Change of concentration distribution with time; $t_1 < t_2 < t_3$.

3. ELECTROPHORESIS AS A SEPARATION METHOD

A basic principle of electrophoresis as a migration separation technique is that a mixture (sample) of different substances in solution is forced by the imposed electric field to make a mechanical movement (migration). Then, after a certain time has elapsed, the mixture is separated into zones of individual substances and each of these, at a given time, occupies a certain position along the migration path. This effect is caused by the differences in the effective mobilities of particular substances which, on a first approximation, result in different migration velocities and, consequently, in different resulting positions of these particular substances after a certain time.

The classification of electrophoretic techniques is usually made according to the type of electrolyte system which predetermines the course of the separation process. The most important feature here is whether the separation proceeds in a continuous or discontinuous electrolyte system.

The continuous electrolyte system is represented by the case where the background electrolyte provides an electrically conducting and buffering medium along the migration path. The problem of whether the result of a separation is a kinetic process or a steady state is determined by the variation of the properties of the background electrolyte system along the migration path. If the composition of the background electrolyte is constant, the electric field and the effective mobilities of the sample components are obviously also constant. Hence, different substances migrate with mutually different constant velocities and the result is a kinetic process. Zone electrophoresis may serve here as an example.

If the composition of the background electrolyte is not constant, then both the electric field and the effective mobilities along the migration path change and, as a result of this, the separation process is strongly affected. Above all, from the macroscopic point of view, the migration of various substances can be, in different places along the separation path, practically stopped. An actual example of such a system is isoelectric focusing.

In the discontinuous electrolyte system a sample migrates between two different electrolytes as a distinct individual zone. The conduction of the electric current in the sample zone is exclusively provided by the analysed substances and the counterionic system. Isotachophoresis is an example of this discontinuous electrolyte system.

3.1. Zone electrophoresis

During zone electrophoresis sample ions migrate in the solution of the background electrolyte, the concentration of which is much higher than that of the sample ions, and which conducts practically all the electric current [15]. From the chemical point of view, the background electrolyte is a buffer with a suitable operational pH region or ligand concentration which, subsequently, results in the

buffering of the effective mobilities of the sample components. For a successful separation the sample components must have mutually different effective mobilities resulting in different migration velocities of their zones during the analysis. Zone electropheretic separation is shown schematically in Fig. 14. The analytical information is given not only by the distance travelled by the separated components but also by their concentration distributions or dispersion [60].

For a quantitative description of the mutual separation of two components the resolution R_n is used, defined as the ratio of the distance between the concentration distributions centroids, Δx, to the mean width of the two peaks at the baseline, taken as $4\bar{\sigma}$, where $\bar{\sigma}$ is the mean standard deviation of their concentration distributions [60].

$$R_n = \frac{\Delta x}{4\bar{\sigma}} \tag{70}$$

To characterize the dispersion proceeding along with the migration, the concept of the number of theoretical plates has been adopted [61]

$$N = L^2/\sigma^2 \tag{71}$$

where L is the distance between the injection point and the detector and σ^2 is the total variance of the concentration profile of the zone. The variance is a result of the superposition of all m dispersion processes j which take place during the analysis. Assuming the mutual independence of these processes, the respective variances are additive [61]

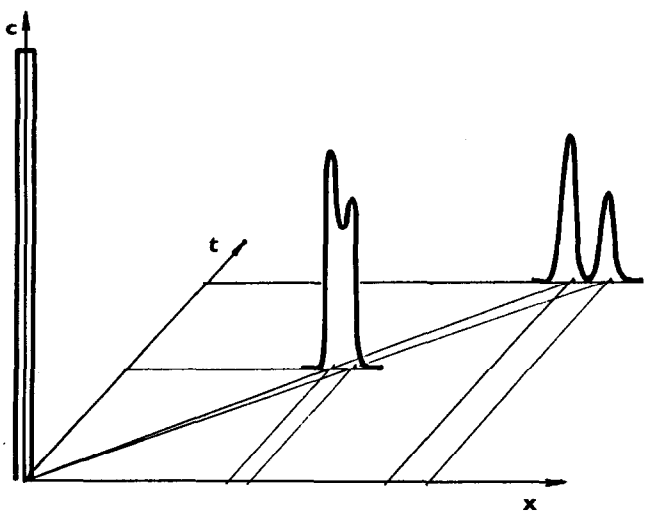

Fig. 14. Space–time scheme of zone electrophoresis separation.

$$\sigma^2 = \sum_{j=1}^{m} \sigma_j^2 = \sum_{j=1}^{m} 2D_j t \cdot \tag{72}$$

where various dispersion processes are characterized by dispersion coefficients D_j.

Assuming the simplified model with the diffusion as the only dispersion phenomenon, the number of theoretical plates can be evaluated from eqns. 69, 71 and the mobility definition eqn. 6 as the dependence of applied voltage $V_a = El$ where l is the total length of the capillary [62]

$$N = uV_a/2D \tag{73}$$

This linear dependence of the separation efficiency was experimentally confirmed for relatively low voltages (less than 30 kV) applied across long and thin capillaries (*ca.* 1 *m* × 75 μm I.D.) [62–65]. In general, however, a non-linear dependence $N = f(V_a)$ with a maximum is obtained [66,67]. Overheating is usually the main reason for the efficiency drop at high voltages shown in Fig. 15.

The radial temperature profile as a result of the Joule heating causes, of course, inhomogeneities in the other physicochemical parameters such as mobilities, pH, density and viscosity. With respect to the already mentioned dependence of the mobilities on temperature (*ca.* 2% per 1 K rise in temperature), the strong effect of Joule heating on the dispersion of zones is clear. The dispersion coefficient due to the Joule heat has been derived in the form [68]

$$D_T = \frac{\varkappa^2 \alpha^2 u^2 E^6 R^6}{3072 D \lambda^2} \tag{74}$$

The dispersion coefficient due to the Joule heat has the same meaning and dimensions as the diffusion coefficient (eqns. 69 and 72).

The dispersion of zones due to electroosmosis is closely related to the instrumental arrangement used. In an open capillary the electroosmotic flow is

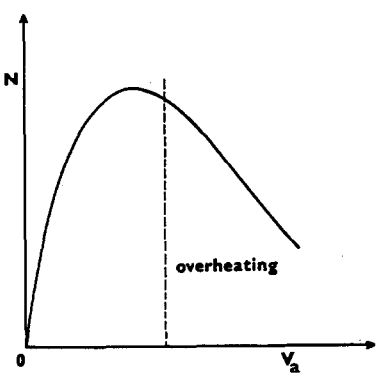

Fig. 15. Dependence of the number of theoretical plates on the applied voltage in zone electrophoresis.

usually considered as non-dispersive. However, in a closed capillary, or in an open capillary with non-uniformly charged walls, the resulting eddy flow can contribute substantially to the dispersion of migrating zones. The respective dispersion coefficient can be calculated by the following relationship [68]

$$D_{eo} = \frac{R^2 \varepsilon^2 \zeta^2 E^2}{48 D \eta^2} \tag{75}$$

Returning to the resolution R_n, it is clear that the difference in peak positions, x, and the mean standard deviations can be evaluated by the mobility definition (eqn. 6) and by the number of theoretical plates (eqn. 71), respectively. Then the resolution R_n is related to the number of theoretical plates by eqn. [60]

$$R_n = \frac{1}{4} \frac{\Delta u}{\langle u \rangle} N^{1/2} \tag{76}$$

where $\Delta u / <u>$ is the relative difference in mobilities of both components. $<u>$ is the average value of the two respective mobilities. Two zones are considered to be well separated if the resolution R_n equals unity. For such a case the required number of theoretical plates can be expressed as follows

$$N_{req} = 16/(\Delta u/\langle u \rangle)^2 \tag{77}$$

It is clear from this equation that the relative difference in effective mobilities, usually called the selectivity, is of key importance for the separation of two substances. For example, the separation of a pair of substances with a relative difference in their effective mobilities of 1% will require 160 000 plates. However, 40 000 plates are sufficient for this separation when the difference is 2% [15].

3.2. Isotachophoresis

For the isotachophoresis a discontinuous electrolyte system is used with the leading electrolyte at the front and the terminating electrolyte at the rear boundary of a sample zone [1,2,5,11].

Various components of the sample are separated and form their own individual zones. After the complete separation is reached, each individual zone contains only one individual substance, and, of course, the common counterion. The zones are attached to one another and migrate at the same velocity, as seen in Fig. 8, therefore the name: iso-tacho = equal speed.

The properties of isotachophoretic zones can be simply described as follows [5]:

1. The zone of ion A migrates behind the leading zone containing the leading

species L; R is the common counter species. Zone A migrates at the same velocity as the leading zone L

$$v = \bar{u}_A E_A = \bar{u}_L E_L \tag{78}$$

2. The driving current density is constant for a constant capillary cross-section

$$i = E_A \varkappa_A = E_L \varkappa_L \tag{79}$$

Isotachophoretic migration represented by a single species in a single zone simplifies the moving boundary equation into the form

$$\frac{\bar{u}_L}{\varkappa_L} = \frac{\bar{u}_A}{\varkappa_A} = ... = \frac{\bar{u}_T}{\varkappa_T} \tag{80}$$

which can be also named as a general isotachophoretic condition.

The evaluation of the separability of substances by isotachophoresis is based on parameters of the $u_{i,j}$ type, *i.e.* the effective mobilities of substance i in the zone of substance j. These values cannot be measured directly and can only be calculated.

The principle of evaluation of the separability of substances on the basis of parameters of the $\bar{u}_{i,j}$ type is basically identical to the principle of the unambiguous determination of the migration sequences of substances. If the migration order can be found unambiguously for a pair of substances i and j and a given electrolyte system, then substances i and j can be separated. A simple criterion can be employed to evaluate the migration order [5].

1. The zone of substance i migrates before the zone of substance j if

$$\bar{u}_{i,j} > \bar{u}_{j,j} \text{ and } \bar{u}_{j,i} < \bar{u}_{i,i} \tag{81}$$

2. The zone of substance j migrates before the zone of substance i if

$$\bar{u}_{i,j} < \bar{u}_{j,j} \text{ and } \bar{u}_{j,i} > \bar{u}_{i,i} \tag{82}$$

If only one inequality is valid for a given pair of substances and selected migration order, then the order cannot be determined unambiguously and the two substances form a stable mixed zone and cannot be separated.

3.3. Isoelectric focusing

Isoelectric focusing is a technique where the sample constituents are separated in a pH gradient according to their isoelectric points [2,6,12,31,69–71]. The pH gradient is generated by the electric current passing through a mixture of carrier

ampholytes, which have isoelectric points in close proximity to each other, placed between electrode chambers containing acidic and basic solutions providing the necessary H^+ and OH^- ions during the run [6]. Any ampholytic substance migrates electrophoretically in the pH gradient in a direction determined by its net charge. After some time, it is focused around a point ξ_i where the pH in the solution is equal to its isoelectric point (pI).

The migration of the substance i in such a system can be described with the help of eqn. 83, expressing the dependence of the mobility u_i on the position along the column [72]

$$u_i = k_i(x - \xi_i) \tag{83}$$

where k_i is a negative constant which reflects the focusing motion bringing a solute back towards a point ξ_i whenever $x \neq \xi_i$. The mobility u_i is positive for $x < \xi_i$ and negative for $x > \xi_i$ (Fig. 16). The constant k_i can be expressed as

$$k_i = \frac{du_i}{d(pH)} \frac{d(pH)}{dx} \tag{84}$$

where $du_i/d(pH)$ is given by the chemical character of the solute and $d(pH)/dx$ describes the slope of the pH gradient and can be regarded as a constant of the apparatus.

After the steady state is reached, the net mass flux is zero and from eqn. 21 with the help of eqn. 12, the following is obtained

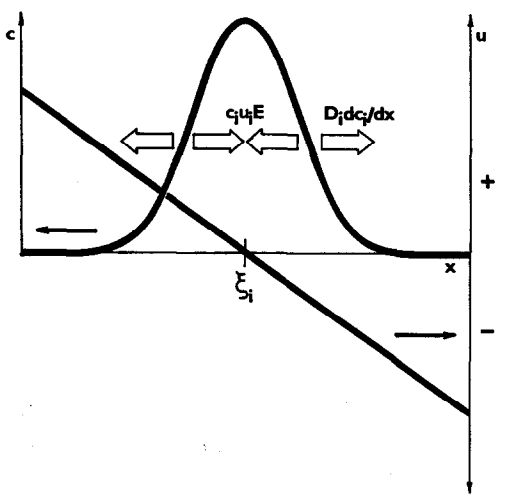

Fig. 16. Dependence of the electrophoretic mobility on distance x (eqn. 83) and the resulting concentration profile of an ampholyte (eqn. 86) in isoelectric focusing.

$$\frac{c(x)u_i(x)I}{S\varkappa} = D_i \frac{dc(x)}{dx} \tag{85}$$

Here the electrophoretic migration and the diffusion are assumed to be the only phenomena affecting particle *i*. By assuming $\varkappa$ to be constant, after integration of this equation, the resulting concentration distribution of the ampholyte is obtained as

$$c/c_0 = \exp\left(-\frac{k_i I(x-\xi_i)^2}{2S\varkappa D_i}\right) \tag{86}$$

Here, c_0 is the concentration at position ξ_i, which is taken as an integration constant. This distribution is a bell-shaped Gaussian curve with the maximum c_0 at position ξ_i (Fig. 16). The outline arrows in Fig. 16 depict the steady state between the flows due to diffusion and electromigration. The variance of this concentration distribution (eqn. 86) is

$$\sigma_i^2 = \frac{D_i S\varkappa}{I\left|\frac{d(pH)}{dx}\right|\left|\frac{du_i}{d(pH)}\right|} \tag{87}$$

The minimum difference in p*I* of two ampholytes which are separated with $R_n = 1$ can be evaluated according to the resolution definition (eqn. 70). By substituting Δx in eqn. 70 with $\Delta pH/[d(pH)/dx]$ and using σ from eqn. 87, $\Delta pH = \Delta pI$ is

$$\Delta pI = 4\left[\frac{D_i\left|\frac{d(pH)}{dx}\right| S\varkappa}{\left|\frac{du_i}{d(pH)}\right| I}\right]^{1/2} \tag{88}$$

The smaller the derivative d(pH)/dx, the smaller the minimum resolvable difference ΔpI in eqn. 88. In practice, with flat pH gradients (1 pH unit difference across the column being several centimetres long), a resolving power of the order of 0.01 pH unit can be reached [6].

3.4. Micellar electrokinetic chromatography

Micellar electrokinetic chromatography (MEKC) is a separation technique combining some of the operational principles of micellar liquid chromatography and capillary zone electrophoresis. This technique may be considered as a type of chromatography with a moving "stationary micellar phase" and with an electroosmotically pumped mobile phase [22,23,26,30,73,74].

When a sample mixture of solutes is injected at one end of the capillary, the migration of solutes in free solution due to both electrophoresis and electroosmosis is superimposed with selective partitioning of the solutes between the free solution and the interior of the micelles. Hence, the complexes formed by the micellar solubilization of solutes provide differences in their effective mobilities and allow their electrophoretic separation. If a micelle possesses an electric charge, even neutral solutes can be separated by electromigration.

At present, micellar phases of sodium dodecyl sulphate and acetyltrimethylammonium bromide are often used for electrophoretic separations. These surfactants are amphophilic molecules composed of a hydrophobic hydrocarbon tail and a hydrophilic head formed by the ionizable group.

In low concentration aqueous solutions the surfactant is mainly present in the monomer form. By the continual addition of the surfactant into the solution the critical micelle concentration can be reached [75,76]; for sodium dodecyl sulphate this is 0.0082 mol/l. At this concentration the monomers start to aggregate on forming micelles. The micelles are roughly spherical and consist typically of 20–100 or more monomers. This number is called the aggregation number. The phenomena occurring in MEKC are schematically depicted in Fig. 17. In aqueous solutions the micelles have the form of drop-like aggregates of surfactant molecules with hydrocarbon tails oriented towards the centre.

The hydrocarbon core of the micelle can serve as a cavity and can reversibly retain a solute. In Fig. 17 it is a neutral, partially solubilized substance P. The polar heads of the surfactant molecules are oriented out of the micelle. Particle N represents here a neutral compound not interacting with micelles which migrates with a velocity given by the electroosmotic flow of only $v_{eo} = Eu_{eo}$ for the respective time t_o. Neutral organic solvents, *e.g.* methanol, used as a sample solvent frequently serve for this purpose. F is a fully solubilized substance. It is completely distributed in the micellar interior and moves with the speed of ionic micelles given by the sum of the electroosmotic mobility and the electrophoretic mobility of micelles $v_m = E(u_{eo} + u_m)$ for time t_m. The directions of the electroosmotic flow and electrophoretic migration of micelles are generally opposite, the elec-

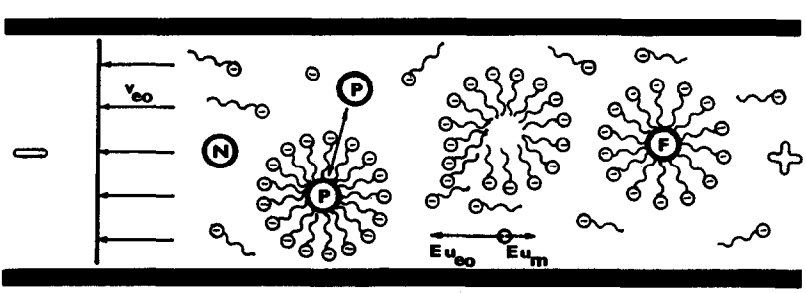

Fig. 17. Schematic representation of the micellar electrokinetic chromatography separation.

troosmotic flow velocity usually being higher than that of electrophoresis of micelles.

The retention time of a neutral substance P can be found by the following consideration. There are a large number of particles P which are distributed between the electrolyte and the micelle cavity in a fast dynamic equilibrium. The fractions in both phases are identical to the respective probabilities $p_e = n_e/(n_e + n_m)$ and $p_m = n_m/(n_e + n_m)$ where n_e and n_m are the total amounts of solute P in the micellar and aqueous phases. Then the mean velocity at which the neutral particle P migrates through the capillary is

$$v_r = p_e v_{eo} + p_m v_m \tag{89}$$

By substituting v_{eo} and v_m with the respective times t_o and t_m and by expressing the probabilities p_e and p_m with the help of capacity factor $\bar{k}' = n_m/n_e$, as is usual in chromatography, the following relationship is obtained for the retention time of substance P [73]

$$t_r = \frac{1 + \bar{k}'}{1 + (t_o/t_m)\bar{k}'} t_o \tag{90}$$

This equation holds true only for non-zero electroosmotic mobility $|u|_{eo}$ which is expected to be higher than $|u|_m$. In the case of a capillary where the electroosmotic flow is completely eliminated, from eqn. 89 for the retention time of substance P, the following is obtained

$$t_r = \frac{(1 + \bar{k}')}{\bar{k}'} t_m \tag{91}$$

This is in fact the effective mobility definition eqn. 13.

3.5. Electrophoresis in sieving media

Electrophoresis in sieving media provides the most powerful method currently available for the analysis of high-molecular-mass molecules of biological origin such as proteins, enzymes, nucleic acids, complex lipids and carbohydrates [3,4,24,27,77]. Despite this, the molecular mechanisms of the separation processes are still not well understood. The present state-of-the-art of the theory gives only a qualitative picture with empirical relationships.

The qualitative description of the movement of biopolymers through a gel network can be divided into three regimes [78]. Fig. 18a shows a polymer coil, with a mean molecular radius, or Stokes' radius R_s, smaller than the average mesh size m. The migration of a polymer chain the size of which is comparable to the distance between the gel fibres (Fig. 18b) is rather different; the random

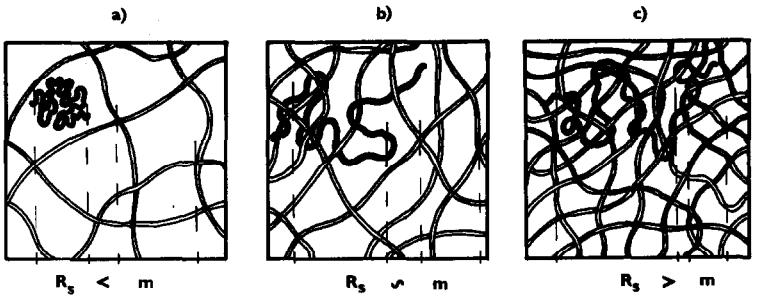

Fig. 18. Illustration of the three entanglement regimes a, b and c. The bold lines represent polymer coils, characterized by a Stokes' radius R_S, migrating among fibres of the gel with an average mesh size m.

polymer coil is weakly entangled in the gel network. A macromolecule much more longer than the average mesh spacing of the gel will be strongly entangled (Fig. 18c). Thus the character of the migration is given not only by the molecular mass of a solute but also by its size, the gel concentration and the degree of cross-linking [78].

Fig. 19 shows schematically the dependence of the electrophoretic mobility of the polyelectrolyte on the molecular mass. The range of molecular masses covers the three regimes of migration in Fig. 18.

The migrating polymer in regime a (Fig. 19) can be considered to be a rigid sphere undergoing biased Brownian movement. The separation, under these conditions, has been explained by a sieving model, where the sphere mobility is regarded as proportional to the probability of the interaction of the molecule with

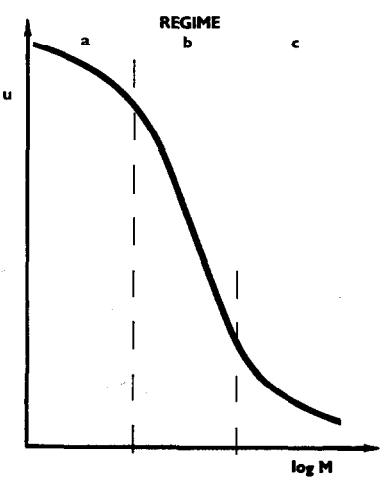

Fig. 19. Schematic dependence of polymer electrophoretic mobility u on log M for the three entanglement regimes a, b and c.

the porous network. As the probability is an exponential function of the gel concentration and the sphere size, the following relationship between electrophoretic mobility and gel concentration has been established [3,4,79,80]

$$\log u = \log u_f - K_r c_g \tag{92}$$

where u_f is the electrophoretic mobility in free electrolyte, c_g is the total gel concentration (monomer + cross-linking agent) and K_r is the retardation coefficient, which is constant for any specified charged solute at a constant percentage of cross-linking of the gel. K_r is dependent on pH and the solvation shell. There is an empirical linear relationship between the retardation coefficient and the molecular mass M [3,4,79,80]

$$K_r = A + BM \tag{93}$$

where A and B are empirical constants.

Eqns. 92 and 93 are based on the assumption that a spherical particle collides with the gel matrix at only one place at a specific time; *i.e.* the polymer chain is not entangled with the matrix [79].

It is evident that such a model cannot describe the polymer chain behaviour in the regimes b and c (Fig. 19). In the latter region, the electrophoretic migration of the macromolecule is strongly affected by the entanglement. It is assumed that the polymer chain undergoes one-dimensional snakelike motion in a tube formed by the pores in the three-dimensional mes of the gel. A general feature of this model, known as a reptation model [24], is that the electrophoretic mobility is inversely proportional to the molecular mass M of the polymer [81]

$$u = A/M \tag{94}$$

where A is constant for a given gel concentration and is approximately inversely proportional to the gel concentration.

The most pronounced drop of the electrophoretic mobility with molecular mass is in regime b (Fig. 19). In other words, the selectivity is the highest here. Experimental results show that both the sieving and reptation theories are inadequate for describing the mobilities of weakly entangled flexible macromolecules.

A very important role is played here by the random regions in the gel that are either very dense, or relatively open. These dense regions can be regarded as entropic barriers to translation. The polymer chain can relax in the open regions where the configurational entropy is high, whereas it must squeeze through denser gel regions where the chain entropy is lower. Thus the entropically regulated transport mechanism brings about such a high sensitivity to the molecular mass [78].

Obviously gel electrophoresis separates molecules on the basis of differences in

both charge and size and the method can be used for measuring molecular mass. Of course, this measurement is not absolute; therefore a suitable calibration is necessary.

There are two principle techniques used. In the first a ligand such as sodium dodecyl sulphate is used to swamp out the initial charges and to give the molecules an approximately constant charge to mass ratio [3,4]. The differences in mobilities of the various solutes are then only due to differences in size. The procedure is easy and is based on adding a sufficient amount of sodium dodecyl sulphate to the protein sample and to the background electrolyte. This ligand is strongly bound to protein (approximately 1.4 g of sodium dodecyl sulphate per 1 g of protein) and gives the protein molecule a net negative charge, making the intrinsic charge on the protein chain insignificant. The resulting net charge per unit mass becomes approximately constant and, under such conditions, the electrophoretic mobility of the polypeptide molecule is dependent only on the effective molecular radius or on the molecular mass. The higher the molecular weight, the lower the electrophoretic mobility.

One of the major theoretical obstacles in the interpretation of the results of this analytical technique is that the detailed structure of the complexes in solution has not been uniquely resolved. Consequently this technique is not an absolute analytical method and calibration is unavoidable for molecular mass determinations.

The second technique is based on mobility measurement in gels of different concentrations, where the pure effect of molecular size can be determined [3,4,82,83]. The basis of this technique is the linear relationship between the gel concentration and the logarithm of the electrophoretic mobility in eqn. 92. If the mobility is measured for various gel concentrations, at least seven for high-precision demands, then the plot of log u *versus* the gel concentration c_g is a straight line with slope K_r. This dependence is known as the Ferguson plot (Fig. 20). This linear region corresponds to the sieving model which is an adequate theoretical description for the migration regime a, as has already been discussed.

The plots in Fig. 20 are typical examples of an analysis of macromolecules A, B, C and D which differ in molecular mass and charge. The higher the slope, or K_r, of the plot, the higher the molecular mass. Compare the lines for molecules C, A and D, B. Molecules A and B have the same molecular mass but the electrical charge of B is higher. The same is true for molecules C and D [4].

It is important that all the conditions, *e.g.* the percentage of cross-linking agent, polymerization conditions, pH, ionic strength, temperature and buffer compositions are kept constant during the measurements in various gel concentrations.

K_r is closely related to the molecular weight (eqn. 93). It was shown experimentally that this relationship is linear over a molecular mass range of at least 45 000–500 000. Eqn. 93 is, in fact, a calibration line and the respective constants A and B are found with the help of molecular mass standards.

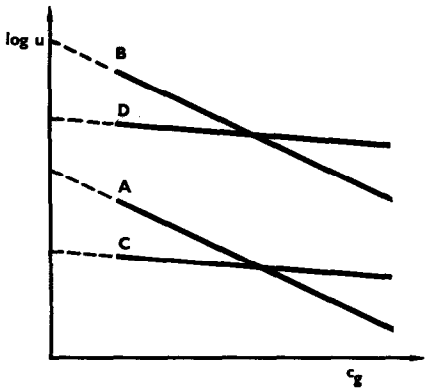

Fig. 20. Ferguson plots of macromolecules A, B, C and D which differ in molecular mass and the charge: $M_B > M_D$; $M_A > M_C$; $z_B > z_A$; $z_D > z_C$.

4. CONCLUSIONS

Electrophoretic methods, the theoretical background of which is described in this paper, are among the most successful analytical methods of the last decade. During this period great attention has been paid to capillary versions of these techniques. Electrophoresis has thus become a competitor or complement to high-performance liquid chromatography. Electrophoretic methods are especially suitable for solving problems in biochemistry, biology, biomedicine, pharmacology and biotechnology. The range of these methods spans from the separation of small inorganic and organic ions and non-ionic solutes, to biopolymers and even whole cells and organic and inorganic particles. Amino acids, nucleic acid bases, nucleosides, nucleotides, peptides, proteins, nucleic acids, subcellular particles, viruses, erythrocytes and other blood cells and latex particles have all been separated using electrophoresis.

A theoretical insight into the problems of any analytical method is unavoidable, not only for those who are working on the design of an apparatus or methodology, but also for users who need to optimize analytical conditions and interpret both correct and unwanted results. No instruction nor apparatus manual mention all the possible situations which arise in practice. Therefore, optimization and troubleshooting are always a result of creative work based on theoretical knowledge and experience.

The objective of any analytical technique is to gain information about the chemical composition of a test sample in a reasonable period of time. To provide such information using separation techniques, there has to be an adequate resolution of the bands of the separated components. In other words, conditions need to be chosen at which the analysis will proceed with a sufficient separation efficiency and selectivity.

In electrophoretic techniques, the electrolyte systems must be optimized first. The selected electrolytes must satisfy the requirements of optimum conductivity,

pH, mobility and concentration to suppress the production of Joule heat and electrophoretic dispersion. Sufficient differences in the effective mobilities of components to be separated must also be provided. In capillary electrophoretic techniques, which are in great progress now, some additional problems may arise from the effects of the inner capillary wall. Here, well pretreated surface showing constant physico-chemical properties (first of all chemical, sorption and electroosmotic activity) is of paramount importance not only for obtaining reproducible experimental results but also for successful applying the theory to optimize separation. It is evident that the black-box approach to this task cannot be entirely successful in these techniques.

SYMBOLS AND UNITS

c	Molar concentration (kmol m^{-3})
$\bar{c}$	Total analytical concentration, eqn. 13 (kmol m^{-3})
c_g	Total gel concentration (%)
D	Diffusion coefficient, eqn. 63 (m^2 s^{-1})
D_T	Dispersion coefficient due to Joule heat, eqn. 74 (m^2 s^{-1})
D_{eo}	Dispersion coefficient due to electroosmosis, eqn. 75 (m^2 s^{-1})
e	Elementary charge of an electron ($1.602 \cdot 10^{-19}$ C)
E	Electric field intensity, eqn. 2 (V m^{-1})
f	Arbitrary function
F	Faraday constant (96 487 C mol^{-1})
F_d	Drag force, eqn. 3 (kg m s^{-2})
F_e	Force induced by electric field, eqn. 1 (kg m s^{-2})
i	Electric current density (A m^{-2})
I	Electric current (A)
J	Mass current density, or mass flux, eqn. 18 (kmol m^{-2} s^{-1})
k	Boltzmann constant ($1.38054 \cdot 10^{-23}$ J K^{-1})
k_i	Proportionality constant
k'	capacity factor (1)
K	acid–base dissociation constant
K_r	Retardation coefficient, eqn. 92
l	Total length of the separation column (m)
L	Distance between injection point and detector (m)
m	Average mesh size of a gel (m)
M	Molecular mass
n_e	Number of particles in electrolyte
n_m	Number of particles in micelles
N	Number of the theoretical plates, eqn. 71
p_e	Probability that the particle is in the electrolyte
p_m	Probability that the particle is in a micelle
P_l	Joule heat production in unit length of column (J m^{-1} s^{-1})
P_v	Joule heat production in unit volume of column, eqn. 56 (J m^{-3} s^{-1})

$\bar{Q}$ Column cooling efficiency, eqn. 57 (K m s J^{-1})
r Cylindrical coordinate, distance from column axis (m)
r_i Sphere radius (m)
R Inner radius of a tube (m)
R_i Molar production of i by a chemical reaction in unit volume, eqn. 20 (kmol m^{-3} s^{-1})
R_s Stokes' radius (m)
R_n Resolution, eqn. 70
S Cross-section (m^2)
t Time (s)
T Temperature (K)
$\bar{T}$ Mean temperature (K)
T_o Temperature of a thermostating medium (K)
U Potential energy (J)
u Electrophoretic mobility, eqn. 6 (m^2 V^{-1} s^{-1})
$\bar{u}$ Effective electrophoretic mobility, eqn. 13 (m^2 V^{-1} s^{-1})
u^o Limiting electrophoretic mobility (at zero concentration) (m^2 V^{-1} s^{-1})
u_{eo} Electroosmotic mobility, eqn. 62 (m^2 V^{-1} s^{-1})
u_f Electrophoretic mobility in free electrolyte, eqn. 92 (m^2 V^{-1} s^{-1})
$<u>$ Average mobility (m^2 V^{-1} s^{-1})
v Velocity (m s^{-1})
v_{eo} Velocity induced by electroosmotic flow, eqn. 62 (m s^{-1})
v_f Velocity of hydrodynamic flow (m s^{-1})
V_a Applied voltage (V)
x Cartesian coordinate (m)
x_i Molar fraction of i, eqn. 13
$\dot{x}$ Velocity (m s^{-1})
y Cartesian coordinate (m)
z_i Valence of ionic species i
α Temperature coefficient of electrophoretic mobility, eqn. 8 (K^{-1})
Δ Difference
ε Permitivity (J^{-1} C^2 m^{-1})
ξ Potential of the double layer at the slipping plane (V)
η Viscosity coefficient (Pa s)
$\varkappa$ Specific conductivity, eqn. 12 (A V^{-1} m^{-1})
λ Temperature conductivity (J s^{-1} m^{-1} K^{-1})
υ Ionic strength (kmol m^{-3})
ξ Focusing point, eqn. 83 (m)
ρ Charge density (C m^{-3})
σ Standard deviation of the concentration distribution (m)
$\bar{\sigma}$ Average standard deviation (m)
ϕ Electric potential (V)
ω Kohlrausch regulating function, eqn. 29 (kmol V s m^{-5})
ω' Regulating function, eqn. 30 (kmol m^{-3})

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