

Multiphasic Zone Electrophoresis. I. Steady-State Moving-Boundary Systems Formed by Different Electrolyte Combinations†

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ABSTRACT: A theory of *multiphasic zone electrophoresis* is developed with which the systematic and rational generation of discontinuous buffer systems for analytical and preparative electrophoresis is made possible. Its basis is the application and extension of the weak electrolyte moving-boundary theory. The primary concept is that of a moving boundary separating homogeneous phases with one common and two dissimilar constituents and generally with different pH's. Such boundaries under steady-state conditions are not subject to degradation by diffusion. It is shown that in addition to the Kohlrausch regulating function for weak electrolytes defined by Dismukes and Alberty [(1954), *J. Amer. Chem. Soc.* 76, 191], $\bar{\omega} = \Sigma \bar{c}_j / r_j$, two other "regulating functions" are constant in successive phases separated by a moving boundary and containing only monovalent ions, acids, and bases: $\omega_1 = \Sigma \bar{c}_j$ and $\omega_2 = \Sigma c_{j(0)}$, where \bar{c}_j and $c_{j(0)}$ are the constituent concentrations and the concentrations of the uncharged

species, respectively, with signs corresponding to the charge of the ionic species of the constituent. Six cases designated as basic "*electrophoretic units*" are described for systems with three constituents comprising two phases and a moving boundary between them. They differ in the combination of constituent classes: weak monovalent and divalent electrolytes, uni- and multivalent ions. For each phase, the concentrations, pH, conductance, and related parameters are specified, and the boundary is defined in terms of ν , the boundary displacement; μ , the ratio of concentrations of the dissimilar constituents in the two phases; and the quantity ω_1 or, in the case of multivalent electrolytes, the moving-boundary equation for the third common constituent. Limits of pH and ionic mobilities are specified within which boundaries remain stable. These results are applied to the generation of integrated buffer systems in the following papers of this series.

The introduction of starch gel (Smithies, 1955) and polyacrylamide gel (Raymond and Weintraub, 1959; Ornstein and Davis, 1962) as supporting media in zone electrophoresis led to the development of analytical and preparative techniques with great resolving power. A "molecular sieving" effect has been presumed chiefly responsible for the improved fractionation of macromolecules, particularly proteins. In the case of so-called "disc electrophoresis," the inherent advantages of the polyacrylamide gel matrix are enhanced by the use of planned buffer discontinuities which lead to the concentration of the sample into a thin starting zone prior to the resolving phase of the electrophoretic experiment. A preliminary theoretical treatment of this technique was given by Ornstein (1964) and stimulated the present more general theoretical development.

The technical aspects and strategy of polyacrylamide gel electrophoresis and the physical-chemical bases for fractionation in restrictive media have been reviewed recently (Chrambach and Rodbard, 1971; Maurer, 1971). It is the purpose of this communication and subsequent parts of this series (Jovin, 1973a-c) to help satisfy the demand for buffer systems throughout the pH range and possessing desired characteristics for use in both preparative and analytical techniques. The term *multiphasic zone electrophoresis* is used rather than "disc electrophoresis" because it is felt that the term is more descriptive of the processes involved and does not imply any particular geometrical configuration. The theory is not in-

herently restricted to methods with rigid supporting media but has equal applicability to "free" electrophoretic techniques. Due to the necessity for involved calculations, it was found imperative to implement the theory in the form of a digital computer program which is described elsewhere (Jovin, 1973c). An output of 4269 systems is available (Jovin *et al.*, 1970; Dante and Chrambach, 1972).

Basic to the following treatment is the application and extension of the weak electrolyte moving-boundary theory developed by Alberty and others (Alberty, 1950; Dismukes and Alberty, 1954; Nichol *et al.*, 1958). Certain aspects of the problem have been treated also by Everaerts and Routs (Everaerts and Routs, 1971; Routs, 1971) in connection with the technique of "isotachopheresis" (Haglund, 1970).

Definitions

The dimensions of the following quantities are indicated within parentheses and define a consistent system of units. Greek letter superscripts indicate the phase to which the quantity applies. An *electrophoretic phase* is defined as a homogeneous solution demarcated by moving and/or stationary boundaries.

\bar{c}_j = constituent concentration of constituent j . Signed quantity, possessing the sign of the ion subspecies of the constituent. A constituent consists of a nondissociable species and all forms in equilibrium with it (M).

$c_{j(i)}$ = concentration of subspecies of constituent j with valence i . Signed (M).

u_j = ion mobility of the ion subspecies of constituent j . Signed according to valence. For divalent weak electrolytes, the mobility of the univalent ion species is designated as

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$u_{j|1|}$ and that of the divalent ion species as $u_{j|2|}$ ($\text{cm}^2 \text{sec}^{-1} \text{V}^{-1}$).

r_j = relative ion mobility of the ion subspecies of constituent j = ratio of u_j to the mobility of the sodium ion determined at the same ionic strength and temperature. Signed. Same convention for divalent weak electrolytes as for u_j (dimensionless).

\bar{u}_j = constituent mobility of constituent j . Signed quantity, representing the average mobility for the entire constituent ($\text{cm}^2 \text{sec}^{-1} \text{V}^{-1}$).

\bar{r}_j = relative constituent mobility of constituent j . Signed (dimensionless).

κ = specific conductance (mhos cm^{-1}).

σ = relative conductance (C cm^{-3}).

ν = boundary displacement, volume swept by a moving-boundary during passage of one coulomb. Signed quantity, positive if the moving-boundary moves in the same direction as the current, *i.e.*, toward the cathode. A double superscript is used to indicate both phases adjoining the moving boundary ($\text{cm}^3 \text{C}^{-1}$).

$\Gamma/2$ = ionic strength.

BV = buffer value (Edsall and Wyman, 1958) (equiv l^{-1}).

E = electric field strength = - potential gradient (V cm^{-1}).

I = current density = current per unit cross-sectional area (A cm^{-2}).

ϕ_j = fraction of constituent j dissociated into ion subspecies of greatest valence (dimensionless).

The following formulas correspond to the definitions given above.

$$\bar{c}_j = \sum^n c_{j(i)} \quad (1)$$

n = number of subspecies of constituent j

$$\bar{u}_j = \frac{\sum^n u_{j(i)} c_{j(i)}}{\bar{c}_j} \quad (2)$$

$$\bar{r}_j = \frac{\sum^n r_{j(i)} c_{j(i)}}{\bar{c}_j} \quad (3)$$

$$\kappa = \frac{F}{1000} \sum^k \sum^n u_{j(i)} c_{j(i)} |i| = 96.5 \sum^k \sum^n u_{j(i)} c_{j(i)} |i| \quad (4)$$

k = number of constituents in the phase

$$\sigma = 96.5 \sum^k \sum^n r_{j(i)} c_{j(i)} |i| \quad (5)$$

$$\kappa \cong \sigma u_{\text{Na}^+} \quad (6)$$

The mobility u_{Na^+} corresponds to the ionic strength and temperature of the phase (Anderson, 1949).

$$\Gamma/2 = \frac{1}{2} \sum^k \sum^n c_{j(i)} |i|^2 \quad (7)$$

$$BV = 2.303 \sum^k \phi_j (1 - \phi_j) |\bar{c}_j| \quad (8)$$

$$E = I/\kappa \quad (9)$$

Assumptions

Implicit in some of the above formulas and necessary for the following theoretical treatment are certain assumptions: (1) constant relative ion mobilities; (2) zero mobilities for uncharged species; (3) additivity of ion mobilities; (4) unitary activity coefficients; (5) negligible contributions of H^+ and OH^- to conductance ($3 < \text{pH} < 11$); (6) constancy of the apparent acid dissociation constants; in practice, values representative for the ionic strengths to be encountered are selected; (7) ideal steady-state moving boundaries; (8) negligible effect of the polyacrylamide supporting medium on buffer ion mobilities (Giddings and Boyack, 1962);¹ (9) constant cross-sectional area; (10) absence of bulk flow of solvent.

Moving-Boundary Equation and Regulating Functions

The moving-boundary equation for weak electrolytes has been derived by Alberty (1950) in the form

$$\bar{u}_j^\alpha \bar{c}_j^\alpha / \kappa^\alpha - \bar{u}_j^\beta \bar{c}_j^\beta / \kappa^\beta = \nu^{\alpha\beta} (\bar{c}_j^\alpha - \bar{c}_j^\beta) \quad (10)$$

Under the assumptions stated above, an alternative expression is

$$\bar{r}_j^\alpha \bar{c}_j^\alpha / \sigma^\alpha - \bar{r}_j^\beta \bar{c}_j^\beta / \sigma^\beta = \nu^{\alpha\beta} (\bar{c}_j^\alpha - \bar{c}_j^\beta) \quad (11)$$

These equations apply to a constituent j in phases α and β separated by a moving boundary with displacement $\nu^{\alpha\beta}$. In essence, they are statements of the conservation of mass.

For systems containing monovalent acids and bases exclusively, Dismukes and Alberty (1954) derived a function $\bar{\omega}$ referred to as the Kohlrausch regulating function for weak electrolytes

$$\bar{\omega} = \sum^k \bar{c}_j / r_j \quad (12)$$

The function $\bar{\omega}$ has the same value in two phases separated by a moving boundary and can be used in place of one moving-boundary equation for calculating the composition of an unknown phase.

It is possible to demonstrate, however, the existence of other "regulating functions" which are simpler in form and provide the same constraint. From eq 1-3 it is seen that for monovalent acids and bases

$$\bar{u}_j \bar{c}_j = u_j c_{j(\pm 1)} \text{ and } \bar{r}_j \bar{c}_j = r_j c_{j(\pm 1)} \quad (13)$$

The moving-boundary eq 11 can be rewritten as

$$r_j c_{j(\pm 1)}^\alpha / \sigma^\alpha - r_j c_{j(\pm 1)}^\beta / \sigma^\beta = \nu^{\alpha\beta} (\bar{c}_j^\alpha - \bar{c}_j^\beta) \quad (14)$$

The equations for all constituents k are summed

$$\sum^k [r_j c_{j(\pm 1)}^\alpha / \sigma^\alpha - r_j c_{j(\pm 1)}^\beta / \sigma^\beta] = \nu^{\alpha\beta} \left[\sum^k \bar{c}_j^\alpha - \sum^k \bar{c}_j^\beta \right] \quad (15)$$

Applying the definition of the relative conductance (eq 5)

$$\sum^k [r_j c_{j(\pm 1)}^\alpha / \sigma^\alpha - r_j c_{j(\pm 1)}^\beta / \sigma^\beta] = \frac{1}{96.5} (\sigma^\alpha / \sigma^\alpha - \sigma^\beta / \sigma^\beta) = 0 \quad (16)$$

¹ A. Chrambach, E. Hearing, J. Lunney, and D. Rodbard, submitted for publication.

It follows, therefore, that

$$\nu^{\alpha\beta} \left[\sum_j^k \bar{c}_j^\alpha - \sum_j^k \bar{c}_j^\beta \right] = 0 \quad (17)$$

In the case of a moving boundary, $\nu^{\alpha\beta} \neq 0$, and thus

$$\sum_j^k \bar{c}_j^\alpha = \sum_j^k \bar{c}_j^\beta \quad (18)$$

This equality defines a new function

$$\omega_1 = \sum_j^k \bar{c}_j \quad (19)$$

which, as is the case with $\bar{\omega}$, is constant in successive phases separated by a moving boundary. Since the constituent concentrations are signed, in their summation the ionic concentrations cancel out due to the electroneutrality restriction. Hence, it follows directly from definition 19 above that the sum of the concentrations of uncharged species is also constant from one phase to the next. It is therefore possible to define another regulating function

$$\omega_2 = \sum_k c_{j(0)} \quad (20)$$

It is interesting that eq 20 follows directly from the moving boundary equation and need not be assumed as has been done previously (Alberty, 1950; Dismukes and Alberty, 1954). Furthermore, it applies to mixtures of weak acids and bases in the same phase. In practice, the function ω_1 is more useful since it immediately yields one constituent concentration if all others in the adjoining phases are known.

It is important to emphasize that none of the regulating functions described above are applicable to systems containing multivalent ions due to the lack of the equivalence expressed in eq 13. In these instances, it is necessary to use all the moving-boundary equations.

An important finding in the derivation of the function $\bar{\omega}$ by Dismukes and Alberty is the fact that all ionic constituent subspecies are diluted by the same factor across a stationary boundary ($\nu^{\alpha\beta} = 0$). It does not follow, however, that the hydrogen ion also obeys this requirement since it is not the sole ionic form of any constituent. As Svensson (1948) has pointed out, it is neither necessary nor expedient to apply the moving-boundary equation to the hydrogen or hydroxyl ion constituents.

Acid-Base Relationships

The purpose of this section is to state relationships which will be useful in the discussion of solutions containing different types of weak electrolytes. The following kinds of buffer constituents are considered: (a) monovalent ions; (b) multivalent ions; (c) monovalent weak acids and bases; (d) divalent weak acids and bases with nonoverlapping dissociations, *i.e.*, $pK_2 - pK_1 > 3$. The nature of a constituent is a function of pH. Thus above pH 8, the acetate ion alone is regarded as a constituent, the conjugate acid being virtually nonexistent. Likewise, in the case of a divalent weak electrolyte at a pH near pK_2 , only the univalent and divalent ion species need be considered.

It is desirable to formulate a theoretical treatment applicable to systems in which the generated moving boundaries migrate toward the anode or the cathode. To this end, the symbol \pm

is used to denote the sign of the ion valence for certain constituents. The + sign identifies constituents as being cations or bases and denotes migration of the moving boundaries toward the cathode. The - sign identifies constituents as anions or acids and indicates migration toward the anode. In all formulas containing \pm symbols, the convention will be to use that sign corresponding to the specified direction of migration. The significance of the reciprocal symbol \mp is obvious.

In the cases described below, only phases with two constituents are considered. All pK values designate apparent acid dissociation constants. The function θ , the absolute value of the ratio of constituent concentrations in a phase, is introduced at this time. Its utility will become apparent in the consideration of interphase relationships. All expressions are derived directly from the definitions, acid-base equilibria and the electroneutrality restriction.

Case I

Constituent	Valences of subspecies	pK
1 uni- or multivalent ion	$\pm m (m \geq 1)$	
2 monovalent weak electrolyte	$0, \mp 1$	pK_2

$$\phi_1 = 1 \quad (21)$$

$$\phi_2 = c_{2(\mp 1)} / \bar{c}_2 = 1 / [1 + 10^{\pm(pK_2 - pH)}] = m / \theta \quad (22)$$

$$pH = pK_2 \pm \log [1 / (1 / \phi_2 - 1)] =$$

$$pK_2 \pm \log [1 / (\theta / m - 1)] \quad (23)$$

$$\theta = -\bar{c}_2 / \bar{c}_1 = m / \phi_2 = m [1 + 10^{\pm(pK_2 - pH)}] \quad (24)$$

$$\Gamma/2 = \frac{1}{2} |\bar{c}_1| m(1 + m) \quad BV = 2.303 |\bar{c}_2| \phi_2 (1 - \phi_2) \quad (24a)$$

Case II

Constituent	Valences of subspecies	pK
1 monovalent weak electrolyte	$0, \pm 1$	pK_1
2 monovalent weak electrolyte	$0, \mp 1$	pK_2

$$\phi_1 = 1 / [1 + 10^{\pm(pH - pK_1)}] = \theta \phi_2 \quad (25)$$

$$\phi_1 = |a| - [a(a + b)]^{1/2} \quad (26)$$

where $a = (1/2)(1 + \theta)/(1 - \rho)$, $b = -2\theta/(1 + \theta)$, and $\rho = 10^{\pm(pK_2 - pK_1)}$. For $pK_1 = pK_2$, $\phi_1 = \theta/(1 + \theta)$.

$$\phi_2 = 1 / [1 + 10^{\pm(pK_2 - pH)}] = \phi_1 / \theta \quad (27)$$

$$\theta = -\bar{c}_2 / \bar{c}_1 = \phi_1 / \phi_2 = \phi_1 [1 + \rho \phi_1 / (1 - \phi_1)] \quad (28)$$

$$pH = pK_1 \pm \log (1 / \phi_1 - 1) = pK_2 \pm \log [1 / (1 / \phi_2 - 1)] \quad (29)$$

$$\Gamma/2 = |\bar{c}_1| \phi_1 \quad BV = 2.303 [|\bar{c}_1| \phi_1 (1 - \phi_1) + |\bar{c}_2| \phi_2 (1 - \phi_2)] \quad (29a)$$

Case III

Constituent	Valences of subspecies	pK
1 divalent weak electrolyte	$\pm 1, \pm 2$	pK_1
2 monovalent weak electrolyte	$0, \mp 1$	pK_2

$$\phi_1 = c_1(\pm 2)/\bar{c}_1 = 1/[1 + 10^{\pm(pH - pK_1)}] = \theta\phi_2 - 1 \quad (30)$$

$$\phi_1 = \left| |a| - [a(a + b)]^{1/2} \right| \quad (31)$$

where $a = (1/2)(\theta + \rho)/(1 - \rho)$, $b = -2(\theta - 1)/(\theta + \rho)$, and $\rho = 10^{\pm(pK_2 - pH)}$. For $pK_1 = pK_2$, $\phi_1 = (\theta - 1)/(\theta + 1)$.

$$\phi_2 = 1/[1 + 10^{\pm(pK_2 - pH)}] = (1 + \phi_1)/\theta \quad (32)$$

$$\theta = -\bar{c}_2/\bar{c}_1 = (1 + \phi_1)/\phi_2 = (1 + \phi_1)[1 + \rho\phi_1/(1 - \phi_1)] \quad (33)$$

$$pH = pK_1 \pm \log(1/\phi_1 - 1) = pK_2 \pm \log[1/(1/\phi_2 - 1)] \quad (34)$$

$$\Gamma/2 = |\bar{c}_1|(1 + 2\phi_1) \quad BV = 2.303[|\bar{c}_1|\phi_1(1 - \phi_1) + |\bar{c}_2|\phi_2(1 - \phi_2)] \quad (34a)$$

Moving-Boundary Systems Formed by Different Electrolyte Combinations: "Electrophoretic Units"

In discontinuous zone electrophoresis systems, several moving boundaries are formed. It is convenient to initially analyze as separate entities the different types of two-phase moving-boundary systems that are encountered and which are designated here as "electrophoretic units."

In all cases, these conditions hold: (a) all phases contain two constituents and one of these is common to the two phases separated by a moving boundary; (b) two constituents disappear across each moving boundary; (c) all moving boundaries migrate under steady-state equilibrium conditions, i.e., they do not demonstrate progressive spreading tendencies.

A phase is considered defined when the nature of the constituents and their constituent concentrations are known since all other parameters are derivable. A constituent is specified by a pK in the case of a weak electrolyte, and the relative ion mobilities and valences of its subspecies. The functions utilized in describing phases are \bar{c} 's, θ , pH, ϕ 's, κ , σ , $\Gamma/2$, BV , and three quantities concerned with the moving boundary and the equilibria across it: ν (boundary displacement), μ (a function derived by condensing the moving-boundary equations for the two constituents disappearing across the boundary), and ω_1 (or the moving-boundary equation for the third common constituent in the case of multivalent electrolytes).

The following systems or electrophoretic units consist of two phases, α (containing constituents 1 and 3) and β (containing constituents 2 and 3), separated by a moving boundary migrating into phase β . The direction of the current is not specified since it depends on which alternative (\pm) for constituent 1 is selected. The field strength in a phase cannot be specified, of course, since it is a function of the applied current.

Case I

Constituent	Valence of subspecies	pK
1 monovalent weak electrolyte	$0, \pm 1$	pK_1
2 uni- or multivalent ion	$\pm m(m \geq 1)$	
3 monovalent weak electrolyte	$0, \mp 1$	pK_2

A. Constituent Concentration Relationships. Due to the fact that \bar{c}_1^β and $\bar{c}_2^\alpha = 0$, the moving-boundary eq 11 applied to constituents 1 and 2 reduces to the form

$$\bar{r}_1^\alpha = \nu^{\alpha\beta}\sigma^\alpha \quad (35)$$

$$\bar{r}_2^\beta = \nu^{\alpha\beta}\sigma^\beta \quad (36)$$

Applying definitions, the electroneutrality restriction and eq 21, it is evident that

$$\bar{r}_1^\alpha = r_1\phi_1^\alpha \quad (37)$$

$$\bar{r}_2^\beta = r_2 \quad (37a)$$

$$\bar{r}_3^\alpha = r_3\phi_3^\alpha \quad \bar{r}_3^\beta = r_3\phi_3^\beta \quad (37b)$$

$$\sigma^\alpha = 96.5\bar{c}_1^\alpha\phi_1^\alpha(r_1 - r_3) \quad (38)$$

$$\sigma^\beta = 96.5m\bar{c}_2^\beta(r_2 - r_3) \quad (39)$$

Division of eq 35 by eq 36, substitution with the expressions 37–39, and rearrangement lead to the equation

$$\mu^{\alpha\beta} \equiv \bar{c}_1^\alpha/\bar{c}_2^\beta = m \frac{(1 - r_3/r_2)}{(1 - r_3/r_1)} \quad (40)$$

This expression defines the function μ which possesses interesting properties in that it is relatively insensitive to (a) temperature, due to the fact that the relative mobilities occur as ratios, (b) r_3 . A good approximation is

$$\mu^{\alpha\beta} \cong \frac{m}{2}(1 + r_1/r_2) \quad (41)$$

for usual values of r_3, r_1, r_2 . By definition

$$\omega_1^{\alpha\beta} = \bar{c}_1^\alpha + \bar{c}_3^\alpha = \bar{c}_2^\beta + \bar{c}_3^\beta \quad (42)$$

for $m = 1$. Equations 40 and 42 permit the calculation of the two constituent concentrations of one phase (α or β) if those of the other phase are known and if constituent 2 is a univalent ion.

In order to derive the corresponding relations for $m \neq 1$, the moving-boundary equation for constituent 3 is written in rearranged form

$$\bar{c}_3^\beta(1 - \bar{r}_3^\beta/\nu^{\alpha\beta}\sigma^\beta) = \bar{c}_3^\alpha(1 - \bar{r}_3^\alpha/\nu^{\alpha\beta}\sigma^\alpha) \quad (43)$$

Substitution with the eq 35, 36, 37 and the electroneutrality restriction lead to the expressions

$$\theta^\alpha = -\bar{c}_3^\alpha/\bar{c}_1^\alpha = 1 + (\theta^\beta - m)/\mu^{\alpha\beta} \quad (44)$$

$$\theta^\beta = -\bar{c}_3^\beta/\bar{c}_2^\beta = m + (\theta^\alpha - 1)\mu^{\alpha\beta} \quad (45)$$

Equations 40, 44 and 45 are used, therefore, to solve for phase constituent concentrations for any m .

The values that θ^α and θ^β may assume are not arbitrary. Equation 24 states that $\theta^\beta = m/\phi_3^\beta$. Since $0 < \phi_3^\beta < 1$, it follows that

$$\theta^\beta > m \quad (46)$$

Substituting this inequality into eq 45 leads to the additional restriction

$$\theta^\alpha > 1 \quad (47)$$

B. pH Relationships. Equations 21–29 present alternative ways of determining the acid–base parameters of each phase provided the proper subscripts and superscripts are used. They also permit the derivation of expressions for the difference in the pH's of the two phases. The following version is derived directly from eq 23, 27, 29, 40, and 45

$$\pm(\text{pH}^\beta - \text{pH}^\alpha) = \log \frac{(\theta^\alpha/\phi_1^\alpha - 1)(1 - r_3/r_1)}{(\theta^\alpha - 1)(1 - r_3/r_2)} \quad (48)$$

It is apparent that the pH difference is independent of m and that it decreases for a decreasing ratio r_2/r_1 and increasing ϕ_1^α . It also follows that

$$r_2/r_1 = \frac{\phi_1^\alpha(\theta^\alpha - 1)}{\theta^\alpha[1 - (1 - \phi_1^\alpha)r_1/r_3] - \phi_1^\alpha} \quad (49)$$

for $\text{pH}^\alpha = \text{pH}^\beta$, and

$$\pm(\text{pH}^\beta - \text{pH}^\alpha) > 0 \quad (50)$$

for $r_2/r_1 > \phi_1^\alpha$.

An important consequence of eq 47 will now be demonstrated

$$\theta^\alpha = \phi_1^\alpha/\phi_3^\alpha = \frac{1 + 10^{\pm(\text{p}K_3 - \text{pH}^\alpha)}}{1 + 10^{\pm(\text{pH}^\alpha - \text{p}K_1)}} > 1$$

and, therefore

$$\pm(\text{p}K_3 + \text{p}K_1 - 2\text{pH}^\alpha) > 0 \quad (51)$$

C. Conditions for Steady-State Moving Boundary. Two conditions must hold in order for a steady-state moving-boundary $\alpha\beta$ to exist: (a) the velocities of constituents 1 and 2 must be greater in phase α than in phase β ; (b) the velocity of constituent 2 must be greater than that of constituent 1 in both phases. It will be demonstrated below (case II) that an equivalent statement of these requirements is

$$\bar{r}_2/\bar{r}_1 > 1 \quad (52)$$

in both phases α and β , or

$$r_2/r_1 > \phi_1^\alpha, \phi_1^\beta \quad (\phi_2^\beta = 1)$$

where ϕ_1^β is the fractional dissociation of a small amount of constituent 1 placed in a solution with the composition and thus the pH of phase β .

From eq 50 it follows directly that for $r_2/r_1 > \phi_1^\alpha$, $\phi_1^\alpha > \phi_1^\beta$. Hence, the necessary and sole requirement for a steady-state moving boundary is

$$r_2/r_1 > \phi_1^\alpha \quad (53)$$

Case II

Constituent	Valence of subspecies	pK
1 monovalent weak electrolyte	0, ± 1	pK ₁
2 monovalent weak electrolyte	0, ± 1	pK ₂
3 monovalent weak electrolyte	0, ∓ 1	pK ₃

A. Constituent Concentration Relationships. Equations 35–45 hold ($m = 1$) with the exception of eq 37a and 39

$$\bar{r}_2^\beta = r_2\phi_2^\beta \quad (54)$$

$$\sigma^\beta = 96.5\bar{c}_2^\beta\phi_2^\beta(r_2 - r_3) \quad (55)$$

The functions θ^α and θ^β can assume values less than 1 but lower limits can be stated as for case I

$$\theta^\alpha > \phi_1^\alpha \text{ and } \theta^\beta > \phi_2^\beta \quad (56)$$

By use of eq 44 both of these restrictions can be stated in terms of θ^α

$$\theta^\alpha > \begin{cases} \phi_1^\alpha \\ 1 - (1 - \phi_2^\beta)/\mu^{\alpha\beta} \end{cases} \quad (57)$$

B. pH Relationships. The acid–base parameters are calculated with eq 25–29. The expression for the difference in pH's of the two phases now has the form

$$\begin{aligned} \pm(\text{pH}^\beta - \text{pH}^\alpha) = \\ \pm(\text{p}K_2 - \text{p}K_1) + \log(1/\phi_2^\beta - 1)/(1/\phi_1^\alpha - 1) \end{aligned} \quad (58)$$

C. Conditions for Steady-State Moving Boundary. Alberty (1950) has stated that the sole condition for a steady-state moving boundary of the type under consideration is that constituent 2 have a higher velocity in the α than in the β phase, or

$$|\bar{u}_2^\alpha E^\alpha| > |\bar{u}_2^\beta E^\beta| \quad (59)$$

where \bar{u}_2^α is the constituent mobility that a trace of constituent 2 would possess when placed in the α phase. Alberty stated further that this inequality cannot be expressed in terms of relative mobilities. That this is not the case and that eq 59 is a necessary but not sufficient condition for a steady-state moving boundary will now be demonstrated.

Moving-boundary eq 10 for constituents 1 and 2 reduces to a form similar to that of eq 35 and 36

$$\bar{u}_1^\alpha = \nu^{\alpha\beta}\kappa^\alpha \text{ and } \bar{u}_2^\beta = \nu^{\alpha\beta}\kappa^\beta \quad (60)$$

In view of relation 9, introduction of the above expressions into inequality 59 leads directly to the statement

$$\bar{u}_2^\alpha/\bar{u}_1^\alpha = \bar{r}_2/\bar{r}_1 > 1 \quad (61)$$

Substituting with eq 37 and 54, it is seen that an equivalent expression for condition 59 is

$$r_2/r_1 > \phi_1^\alpha/\phi_2^\alpha \quad (62)$$

But constituent 1 also disappears across the moving boundary $\alpha\beta$ and it follows that a necessary condition is that its velocity be greater in the α phase than in the β phase, or

$$|\bar{u}_1^\alpha E^\alpha| > |\bar{u}_1^\beta E^\beta| \quad (63)$$

By a process identical to that above, this inequality reduces to the form

$$r_2/r_1 > \phi_1^\beta/\phi_2^\beta \quad (64)$$

It is now possible to state the *general requirement for a steady-state moving boundary* as

$$r_2/r_1 > \phi_1/\phi_2 \quad (65)$$

in both phases α and β . Alternative forms of expression 65, obtained by use of eq 29, will be useful later in the discussion

$$\pm(pK_2 - pK_1) > \log \frac{(r_1/r_2 - \phi_2)}{(1 - \phi_2)} \quad (66)$$

$$\pm(pK_2 - pK_1) > \log \frac{(1 - \phi_1)}{(r_2/r_1 - \phi_1)} \quad (67)$$

in both phases α and β . These two inequalities are equivalent but eq 66 is more applicable to phase β and eq 67 to phase α . It is evident from eq 67 that the ratio r_2/r_1 must be greater than the largest value for ϕ_1 , whether that be achieved in phase α or β .

Case III

Constituent	Valence of subspecies	pK
1 monovalent weak electrolyte	0, ± 1	pK_1
2 divalent weak electrolyte	± 1 , ± 2	pK_2
3 monovalent weak electrolyte	0, ∓ 1	pK_3

A. Constituent Concentration Relationships. The expressions for the relative constituent mobility of constituent 1 and the conductance of phase α are identical to these for case I. Equation 36 holds for constituent 2 as well but two new relationships follow directly from the definitions and the electroneutrality restriction.

$$\bar{r}_2^\beta = r_{21}(1 - \phi_2^\beta) + r_{22}\phi_2^\beta = r_{21} + (r_{22} - r_{21})\phi_2^\beta \quad (68)$$

$$\sigma^\beta = 96.5\bar{c}_2^\beta[r_{21} - r_3 + (2r_{22} - r_{21} - r_3)\phi_2^\beta] \quad (69)$$

Division of eq 35 by 36 and substitution with the expressions for the relative constituent mobilities and relative conductances lead to an equation corresponding to 40 but which unlike the latter is a function of pH

$$\mu^{\alpha\beta} \equiv \bar{c}_1^\alpha/\bar{c}_2^\beta = \frac{[1 + b + (2a + b - 1)\phi_2^\beta]}{[1 + (a - 1)\phi_2^\beta](1 + c)} \quad (70)$$

where $a = r_{22}/r_{21}$, $b = -r_3/r_{21}$, and $c = -r_3/r_1$. The problem in evaluating eq 70 is that in general, ϕ_2^β is not known initially. It is possible, however, to obtain an expression for ϕ_2^β which is only a function of the relative mobilities, pK 's, and θ^α . Combining eq 28, 33, 35, 36, 43, 68, and 70, and applying the electroneutrality restriction, the following cubic equation for ϕ_2^β is derived

$$(\phi_2^\beta)^3 + \frac{B}{A}(\phi_2^\beta)^2 + \frac{C}{A}\phi_2^\beta + \frac{D}{A} = 0 \quad (71)$$

where $a = r_{22}/r_{21}$, $b = r_3/r_{21}$, $c = -r_3/r_1$, $d = -(c + \theta^\alpha)/(1 + c)$, $e = d(1 + b)$, $f = d(2a + b - 1)$, $\rho = 10^{\pm(pK_3 - pK_2)}$, $A = (a - 1)(1 - \rho)$, $B = 1 + b + f - a\rho$, $C = 1 + b + f - a\rho$, $D = 1 + e - a - f - \rho$, $D = -(1 + b + e)$. This equation is solved numerically (e.g., by Newton's method) to yield ϕ_2^β . It is then possible to evaluate $\mu^{\alpha\beta}$.

The derivation of eq 71 also yields the following relations

$$\theta^\beta = -\bar{c}_3^\beta/\bar{c}_2^\beta = \mu^{\alpha\beta}(c + \theta^\alpha) - b(1 + \phi_2^\beta)/[1 + (a - 1)\phi_2^\beta] \quad (72)$$

$$\theta^\alpha = -\bar{c}_3^\alpha/\bar{c}_1^\alpha = \{\theta^\beta + b(1 + \phi_2^\beta)/[1 + (a - 1)\phi_2^\beta]\}/\mu^{\alpha\beta} - c \quad (73)$$

where a , b , and c have the values defined in eq 71. Alternatively, it is possible to calculate θ^β by using ϕ_2^β to obtain pH^β (eq 34), computing ϕ_3^β (eq 32), and substituting into expression 33. This method is unreliable for a value of ϕ_2^β close to 1, however, and in this instance it is preferable to use eq 72, then compute ϕ_3^β and pH^β .

Lower bounds for the θ functions are apparent by inspection of eq 28 and 33

$$\theta^\beta > 1 + \phi_2^\beta \quad (74)$$

$$\theta^\alpha > \phi_1^\alpha \quad (75)$$

By use of eq 71, both of these restrictions can be stated in terms of θ^α

$$\theta^\alpha > \left\{ \frac{\phi_1^\alpha}{[1 + b + (a - 1)\phi_2^\beta](1 + \phi_2^\beta)(1 + c)} - c \right\} \equiv G \quad (76)$$

where a , b , and c have the values defined in eq 71. It is important to note that

$$\lim G = 1 \text{ as } \phi_2^\beta \rightarrow 0 \text{ or } 1 \quad (77)$$

and

$$G < 1 \text{ for } 0 < \phi_2^\beta < 1 \quad (78)$$

By differentiating G with respect to θ_2^β , it is found that a minimal value for G is achieved with

$$\phi_2^\beta = [h(1 + h)]^{1/2} - h \equiv H \quad (79)$$

where $h = (1 + b)/(2a + b - 1)$. Utilizing various values for a and b , a remarkable constancy in H is revealed

$$H = 0.37 \pm 0.03 \quad (80)$$

Introducing this value for ϕ_2^β in eq 76, it can be demonstrated that values for G do not decrease appreciably below 0.9, *i.e.*

$$\theta^\alpha > G \geq 0.9 \quad (81)$$

B. pH Relationships. The acid-base parameters are calculated with eq 25–34. Equation 58 holds as well.

C. Conditions for Steady-State Moving Boundary. The same arguments used in case II lead to the conclusion that a steady-state moving boundary is achieved with

$$\bar{r}_2/\bar{r}_1 > 1 \text{ in both phases } \alpha \text{ and } \beta \quad (82)$$

or

$$[r_{21} + (r_{22} - r_{21})\phi_2]/r_1\phi_1 > 1 \quad (83)$$

Equations similar in form to 66 and 67 follow directly from eq 83

$$\pm(pK_2 - pK_1) > \log \frac{(r_2 - r_{21}/\phi_1)(1 - \phi_1)}{(r_{22} - r_1\phi_1)} \quad (84)$$

$$\pm(pK_2 - pK_1) > \log \frac{(r_1/[r_{21} + (r_{22} - r_{21})\phi_2] - 1)}{(1/\phi_2 - 1)} \quad (85)$$

Equations 84 and 85 are equivalent, but the former is more useful for phase α and the latter for phase β . In practice, it is not difficult to fulfill these conditions due to the nature of the electrolytes. It is evident that steady-state conditions are met regardless of pK_2 if $r_{21}/r_1 > 1$.

Case IV

Constituent	Valence of subspecies	pK
1 divalent weak electrolyte	$\pm 1, \pm 2$	pK_1
2 uni- or multivalent ion	$\pm m (m \geq 1)$	
3 monovalent weak electrolyte	$0, \mp 1$	pK_3

A. Constituent Concentration Relationships. Equations 35, 36, and 39 apply in intact form but obvious modifications must be made in 68 and 69

$$\bar{r}_1^\alpha = r_{11}(1 - \phi^\alpha) + r_{12}\phi_1^\alpha = r_{11} + (r_{12} - r_{11})\phi_1^\alpha \quad (86)$$

$$\sigma^\alpha = 96.5\bar{c}_1^\alpha[r_{11} - r_3 + (2r_{12} - r_{11} - r_3)\phi_1^\alpha] \quad (87)$$

The same process described for the previous cases leads to

$$\mu^{\alpha\beta} \equiv \bar{c}_1^\alpha/\bar{c}_2^\beta = m \frac{[1 + (a - 1)\phi_1^\alpha](1 + c)}{[1 + b + (2a + b - 1)\phi_1^\alpha]} \quad (88)$$

where $a = r_{12}/r_{11}$, $b = -r_3/r_{11}$ and $c = -r_3/r_2$. If ϕ_1^α is specified, as will be the case in the later discussion, the direct evaluation of eq 88 is possible. One can also derive a cubic equation for ϕ_1^α in terms of θ^β , similar to eq 71 except that in the expression for d , the value m appears as a coefficient of c .

Substitution of the above relations into eq 43, as in the previous cases, provides the θ functions

$$\theta^\beta = \mu^{\alpha\beta}\{\theta^\alpha + b(1 + \phi_1^\alpha)/[1 + (a - 1)\phi_1^\alpha]\} - mc \quad (89)$$

$$\theta^\alpha = (\theta^\beta + mc)/\mu^{\alpha\beta} - b(1 + \phi_1^\alpha)/[1 + (a - 1)\phi_1^\alpha] \quad (90)$$

where a , b , and c are defined in eq 88.

Lower bounds for these functions are

$$\theta^\beta > m \quad (91)$$

$$\theta^\alpha > 1 + \phi_1^\alpha \quad (92)$$

B. pH Relationships (same as for Case III).

C. Conditions for Steady-State Moving Boundary.

In this case, the general restriction 61 reduces to

$$r_2/\bar{r}_1 > 1 \quad (93)$$

or

$$r_2/r_{11} > 1 + (r_{12}/r_{11} - 1)\phi_1 \quad (94)$$

in both phases α and β .

Since $r_{12}/r_{11} > 1$, it follows that $r_2/r_{11} > 1$ is a minimal condition and that furthermore, for $r_2/r_{12} > 1$, a steady-state moving boundary will exist regardless of phase pH's. By using eq 30 for ϕ_1^β and 34 for pH^α , two additional versions of eq 94 can be derived

$$\pm(pH - pK_1) > \log [(r_{12}/r_{11} - r_2/r_{11})/(r_2/r_{11} - 1)] \quad (95)$$

in both phases α and β .

$$\pm(pH^\beta - pH^\alpha) > \log \frac{(r_{12}/r_{11} - r_2/r_{11})}{(r_2/r_{11} - 1)(1/\phi_1^\alpha - 1)} \quad (96)$$

Inequality 96 states the necessary condition for satisfaction of 93 in phase β alone and does not imply a corresponding fulfillment in phase α .

In the discussion to follow, ϕ_1^α will in general be known and the problem will be to select a constituent 2 which can fulfill the above conditions. Since pH^β is itself a function of r_2 , eq 94–96 are not directly applicable to phase β . This difficulty is overcome by eliminating pH from eq 95 through the use of relation 23 which supplies pH^β as a function of θ^β . Substitution with eq 89 and 86 leads to the expression

$$r_2/r_{11} > p + [p^2 + ab/(1 + q)]^{1/2} \quad (97)$$

where

$$q = \frac{[1 + b + (2a + b - 1)\phi_1^\alpha]10^{\pm(pK_2 - pK_1)}}{[1 + (a - 1)\phi_1^\alpha](\theta^\alpha - 1) - a\phi_1^\alpha}$$

$$p = \frac{1}{2}(q + b - a)/(1 + q)$$

a , b defined in eq 88

The conditions for a steady-state moving boundary are thus represented by eq 94 (with $\phi_1 = \phi_1^\alpha$) and 97, the former applying to phase α and the latter to phase β . Both inequalities must be satisfied.

Although m does not appear explicitly in the above relations, its significance becomes apparent in the fact that r_2 tends to be greater with increasing m .

Case V

Constituent	Valence of subspecies	pK
1 uni- or multivalent ion	$\pm m_1 (m_1 \geq 1)$	
2 uni- or multivalent ion	$\pm m_2 (m_2 \geq 1)$	
3 monovalent weak electrolyte	0, ∓ 1	pK ₃

For this case, only expressions for $\mu^{\alpha\beta}$ and θ^β will be derived. Equations 35 and 36 apply as they have for each preceding case. The other relationships are

$$\bar{r}_1^\alpha = r_1 \quad (98)$$

$$\bar{r}_2^\beta = r_2$$

$$\sigma^\alpha = 96.5 m_1 \bar{c}_1^\alpha (r_1 - r_3) \quad (99)$$

$$\sigma^\beta = 96.5 m_2 \bar{c}_2^\beta (r_2 - r_3) \quad (100)$$

These equations yield

$$\mu^{\alpha\beta} = \bar{c}_1^\alpha / \bar{c}_2^\beta = \frac{m_2(1 - r_3/r_2)}{m_1(1 - r_3/r_1)} \quad (101)$$

Substitution of eq 35, 36, 98, and 101 into eq 43, the third moving-boundary equation, yields

$$\begin{aligned} \theta^\beta &= \mu^{\alpha\beta} (\theta^\alpha - m_1 r_3/r_1) + m_2 r_3/r_2 \\ &= \left[\frac{(\theta^\alpha/m_1 - r_3/r_1)(1 - r_3/r_2)}{(1 - r_3/r_1)} + r_3/r_2 \right] m_2 \end{aligned} \quad (102)$$

In all the five cases discussed above, the common constituent 3 has been a monovalent weak electrolyte. It is possible, however, to use an ion as constituent 3 under certain conditions (case VI), a fact of considerable practical importance because it allows the generation of complete electrophoretic buffer systems which are simpler chemically and potentially less harmful to sensitive biological materials.

Case VI

Constituent	Valence of subspecies	pK
1 monovalent weak electrolyte	0, ± 1	pK ₁
2 monovalent weak electrolyte	0, ± 1	pK ₂
3 uni- or multivalent ion	$\mp n$	

This case is indistinguishable from case II except that certain relationships are necessarily altered

$$\begin{aligned} \bar{r}_3^\alpha &= \bar{r}_3^\beta = r_3 \text{ since } \phi_3 \equiv 1 \\ \theta^\alpha &= \phi_1/n \text{ and } \theta^\beta = \phi_2/n \end{aligned} \quad (103)$$

Thus, the conditions analogous to 57 but with a reversal in the inequality are

$$\theta^\alpha < \begin{cases} 1/n \\ 1 + (1/n - 1)/\mu^{\alpha\beta} \end{cases} \quad (104)$$

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Appendix

In order to illustrate the preceding theoretical discussion with a concrete example, I shall analyze a specific instance of a case I electrophoretic unit. Let the constituents and their pertinent characteristics be as follows

Constituent	Valence of subspecies	pK	r
1 glycine	0, -1	9.74	-0.72
2 Cl ⁻	-1		-1.55
3 Tris	0, +1	8.07	+0.50

where the pK and relative ion mobilities r apply to 25° and $\Gamma/2 = 0.01$ (Jovin, 1973c).

Consider a solution (phase β) of composition 0.0617 M Tris and 0.06 M HCl. It follows from the definitions that $\bar{c}_2^\beta = -0.06$, $\bar{c}_3^\beta = 0.0617$, and thus $\theta^\beta = -\bar{c}_3^\beta/\bar{c}_2^\beta = 1.028$ (eq 24), and $\text{pH}^\beta = 6.52$ (eq 23). Also $\phi_2^\beta = 1$ (eq 21) and $r_2^\beta = r_2 = -1.55$ (37a).

A second solution containing Tris and glycine at arbitrary concentrations is now layered over phase β and a current is applied with the anode in contact with the original solution. Assume for the moment that a steady-state moving boundary forms which progressively sweeps out a new phase (designated α) as it traverses phase β toward the anode. The boundary separates constituents 1 and 2 and thus phase α contains the constituents 1 and 3, that is, glycine and Tris. The characteristics of phase α are solely determined by those of phase β . The function $\mu^{\alpha\beta} = \bar{c}_1^\alpha/\bar{c}_2^\beta = (1 - r_3/r_2)/(1 - r_3/r_1) = 0.78$ (eq 40) and thus $\bar{c}_1^\alpha = -0.06 \times 0.78 = -0.0468$, $\bar{c}_3^\alpha = \bar{c}_2^\beta + \bar{c}_3^\beta - \bar{c}_1^\alpha = 0.0485$ (eq 42), and hence $\theta^\alpha = -0.0485/(-0.0468) = 1.036$. (Alternatively, eq 44 could have been used to obtain θ^α .) It follows directly that $\phi_1^\alpha = 0.13$ (eq 26) and $\text{pH}^\alpha = 8.91$ (eq 29).

The original steady-state moving-boundary assumption is confirmed since we note that the condition (eq 53) $r_2/r_1 = 2.15 > \phi_1^\alpha = 0.13$ has been fulfilled.

All other parameters for phases α and β are calculated readily from the appropriate formulas, and the boundary displacement $v^{\alpha\beta}$ is obtained from either eq 35 or 36.

The above example corresponds in actual fact to part of the well-known discontinuous buffer system for polyacrylamide gel electrophoresis described by Ornstein (1964) and Davis (1964). The given phase β has the composition of the gel unit designated in the literature by the various terms "spacer, upper, stacking, or concentrating gel." It should be noted, however, that the phase α resulting from phase β , as calculated above, does not correspond to the Tris-glycine "upper buffer" originally specified by Ornstein and Davis with a pH of 8.3. The same phase α forms independently of the upper buffer, but that it cannot achieve pH 8.3, regardless of the composition of phase β , is seen immediately from eq 51 which states that the minimum pH $^\alpha$ possible (corresponding to $\theta = 1$) is $(\text{pK}_1 + \text{pK}_3)/2 = 8.9$, a value necessarily close to that actually calculated for $\theta^\alpha = 1.036$.

It is precisely this feature of "retrograde regulation" which is most characteristic of the systems or electrophoretic units described above. That is, the phase trailing the moving boundary is uniquely defined by the phase into which the

boundary migrates. It remains to be noted with respect to the above example that altering the overall concentration of a phase but not the ratio of the constituents, *i.e.*, θ , obviously does not affect those quantities which depend only on θ such as the ϕ 's and pH. Thus doubling the concentration of phase β would lead to a corresponding doubling of the constituent concentrations of the calculated phase α and a halving of the boundary displacement $v^{\alpha\beta}$.

To avoid confusion, it is worthwhile to point out that constituent 3, phase α , and the original Tris-glycine solution in the above example correspond to constituent 6, phase ζ , and phase α , respectively, in the nomenclature used in the subsequent paper of this series (Jovin, 1973a).²

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² The Tris-glycine system is discussed further in Jovin (1973a).

Multiphasic Zone Electrophoresis. II. Design of Integrated Discontinuous Buffer Systems for Analytical and Preparative Fractionation†

Thomas M. Jovin

ABSTRACT: The theory of multiphasic zone electrophoresis introduced previously is applied to the design of discontinuous buffer systems for the analytical and preparative separation of macromolecules. The features of stacking or preconcentration of the sample into a thin starting zone and the requirements for the subsequent unstacking and resolution phase of electrophoresis are treated extensively. The design of an integrated system proceeds from the specification of desired characteristics to the selection of the necessary ion and

buffer constituents, and, finally, to the optimization of the operational conditions. Two new classes of buffer systems are considered. In the first, the stacking process occurs between phases in which the leading and trailing constituents are both monovalent weak electrolytes. The additional feature in the second case is the use of an ion as the common constituent throughout the system. In both instances, the systems have favorable functional characteristics and are simpler in design and use than the conventional system.

Since its inception, polyacrylamide gel electrophoresis has become one of the most potent tools in the analytical and preparative repertoire of the biochemist (Chrambach and Rodbard, 1971). The great resolution of the technique derives both from the phenomenon of restricted migration, responsive to molecular size and shape (Rodbard and Chrambach, 1970), and to the use of multicomponent discontinuous buffer sys-

tems which lead to sample preconcentration (Ornstein, 1964; Davis, 1964).

General qualitative guidelines for the design of discontinuous buffer systems have been given by Williams and Reisfeld (1964). Owing to the nature of the electrophoretic process in multicomponent chemical systems, however, it is necessary to apply quantitative methods of analysis in order to specify adequately any given system.

It is the purpose of this communication to describe a procedure for the systematic design of discontinuous buffer systems which operate satisfactorily throughout the pH range. Such systems consist essentially of various phases sep-

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