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 $\nu^{\alpha\beta}$.

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]

References

Alberty, R. A. (1950), *J. Amer. Chem. Soc. 72*, 2361.

Anderson, E. A. (1949), Ph.D. Dissertation, Univ. of Wisconsin, Madison, Wis.

Chrambach, A., and Rodbard, D. (1971), *Science 172*, 440.

Dante, M. L., and Chrambach, A. (1972), *Science 175*, 95.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Dismukes, E. B., and Alberty, R. A. (1954), *J. Amer. Chem. Soc. 76*, 191.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, New York, N. Y., Academic Press.

Everaerts, F. M., and Routs, R. J. (1971), *J. Chromatogr. 58*, 181.

Giddings, J. C., and Boyack, J. R. (1962), *J. Theoret. Biol. 2*, 1.

Haglund, H. (1970), *Sci. Tools 17*, 2.

Jovin, T. M. (1973a), *Biochemistry 12*, 879.

Jovin, T. M. (1973b), *Biochemistry 12*, 890.

Jovin, T. M. (1973c), *Ann. N. Y. Acad. Sci.* (in press).

Jovin, T. M., Dante, M. L., and Chrambach, A. (1970), Multiphasic Buffer Systems Output, Springfield, Va., National Technical Information Service, PB No. 196085 to 196092 and 203016.

Maurer, H. R. (1971), Disc Electrophoresis, New York, N. Y., Walter de Gruyter.

Nichol, J. C., Dismukes, E. B., and Alberty, R. A. (1958), *J. Amer. Chem. Soc. 80*, 2610.

Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.

Ornstein, L., and Davis, B. J. (1962), *in* Disc Electrophoresis, Rochester, N. Y., Distillation Products Industries.

Raymond, S., and Weintraub, L. (1959), *Science 130*, 711.

Routs, R. J. (1971), Ph.D. Dissertation, Eindhoven University of Technology, Eindhoven, Netherlands.

Smithies, O. (1955), *Biochem. J. 61*, 629.

Svensson, H. (1948), *Acta Chem. Scand. 2*, 841.

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

General qualitive 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-

† From the Abteilung Molekulare Biologie, Max-Planck-Institut fuer Biophysikalische Chemie, 34 Goettingen, Germany. *Received May 23, 1972*. This work was done in part during the tenure of an Established Investigatorship of the American Heart Association.

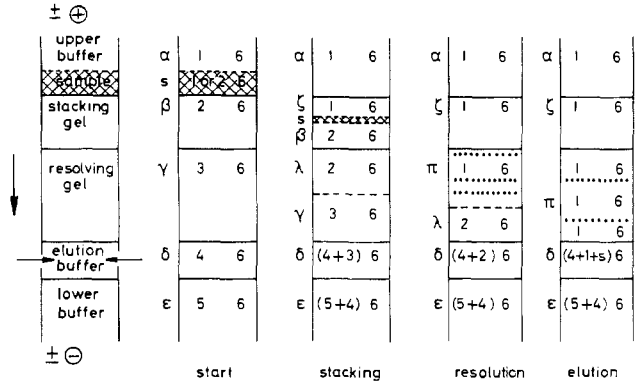

FIGURE 1: Stages in multiphasic analytical or preparative zone electrophoresis. Polyacrylamide gels are assumed to be the supporting media. Numbers 1–6 refer to constituents. Straight lines represent stationary boundaries, dashed lines are moving boundaries, and dotted lines are individual bands or independently migrating sample components. The arrow designates the direction of migration and not current. Further explanations are given in the text.

arated by moving boundaries. The basic element, therefore, can be considered to be each individual boundary and the two phases with which it is associated. Six configurations of this "electrophoretic unit," according to the nature of the constituent electrolytes, have been analyzed (Jovin, 1973a).

The major application of discontinuous buffer systems is undoubtedly in the area of polyacrylamide gel electrophoresis. The theory is not restricted inherently, however, to the use of any particular supporting medium but has equal applicability to "free" electrophoretic techniques.

A schematic representation of an electrophoretic system is shown in Figure 1. The physically distinct component parts are indicated by the diagram at the left. The designated arrangement refers specifically to a preparative device described previously (Jovin *et al.*, 1964) but by omission of the elution buffer segment it applies equally well to analytical methods, such as the conventional "disc" electrophoresis. The designations "lower" and "upper" do not necessarily imply orientation with respect to gravity but define the intrinsic polarity of the system: all moving boundaries migrate "downward." Reference is made to stacking and resolving *gels* because this discussion is designed primarily for application to polyacrylamide gel electrophoresis. The sample is interposed between the upper buffer and the stacking gel after density stabilization by addition of a nonelectrolyte or by the less desirable procedure of copolymerization with acrylamide in a "sample gel" (Davis, 1964). Concentration of the sample takes place in the stacking gel, the matrix of which serves primarily as an anticonvective medium with no conscious attempt made to superimpose a molecular sieving effect. The sample is fractionated in the resolving gel. In preparative equipment, the bands are made to migrate into an elution chamber interposed between the resolving gel and the lower buffer reservoir.

The symbol ± placed before the two pole designations has the significance defined previously (Jovin, 1973a). In the case of the + alternative, boundaries and bands migrate toward the cathode and constituents 1–5 are cations, monoacidic and diacidic bases. It follows that the − symbol indicates migration toward the anode and selection of constituents 1–5 from anions and monobasic and dibasic acids.

It should be noted that two types of constituent combinations are suitable for the design of a system. In the first instance, and the one corresponding to all published systems to date, constituent 6 is a monovalent weak electrolyte, as is constituent 1. Constituent 2 can be either an ion or a monovalent or divalent weak electrolyte. Once the combination of constituents 1, 2, and 6 is fixed, constituent 3 is selected on a relatively flexible basis. The second and new configuration for discontinuous buffer systems involves the choice of an *ion* as constituent 6 but this possibility presents itself only in combination with a monovalent weak electrolyte as constituent 2. This arrangement of buffers is much more flexible and less demanding in the choice of constituents. In certain cases, however, it can be considered to be a degenerate version of the first type of configuration, namely if one uses a monovalent weak electrolyte as constituent 6 at pH's far removed from its pK so that only the ionized species exists.

In terms of electrophoretic phases, the different configurations of the system at the four major stages in the electrophoretic experiment are shown to the right in Figure 1. Phases are labeled with greek letters and the symbol s (for sample). The electrophoretic definition of a phase applies with two exceptions: phases δ and ϵ exist only by virtue of physical boundaries and do not fulfill the homogeneity criterion. In addition, the sample phase s is in actuality heterogeneous in that it initially consists of a polydisperse mixture and later, in the "stacked" configuration, comprises a number of contiguous distinct, though minute, subphases. By the same token, each band migrating through the resolving gel constitutes a separate phase. These distinctions, however, have no bearing on the calculation of the buffer systems. The buffer constituents are designated by the numbers 1–6. It is seen in Figure 1 that constituent 6 is common to all phases, an obvious requirement in the absence of moving boundaries migrating "backward."[1] Stationary boundaries, electophoretic and/or physical, are indicated by solid lines in the diagrams, moving boundaries by dashed lines, and sample components by cross-hatching (while stacking) and dotted lines or "bands" (during separation).

In this discussion, assumptions stated previously (Jovin, 1973a) are considered to hold. A further and more questionable assumption is that polymerizing agents have a negligible effect on the buffer systems and their operation.[2]

The procedure involved in the design of a buffer system will be considered in sections I to IV.

Further considerations, applications, and procedures are described in the next paper of this series (Jovin, 1973b), in which actual representative systems are analyzed, including the original "Tris–glycine" system of Ornstein (1964) and Davis (1964). The computer implementation of the theory is described elsewhere (Jovin, 1973c).

I. Design Parameters

The use of electrophoresis as an analytical or preparative laboratory procedure involves the selection of (1) supporting medium; (2) operating pH; (3) polarity of migration, *i.e.*, anodic or cathodic in the case of a unidirectional apparatus; (4) operating temperature. The first of these factors will not be considered here, save for the statement that with polyacrylamide gels, the monomer concentration and degree of crosslinking are selected on the basis of desired "pore size" (Rod-

[1] So-called "cross-boundary" systems with pairs of oppositely migrating boundaries are discussed in the following paper (Jovin, 1973b).

[2] A. Chrambach, E. Hearing, J. Lunney, and D. Rodbard, submitted for publication.

bard and Chrambach, 1970, 1971; Rodbard *et al.*, 1971; Lunney *et al.*, 1971). The operating temperature is pertinent in that it determines which pK and mobility values are to be used in the calculations. In general, therefore, a computed system corresponds to a specific temperature. Reference to Figure 1 indicates that fractionation of the sample takes place in the π phase. It follows that:

$$\text{pH}^{\pi} = \text{desired operating pH} \pm \text{allowable tolerance}^{3} \quad (105)$$

A degree of flexibility is introduced in eq 105 due to frequent paucity of available constituents. The polarity of migration is selected on the basis of known or probable sample characteristics. Thus, for alkaline pH's, the − mode is usual since most proteins, for example, will be above the isoelectric point and hence migrate toward the anode. Conversely, the + mode applies at most acid pH's.

Little attention is given frequently to the ionic strength of buffers used in electrophoresis; concentrations are changed empirically on the basis of the heat dissipation characteristics of the apparatus. In our case, the calculations require the initial selection of an "operating ionic strength." A convenient value from both a practical and theoretical point of view is

$$(\Gamma/2)^{\pi} = 0.015 \quad (106)$$

Once a system is computed, other values for the ionic strength $(\Gamma/2)^{\pi}$ can be achieved simply by altering all constituents by the factor $(\Gamma/2)^{\pi}/0.015$. Additional observations regarding the ionic strength will be made below.

II. Selection of Constituents

For the purpose of this discussion a constituent is defined in terms of valences, mobilities, and a pK. Characteristics such as chemical stability, solubility, specific binding properties, aggregation tendencies, oxidation or reduction potentials are not considered (except for solubility in the case of constituent 6) since their effect is to limit the array of available constituents and not to affect the calculations.

Constituents 1–6 are not selected in an arbitrary order. Overlapping restrictions suggest the following sequence.

Constituent 1. An explicit description of the sample concentration or "stacking" procedure must be made at this time. It has been shown that the velocities of different constituents in a phase can be compared in terms of their relative constituent mobilities. Concentration of the sample occurs due to the fact that (a) all sample molecules have greater velocities than constituent 1 in phase ζ, (b) all sample molecules have lesser velocities than constituent 2 in phase β, and (c) the velocity of moving boundary ζs is consequently greater than the velocity of the sβ moving boundary until a steady state is reached. The requirements for achieving these conditions are

$$|\bar{r}_1^{\zeta}| \leqslant r_{\min}{}^{\zeta} < |\text{relative constituent mobilities}$$

$$\text{of sample placed in phase } \zeta\ | < |\bar{r}_2^{\beta}| \quad (107)$$

$$|\bar{r}_2^{\beta}| \geqslant r_{\max}{}^{\beta} > |\text{relative constituent mobilities}$$

$$\text{of sample placed in phase } \beta|.$$

[3] Equations with numbers prior to 105 are found in the preceding paper of this series (Jovin, 1973a).

In general, the range of constituent mobilities of the sample is not known and appropriate values for $r_{\min}{}^{\zeta}$ and $r_{\max}{}^{\beta}$ must be selected empirically. Useful quantities for most purpose are

$$r_{\min}{}^{\zeta} = 0.06 \qquad r_{\max}{}^{\beta} = 0.4 \quad (108)$$

Equation 107 and the values indicated in eq 108 lead to the conclusion that *only monovalent weak electrolytes can be utilized for constituent 1.*

It should be obvious that in certain instances, conditions 107 and 108 will not be met due to the fact that some sample constituents may have mobilities such that they migrate in a direction opposite to that of constituent 1. The solution to this problem is to utilize more than one system in order to ensure that all constituents are being observed. For example, it is possible to generate "mirror" systems, *i.e.*, pairs of systems with the same phase ζ but opposite polarity (Jovin, 1973b,c).

The stacking procedure is completed when all sample constituents are arranged in a sequence of homogeneous phases demarcated by moving boundaries. The diffusion of a compound into adjacent phases is counteracted by electrophoretic restoration due to differential velocities as described above. There results a stepwise progression of constitutent mobilities which, however, need not be strictly monotonic through the stack. Consider the moving boundary sβ. If one assumes that the sample constituent s1 directly adjacent to the boundary is a polyvalent ion with |valence| m_{s1} and relative mobility r_{s1}, an estimate of the constituent concentration of this sample component can be obtained with eq 101[4]

$$\bar{c}_{s1}/\bar{c}_2^{\beta} = \frac{(1 - r_6/r_2)}{m_{s1}(1 - r_6/r_{s1})} \quad (109)$$

for constituent 2, a univalent ion (or monovalent weak electrolyte). Assuming $r_6/r_2 = -1$, $r_6/r_1 = -3$, eq 109 reduces to

$$\bar{c}_{s1}(M) = \bar{c}_2^{\beta}/2m_{s1}$$

or

$$\bar{c}_{s1}\ (\text{mg ml}^{-1}) = \bar{c}_2^{\beta}\ [\text{M}_w]_{s1}/2m_{s1}$$

For $m_{s1} = 5$, $[\text{M}_w]_{s1} = 50{,}000$, $|\bar{c}_2^{\beta}| = 0.04$, it is computed that $|\bar{c}_{s1}| = 200$ mg/ml. Thus for proteins or other high molecular weight substances, large constituent concentrations are achieved once stacking is completed, a fact that is reflected in the compactness of the fully concentrated phase s.

It will now be demonstrated that another consequence of the stacking process is the establishment of an equilibrium relationship between phases ζ and β identical with that which would ensue if they were physically adjacent to a common moving boundary. The fact that the stacking sample phase s migrates as a unit implies that the velocities of each individual unit in the stack are identical. By definition of mobility, an equivalent statement of this condition is that the product of electric field strength and constituent mobility is constant in phases ζ, s, and β

$$E\bar{u} = E^{\zeta}\bar{u}_1{}^{\zeta} = E^{sn}\bar{u}_{sn}{}^{sn} = E^{\beta}\bar{u}_2{}^{\beta} \quad (110)$$

$$n = 1, 2, \ldots k$$

[4] Equations 1 to 104 appear in paper I of this series (Jovin, 1973a).

where constituent s1 is adjacent to moving boundary sβ and constituent sk is adjacent to moving boundary ζs. This velocity expression can be regarded, therefore, as the "regulating function" for the stacked sample. It is evident by inspection that the gradient in constituent mobilities, described as a basis for the stacking process, is accompanied by an inverse gradient in electric field strength which tends to maintain the steady state migration of phase s.[5] Using relationships 2, 4, 9, and the electroneutrality restriction, eq 110 leads to

$$\bar{c}_1^{\zeta}/\kappa^{\beta} = 1/96.5\bar{u}_2^{\beta}(1 - \mu_6/\mu_1) \quad (111)$$

Invoking the assumption that ratios of ion mobilities are equivalent to the ratios of the corresponding relative mobilities if the ion mobilities refer to the same phase, eq 111 can be transformed into the equivalent expression

$$\bar{c}_1^{\zeta}/\sigma^{\beta} = 1/96.5\bar{r}_2^{\beta}(1 - r_6/r_1) \quad (112)$$

Depending upon the nature of constituent 2, this relationship can be readily shown to correspond to eq 40 or 70, thus affirming that $\bar{c}_1^{\zeta}/\bar{c}_2^{\beta} = \mu^{\zeta\beta}$.

Since all boundaries between and including ζs and sβ are necessarily moving boundaries, the general moving boundary eq 43 can be written for constituent 6 across each boundary. Every phase is represented in the two equations for the moving boundaries constituting its limits and it therefore follows that the quantity $\bar{c}_6(1 - \bar{r}_6/\nu\sigma)$ is constant from one phase to the next. Thus

$$\bar{c}_6^{\zeta}(1 - \bar{r}_6^{\zeta}/\nu^{\zeta s}\sigma^{\zeta}) = \bar{c}_6^{\beta}(1 - \bar{r}_6^{\beta}/\nu^{s\beta}\sigma^{\beta}) \quad (113)$$

Equations 112 and 113 indicate that *phase s does not alter the relationships existing between phases ζ and β in the absence of any sample.*

As phase s migrates into the resolving gel, changes in relative constituent mobilities occur due to pH and ionic strength differentials across the stationary boundary and due to the restrictive effect of the gel matrix on large molecules. It has been demonstrated that well-defined relationships exist between phases separated by a steady-state moving boundary (Jovin, 1973a). Thus the composition of phase λ, formed when constituent 2 migrates into the resolving gel, is dependent upon the nature of phase γ. Likewise the migration of the ζs boundary into the resolving gel is accompanied by the creation of a new phase π, the composition of which is in turn determined by the characteristics of the phase λ due to the "transmission" effect of the stacked sample demonstrated in the previous paragraph. In order for the individual components of the sample to migrate as discrete bands it is necessary to reverse the conditions established in order to achieve the stacking process. This is accomplished by constructing the system so as to obtain: pH^{π} designated in eq 105, $(\Gamma/2)^{\pi}$ designated in eq 106, and

$$|\bar{r}_1^{\pi}| \gtrless r_{max}{}^{\pi} > |\text{relative constituent mobilities of sample placed in phase } \pi| \quad (114)$$

In the event that condition 114 is satisified, the velocity of constituent 1 in phase π exceeds that of all sample components. Hence, moving boundary $\pi\lambda$ forms and migrates ahead while the stacked sample, now in a homogeneous buffer phase, is resolved into discrete bands migrating under the influence of a uniform electric field strength E^{π}.[6] Selection of the proper value for $r_{max}{}^{\pi}$ is again empirical unless specific information regarding the sample is available. It is possible to use a value less than that of $r_{max}{}^{\beta}$ because the molecular sieving effect of the gel tends to decrease sample constituent mobilities appreciably although the pH changes operate so as to increase molecular charge. For most purposes, one can employ

$$r_{max}{}^{\pi} = 0.3 \quad (115)$$

An operational check for the suitability of the value selected for $\bar{r}_1{}^{\pi}$ is provided by moving boundary $\pi\lambda$. When a dye is used as a marker, it becomes concentrated at this boundary because its constituent mobility lies between $\bar{r}_1{}^{\pi}$ and $\bar{r}_2{}^{\lambda}$. Any sample components with constituent mobilities in this intermediate range will likewise become stacked at the $\pi\lambda$ boundary. Thus in the case of proteins, a heavy band at the site of the tracking dye indicates that the value of $|\bar{r}_1{}^{\pi}|$ is too low. The effect is commonly encountered with heterogeneous protein mixtures. Unless demonstrated otherwise, it is safe to assume that a band at the position of the dye is heterogeneous and that its components are relatively low molecular weight proteins or polypeptides, possessing a relatively high |constituent mobility|.

It is necessary to digress at this time in order to consider limitations on constituent 6, in the event it is a monovalent weak electrolyte, which have a bearing on the selection of constituent 1.

Equation 28 states that

$$\theta^{\zeta} = \phi_1{}^{\zeta}[1 + \rho\phi_1{}^{\zeta}/(1 - \phi_1{}^{\zeta})] \quad (116)$$

where $\rho = 10^{\pm(pK_6 - pK_1)}$. From eq 47, 57, and 76, it is seen that

$$\theta^{\zeta} > Q \quad (117)$$

where Q is dependent upon the nature of constituent 2.

Constituent 2	Q_{min}
uni- or multivalent ion	1
monovalent weak electrolyte	$1 - (1 - \phi_2{}^{\beta})/\mu^{\alpha\beta}$
divalent weak electrolyte	~ 0.9

Furthermore, from eq 107, 108, 114, and 37

$$\phi_1{}^{\zeta} = r_{min}{}^{\zeta}/|r_1|$$

$$\phi_1{}^{\pi} = r_{max}{}^{\pi}/|r_1| \quad (118)$$

$$\phi_2{}^{\beta} \gtrless r_{max}{}^{\beta}/|r_2|$$

for constituent 2 a monovalent weak electrolyte. Combining eq 116 and 117 with eq 29, which relates pH^{π} to $\phi_1{}^{\pi}$, one obtains

[5] It should be noted, however, that the stepwise changes in pH, $\bar{u}$, and E within a stack need not be strictly monotonous.

[6] The differential voltage gradients within a band and within the adjacent buffer phase (Ornstein, 1964) are neglected in this discussion.

$$\pm(pK_6 - pH^\pi) > P \equiv \log [(Q/\phi_1^\zeta - 1)(1/\phi_1^\zeta - 1)/(1/\phi_1^\pi - 1)] \quad (119)$$

The importance of this equation is revealed by considering a solubility restriction on constituent 6 in phase π

$$|\bar{c}_6^\pi| = |c_{6^{(\neq 1)}}^\pi|/\phi_6^\pi = (\Gamma/2)^\pi/\phi_6^\pi \quad (120)$$

$$|c_{6^{(0)}}^\pi| = |\bar{c}_6^\pi|(1 - \phi_6^\pi) = (\Gamma/2)^\pi(1/\phi_6^\pi - 1) = (\Gamma/2)^\pi 10^{\pm(pK_6 - pH^\pi)}$$

It is obvious that $|c_{6^{(0)}}^\pi|$, the concentration of the uncharged subspecies of constituent 6 in phase π, must not exceed the molar solubility S_6 of this constituent in an aqueous solution at the specified temperature. Thus

$$\pm(pK_6 - pH^\pi) \leqslant \log [S_6/(\Gamma/2)^\pi] \quad (121)$$

This expression and eq 119 can be combined to provide the *major basis for the selection of constituent 6 in the case of a monovalent weak electrolyte*

$$P < \pm (pK_6 - pH^\pi) \leqslant \log [S_6/(\Gamma/2)^\pi] \quad (122)$$

In order to liberalize the restrictions stated in eq 122 it is necessary to seek conditions leading to a minimal value for P. Utilizing the relationships in eq 118, an equivalent statement for P is obtained

$$P = \log [(Q|r_1|/r_{min}^\zeta - 1)(|r_1|/r_{min}^\zeta - 1)/(|r_1|/r_{max}^\pi - 1)] \quad (123)$$

By inspection it is seen that P is reduced by (a) increasing r_{min}^ζ, (b) decreasing r_{max}^π, (c) decreasing Q, and (d) optimizing $|r_1|$ in terms of r_{min}^ζ and r_{max}^π, and Q. By differentiation of P with respect to $|r_1|$, one obtains

$$|r_1|_{opt} = r_{max}^\pi + [(r_{max}^\pi - r_{min}^\zeta)(r_{max}^\pi - r_{min}^\zeta/Q)]^{1/2} \quad (124)$$

The relative advantages and disadvantages of each different type of constituent 2 will be discussed later. At this point, it is necessary only to state that the best procedure is to attempt the calculation of systems for the three alternatives noted and to determine the applicability of the individual systems at the conclusion of the computations. Solely from the standpoint of flexibility in the selection of constituent 6, it is evident from the relationships listed in eq 117 that for constituent 2 a monovalent weak electrolyte, Q and therefore P in eq 123 are smaller than in other cases.

The procedure for selecting constituent 1 will now be recapitulated as a series of steps: (1) establish values for r_{min}^ζ, r_{max}^β, r_{max}^π (see eq 107 and 115); (2) select monovalent weak electrolytes as potential choices for constituent 1 on the basis of the equation below and the minimal requirement $|r_1| > r_{max}^\pi$

$$pK_1 \cong pH^\pi \pm \log [1/(|r_1|/r_{max}^\pi - 1)] \quad (125)$$

(3) compute $|r_1|_{opt}$ (eq 124) using appropriate Q values for each case listed in eq 117; (4) select the final choice (for each type of constituent 2) on the basis of the degree of correspondence between $|r_1|$ and $|r_1|_{opt}$, and the array of potential constituents 6 according to eq 122; (5) in the event constituent 2 is a monovalent weak electrolyte, then it is possible to make constituent 6 an ion (case VI, Jovin, 1973a), and it follows that eq 122 is no longer applicable.

Constituent 6. The following quantities are known at this time: r_{min}^ζ, r_{max}^β, r_{max}^π, pH^π, $(\Gamma/2)^\pi$, pK_1, and r_1.

The basis for selecting a monovalent weak electrolyte as constituent 6 has already been described. As in the case of constituent 1, it is advisable to select a constituent 6 for each potential type of constituent 2. This is accomplished by introducing the corresponding Q in eq 123 for P, thus defining different ranges for the quantity $\pm(pK_6 - pH^\pi)$ in eq 122.

It will be demonstrated later that optimization of the stacking process is achieved if $\pm(pK_6 - pH^\pi)$ is maximized, subject to the solubility restriction in eq 121. In addition, it is also desirable to minimize $|r_6|$. The rationale for this assertion involves the question of ohmic heating in the electrophoretic system. It is of practical advantage to reduce heat production without lowering the velocity of migration. In specific terms, this goal is achieved by minimizing the ratio of power per unit field strength.

$$\text{applied electrical power} = EI = E^2\kappa \quad (126)$$

Thus power/unit voltage gradient $= \kappa$. Since the conductance κ varies directly with $|r_6|$, it follows that low values for $|r_6|$ are preferable.

In systems designed for use in preparative devices, the use of a volatile elution buffer makes possible concentration procedures such as lyophilization. Since constituent 6 is the major component of the elution buffer, a volatile uncharged subspecies is desirable.

A summary of the procedure for selecting constituent 6 is, therefore: (1) compute a P (eq 123) for each type of constituent 2; (2) select monovalent weak electrolytes as potential choices on the basis of eq 122; (3) for constituent 2 a monovalent weak electrolyte, an ion of opposite charge can also be used for constituent 6; (4) select the final choice (for each type of constituent 2) after attempting to (a) maximize $\pm(pK_6 - pH^\pi)$ as necessary; (b) minimize $|r_6|$; and (c) provide volatility if desired.

Constituent 2. The following quantities are known at this time: r_{min}^ζ, r_{max}^β, r_{max}^π, pH^π, $(\Gamma/2)^\pi$, pK_1, r_1, (pK_6), and r_6. It is necessary to compute (a) ϕ_1^ζ (eq 118), (b) pH^ζ (eq 29), (c) θ^ζ (eq 28), (d) ϕ_1^π (eq 118), and (e) θ^π (eq 28).

Three types of electrolytes will be considered as choices for constituent 2: uni- or multivalent ions, monovalent weak electrolytes, and divalent weak electrolytes. Which type is selected for use in a particular system depends upon the availability of potential choices and the specific application projected for the system. Certain advantages and disadvantages can be cited for each case.

UNI- OR MULTIVALENT IONS. These constituents tend to provide chemical stability and a large value for $|\bar{r}_2^\beta|$, a desirable feature when the components of the sample tend to have correspondingly high constituent mobilities. On the other hand, it is relatively difficult to achieve low values for $|\bar{r}_1^\zeta|$ which makes stacking difficult for components with low constituent mobilities. In addition, large pH differentials between phases ζ and β are necessarily created and phase β is very poorly buffered.

MONOVALENT WEAK ELECTROLYTES. Under ideal conditions, this type of constituent 2 is the most desirable because it per-

mits low values for $|\bar{r}_1^{\zeta}|$, provides a relatively high buffer value for phase β, and does not lead to large pH differences between phases ζ and β. On the other hand, it is difficult to achieve very large values for $|\bar{r}_2^{\beta}|$ and the availability of compounds is sometimes limited. An additional advantage is that constituent 6 can be an ion.

DIVALENT WEAK ELECTROLYTES. Approximately the same considerations apply for this type of constituent as for uni- or multivalent ions with the exception that, under proper circumstances, it is possible to buffer phase β adequately. Availability is often the major difficulty due to the restriction of the theory to compounds with nonoverlapping dissociations, although generalization of the equations is quite feasible.

The procedure for selecting constituent 2 will be described separately for each type of electrolyte, the assumption being made that constituents 1 and 6 have been chosen in a corresponding manner.

Case I. Constituent 2, a Uni- or Multivalent Ion (Valence = $\pm m_2$). Phases ζ and β, and phases π and λ constitute two systems entirely analogous to the prototype system or electrophoretic unit, case I, described previously (Jovin, 1973a). The selection of constituent 2, therefore, can be made by considering the restrictions that apply to such systems. These are:

$$r_2/r_1 > \phi_1^{\zeta} \text{ and } \phi_1^{\pi} \text{ (from eq 53)}$$
$$|r_2| \geqslant r_{\max}{}^{\beta} \text{ (from eq 107)} \tag{127}$$

$$\pm(\mathrm{pH}^{\beta} - \mathrm{pH}^{\zeta}) \leqslant L \tag{128}$$

The latter is an arbitrary restriction which may be imposed, if desired, in order to prevent unduly large pH differences between phases ζ and β. From eq 50, it is evident that L must be a number greater than 0. Introducing eq 23, 40, and 45 into 128 and rearranging, a lower limit for the value of $|r_2|$ is obtained. In addition, a restatement of condition 127 can be made by reference to eq 118 and from the knowledge that $r_{\max}{}^{\pi} > r_{\min}{}^{\zeta}$. In summary, the procedure for selecting constituent 2 in this case is (1) select a value for the pH limit L (condition 128); (2) select constituent 2 on the basis of the expression

$$|r_6| \Big/ \left\{ \frac{(1 - r_6/r_1)10^{[\pm(\mathrm{pK}_6 - \mathrm{pH}^{\zeta}) - L]}}{\theta^{\zeta} - 1} - 1 \right\} \geqslant |r_2| \begin{cases} > r_{\max}{}^{\pi} \\ \geqslant r_{\max}{}^{\beta} \end{cases} \tag{129}$$

where the valence m_2 serves only as a means of finding an ion with the desired r_2. Note that if the left hand side of expression 129 is excessively small, *i.e.*, $\leqslant r_{\max}{}^{\pi}$ or $r_{\max}{}^{\beta}$, a less restrictive (*i.e.*, larger) value for L must be used.

Case II. Constituent 2, a Monovalent Weak Electrolyte. In this case, phases ζ and β, and phases π and λ constituent two systems analogous to the prototype systems or electrophoretic units, cases II or VI, described previously (Jovin, 1973a), depending upon the nature of constituent 6. The corresponding restrictions are

$$\text{(a)} \quad \pm(\mathrm{pK}_2 - \mathrm{pK}_1) > \log \frac{(r_1/r_2 - \phi_2)}{(1 - \phi_2)} \equiv S \text{ (eq 66)} \tag{130}$$

or

$$\pm(\mathrm{pK}_2 - \mathrm{pK}_1) > \log \frac{(1 - \phi_1)}{(r_2/r_1 - \phi_1)} \equiv T \text{ (eq 67)} \tag{131}$$

in phases ζ, β, π, λ.

$$\text{(b)} \quad \phi_2^{\beta} \geqslant r_{\max}{}^{\beta}/|r_2|$$

Obviously $|r_2| > r_{\max}{}^{\beta}$.

$$\text{(c)} \quad \pm(\mathrm{pH}^{\beta} - \mathrm{pH}^{\zeta}) \leqslant L$$

This is an arbitrary restriction identical with eq 128. In this case, however, L is not necessarily limited to values greater than 0. Introducing eq 58 into the inequality, an equivalent statement is derived.

$$\pm(\mathrm{pK}_2 - \mathrm{pK}_1) \leqslant L + \log [(1/\phi_1^{\zeta} - 1)/(1/\phi_2^{\beta} - 1)] \tag{132}$$

The steady-state moving-boundary condition 131 is of immediate applicability to phases ζ and π because ϕ_1^{ζ} and ϕ_1^{π} are known quantities, while condition 130 is more useful for phases β and λ due to the fact that it is formulated in terms of ϕ_2. Equations 40 and 45 supply the following relationships

$$\theta^{\beta} = -\bar{c}_6^{\beta}/\bar{c}_2^{\beta} = 1 + (\theta^{\zeta} - 1)(1 - r_6/r_2)/(1 - r_6/r_1)$$
$$\theta^{\lambda} = -\bar{c}_6^{\lambda}/\bar{c}_2^{\lambda} = 1 + (\theta^{\pi} - 1)(1 - r_6/r_1)/(1 - r_6/r_2) \tag{133}$$

Hence for every potential constituent 2, θ^{β} and θ^{λ} can be computed, leading immediately to values for ϕ_2^{β} and ϕ_2^{λ} through eq 26 in which $\rho = 10^{\pm(\mathrm{pK}_6 - \mathrm{pK}_2)}$, or eq 103 for the case of constituent 6 being an ion.

From the standpoint of flexibility, one further observation can be made regarding conditions 130 and 131. By inspection it can be seen that S and T vary inversely with respect to $|r_2|$. That is, the pK restrictions that limit the choice of constituent 2 are minimized for large values of $|r_2|$. In addition, a higher $|\bar{r}_2^{\beta}|$ is made feasible, a desirable feature discussed earlier.

In summary, the procedure for selecting constituent 2 in this case is (1) select a value for the pH limit L (condition 128); (2) consider a suitable range of monovalent weak electrolytes as potential choices for constituent 2. (A useful guide in this regard is the pK since most substances found appropriate will possess pK values in the general vicinity of pH^{ζ}.) In addition, it is necessary that $|r_2| > r_{\max}{}^{\beta}$. Compute for each possibility: (a) ϕ_2^{β} (eq 133 and 26). Reject all compounds for which $|r_2|\phi_2^{\beta} < r_{\max}{}^{\beta}$. (3) Ascertain that for each possibility, pK_2 lies in the range specified by equations 130 and 132; (4) select the final choice on the basis of: (a) maximal $|\bar{r}_2^{\beta}| = |r_2|\phi_2^{\beta}$ and (b) proximity of ϕ_2^{β} to optimal (from buffering standpoint) value of 0.5.

Case III. Constituent 2, a Divalent Weak Electrolyte. The corresponding prototype system or electrophoretic unit is case III, discussed earlier (Jovin, 1973a). The divalent weak electrolyte is presumed to have nonoverlapping dissociations and only the second is considered, thus implying the existence of ion subspecies exclusively. The following restrictions are imposed (eq 84 and 85):

$$\text{(a)} \quad \pm(\mathrm{pK}_2 - \mathrm{pK}_1) > \log \frac{(r_1 - r_{21}/\phi_1)(1 - \phi_1)}{(r_{22} - r_1\phi_1)}$$

or

$$\pm(\mathrm{pK}_2 - \mathrm{pK}_1) > \log \frac{(r_1/[r_{21} + (r_{22} - r_{21})\phi_2] - 1)}{(1/\phi_2 - 1)} \tag{134}$$

in phases $\zeta, \beta, \pi, \lambda$.

(b) $$|\bar{r}_2^{\beta}| \geqslant r_{max}^{\beta}$$

(c) $$\pm(pH^{\beta} - pH^{\zeta}) \leqslant L$$

In the application of the steady-state moving-boundary conditions 134, ϕ_1^{ζ} and ϕ_1^{π} are known quantities, but it is necessary to compute ϕ_2^{β} and ϕ_2^{λ} by use of eq 71 for which the appropriate parameters are: $b = -r_6/r_{21}$; $c = -r_6/r_1$; $d = -(c + \theta)/(1 + c)$, where $\theta = \theta^{\zeta}$ in the case of ϕ_2^{β}, and $\theta = \theta^{\pi}$ in the case of ϕ_2^{λ}; $\rho = 10^{\pm(pK_6 - pK_2)}$. All other quantities are as stated.

It is now possible to summarize the procedure for selecting constituent 2 in this case. (1) Select a value for the pH limit L. (2) Consider a suitable array of divalent weak electrolytes. As in case II, a useful guide is the pK value, a convenient range for which can be centered about pH^{ζ} (as a consequence of (1) and the desire to provide buffering for phase β). For each possibility, compute (a) ϕ_2^{β} (eq 71); (b) $|\bar{r}_2^{\beta}|$ (eq 68); reject compounds for which $|\bar{r}_2^{\beta}| < r_{max}^{\beta}$; (c) ϕ_2^{λ} (eq 71 with parameters indicated above). (3) Ascertain that for each possibility, pK_2 lies in the range specified by eq 134. (4) Select a final choice as constituent 2 on the basis of (a) maximal $|\bar{r}_2^{\beta}|$ and (b) proximity of ϕ_2^{β} to the optimal value for H (eq 79 and 80).

Optimization of phases ζ and β. Once constituents 1, 6, and 2 have been selected, it is possible to reexamine the characteristics of phases ζ and β with the view of optimizing the stacking process if possible. In this instance, optimization consists of minimizing $|\bar{r}_1^{\zeta}|$ and/or maximizing $|\bar{r}_2^{\beta}|$. The procedure can be considered separately for each type of constituent 2.

Case I. Constituent 2, a Uni- or Multivalent Ion. In this case, $\bar{r}_2^{\beta} = r_2$ and is therefore not susceptible to alteration. It is possible, however, to alter pH^{ζ} within the limits imposed by eq 51. Thus

$$pH^{\zeta} < \frac{1}{2}(pK_1 + pK_6) \text{ for the } + \text{ case}$$
$$pH^{\zeta} > \frac{1}{2}(pK_1 + pK_6) \text{ for the } - \text{ case} \qquad (135)$$

Adjusting pH^{ζ} toward these limits leads to a minimal value for ϕ_1^{ζ} and thus $|\bar{r}_1^{\zeta}|$. That this is so is seen readily by considering eq 25 which states that ϕ_1^{ζ} decreases with increasing values of pH^{ζ} (+ case) or decreasing values (− case). These considerations also explain the statement made in the discussion of the selection of constituent 6 to the effect that maximizing the quantity $\pm(pK_6 - pH^{\pi})$ was desirable, since this is equivalent to maximizing $\pm(pK_6 + pK_1)$. One direct effect of altering pH^{ζ} in this manner is to increase the pH differential between phases ζ and β (eq 48), thus possibly exceeding the arbitrary limit L imposed in the selection of constituent 2. Hence it is necessary to weigh these opposing tendencies in the light of the intended purpose for the system. It should also be noted that if changes are made in the direction of decreasing ϕ_1^{ζ} (and thus r_{min}^{ζ}), steady-state conditions will necessarily hold as a consequence of condition 53 which was applied originally.

Case II. Constituent 2, a Monovalent Weak Electrolyte. In this instance, $\bar{r}_1^{\zeta}$ and $\bar{r}_2^{\beta}$ are related in a direct way and an attempt to alter one in a given direction results in a corresponding change in the other although the magnitudes of the differences generally will not be the same. The decision, therefore, is whether it is desirable to achieve a lower $|\bar{r}_1^{\zeta}|$ at the expense of a lower $|\bar{r}_2^{\beta}|$ or a higher $|\bar{r}_2^{\beta}|$ at the expense of a higher $|\bar{r}_1^{\zeta}|$. The problem must ultimately be resolved according to the characteristics of the sample and some attention to this will be given in a later section dealing with required stacking gel lengths. The computations relating $\bar{r}_1^{\zeta}$ and $\bar{r}_2^{\beta}$ are carried out by the use of eq 26, 28, 37, 44, 45, 54, 72, 73, and 118. Equation 40 which defines $\mu^{\zeta\beta}$ is needed only once since this quantity is invariant for given constituents 1, 6 and 2. The sequence in calculations required (for constituent 6 a monovalent weak electrolyte)[7] in going from a value of $\bar{r}_1^{\zeta}$ to the corresponding $\bar{r}_2^{\beta}$ is the following: $\bar{r}_1^{\zeta}$ (or r_{min}^{ζ}), ϕ_1^{ζ}(118), θ^{ζ}(28), θ^{β}(72), ϕ_2^{β}(26), $\bar{r}_2^{\beta}$(54). The reverse process is identical: $\bar{r}_2^{\beta}$, ϕ_2^{β}(118), θ^{β}(28), θ^{ζ}(73), ϕ_1^{ζ}(26), $\bar{r}_1^{\zeta}$(37). Also of consideration in this process are the questions of buffering and pH in phase β, implying that ϕ_2^{β} should not be allowed to assume extreme values. It is necessary to indicate that alterations in the systems must be checked against the steady-state moving-boundary restrictions in eq 130–131 since compliance is no longer assured.

Case III. Constituent 2, a Divalent Weak Electrolyte. In this case, as in case I, $\bar{r}_2^{\beta}$ is usually more than adequate and the problem is to optimize $\bar{r}_1^{\zeta}$. It has been demonstrated that θ^{ζ} is minimized by utilizing the value for ϕ_2^{β} computed in eq 79. The corresponding θ^{ζ} can be determined from eq 73, from which ϕ_1^{ζ} and thus $\bar{r}_1^{\zeta}$ follow directly as shown above. If changes are instituted, eq 134 must be reevaluated in order to establish fulfillment of the steady-state moving-boundary condition.

If changes are made in accordance with the above considerations, revised values for θ^{ζ}, pH^{ζ}, ϕ_1^{ζ}, ϕ_2^{β}, r_{min}^{ζ}, $\bar{r}_1^{\zeta}$, and $\bar{r}_2^{\beta}$ must be specifically indicated.

Constituent 3. The following quantities are known at this time: pK_1, (pK_6), (pK_2), r_1, r_6, r_2 (or r_{22} and r_{21}), $\bar{r}_1^{\zeta}$, $\bar{r}_2^{\beta}$, $\bar{r}_1^{\pi}$, pH^{ζ}, pH^{π}, ϕ_1^{ζ}, ϕ_1^{π}, ϕ_2^{β}, ϕ_2^{λ}, θ^{ζ}, θ^{β} (except for constituent 2 an ion), θ^{π}, θ^{λ} (except for constituent 2 an ion), $(\Gamma/2)^{\pi}$. For constituent 2 an ion, θ^{β} and θ^{λ} are computed from eq 40 and 45.

Phase γ is the original buffer phase corresponding to the resolving gel at the time of polymerization. From a practical standpoint, it is desirable to avoid very large pH gradients between phase γ and phase β (the stacking gel). Thus in selecting constituent 3, the attempt is made to minimize the quantity $\pm(pH^{\beta} - pH^{\gamma})$.

For *constituent 6 an ion*, constituent 3 is arbitrarily made the same as constituent 2 in practical systems computed to date. If desired, however, other appropriate weak electrolytes can be selected. For *constituent 6 a monovalent weak electrolyte*, $|\bar{c}_6^{\gamma}|$ will be in general quite large, thus providing an adequate buffer value despite a low ϕ_6^{γ}. It follows that a weak electrolyte is not generally required for constituent 3. For the sake of computational ease and maximal availability, the following discussion will consequently be limited to uni- or multivalent ions as choices for this constituent with the exception of the degenerate case of constituent 3 equal to constituent 2.

The composition of phase λ is a function of phase γ parameters since the two phases are in equilibrium across the

[7] In most practical instances, the same equations developed for the case of a monovalent weak electrolyte as constituent 6 can be used in the event an ion is selected for the latter. One merely assigns to the ion a fictitious pK far removed from all pH's in the system, *i.e.*, higher in the case of − systems and lower in the case of + systems.

moving boundary $\lambda\gamma$. The selection of constituent 3 has to be considered separately, therefore, for the different types of constituent 2.

Case I. Constituent 2, a Uni- or Multivalent Ion (Valence = $\pm m_2$). In this case, the easiest procedure is to make constituent 3 the same as constituent 2, thus eliminating the moving boundary $\lambda\gamma$ and creating a phase λ identical with phase γ. In general, however, this procedure will not minimize the quantity $\pm(\mathrm{pH}^\beta - \mathrm{pH}^\gamma)$. It is therefore of interest to consider the case of constituent 3 different from constituent 2 and the optimal characteristics it should possess.

The two following expressions follow directly from eq 23

$$\begin{aligned} \pm(\mathrm{pH}^\gamma - \mathrm{p}K_6) &= \log [1/(\theta^\gamma/m_3 - 1)] \\ \pm(\mathrm{pH}^\beta - \mathrm{p}K_6) &= \log [1/(1/\phi_2{}^\beta - 1)] \end{aligned} \tag{136}$$

From eq 102, it is seen that

$$(\theta^\gamma/m_3 - 1) = (\theta^\lambda/m_2 - 1)(1 - r_6/r_3)/(1 - r_6/r_2) \tag{137}$$

Combining eq 136 and 137

$$\pm(\mathrm{pH}^\beta - \mathrm{pH}^\gamma) = \log \left[\frac{(\theta^\lambda/m_2 - 1)(1 - r_6/r_3)}{(1 - r_6/r_2)(1/\phi_2{}^\beta - 1)} \right] \tag{138}$$

By inspection of eq 138 it is seen that $\pm(\mathrm{pH}^\beta - \mathrm{pH}^\gamma)$ is minimized for large values of $|r_3|$. This is fortunate, since the steady-state moving-boundary requirement for this system is $r_3/r_2 > 1$.

A summary of the procedure for selecting constituent 3 in this case is (1) select an ion with a maximal value for $|r_3|$, the minimal requirement being $r_3/r_2 > 1$, or (2) make constituent 3 the same as constituent 2.

Case II. Constituent 2, a Monovalent Weak Electrolyte. Equation 138 is equally applicable in this case since for $m_2 = 1$, eq 137 reduces to a form identical with the combination of 40 and 45. Hence it is desirable to select a high $|r_3|$ as before. The steady-state moving-boundary restriction is now given by eq 53, $r_3/r_2 > \phi_2{}^\lambda$. If the pH differentials between phases β and γ become too great in the case of an ion as constituent 6, it is always possible to make constituents 3 and 2 the same.

Case III. Constituent 2, a Divalent Weak Electrolyte. The prototype system or electrophoretic unit that corresponds to this case is case IV discussed earlier (Jovin, 1973a).

Inspection of eq 88 and 89 indicates that an expression similar to 137 can be derived

$$(\theta^\gamma/m_3 - 1) = K(1 - r_6/r_3) \tag{139}$$

where K is a positive quantity dependent solely upon r_{21}, r_{22}, θ^λ, and $\phi_2{}^\lambda$. It therefore follows that the same conclusion holds as before, namely that the pH differential between phases β and γ is minimized by selecting a high value for $|r_3|$.

The steady-state moving-boundary restrictions are given by eq 94 and 97.

$$r_3/r_{21} > \begin{cases} 1 + (a - 1)\phi_2{}^\lambda \\ p + [p^2 + ab/(1 + q)]^{1/2} \end{cases} \tag{140}$$

where

$$a = r_{22}/r_{21}$$

$$b = -r_6/r_{21}$$

$$q = \frac{[1 + b + (2a + b - 1)\phi_2{}^\lambda]10^{\pm(\mathrm{p}K_6 - \mathrm{p}K_2)}}{[1 + (a - 1)\phi_2{}^\lambda](\theta^\lambda - 1) - a\phi_2{}^\lambda}$$

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

In summary, for selecting constituent 3 in this case one seeks an ion with a maximal value of $|r_3|$, the minimal requirements being specified in relation 140.

It should be pointed out that selecting a constituent 3 with a high mobility does not have the unfavorable implications regarding heat production described in the section dealing with constituent 6. In fact, just the opposite holds. The rationale for this assertion is the following. The voltage gradients of interest are those determining the velocities of the sample constituents in both the stacking and resolving stages of electrophoresis, that is, $|E^\zeta| < |E^{\delta\eta}| < |E^\beta|$ and $|E^\pi|$. If the physical apparatus imposes an upper limit on the allowable power per unit length of gel $P_{\max}$, then the specified voltage gradients determine a maximal current $I_{\max}$ that can pass through the entire system. The heat production in other elements of the system is proportional to $I_{\max}{}^2/\kappa$ and is therefore minimized by increasing the conductance in the corresponding phases.

Constituent 4. It has been pointed out that phase δ is not a true electrophoretic phase but exists by virtue of physically placing an elution chamber in contact with the resolving gel. Thus the $\gamma\delta$ and later the $\pi\delta$ boundaries do not constitute true electrophoretic stationary boundaries.

Since constituent 4 migrates toward the ϵ phase, its characteristics do not affect the processes occurring in the gel. It can therefore be selected on the basis of desirable physical characteristics. In the discussion of constituent 6, the question of elution buffer volatility was mentioned briefly. It is obvious that a fully volatile buffer in phase δ is achieved only if both constituents 6 and 4 are volatile. The implication is that constituent 4 must consist of a volatile monovalent weak electrolyte or (depending upon pH^δ) a univalent ion whose conjugate acid or base is volatile. It is true, however, that the elution buffer leaving the system is necessarily "contaminated" with constituents 3 and 2 and later with constituent 1 (together with the desired bands) although the concentration of these components will be relatively low. Nonetheless, it may be desirable to select a volatile constituent 1 if available. To summarize, constituent 4 will consist of the following electrolyte type: (1) volatile monovalent weak electrolyte or univalent ion with volatile conjugate acid or base; (2) uni- or multivalent ion with a high $|r_4|$.

In the case of the monovalent weak electrolyte, some specification of pK can be made. It is desirable to maintain $\pm(\mathrm{pH}^\delta - \mathrm{pH}^\pi) > 0$ for two reasons: (a) the necessary concentration of constituent 6 required to achieve a given ionic strength is reduced (in the event this constituent is a monovalent weak electrolyte); (b) the constituent mobilities of the compounds eluting from the resolving gel are reduced in magnitude, thereby minimizing migration toward phase ϵ which in general is separated from phase δ by a semipermeable membrane. Another limit is given by $\pm(\mathrm{pH}^\zeta - \mathrm{pH}^\delta) \geqslant 0$, the justification for which is based on the necessity for avoiding pH ranges in which the isoelectric points of sample constituent are reached or exceeded, thus possibly hindering their elution

from the resolving gel. Taken together, the two pH restrictions specify

$$\begin{aligned} &pH^{\pi} < pH^{\delta} \leqslant pH^{\zeta} \text{ (for the + case)} \\ &pH^{\zeta} \leqslant pH^{\delta} < pH^{\pi} \text{ (for the − case)} \end{aligned} \tag{141}$$

The desirability of providing buffering action if possible, yet minimizing the required $|\bar{c}_4{}^{\delta}|$ for a given $(\Gamma/2)^{\delta}$, leads to the specification $\phi_4{}^{\delta} \geqslant 0.5$, or

$$\pm(pH^{\delta} - pK_4) \leqslant 0 \tag{142}$$

It is evident that large values of $\pm(pK_4 - pH^{\delta})$ are actually equivalent to the selection of a univalent ion for which the conjugate acid or base is a monovalent weak electrolyte.

A summary for the selection of constituent 4 is therefore: (1) select pH^{δ} according to the specification 141; (2) select the type of electrolyte according to whether volatility is desired: (a) volatility: monovalent weak electrolyte with a pK fulfilling condition 142; (b) nonvolatility: uni- or multivalent ion with a high value for $|r_4|$.

Constituent 5. This constituent is located in phase ϵ, the "lower buffer." Nonvolatility is desirable. In order to prevent large pH gradients across the membrane separating phases δ and ϵ, it is desirable to make

$$pH^{\epsilon} \cong pH^{\delta} \tag{143}$$

The type of electrolyte can be selected arbitrarily. If buffering action additional to that supplied by constituent 6 is desired, a monovalent or divalent weak electrolyte can be selected with $pK_5 \cong pH^{\epsilon}$. Otherwise, a uni- or multivalent ion can be employed, preferably possessing a high $|r_5|$ so as to minimize the voltage drop across the lower buffer.

III. Problems Related to Specific Phases

A. Phase α. From a theoretical standpoint, within the assumptions of this discussion, the characteristics of phase α are arbitrary save for the specification that it must contain constituents 1 and 6. Any discrepancies between phase α and phase ζ (the composition of which is determined by phase β) result in the formation of a stationary boundary $\alpha\zeta$. This is easily confirmed by considering moving boundary eq 11 for constituent 1 and boundary $\alpha\zeta$ across which no constituent disappears.

$$\bar{r}_1{}^{\alpha}\bar{c}_1{}^{\alpha}/\sigma^{\alpha} - \bar{r}_1{}^{\zeta}\bar{c}_1{}^{\zeta}/\sigma^{\zeta} = \nu^{\alpha\zeta}(\bar{c}_1{}^{\alpha} - \bar{c}_1{}^{\zeta}) \tag{144}$$

Using the relationships $\bar{r}_1 = r_1\phi_1$, and $\sigma = 96.5\bar{c}_1\phi_1(r_1 - r_6)$, it is seen that

$$\bar{r}_1{}^{\alpha}\bar{c}_1{}^{\alpha}/\sigma^{\alpha} - \bar{r}_1{}^{\zeta}\bar{c}_1{}^{\zeta}/\sigma^{\zeta} = \frac{1}{96.5}\left[\frac{r_1}{(r_1 - r_6)} - \frac{r_1}{(r_1 - r_6)}\right] = 0 \tag{145}$$

It follows that for $\bar{c}_1{}^{\alpha} \neq \bar{c}_1{}^{\zeta}$, $\nu^{\alpha\zeta} = 0$ from eq 144, thereby establishing that boundary $\alpha\zeta$ is stationary. If $\bar{c}_1{}^{\alpha} = \bar{c}_1{}^{\zeta}$, then phases α and ζ are identical and no boundary of any kind exists between them.

Despite the theoretical flexibility in the composition of phase α, the fact is that phase ζ does not achieve the composition predicted from the characteristics of phase β until the stacking process is completed. If follows, therefore, that particularly in the early stages, facilitation of stacking can be brought about by making phase α identical with the theoretical and predicted phase ζ.

B. Phase β. The considerations discussed with reference to phase α also lead to the conclusion that, relative to phase λ, the composition of phase β is arbitrary (save for the specification of constituent 6 and 2) due to the existence of the stationary boundary $\beta\lambda$. Specifically, the implication is that $(\Gamma/2)^{\beta}$ bears no necessary relation to $(\Gamma/2)^{\pi}$. This degree of freedom can be considered in terms of $\bar{c}_2{}^{\beta}$ as an independent variable. In eq 109 it is shown that the length of the stacked sample is inversely proportional to $|\bar{c}_2{}^{\beta}|$. Thus a relatively high value for the latter quantity is desirable although upper limits are imposed by solubility restrictions and the fact that boundary velocities also vary inversely with the concentration of phase β at a given current level. It has been found experimentally that good stacking is achieved with the correlated quantity

$$|\bar{c}_1{}^{\zeta}| = 0.04 \pm 0.02 \tag{146}$$

Thus one arbitrary approach is to select a value in the above range.

Another approach is to consider that situation in which the boundary displacements for the moving boundaries $s\beta$ and $\pi\lambda$ are equal. The advantage of such a condition is a practical one in that once stacking is completed and the velocity of the tracking dye (if present) calculated ($v^{\zeta\beta} = \nu^{\zeta\beta} \cdot I$) and/or measured, the time required for the dye band to reach any particular point in the resolving gel can be computed easily. If, however, the boundary displacements of the two moving boundaries are different, then it is necessary to measure the lengths of both the stacking and resolving gels and the corresponding velocities in order to arrive at the same quantity. From eq 35 and 37 it follows that the stipulation of equal velocities implies

$$\bar{r}_1{}^{\zeta}/\sigma^{\zeta} = \bar{r}_1{}^{\pi}/\sigma^{\pi} = 1/96.5\bar{c}_1{}^{\zeta}(1 - r_6/r_1) = 1/96.5\bar{c}_1{}^{\pi}(1 - r_6/r_1) \tag{147}$$

or

$$\bar{c}_1{}^{\zeta} = \bar{c}_1{}^{\pi} = (\Gamma/2)^{\pi}/\phi_1{}^{\pi}$$

and

$$\nu^{\zeta\beta} = \nu^{\pi\lambda}$$

In the final analysis, the optimal value for $\bar{c}_1{}^{\zeta}$ or $\bar{c}_2{}^{\beta}$ has to be determined empirically. Experimentally, this is easily done with analytical gels by successive dilution of a concentrated stacking gel solution with a solution containing only the components of the polymerizing system. Phase α must also be varied in a corresponding manner.

C. "Phase η" Phenomenon. For the sake of theoretical consistency it is necessary to consider the nature of the stationary boundary $\beta\lambda$ for constituent 2 a divalent weak electrolyte. The same rationale used to derive eq 145 demonstrating the existence of a stationary boundary $\alpha\zeta$ between dissimilar phases α and ζ applies to the boundary $\beta\lambda$ for constituent 2 an ion or monovalent weak electrolyte. However, this is no longer the case for constituent 2 a divalent weak electrolyte due to the fact that the quantity $\bar{r}_2\bar{c}_2/\sigma$ is not a constant (as-

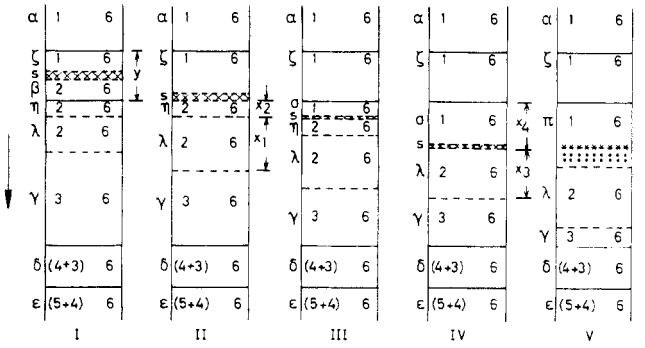

FIGURE 2: Phase "η" phenomenon for constituent 2 a divalent weak electrolyte. Details are given in the text.

suming invariant relative ion mobilities) but a function of pH. From eq 68 and 69, it is seen that

$$\bar{r}_2\bar{c}_2/\sigma = \frac{1 + (a - 1)\phi_2}{96.5[1 + b + (2a + b - 1)\phi_2]} \qquad (148)$$

where $a = r_{22}/r_{21}$ and $b = -r_6/r_{21}$. Since $pH^\beta \neq pH^\lambda$, it is seen that the left-hand side of the moving-boundary eq 11 for constituent 2 in relation to boundary $\beta\lambda$ no longer is equal to zero. It follows immediately that $\nu^{\beta\lambda} \neq 0$, thereby negating the existence of a stationary boundary $\beta\lambda$.

What in fact occurs is the formation of a new moving boundary sweeping out a phase designated as η, which is a concentrated version of phase β from which it is separated by stationary boundary $\beta\eta$ at the stacking gel–resolving gel interface. These relationships are indicated in Figure 2, diagram I, in which the stacking stage is depicted. From eq 148, it is seen that the requirement for the existence of a stationary boundary $\beta\eta$ is that $pH^\beta = pH^\eta$. Since the function θ is a function of pH alone, $\theta^\beta = \theta^\eta$. Hence, all constituent concentrations are diluted by the same factor across boundary $\beta\eta$. Constituent 2 does not disappear across the moving boundary $\eta\lambda$ since it is present in phase λ, the composition of which is determined, as described previously, by the nature of phase γ. This type of system is entirely analogous to a free electrophoresis experiment reported by Nichol (1950) in which a phosphate constituent migrated behind an iodide constituent, giving rise to two moving boundaries across one of which the phosphate constituent did not disappear. The equations which will be developed below accurately predict the relative magnitudes of the displacements of the two moving boundaries, as represented in Figure 6 of the cited paper.

Reference to diagram II in Figure 2 indicates that when the stacked sample, *i.e.*, moving boundary $s\beta$, reaches the stacking gel–resolving gel interface, phase β ceases to exist. At this point, moving boundary $\lambda\gamma$ has migrated a distance $x_2 + x_1$ from the gel interface, and moving boundary $\eta\lambda$ has migrated a distance x_2. In III, the stack has migrated into the resolving gel, possibly widening somewhat due to the fact that the gel matrix tends to reduce the constituent mobilities of the sample components so that some may unstack. A phase *o*, analogous to phase ζ in the same way that phase η is analogous to phase β, forms behind the sample. However, moving boundary $s\eta$ possesses a greater velocity than moving boundary $\eta\lambda$ and "catches up," as shown in IV, with the consequence that phase η is obliterated. At this stage, phase λ directs the characteristics of the sample phases and the phase behind containing constituent 1. As a consequence, phase *o* is replaced by a phase π, possessing characteristics directed by phase λ. Hence in the manner described previously, constituent 1 acquires a constituent mobility greater than that of the sample components and migrates ahead, forming moving boundary $\pi\lambda$. By reference to V it is seen that the net effect of this entire process is to displace the "starting" boundary for the resolution stage of the electrophoretic experiment from the gel interface to a position located in the resolving gel distance x_4 away and indicated, in diagram V, by the line of * symbols. In addition, the stacked phase s may tend to widen somewhat prior to the "destacking" process.

Equations describing this phenomenon will now be derived and will quantitatively indicate that for systems designed according to all the preceeding considerations, the "phase η" effect is quite minor. In other words, it is found that distance x_4 is extremely small relative to the length y of the stacking gel and the maximal length x_3 of phase λ.

As a direct consequence of the observations made regarding the stationary boundary $\beta\eta$, the following relationships can be written

$$\begin{aligned} \bar{c}_6^\eta &= w\bar{c}_6^\beta & \bar{r}_6^\eta &= \bar{r}_6^\beta \\ \bar{c}_2^\eta &= w\bar{c}_2^\beta & \bar{r}_2^\eta &= \bar{r}_2^\beta \qquad \nu^{s\eta} = \nu^{s\beta}/w = \nu^{\delta\beta}/w \\ \sigma^\eta &= w\sigma^\beta & \theta^\eta &= \theta^\beta \end{aligned} \qquad (149)$$

where w is the dilution factor across the stationary boundary $\beta\eta$. Moving boundary equations can be written for both constituents 2 and 6 across the moving boundary $\eta\lambda$.

$$\begin{aligned} \bar{r}_2^\eta\bar{c}_2^\eta/\sigma^\eta - \bar{r}_2^\lambda\bar{c}_2^\lambda/\sigma^\lambda &= \nu^{\eta\lambda}(\bar{c}_2^\eta - \bar{c}_2^\lambda) \\ \bar{r}_6^\eta\bar{c}_6^\eta/\sigma^\eta - \bar{r}_6^\lambda\bar{c}_6^\lambda/\sigma^\lambda &= \nu^{\eta\lambda}(\bar{c}_6^\eta - \bar{c}_6^\lambda) \end{aligned} \qquad (150)$$

It is assumed that the entire electrophoretic system has been calculated according to the procedure described in section IV. Substituting the relations of eq 149 into eq 150, therefore, leads to the following equations in which p and q can be immediately calculated since all the quantities on the left are known.

$$\begin{aligned} \bar{r}_2^\beta\bar{c}_2^\beta/\sigma^\beta - \bar{r}_2^\lambda\bar{c}_2^\lambda/\sigma^\lambda &= p = \nu^{\eta\lambda}(w\bar{c}_2^\beta - \bar{c}_2^\lambda) \\ \bar{r}_6^\beta\bar{c}_6^\beta/\sigma^\beta - \bar{r}_6^\lambda\bar{c}_6^\lambda/\sigma^\lambda &= q = \nu^{\eta\lambda}(w\bar{c}_6^\beta - \bar{c}_6^\lambda) \end{aligned} \qquad (151)$$

Solving for w

$$w = \frac{\bar{c}_2^\lambda - \bar{c}_6^\lambda(p/q)}{\bar{c}_2^\beta - \bar{c}_6^\beta(p/q)}$$

Equations 151 now yield a relation for $\nu^{\eta\lambda}$ in which all quantities are known

$$\nu^{\eta\lambda} = p/(w\bar{c}_2^\beta - \bar{c}_2^\lambda) \qquad (152)$$

Using the relation $\nu^{\lambda\gamma} = \bar{r}_2^\lambda/\sigma^\lambda$, the ratio of the displacement values for the two moving boundaries $\eta\lambda$ and $\lambda\gamma$ is obtained, and the equality with $x_2/(x_1 + x_2)$ follows from Figure 2.

$$\nu^{\eta\lambda}/\nu^{\lambda\gamma} = x_2/(x_1 + x_2) = p\sigma^\lambda/\bar{r}_2^\lambda(w\bar{c}_2^\beta - \bar{c}_2^\lambda) \qquad (153)$$

By reference to Figure 2, it is also evident that

$$x_4/\nu^{\zeta\eta} = (x_4 - x_2)/\nu^{\eta\lambda} \tag{154}$$

Conservation of mass dictates that

$$\bar{c}_2{}^{\beta}y = \bar{c}_2{}^{\eta}x_2 + \bar{c}_2{}^{\lambda}x_1 = \bar{c}_2{}^{\lambda}x_3 \tag{155}$$

Combining eq 149, 153, 154, and 155, one obtains

$$\begin{aligned} x_1/y &= 1/[w/(\nu^{\lambda\gamma}/\nu^{\eta\lambda} - 1) + \bar{c}_2{}^{\lambda}/\bar{c}_2{}^{\beta}] \\ x_2/y &= 1/[w + \bar{c}_2{}^{\lambda}(\nu^{\lambda\gamma}/\nu^{\eta\lambda} - 1)/\bar{c}_2{}^{\beta}] \\ x_3/y &= \bar{c}_2{}^{\beta}/\bar{c}_2{}^{\lambda} \\ x_4/y &= 1/(1 - w\nu^{\eta\lambda}/\nu^{\zeta\beta})[w + \bar{c}_2{}^{\lambda}(\nu^{\lambda\gamma}/\nu^{\eta\lambda} - 1)/\bar{c}_2{}^{\beta}] \end{aligned} \tag{156}$$

Calculations for the original and still widely used "Tris–glycine" system of Ornstein (1964) and Davis (1964), to be discussed in the next paper (Jovin, 1973b), indicate that x_1/y and $x_4/y \ll 1$. This condition should be confirmed for every designed system employing a divalent weak electrolyte as constituent 2.

D. Phase δ. It is necessary to specify a value for $(\Gamma/2)^{\delta}$. In order to facilitate the elution process, that is, the removal of the desired components from the electrical field, it is desirable to provide a minimal voltage gradient in the elution chamber. This can be achieved by establishing a high $(\Gamma/2)^{\delta}$, so that in general

$$(\Gamma/2)^{\delta} > (\Gamma/2)^{\pi} \tag{157}$$

An upper limit for the ionic strength is predicted on the basis of two considerations: (a) a very concentrated elution buffer will necessarily diffuse into the resolving gel, thus tending to diminish the voltage gradient and unfavorably retard the migration of bands out of the gel; (b) solubility limits may be exceeded. Determination of the optimal $(\Gamma/2)^{\delta}$ is ultimately an empirical process, but on the basis of the above discussion, the following limits are proposed

$$1 < [(\Gamma/2)^{\delta}/(\Gamma/2)^{\pi}] < 10 \tag{158}$$

E. Phase ε. It is also necessary to specify an ionic strength for this phase. Low values are undesirable since they lead to large voltage drops across the lower buffer. An arbitrary guide might be

$$(\Gamma/2)^{\pi} < (\Gamma/2)^{\epsilon} \leqslant (\Gamma/2)^{\delta} \tag{159}$$

It should be emphasized that a buffer solution with the composition of phase ζ is a poor choice for phase ε due to the fact that $(\Gamma/2)^{\zeta}$ tends to be very low.

F. $(\Gamma/2)^{\pi}$. Many considerations enter into the selection of this quantity though final optimization usually must be accomplished empirically. A lower limit is obviously determined by several parameters: loss of buffering value, failure of the assumption regarding negligible contributions of H^+ and OH^- to conductance, and increased bandwidths. Nonetheless, a *relatively* low value for the ionic strength is desirable from the standpoint of maximizing the electric field strength and thereby the velocities of migration. Also, relative electrophoretic mobilities of macroions tend to increase with decreasing ionic strength in accordance with theoretical prediction (Overbeek, 1950). Thus the R_F of a protein band, for example, is often seen to vary inversely with $(\Gamma/2)^{\pi}$ (Ornstein, 1964). The term R_F is used in this context to indicate the ratio of displacements of a given band and the moving boundary $\pi\lambda$, respectively, from the stacking gel–resolving gel interface.

It is easily shown that for a given power level, the following relationship exists between voltage gradient and ionic strength in phase π.

$$E_2{}^{\pi}/E_1{}^{\pi} = \left[\frac{(\Gamma/2)_1{}^{\pi}}{(\Gamma/2)_2{}^{\pi}}\right]^{1/2} = I_1/I_2 \tag{160}$$

Thus, decreasing the ionic strength by a factor of 2 leads to an increase in the voltage gradient by a factor of $\sqrt{2}$, while the current is diminished by the same factor.

In practice, alterations in $(\Gamma/2)^{\pi}$ are accomplished easily without necessity of recalculating the system. Constituent concentrations in phases α, ζ, β, λ, γ, and π are changed by the same ratio or, in other words, the corresponding buffer solutions are concentrated or diluted by the given factor.

By manipulating this quantity, therefore, alone or in combination with the composition of the gel matrix, it is possible to achieve striking alterations in the electrophoretic patterns. From the standpoint of preparative procedures, it is often possible to greatly accelerate the migration and hence elution of desired bands by modest decrements in the ionic strength and effective gel "pore size."

IV. Calculation of Phase Compositions and Parameters[7]

The following quantities are known at this time: (a) pK_1, (pK_2), (pK_4), (pK_5), (pK_6); (b) r_1, r_2 (or r_{21}, r_{22}), r_3, r_4, r_5, r_6; (c) θ^{ζ}, θ^{β}, θ^{π}, θ^{λ}; (d) $\phi_1{}^{\zeta}$, $\phi_1{}^{\pi}$, $\phi_2{}^{\beta}$, $\phi_2{}^{\lambda}$, $\phi_3{}^{\gamma}$; (e) pH^{ζ}, pH^{π}, pH^{δ}, pH^{ϵ}; (f) $(\Gamma/2)^{\pi}$, $(\Gamma/2)^{\delta}$, $(\Gamma/2)^{\epsilon}$; (g) $\bar{r}_1{}^{\zeta}$, $\bar{r}_1{}^{\pi}$, $\bar{r}_2{}^{\beta}$, $\bar{r}_3{}^{\gamma}$.

It is necessary to compute the following quantities for each phase: all $\bar{c}$'s, all $\bar{r}$'s, pH, all ϕ's, θ, $\Gamma/2$, σ, κ, BV, ν.

A suitable sequence for performing the calculations will now be described. The appropriate equation number will be indicated in parentheses following every quantity. For equations prior to and including 104, determination of the proper parameters is made by comparing the prototype system to the corresponding phases and boundaries in Figure 1.

A. Phase π. $\bar{c}_1{}^{\pi}$(147), $\bar{c}_6{}^{\pi}$(28), $\phi_6{}^{\pi}$(28), $\bar{r}_6{}^{\pi}$(37b), σ^{π}(38), κ^{π}(6), BV^{π}(29a), $\nu^{\pi\lambda}$(35).

B. Phase β. $\mu^{\zeta\beta}$ and $\bar{c}_3{}^{\beta}$(40 or 70), $\bar{c}_6{}^{\beta}$(24, 28 or 33), $\phi_6{}^{\beta}$(22, 27 or 32), $\bar{r}_6{}^{\beta}$(37b), pH^{β}(23, 29, or 34), $(\Gamma/2)^{\beta}$ and BV^{β}(24a, 29a, or 34a), σ^{β}(39, 55, or 69), κ^{β}(6), $\nu^{\zeta\beta}$(36).

C. Phase ζ. $\bar{c}_1{}^{\zeta}$(146), $\bar{c}_6{}^{\zeta}$(28), $\phi_6{}^{\zeta}$(27), $\bar{r}_6{}^{\zeta}$(37b), $(\Gamma/2)^{\zeta}$ and BV^{ζ}(29a), σ^{ζ}(38), κ^{ζ}(6), $\nu^{\zeta\beta}$(35).

D. Phase α. Same as phase ζ except no corresponding ν.

E. Phase λ. $\mu^{\pi\lambda}$(40 or 70), $\bar{c}_2{}^{\lambda}$(40 or 70), $\bar{c}_6{}^{\lambda}$(24, 28, or 33), $\phi_6{}^{\lambda}$(22, 27, or 32), pH^{λ}(23, 29, or 34), $(\Gamma/2)^{\lambda}$ and BV^{λ}(24a, 29a, or 34a), σ^{λ}(39, 55, or 69), κ^{λ}(6), $\nu^{\lambda\gamma}$(36), $\bar{r}_2{}^{\lambda}$(37, 54, or 68), $\bar{r}_6{}^{\lambda}$(37b).

F. Phase γ. $\mu^{\lambda\gamma}$(101, 40, or 88), $\bar{c}_3{}^{\gamma}$(101, 40, or 88), θ^{γ}(102, 45, or 89), $\bar{c}_6{}^{\gamma}$(102, 45, or 89), $\phi_6{}^{\gamma}$(22), pH^{γ}(23), $\bar{r}_6{}^{\gamma}$(37b), $(\Gamma/2)^{\gamma}$ and BV^{γ}(24a, 29a, or 34a), σ^{γ}(39), κ^{γ}(6), $\nu^{\lambda\gamma}$(36).

G. Phase δ. $\phi_6{}^{\delta}$(22 or 27), $\bar{c}_6{}^{\delta}$(7 and 24 or 28), $\phi_4{}^{\delta}$(21 or 25), θ^{δ}(24 or 28), $\bar{c}_4{}^{\delta}$(24 or 28), σ^{δ}(39 or 55), κ^{δ}(6), BV^{δ}(24a).

H. Phase ε. $\phi_6{}^{\epsilon}$(22, 27, or 32), $\phi_5{}^{\epsilon}$(21, 25, or 30), $\bar{c}_6{}^{\epsilon}$(24a or 34a), θ^{ϵ}(24, 28, or 33), $\bar{c}_5{}^{\epsilon}$(24, 28, or 33), σ^{ϵ}(39, 55, or 69), κ^{ϵ}(6), BV^{ϵ}(24a).

I. Phase η (for constituent 2 a divalent weak electrolyte). w(151), $\nu^{\eta\lambda}$(152), x_2/y(156), x_4/y(156).

Acknowledgments

I thank Drs. A. Chrambach, D. Rodbard, and J. Lunney for the critical reading of earlier versions of this manuscript.

References

Chrambach, A., and Rodbard, D. (1971), *Science 172*, 440.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Jovin, T. M. (1973a), *Biochemistry 12*, 871.
Jovin, T. M. (1973b), *Biochemistry 12*, 890.
Jovin, T. M. (1973c), *Ann. N. Y. Acad. Sci.* (in press).
Jovin, T. M., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9*, 351.
Lunney, J., Chrambach, A., and Rodbard, D. (1971), *Anal. Biochem. 40*, 158.
Nichol, J. C. (1950), *J. Amer. Chem. Soc. 72*, 2367.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Overbeek, J. T. G. (1950), *Advan. Colloid Sci. 3*, 97.
Rodbard, D., and Chrambach, A. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 970.
Rodbard, D., and Chrambach, A. (1971), *Anal. Biochem. 40*, 95.
Rodbard, D., Kapadia, G., and Chrambach, A. (1971), *Anal. Biochem. 40*, 135.
Williams, D. E., and Reisfeld, R. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 373.

Multiphasic Zone Electrophoresis. III. Further Analysis and New Forms of Discontinuous Buffer Systems†

Thomas M. Jovin

ABSTRACT: In the conventional discontinuous buffer system for analytical and preparative electrophoresis, the sample is concentrated or "stacked" into a thin zone prior to resolution in a supporting medium such as polyacrylamide gel. In this paper, the theory of multiphasic zone electrophoresis developed previously is applied, first of all, to certain procedures related to the stacking process: (a) the selection and use of a *tracking dye;* (b) *preequilibration of sample* and selection of the required length of stacking gel; (c) *selective stacking* of desired components by suitable alteration of the pH and composition of the resolving phase; (d) *selective restacking* of slowly migrating bands by the generation of a new moving boundary after separation has been effected; and (e) *steady-state stacking* or *isotachophoresis* in which a relatively extended stack is generated with or without the inclusion of ampholytes as "spacers." In subsequent sections, special topics and new applications of the theory of multiphasic zone electrophoresis are discussed: (a) electrophoresis in *pH gradients* as in the technique of isoelectric focusing. Such gradients are created by polymerizing a resolving gel containing a gradation of buffer concentrations; (b) *bidirectional electrophoresis* in which are used pairs of electrophoretic systems having the same upper buffer but opposite polarity. So-called mirror systems of this type permit the analysis of heterogeneous sample populations containing macromolecules of opposite net charge; and (c) *cross-boundary electrophoresis* with which stacking and separation are effected in a single gel. Such systems employ a pair of moving boundaries migrating in opposite directions. Finally the complete analysis of two electrophoretic buffer systems is presented, one of which is the original "Tris–glycine" system for disc electrophoresis. The second is one of the 4269 systems generated by computer according to the present theory.

A. Tracking Dye

It has been found useful to utilize a dye as a visible marker for the moving boundaries $s\beta$ and $\pi\lambda$.[1] By introducing it into phases initially, the progress of the stacking process can be readily observed, providing the dye possesses characteristics which lead to its being stacked between the sample and constituent 2. That is

$$\bar{r}_{\mathrm{dye}}{}^{s}/\bar{r}_{s1}{}^{s} > 1 \qquad \bar{r}_{\mathrm{dye}}{}^{\beta}/\bar{r}_{2}{}^{\beta} < 1 \tag{161}$$

The corresponding requirement for use of the dye as a marker of the moving boundary $\pi\lambda$ is

$$\bar{r}_{\mathrm{dye}}{}^{\pi}/\bar{r}_{1}{}^{\pi} > 1 \qquad \bar{r}_{\mathrm{dye}}{}^{\lambda}/\bar{r}_{2}{}^{\lambda} < 1 \tag{162}$$

In systems utilizing a weak monovalent electrolyte as constituent 2, it may be found that for certain dyes the second condition in 161 is not met and hence the dye does not stack properly. However, condition 162 may be fulfilled with the consequence that the dye is still useful since this boundary is the one used for the calculation of R_F values.

The practice of placing the dye with the sample is preferable to introducing it into the entire upper buffer. In the latter case, it does not become entirely stacked at the moving boundary and tends to trail in phase π, although this fact may not be apparent to the naked eye. Thus, it is possible to determine the minimal amount of dye required for a visible sharp band and place this in the sample or, alternatively, in the stacking gel from which it will also become stacked.

The moving boundary $\pi\lambda$ with which the tracking dye is associated can provide quantitative information about the constituent mobility of a given band migrating in the π phase. If the distance such a band has migrated in the π phase in a certain time, relative to the corresponding excursion of the tracking dye, is denoted by R_F, then the relative constituent mobility of the band $= R_F\bar{r}_1{}^{\pi}$ and the absolute mobility can

† From the Abteilung Molekulare Biologie, Max-Planck-Institut fuer Biophysikalische Chemie, 34 Goettingen, Germany. *Received May 23, 1972.* This work was done in part during the tenure of an Established Investigatorship of the American Heart Association.

[1] Definitions of nomenclature and equations with numbers prior to 161 are found in the preceding papers of this series (Jovin, 1973a,b).