Acknowledgments

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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

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

be estimated by multiplication with the sodium ion mobility appropriate for the temperature and ionic strength used (Rodbard and Chrambach, 1971).[2]

B. Sample Equilibrium and Required Stacking Gel Length

The velocity of a moving boundary is directly related to the boundary displacement by the expression

$$\text{velocity (cm sec}^{-1}) = \nu I \tag{163}$$

where I is the current density. Thus the ratio of velocities for different boundaries is identical with the ratio of the corresponding boundary displacements.

Consider for the moment a sample of volume y which is equilibrated against a buffer solution identical in composition with phase β. It follows that

$$\nu^{\zeta s} = \nu^{\zeta\beta} = \bar{r}_2^{\beta}/\sigma^{\beta} \text{ and } \nu^{s\beta} = \bar{r}_{s1}{}^{\beta}/\sigma^{\beta} \tag{164}$$

Thus the ratio of the velocity of the upper and lower boundaries of phase s is given by

$$\nu^{\zeta s}/\nu^{s\beta} = \bar{r}_2^{\beta}/\bar{r}_{s1}{}^{\beta} \tag{165}$$

The condition for complete stacking can be stated approximately as the necessity for the moving boundary ζs to sweep out a volume larger by y than the volume traversed by moving boundary sβ. That is, boundary ζs must "catch up" to boundary sβ. Consider the volume of stacking gel required in order for this process to take place as x. It follows that

$$(x + y)/\nu^{\zeta s} \cong x/\nu^{s\beta} \tag{166}$$

or $x/y \cong 1/(\bar{r}_2^{\beta}/\bar{r}_{s1}{}^{\beta} - 1)$.

Now let us consider the same sample of volume y equilibrated against a buffer solution with composition equal to that of phase ζ. In this case,

$$\nu^{\zeta s} = \bar{r}_{sk}{}^{\zeta}/\sigma^{\zeta} \qquad \nu^{s\beta} = \nu^{\zeta\beta} = \bar{r}_1{}^{\zeta}/\sigma^{\zeta}$$

It follows that the ratio of velocities of the upper and lower boundaries of phase s is given by

$$\nu^{\zeta s}/\nu^{s\beta} = \bar{r}_{sk}{}^{\zeta}/\bar{r}_1{}^{\zeta} \tag{167}$$

In this case, eq 166 reduces to

$$x/y \cong 1/(\bar{r}_{sk}{}^{\zeta}/\bar{r}_1{}^{\zeta} - 1) \tag{168}$$

In the above discussion, $\bar{r}_{s1}$ is the constituent mobility of the "fastest" sample component, implying that in the stacked sample, constituent s1 is adjacent to the moving boundary sβ. On the other hand, $\bar{r}_{sk}$ refers to the "slowest" sample component, that is, the one that lies adjacent to the moving boundary ζs in the stacked sample.

Equations 166 and 168 provide a rationale for determining whether a sample of relatively large volume should be equilibrated against upper buffer or phase β buffer. Systems in which constituent 2 is an ion or a divalent weak electrolyte generally will have a large value for $|\bar{r}_2^{\beta}|$, whereas the corresponding value for $|\bar{r}_1{}^{\zeta}|$ may be such that

$$\bar{r}_2^{\beta}/\bar{r}_{s1}{}^{\beta} > \bar{r}_{sk}{}^{\zeta}/\bar{r}_1{}^{\zeta} \tag{169}$$

If this condition holds, then it is preferable to equilibrate the sample against phase β since the ratio x/y will be smaller. Note that in order to achieve a value of $x/y \leqslant 1$, it is necessary that $\bar{r}_2^{\beta}/\bar{r}_{s1}{}^{\beta} \geqslant 2$.

In systems utilizing a monovalent weak electrolyte as constituent 2, $|\bar{r}_2^{\beta}|$ is generally relatively small, but very low values for $|\bar{r}_1{}^{\zeta}|$ are possible. In such a case, condition 169 may not hold with the implication that the sample should be equilibrated against the upper buffer in order to minimize the required length of stacking gel.

In each specific case, the characteristics of the system and the sample will determine the optimal procedure. It is obvious that for very small sample volumes, the problem is not critical and assuming the absence of large amounts of electrolyte in the sample, it may not be necessary to preequilibrate against any particular buffer.

It remains to point out the obvious fact that boundary velocity calculations according to eq 163 allow estimates as to the necessary time for each stage of the electrophoretic experiment to take place, provided the current through the system is maintained constant. A current regulated power supply is required due to the fact that the total voltage drop across the gel is a function of time.

$$\text{total voltage drop} = I\sum_i L_i(t)/\kappa_i \tag{170}$$

where $L_i(t)$ and κ_i are the length (time dependent) and conductance, respectively, of a given phase and the summation is over all phases, i.

C. Selective Stacking

Let it be assumed that for a given system, a certain band (component k) migrates in phase π with $R_F = \bar{r}_k{}^{\pi}/\bar{r}_1{}^{\pi}$. In the case of a preparative system, elution of this band may be difficult without a large dilution factor if the R_F value is relatively small. If the gel pattern is such that most of the other sample components possess even lower R_F values, it is possible to alter the system so that the band of interest migrates in a stacked configuration and is thereby eluted promptly and in a concentrated form. In order to accomplish this condition, it is first necessary to change the parameters of phase π so that

$$(\bar{r}_1{}^{\pi})_2 = (x)R_F(\bar{r}_1{}^{\pi})_1 \tag{171}$$

In eq 171, the subscripts 2 and 1 refer to the altered and original system, respectively. The factor x is less than 1, *i.e.*, $(\bar{r}_1{}^{\pi})_2/(\bar{r}_k{}^{\pi})_1 \leqslant 1$ since the required alteration of pH$^{\pi}$ will in general tend to decrease the value of $|\bar{r}_s{}^{\pi}|$ although the magnitude of the change in most instances will be quite small relative to the corresponding alteration in $\bar{r}_1{}^{\pi}$. Thus the value for x must be determined empirically for each specific case. Initially, a suitable value might be $x = 0.8$.

Equation 171 is the expression, then, of the need to have

$$(\bar{r}_k{}^{\pi})_2/(\bar{r}_1{}^{\pi})_2 > 1 \qquad (\bar{r}_k{}^{\lambda})_2/(\bar{r}_2{}^{\lambda})_2 < 1 \tag{172}$$

in order to maintain component k stacked at the moving boundary $\pi\lambda$ while the other components with lower R_F values migrate in phase π and are thereby separated from the desired substance. It is of course obvious that all other com-

[2] A. Chrambach and T. Jovin, unpublished data.

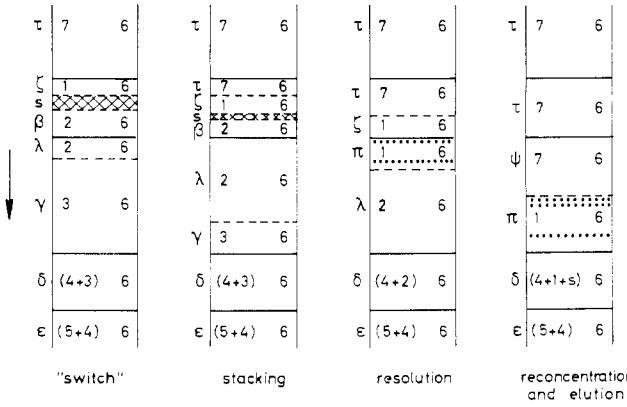

FIGURE 1: Stages in the "restacking" process. Details are given in the text.

ponents meeting conditions 172 will also be stacked at the moving boundary. In general, these components will correspond to those with R_F values greater than that of the desired band in the original system.

The change stipulated by eq 171 implies the necessity for recalculating the parameters of phases π, λ, and γ. The proper sequence of computations will now be given in a form similar to one given previously for the entire system (Jovin, 1973b).

Phase π. Let $(\Gamma/2)_2^{\pi} = (\Gamma/2)_1^{\pi}$; $\phi_1^{\pi}(37)$, $pH^{\pi}(23)$, $\theta^{\pi}(28)$. Other quantities are calculated in the sequence described previously for this phase.

Phase λ. For constituent 2 a divalent weak electrolyte: $\phi_2^{\lambda}(71)$, $\theta^{\lambda}(72)$. For constituent 2 an ion or monovalent weak electrolyte: $\theta^{\lambda}(133)$, $\phi_2^{\lambda}(21$ or $26)$. The additional calculations have been described previously for this phase.

Phase γ. Calculate as described previously for this phase.

The other phases (α, ζ, β, δ, ϵ) remain unaltered except it should be noted that the $\bar{c}_1^{\zeta}$ originally computed on the basis of eq 147[3] will no longer be equal to $\bar{c}_1^{\tau}$. If desired, phases ζ and β can be altered in correspondence with the new value of $\bar{c}_1^{\tau}$, but from a practical standpoint, it may not be necessary to do so.

In general, the conditions specified in 172 will be fulfilled without providing much of a differential in the constituent mobilities of the sample component and constituent 1. In other words, the steady-state equilibrium which maintains the component stacked at the moving boundary may be precarious. It is therefore advisable to operate the system at relatively low current levels in order to prevent disruption of the labile equilibrium.

D. "*Restacking*"

In many instances, desired components s migrate as bands with lower R_F values than many of the other sample constituents. In these instances, elution from a preparative device is a time consuming process and usually involves inordinate dilution of the material. The selective stacking procedure described above will not be applicable due to the presence of many components with larger R_F values.

Under suitable conditions, it is possible to generate a second moving boundary which migrates behind boundary $\pi\lambda$ at a fixed distance and at which the desired bands with low mobilities will stack, thus leading to their prompt elution in concentrated form. This is accomplished by removing the original upper buffer phase α after a specified time of migration of constituent 1 into the resolving gel, and replacing it with a new buffer phase τ. The brief interruption of current flow does not lead to adverse effects since the sample will immediately concentrate maximally when electrophoresis is resumed.

For this procedure, it is necessary that the sample (if of large volume) be equilibrated against phase β in order that the stacking process will have progressed as far as possible toward completion by the time constituent 1 begins to migrate into the resolving gel.

The various stages in this procedure are shown in Figure 1. The "switch" stage corresponds to the stacking stage in Figure 1 (Jovin, 1973b). All of the sample is in the stacking gel but may not be completely stacked. Phase α is removed and phase τ substituted. In the subsequent stacking stage, phase s has been maximally concentrated and moving boundary $\tau\zeta$ is migrating in the stacking gel. The depicted resolution stage is equivalent to that shown before, but in the reconcentration stage, it is seen that moving boundary $\psi\pi$ is carrying with it those components with low R_F values in the conventional system. At the termination of the elution phase, little or no sample material is left in the resolving gel.

From the same considerations that apply to constituent 1, it is evident that constituent 7 must be a monovalent weak electrolyte for which

$$\bar{r}_s^{\psi}/\bar{r}_7^{\psi} > 1 \qquad \bar{r}_7^{\pi}/\bar{r}_1^{\pi} < 1 \tag{173}$$

It is already known that $\bar{r}_s^{\pi}/\bar{r}_1^{\pi} < 1$ by virtue of the fact that the desired components s are migrating in phase π to begin with.

In order to avoid retardation of components s due to a pH effect, *i.e.*, in order to have $\bar{r}_s^{\psi}/\bar{r}_s^{\pi} \geq 1$, a further condition is

$$\pm(pH^{\pi} - pH^{\psi}) \geq 0 \tag{174}$$

A value for $\bar{r}_7^{\psi}$ can be stipulated on the basis of the first condition in 173

$$\bar{r}_7^{\psi}/\bar{r}_1^{\pi} \leq (R_F)_{\min} \tag{175}$$

where $(R_F)_{\min}$ refers to the minimal R_F value of the desired component or components, and will simply be referred to as R_F in the following discussion.

Taken together, conditions 174 and 175 assure the satisfaction of steady-state conditions for the moving boundary $\psi\pi$.

Condition 175 can be restated in terms of ϕ_7^{ψ}

$$\phi_7^{\psi} \leq R_F\bar{r}_1^{\pi}/r_7 \tag{176}$$

Combination of conditions 176 and 174 with eq 25 yields

$$\pm(pH^{\pi} - pK_7) \geq \log[(r_7/\bar{r}_1^{\pi}R_F) - 1] \tag{177}$$

This equation supplies the basis for the selection of constituent 7. However, the composition of phase ψ is not arbitrary but is determined by the characteristics of phase π. It is therefore necessary for each potential choice of constituent 7 to compute $\mu^{\psi\pi}$ from eq 40, then θ^{ψ} (eq 44), and thereby ϕ_7^{ψ} (eq 26). This computed value for ϕ_7^{ψ} is checked against

[3] For eq 1 to 104, see paper I of this series (Jovin, 1973a); for eq 105 to 160 see paper II of this series (Jovin, 1973b).

eq 176, and final selection of the constituent is made according to the degree to which the equality condition can be achieved in that equation.

The following is the sequence of computations for the determination of phases ψ and τ.

Phase ψ. pK_7, r_7, ϕ_7^{ψ}, θ^{ψ}, $\mu^{\psi\pi}$ are known. $\bar{c}_7^{\psi}(40)$, $\bar{c}_6^{\psi}(28)$, $\phi_6^{\psi}(28)$, $\bar{r}_7^{\psi}(35)$, $\bar{r}_6^{\psi}(37b)$, $pH^{\psi}(29)$, $(\Gamma/2)^{\psi}$ and $BV^{\psi}(29a)$, $\sigma^{\psi}(38)$, $\kappa^{\psi}(6)$, $\nu^{\psi\pi}(35)$.

Phase τ. $\mu^{\tau\zeta}(=\mu^{\psi\pi})$, $\theta^{\tau}(44)$, $\bar{c}_7^{\tau}(40)$, $\bar{c}_6^{\tau}(28)$, $\phi_7^{\tau}(26)$, $\phi_6^{\tau}(28)$, $\bar{r}_7^{\tau}(35)$, $\bar{r}_6^{\tau}(37b)$, $pH^{\tau}(29)$, $(\Gamma/2)^{\tau}$ and $BV^{\tau}(29a)$, $\sigma^{\tau}(38)$, $\kappa^{\tau}(6)$, $\nu^{\tau\zeta}(35)$.

In general, the considerations involved in the selection of constituent 7 should ensure compliance with steady-state moving-boundary restrictions for the boundary $\tau\zeta$. Confirmation of the fact is readily made by reference to eq 66 and 67. The value for $(\Gamma/2)^{\tau}$ will tend to be quite low, giving rise to large voltage drops and increased heat production. To counteract this effect, it may be possible to utilize a more concentrated version of phase τ as the upper buffer. An optimal value can be determined empirically.

It is possible to estimate the separation between the two moving boundaries $\psi\pi$ and $\pi\lambda$ by applying the conservation of mass principle to constituent 1. Thus, if y = separation between the two moving boundaries $\tau\zeta$ and ζs in the stacking gel, the corresponding separation x in the resolving gel will obey the relation

$$x/y = \bar{c}_1^{\zeta}/\bar{c}_1^{\pi} \tag{178}$$

This same consideration leads immediately to the conclusion that the two moving boundaries have the same velocities in both stacking and resolving gels. That is

$$\nu^{\tau\zeta} = \nu^{\zeta s} = \nu^{\zeta\beta} \qquad \nu^{\psi\pi} = \nu^{\pi\lambda} \tag{179}$$

In many instances, it may be desirable to elute one or more bands from the resolving gel before the second stacking moving boundary $\psi\pi$ "catches up" to them and reverses the entire resolving phase of the experiment. One can derive an expression relating the required length of resolving gel for which bands with R_F values greater than a given value R will elute before moving boundary $\psi\pi$ reaches the end of the gel. The quantity x described above provides the linear distance of gel separating moving boundaries $\tau\pi$ and $\pi\lambda$. Thus when boundary $\tau\pi$ is at the instant of departure from the stacking gel–resolving gel interface, boundary $\pi\lambda$ will have migrated distance x into the resolving gel and a band with $R_F = R$ will have migrated a distance of Rx. If the total length of resolving gel is L, it follows that

$$(L - x)/R\nu^{\pi\lambda} = L/\nu^{\psi\pi} \tag{180}$$

where the denominators are the displacement values for the band in phase π and moving boundary $\psi\pi$, respectively. From relations 179, it follows that

$$L/x = 1/(1 - R) \tag{181}$$

It follows, therefore, that in order to prevent merging of the moving boundary $\psi\pi$ and bands with R_F values $\geqslant R$

$$L < x/(1 - R) \tag{182}$$

or

$$x > L(1 - R)$$

These calculations involve many assumptions and must be employed merely to provide approximate values for the quantities involved.

It should be mentioned that the physical "switching" operation can be avoided by placing the desired amount of phase α (including sucrose or another nonelectrolyte) over the sample and layering phase τ over phase α initially.

E. Steady-State Stacking and Isotachophoresis

Reference is made here to procedures for (1) concentrating dilute samples such as column eluates, and (2) creating long stacks for analytical or preparative purposes. It is necessary to differentiate between the use of samples without additional components, *i.e.*, steady-state stacking (first demonstrated by Ornstein in 1963[4]), and a similar procedure in which additional ampholytes are introduced for the purpose of creating "spacer" zones between sample constituents, *i.e.*, isotachophoresis (Haglund, 1970; Everarerts and Routs, 1971; Routs, 1971; Chrambach *et al.*, 1973).

Sample concentration methods do not require the development of any new principles. In practice, the required length of stacking gel can be determined according to the considerations given in the preceding section B. Continuous elution is not required but the preparative apparatus must possess a chamber adjacent to the gel from which the sample can be easily recovered after the stack has migrated out. The same considerations apply for analytical-scale procedures in which the aim is either to assess the effectiveness of the initial stacking process prior to subsequent fractionation or to concentrate the sample for immunological, enzymatic, or other biochemical determinations on the stack itself.

The preparative stacking procedure requires the calculation of the necessary concentrations for phase β (and consequently phase ζ) relative to the amount of sample and the geometrical characteristics of the apparatus. The purpose is to utilize the stacking process not for its concentrating potential primarily but as a means of fractionation on the basis of distinct constituent mobilities for each component of the heterogeneous sample mixture. If the stacked sample achieves an overall length L in the stacking gel and migrates at a velocity v it will elute entirely in a period of time $t = L/v$. Each component will elute in a period of time $t_i = tL_i/L$, where L_i is the length of the stack occupied by that component. Hence successive fractions will contain the individual components with heterogeneity only at the point of overlap. The great advantages of this procedure derive from the fact that the components elute in concentrated form at a constant velocity, including substances that migrate extremely slowly under the influence of a uniform electrical field. It has been already stated in eq 110 and its corresponding discussion that there is a gradient in electric field strength within the stack accounting for the unit velocity. From eq 109 it is seen that L varies inversely with $|\bar{c}_2^{\beta}|$ for a given amount of material. Thus one must first select the proper $\bar{c}_2^{\beta}$. This allows the calculation of the required concentrations for phase ζ (and phase α). It is obvious, in addition, that the necessary length of stacking gel $> L$. The same considerations regarding con-

[4] L. Ornstein, exhibit of the Canal Industrial Corp., 47th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1963.

centrations apply to analytical procedures. In the latter instance, however, there is the problem of visualizing individual components by some functional test since by simple protein staining, for example, only an uninterrupted column will be observed.

The procedure of isotachophoresis represents an attempt to separate components within the stack by addition of ampholytes with electrophoretic mobilities and titration curves such that they achieve equilibrium positions within the stack. Thus upon elution or staining, a certain degree of discrimination between sample components is achieved.

In all cases where a relatively continuous series of components with differing electrophoretic mobilities is present, the stack will generally be in a labile equilibrium since the regulating process establishing and maintaining the individual moving boundaries depends upon how large the relative steps in mobility and electric field are. Thus while high fields sharpen the boundaries (Ornstein, 1964), the attendant Joule heating and thermal convection tend to produce mixing between zones. In addition, proteins at high concentrations tend to participate in complex interactions which lead often to precipitation. This is particularly true if during the formation of the stack, in which a pH gradient as well as a gradient in the electric field is established, a particular component is titrated to its isoelectric point.

F. pH Gradients and Isoelectric Focusing

The basis of isoelectric focusing techniques is the use of ampholytes which under the influence of an electric field assume an equilibrium distribution about their isoelectric point in a pH gradient (Svensson, 1961; Finlayson and Chrambach, 1971). If a series of compounds with different isoelectric points are selected, a pH gradient in the desired range can be established.

The theory of multiphasic zone electrophoresis suggests an alternative procedure for creating relatively stable pH gradients using only simple buffers. In the conventional system described above (Jovin, 1973b), the resolution phase π is homogeneous with respect to composition and pH. If, however, the resolving gel, phase γ, is prepared with an appropriate gradient in the relative concentrations of constituent 3 and 6, the phases λ and π which form subsequently will also reflect the gradient in their properties. Consider a system with polarity $-$ (migration to the anode). By the methods already described one can calculate the two compositions of phase γ required to ultimately achieve the corresponding and desired pH's in phase π, that is, during resolution. In the preparation of the resolving gel, therefore, one merely creates a gradient by the conventional technique of appropriately mixing two solutions consisting of the components required for polymerizing the gel and the two phase γ buffers. In the case of the $-$ polarity system, the phase γ buffer with the highest pH is at the most distal point of the resolving gel.

In such a system, the stacking or concentration phase proceeds normally but as the $\pi\lambda$ boundary traverses the resolving gel, a linear gradient in pH is created and individual sample components successively unstack and migrate to their isoelectric or equilibrium positions in the gel. The pH gradient is subject to degradation by diffusion and thus the gel concentration will largely influence the time over which the procedure is feasible. Obviously, in such a system, it is not desirable to impose the molecular sieving effect generally associated with gel electrophoresis and the gel matrix can be optimized with respect to its anticonvective function alone. Initial experiments with this technique have been reported.[5]

G. "Mirror Systems" for Bidirectional Electrophoresis

In a heterogeneous mixture of sample components, *e.g.*, proteins, the selection of a certain system with a given pH^{π} and polarity necessarily limits the range of mobilities within which stacking and resolution are effected. Components with isoelectric points such that they migrate in the opposite direction at pH^{ζ} will not be detected.

To overcome this limitation, particularly at neutral pH's where the problem is most acute, it is possible to generate a complementary pair of systems by which all components will be observed. Such systems must have the following properties: (1) opposite polarity and (2) identical phase ζ, *i.e.*, upper stacking phase.

These conditions, if fulfilled, imply that the systems are "mirrors" of each other in the sense that constituents 1 and 6 of the one correspond to constituents 6 and 1, respectively, of the other. Both constituents are monovalent weak electrolytes, and furthermore, for efficient stacking in both directions, both must have low enough absolute values for their constituent mobilities in phase ζ. The resolving pH^{π} for the two systems can be selected at will within the limitations of each system. Thus the procedure is to run a given sample in the two systems separately or to physically link the gels with the sample compartment between them. For each system the selection of constituents 2 and 3 is also made independently and according to the criteria described previously (Jovin, 1973b).

An example of such a pair of systems out of many generated by computer is given elsewhere (Jovin, 1973c). A more empirical system was reported by Raeusen (1967).

H. Cross-Boundary Electrophoresis

One disadvantage of discontinuous buffer systems is the requirement for two gels with different formulations which because of diffusion cannot be prepared and stored indefinitely. It is therefore worthwhile to consider the possibility of applying the basic principles in such a way that only one gel need be used. It is not desirable to simply omit the stacking gel because the concentration process due to the planned buffer discontinuities will not fully take place before some of the sample enters the resolving gel. One solution to this problem is the creation of a buffer system in which *two* moving boundaries with *opposite* directions of migration cross and subsequently generate the desired resolving phase.[6] Such a *cross-boundary* system is shown in Figure 2.

There is a single gel (phase β) with constituents 2 and 6, and upper and lower buffers (phases α and α', respectively) with constituent pairs 1 and 6 and 2 and 8. The sample is interposed, as usual, between phases α and β, and stacking ensues upon application of current, with the formation of phase ζ in the gel. At the same time, a moving boundary forms and migrates in the opposite direction, creating phase ζ' in the lower half of the gel. When the boundaries meet (Figure 2) phase β ceases to exist. Under proper conditions, a different phase π is created as two new moving boundaries form and migrate away from each other. Thus if phase π has the characteristics described for the conventional type of system, the sample components will unstack from the $\pi\zeta'$ boundary and separate into bands. The origin of the resolving phase is thus the point of crossing of the two original boundaries, denoted by the row of stars in the last diagram of Figure 2.

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

[6] L. Ornstein, personal communication.

TABLE I: Correspondence between Parameters in the Cross-Boundary System of Figure 2 with Elements of Its Constituent Subsystems.[a]

Subsystem	Polarity	Phase		Constituent		
		α	β	1	2	3
I	$\pm$	ζ	β	1	2	6
II	$\mp$	ζ'	β	8	6	2
III	$\pm$	π	ζ'	1	2	8
IV	$\mp$	π	ζ	8	6	1

[a] Each subsystem is comprised of one moving boundary and two phases and constitutes a class II electrophoretic unit described in Jovin (1973a), eq 54–57.

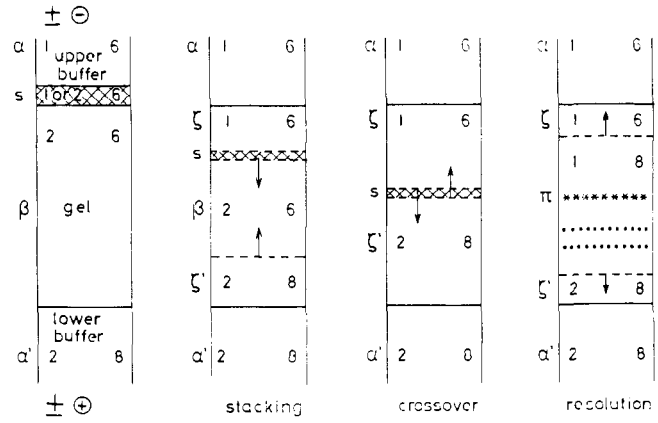

FIGURE 2: Cross-boundary electrophoresis. Details are given in the text and in Table I.

To design such a system is difficult due to the complicated interrelationships between the constituents and the properties of the phases. In order to take advantage of the formalism developed for the conventional systems, one may regard the cross-boundary system as a combination of four subsystems corresponding to class II electrophoretic units (Jovin, 1973a). The correspondence between constituents and phases is shown in Table I. For the usual stacking and unstacking conditions to hold, of course, it is necessary that $\pm(\mathrm{pH}^{\zeta} - \mathrm{pH}^{\pi}) > 0$, so that $|\bar{r}_1^{\zeta}| \leqslant r_{\min}^{\zeta}$ (eq 107) and $|\bar{r}_1^{\pi}| \geqslant r_{\max}^{\pi}$ (eq 114).

Since it is required that stacking and resolution take place in the same gel, certain compromises in the design of the gel matrix must be made. Thus if a high polyacrylamide content is desired to maximize molecular sieving effects, the stacking process will be correspondingly difficult since the constituent mobilities of the sample will tend to be small in magnitude. In such cases, constituent 1 and pH^{ζ} have to be chosen with care so as to achieve the desired limit. Furthermore, because no physical boundary demarcates the point at which the two original boundaries cross and thus define the beginning of phase π, the subsequent calculation of R_F values is rendered somewhat difficult. For a given system, however, the point of crossing will occur at a distance from the top of the gel equal to a certain fraction of its total length. This fraction should be approximately equal to the ratio of the boundary displacements, $\nu^{\zeta\beta}/\nu^{\zeta'\beta}$. It is advantageous to have this ratio as small as possible in order to minimize the time of migration of the stacked sample after it has achieved the final degree of concentration. Since the stacking conditions determine $\nu^{\zeta\beta}$, one can only attempt to maximize $\nu^{\zeta'\beta}$. By examination of eq 35 and 38, it is seen that $\nu^{\zeta'\beta}$ is maximized by making $|r_1|$ as large and $|r_2|$ as small as possible, through appropriate selection of constituents 1 and 2.

I. Influence of Gel Structure and Inclusion of Nonconstituents

For the purpose of this discussion, all specific effects of gel structure upon the separation of specific molecular species can be regarded as alterations of the constituent mobilities during stacking and resolution. The more relevant interpretation of mobilities in terms of molecular parameters such as size and shape have been considered elsewhere (Rodbard and Chrambach, 1971; Rodbard *et al.*, 1971; Lunney *et al.*, 1971).

In addition to the total polyacrylamide concentration and degree of cross-linking, profound effects on the mobility of certain compounds can result from copolymerization of acrylamide with charged monomers or other substances with the capacity to interact in specific ways, *e.g.*, nucleic acids.

The introduction of urea or other nonelectrolytes into the gel also leads to complicated effects. Besides the interactions with solute molecules, there is a perturbation of the solvent system itself with consequent changes in relevant physical parameters such as pK's. Thus while the empirical approach of simply adding urea, for example, to a calculated system often results in success, it does not follow that the system parameters remain unaffected. This is even more true of charged molecules such as mercaptoacetic acid or sodium dodecyl sulfate. Since both components participate in the stacking process, they will not be distributed uniformly throughout the system. It has, however, been the experience that sodium dodecyl sulfate works well in combination with discontinuous buffer systems (Neville, 1971; Neville and Glossmann, 1971). To fully account for such cases, the theory must be extended.

J. Analysis of "Tris–Glycine" System

The original system for "disc" electrophoresis reported by Ornstein and Davis (1962) has been used extensively for a variety of purposes in both analytical and preparative equipment. It is a simple matter to analyze this system according to the general theory I have described so as to determine the operating characteristics of every phase. This has been done with a general computer program described elsewhere (Jovin, 1973c). Other published systems have also been analyzed (Jovin, 1973c).

The input information is given in Figure 3 using the terminology in the present theory. The temperature was chosen to be 25° in this case; the analysis has also been made for 0° with somewhat different results due to the temperature dependence of the physical constants.

The complete description of the system is given in Figure 4 together with recipes for the convenient preparation of the buffer solutions required for setting up the system. Phase π is also specified for the sake of those users desiring to test the stability of an enzyme or other labile biological substance under actual conditions of separation. From the standpoint of the stacking process, it is seen that $\bar{r}_2^{\beta}$ is quite adequate, as one would expect, but $|\bar{r}_1^{\zeta}|$ is somewhat larger than desirable according to specification 108. The value of θ^{ζ} is in accordance with the theory which predicts an approximate lower limit of 1 for constituent 2 a divalent weak electrolyte (condition 81). It is interesting to note that the value for ϕ_2^{β} differs

```
                    SYSTEM NUMBER  Ornstein and Davis,1962.
      INPUT DATA

DATE = MAY 3/72     COMPUTER SYSTEM NUMBER = JOVIN
POLARITY = - (MIGRATION TOWARD ANODE)      TEMPERATURE =  25  DEG. C.


          SPECIFIED CONSTITUENTS
CONSTITUENT 1 = NO.   29 , GLYCINE
CONSTITUENT 2 = NO.   92 , PHOSPHATE-DIBASIC
CONSTITUENT 3 = NO.   99 , CHLORIDE -
CONSTITUENT 4 = NO.   99 , CHLORIDE -
CONSTITUENT 5 = NO.   99 , CHLORIDE -
CONSTITUENT 6 = NO.   12 , TRIS

          SPECIFIED CONCENTRATIONS
PHASE ALPHA(1) -    C1 =       .03840        C6 =       .30495
PHASE  BETA(2) -    C2 =       .03200        C6 =       .05890
PHASE GAMMA(3) -    C3 =       .06000        C6 =       .37780

          PHASE DELTA(10) - ELUTION BUFFER
RATIO IONIC STRENGTHS IS(10)/IS(9) =      1.0
MIN PH =      8.5
MAX PH =     10.5

          PHASE EPSILON(11) - LOWER BUFFER
IS =          .05
PHI(6) =      .90

          PHASE PSI(5) AND TAU(6) - RESTACKING PARAMETERS
PFMAX =     .80
MAX ABS(PH(5) - PH(9)) =  2.00
```

FIGURE 3: Input information for the computer analysis of the "Tris–glycine" electrophoretic system. Constituent numbers and phase designations correspond to the text. The additional specification of phases in terms of numbers is for the internal use of the computer. IS is ionic strength and RFMAX is an altered designation of the parameter $(R_F)_{min}$ specified in eq 175. The systems 4062 and 4192 referred to are duplicate systems found in the general computer output (Jovin *et al.*, 1970; Jovin, 1973c).

appreciably from the "optimal" value given by eq 79 and 80. For phosphoric acid, $H = 0.38$ (eq 79) but alteration of phase β so as to achieve this value leads only to a modest decrease in θ^ζ, pH^ζ, $\phi_1{}^\zeta$, and thus $|\bar{r}_1{}^\zeta|$. It is seen that pH^ζ is considerably higher than the value predicted by Ornstein originally and the pH of his phase α or the upper buffer, also shown in Figure 4. This discrepancy is accounted for by the fact that the original treatment of Ornstein and Davis (1962) did not adequately consider the transport of constituent 6 and the assumption underlying equation 11 of their discussion[7] can be shown to be at variance with the conditions imposed by the moving-boundary equation for that constituent. This question is of practical importance because the relative conductance of phase α is approximately one-third that of phase ζ with the consequence that the voltage drop across the upper buffer and heat production are three times as great as in the optimized system. No advantage derives from the lower pH since it has been demonstrated that phase α has no influence on phase ζ. It has been found that with the preparative apparatus described previously (Jovin *et al.*, 1964), the overall potential drop across the system is reduced by one-third (for equal current levels) by substituting phase α with an upper buffer having the composition of phase ζ.

```
                    SYSTEM NUMBER  Ornstein,L. Davis,B.J.,"Disc Electro-
                                   phoresis," Dist.Prod.Ind.,Rochester,1962.
DATE = MAY 3/72     COMPUTER SYSTEM NUMBER = JOVIN      (4062,4192)
POLARITY = - (MIGRATION TOWARD ANODE)      TEMPERATURE =  25  DEG. C.

CONSTITUENT 1 = NO.   29 , GLYCINE
CONSTITUENT 2 = NO.   92 , PHOSPHATE-DIBASIC
CONSTITUENT 3 = NO.   99 , CHLORIDE -
CONSTITUENT 4 = NO.   12 , TRIS

                              PHASES
          ALPHA(1)    BETA(2)     BETA(2)     PI(9)    LAMBDA(5)   GAMMA(3)

C1          .0384       .0513                .0468
C2                                   .0320                .0262
C3                                                                    .3600
C6          .3049       .0542       .0588    .3646       .3699       .3778
THETA        .120       1.067       1.937    7.796      14.136       6.297

PHI(1)       .042        .132                 .327
PHI(2)                               .629                .988
PHI(3)                                                                1.000
PHI(4)       .327        .123        .986     .042        .181        .159

PF(1)       -.030       -.095                -.236
PF(2)                                -.926               -.936
PF(3)                                                              -1.550
PF(6)        .367        .062        .843     .021        .073        .079

PH           8.38        8.62        7.38     9.43        8.86        6.79

ION.STR.    .0216       .0068       .0322    .0153       .0179       .0600
SIGMA        .391        .706       5.929    1.806       7.318      11.865
KAPPA         94.        388.       3320.     956.       3174.       5236.
MU          -.159       -.119       -.119    -.131       -.131       -.131
BV           .004        .027        .031     .058        .109        .116

PHASE ETA(3)       Y1= 1.071   Y2=  .024   Y3= 1.223   Y4=  .329

          RECIPES FOR BUFFERS OF PHASES ZETA(4),BETA(2),GAMMA(3),ZI(9)
                                        1Y          4X          4X          4X
CONSTITUENT                         PHASE 4     PHASE 2     PHASE 3     PHASE 9

  GLYCINE              GM            3.85                                  1.41
  1 M HIPPA            ML                        12.80
  1 M HCL              ML                                    24.00
  TRIS                 GM                                                 17.66
  H2O TO                          1 LITER     100 ML      100 ML      100 ML

AT FINAL CONCENTRATION =
  PH(25 DEG.C.)                      8.62        7.38        8.79        6.43
  KAPPA(25 DEG.C.)                    388.       3020.       5234.        855.
```

FIGURE 4: Complete description of the "Tris–glycine" system. The parameter designations correspond to the nomenclature in the text. The units of KAPPA (specific conductance) are μmhos cm^{-1}.

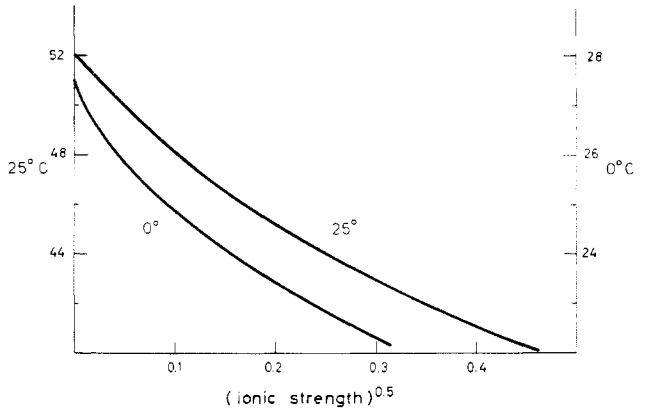

FIGURE 5: Sodium ion mobility as a function of temperature and ionic strength. The data are calculated from conductivity and cation transport numbers in Robinson and Stokes (1959). A functional form expressed as a third-order polynomial is given in Figure 4 of Jovin (1973c).

The parameters X2 and X4 listed in Figure 4 correspond to the quantities x_2/y and x_4/y in eq 156. From their small magnitude it can be concluded that the "phase η" phenomenon is not of major importance in this system. It does, however, affect the calculation of R_F values for components with low velocities relative to the moving boundary $\pi\lambda$. Thus, the true R_F value can be determined by the following equation which takes into account the displacement of the "starting zone" from the stacking gel–resolving gel interface.

$$R_F(\text{true}) = (d_s - 0.028y)/(d_{\pi\lambda} - 0.028y) \qquad (183)$$

where d_s = distance of migration from the stacking gel–resolving gel interface for the particular band; $d_{\pi\lambda}$ = distance of migration from the gel interface for the moving boundary, *i.e.*, tracking dye; y = length of stacking gel. It is evident from eq 183 that the correction can be appreciable for large values of y. No implication is made to the effect that sample components cannot be found at or within $0.028y$ of the gel interface. The effect of the gel matrix on certain substances may be such that they exhibit very low velocities in the resolving gel and do not remain stacked when phase s migrates into the gel. The above correction does assume that the band under consideration remains stacked until the annihilation of

[7] This point is discussed by Ornstein (1964).

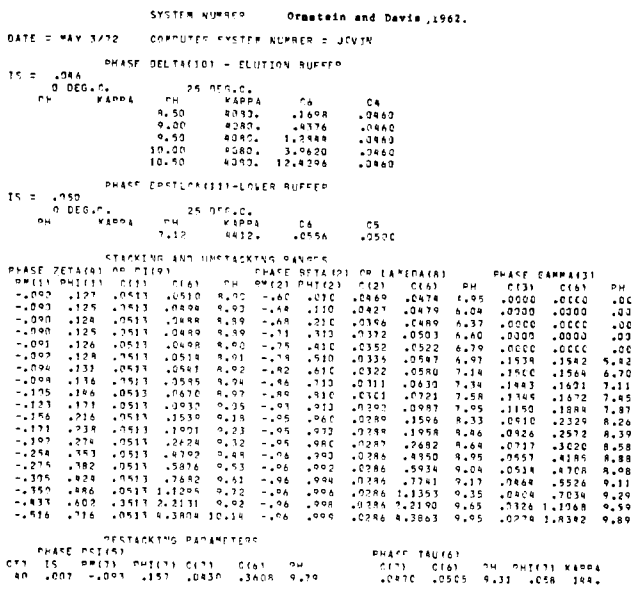

FIGURE 6: Further computer generated analysis of the "Tris–glycine" system. Details are given in the text.

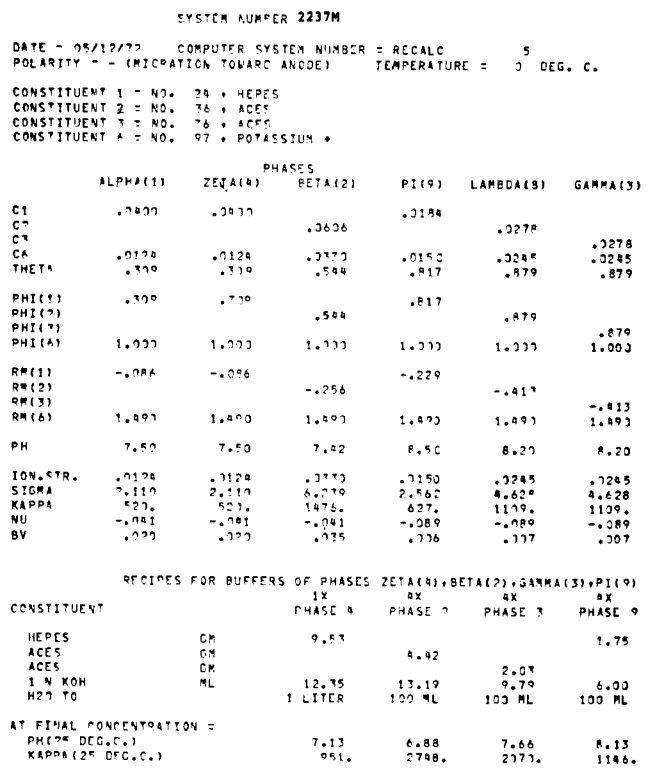

FIGURE 7: Description of a computer generated system in which constituent 6 is an ion. Details are given in the text.

phase η is completed. In view of the value for $\bar{r}_1^{\zeta}$ and $\bar{r}_1^{\pi}$, this condition may hold only for bands with R_F values greater than 0.095/0.24 = 0.4. Hence in actuality, it is rarely necessary to utilize eq 183 unless very long stacking gels relative to the resolving gels are used, such as for very dilute samples, *e.g.*, column eluates.

The mobility of the Na^+ ion as a function of ionic strength and temperature is given in Figure 5. These values are required for the computation of relative conductances according to eq 5.

More information about the system is given in the last page of the computer output, shown in Figure 6. Various possibilities for elution buffers (phase δ) are given at the top of the page, followed by the lower buffer (phase ϵ) with characteristics specified in the input. The table of stacking and unstacking ranges indicates to what degree the system can be varied and still fulfill the steady-state moving-boundary restrictions.

Columns 6 to 10 give data about phase β, notably the pH^{β} and $\bar{r}_2^{\beta}$ corresponding to the indicated concentrations of constituents 2 and 6. In columns 1–5 along a given line are the data for the phase ζ, generated under the influence of the particular phase β shown on the same line. Thus it is a simple matter to determine the necessary stacking gel buffer for any desired conditions in phase ζ, *e.g.*, higher or lower $|\bar{r}_1^{\zeta}|$ or pH^{ζ}. Columns 11–13 give information about the lower gel buffer, phase γ. Information about phase π is also available in columns 1–6. Thus, if one desires a specific pH^{π} or $\bar{r}_1^{\pi}$, the appropriate line in columns 1–6 is sought. Then the composition of phase γ required to achieve this phase π appears on the same line in the last three columns of the table (the presence of 0's implies the condition is impossible). In summary, it is possible to generate subsystems with the same constituents within a rather large range of pH and mobility conditions, thereby greatly enhancing the utility of the method. The parameters which are kept constant in all cases are $\bar{c}_1^{\zeta}$ and $(\Gamma/2)^{\pi}$.

The last item in Figure 6 gives information about restacking possibilities, as described in section D of this paper. The computer program identifies buffer 40 (γ-aminobutyric acid) as a possible candidate for constituent 7. Phase τ is given at the right and phase ψ at the left. It can be seen that $|\bar{r}_1^{\psi}|$ equals 0.093 and pH^{ψ} is 9.79, as compared to the corresponding values in phase π: $|\bar{r}_1^{\pi}|$ = 0.236 and pH 9.43 (Figure 4). Such conditions should be quite favorable for achieving the objectives of the restacking procedure.

The experimental verification of the parameters given in Figure 5 is reported elsewhere.[5]

K. Analysis of a Newly Generated Buffer System

With the computer program referred to in section J, a library of 4269 buffer systems for 0 and 25° and for pH's in the range 2.5–11 has been generated (Jovin *et al.*, 1970).

A representative system (2237 M) from this library is shown in Figures 7 and 8. Its most notable feature is the use of the ion K^+ as constituent 6. In addition to simplifying the system, it has been found that the pH difference between phases ζ and β is generally smaller using K^+ than in the case of a monovalent weak electrolyte as constituent 6. In fact, system 2237 is an example of what one might term an "iso pH" system since there are conditions under which all the pH's in the various phases are virtually the same (lines 5, 10, and 13 of the table in Figure 8). If the molecular sieving effect of the resolving gel upon the macromolecules in the sample is greater than upon the buffer ions, as will generally be the case, and if the differential effect is large enough so that the sample constituent mobilities $|\bar{r}_s^{\pi}|$ are less than $|\bar{r}_1^{\pi}|$, unstacking can take place without the usual programmed change in pH from pH^{ζ} to pH^{π}. Thus phase ζ and π can be made identical by setting phase β the same as phase γ.

The stacking and unstacking conditions in this system are quite suitable for most purposes and the resolving pH^{π} of 8.5

SYSTEM NUMBER 2237M

DATE = 05/12/72 COMPUTER SYSTEM NUMBER = RECALC 5

STACKING AND UNSTACKING RANGES

PHASE ZETA(8) OR PI(9)					PHASE BETA(2) OR LAMBDA(8)					PHASE GAMMA(3)		
RM(1)	PHI(1)	C(1)	C(6)	PH	RM(2)	PHI(2)	C(2)	C(6)	PH	C(3)	C(6)	PH
-.013	.010	.0400	.0004	5.85	-.16	.347	.0606	.0210	7.07	2.2738	.7888	7.07
-.017	.060	.0400	.0024	6.66	-.18	.380	.0606	.0230	7.13	.3790	.1440	7.13
-.031	.110	.0400	.0044	6.94	-.19	.413	.0606	.0250	7.19	.2067	.0853	7.19
-.045	.160	.0400	.0064	7.13	-.21	.446	.0606	.0270	7.25	.1421	.0634	7.25
-.059	.210	.0400	.0084	7.27	-.23	.479	.0606	.0290	7.30	.1083	.0518	7.30
-.073	.260	.0400	.0104	7.40	-.24	.512	.0606	.0310	7.36	.0875	.0448	7.36
-.087	.310	.0400	.0124	7.50	-.26	.545	.0606	.0330	7.42	.0733	.0400	7.42
-.101	.360	.0400	.0144	7.60	-.27	.578	.0606	.0350	7.48	.0632	.0365	7.48
-.115	.410	.0400	.0164	7.69	-.29	.611	.0606	.0370	7.54	.0555	.0339	7.54
-.129	.460	.0400	.0184	7.78	-.30	.644	.0606	.0390	7.60	.0494	.0318	7.60
-.143	.510	.0400	.0204	7.87	-.32	.677	.0606	.0410	7.66	.0446	.0302	7.66
-.157	.560	.0400	.0224	7.95	-.33	.710	.0606	.0430	7.73	.0406	.0288	7.73
-.171	.610	.0400	.0244	8.04	-.35	.743	.0606	.0450	7.80	.0373	.0277	7.80
-.185	.660	.0400	.0264	8.14	-.36	.776	.0606	.0470	7.88	.0345	.0267	7.88
-.199	.710	.0400	.0284	8.24	-.38	.809	.0606	.0490	7.97	.0320	.0259	7.97
-.213	.760	.0400	.0304	8.35	-.40	.842	.0606	.0510	8.07	.0299	.0252	8.07
-.227	.810	.0400	.0324	8.48	-.41	.875	.0606	.0530	8.18	.0281	.0246	8.18
-.241	.860	.0400	.0344	8.64	-.43	.908	.0606	.0550	8.33	.0264	.0240	8.33
-.255	.910	.0400	.0364	8.85	-.44	.941	.0606	.0570	8.54	.0250	.0235	8.54
-.269	.960	.0400	.0384	9.23	-.46	.974	.0606	.0590	8.91	.0237	.0231	8.91
-.272	.970	.0400	.0388	9.36	-.46	.980	.0606	.0594	9.03	.0234	.0230	9.03
-.274	.980	.0400	.0392	9.54	-.46	.987	.0606	.0598	9.21	.0232	.0229	9.21
-.277	.990	.0400	.0396	9.85	-.47	.993	.0606	.0602	9.52	.0230	.0228	9.52
-.278	.993	.0400	.0397	10.00	-.47	.995	.0606	.0604	9.67	.0229	.0228	9.67
-.279	.996	.0400	.0398	10.25	-.47	.997	.0606	.0605	9.92	.0228	.0228	9.92
-.280	.999	.0400	.0400	10.85	-.47	.999	.0606	.0606	10.53	.0228	.0227	10.53

RESTACKING PARAMETERS

PHASE PSI(5)						PHASE TAU(6)				
CT7	IS	RM(7)	PHI(7)	C(7)	C(6)	PH	C(7)	C(6)	PH	PHI(7) KAPPA
NO CONSTITUENT FOUND										

FIGURE 8: Table of stacking and unstacking ranges for the system described in Figure 7.

is more appropriate for labile substances than the high pH of the Tris–glycine system described in section J (the pH^{π} of which is 10.7 at 0°). The extensive table of stacking and unstacking ranges (Figure 8) indicates the great flexibility characteristic of this type of system in which constituent 2 is a monovalent weak electrolyte.

No elution or lower buffers are given in Figure 8 since any potassium salt can be used at an appropriate ionic strength. It is also seen that no constituent 7 was found which could fulfill the restacking requirements for this particular system.

Relative disadvantages are the small magnitudes of BV^{π} and $\nu^{\pi\lambda}$ due to the lack of buffering by constituent 6 and the high mobility of the Na^{+} ion, respectively.

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