Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Chrambach, A. , Hearing, E. , Lunney, J. and Rodbard, D.(1972) 'Experimental Validation of the Predicted Properties of a Multiphasic Buffer System Applied to Polyacrylamide Gel Electrophoresis', Separation Science and Technology, 7: 6, 725 — 745
To link to this Article: DOI: 10.1080/00372367208057979
URL: http://dx.doi.org/10.1080/00372367208057979

SEPARATION SCIENCE, 7(6), pp. 725–745, 1972

Experimental Validation of the Predicted Properties of a Multiphasic Buffer System Applied to Polyacrylamide Gel Electrophoresis

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Summary

The properties of a buffer system predicted by T. M. Jovin's theory of multiphasic (discontinuous) buffer systems have been tested experimentally both in free solution and in polyacrylamide gels. The most widely applied multiphasic buffer system, viz., the Tris–glycine system of Ornstein and Davis was used for this purpose. The properties of this system were computed on the basis of the Jovin theory. The pH, specific conductance, and boundary displacement were measured in the buffers corresponding to the original and the operative buffers of both the stacking (upper) gel and the separation (lower) gel in polyacrylamide gel electrophoresis. Values of pH and specific conductance in the absence of gels were found to be in reasonable agreement with theory. In polyacrylamide gels, after removal of residual reactants of the polymerization reaction by equilibration with the appropriate buffer, there was good agreement between observed and predicted values of pH and boundary displacement. However, the observed values for specific conductance were lower than predicted. The effects of pre-electrophoresis and of gel concentration on pH, conductance, and boundary displacement were also studied.

A new method has been developed for formation of stable pH gradients in polyacrylamide gel electrophoresis using multiphasic buffer systems. A concentration and pH gradient in the upper (stacking) gel as formed results in a

pH gradient in the operative stacking gel (after passage of the stack). This pH gradient is stable for at least 8 h in the system investigated, and may be applicable to protein fractionation.

INTRODUCTION

The genealogy of multiphasic (discontinuous, disc, or "isotachophoresis") buffer systems has been previously reviewed (*1*, *2*). These buffer systems were first developed and studied at the turn of the century. For several decades thereafter their application remained restricted to the separation of simple ions in a few physical-chemical laboratories. The importance of multiphasic buffer systems for the electrophoretic fractionation of proteins and other macromolecules was discovered and widely popularized by Ornstein and Davis (*1*, *3*), who developed the "Tris system" for this purpose. This system provided the field of macromolecular fractionation with a powerful fractionation tool, when applied in conjunction with polyacrylamide gel electrophoresis (PAGE); it proved to possess remarkably wide applicability (*4*), particularly when used with gels of variable pore sizes. However, the theoretical treatment of multiphasic buffer systems by Ornstein (1) has been used to generate only a few buffer systems operative at pH values other than the Tris system (*5*, *6*). Ornstein's theory does not provide an exact or explicit description of the several buffer phases across the moving boundaries (stacks) which appear in both the stacking gel and the separation gel.

These deficiencies were remedied by T. M. Jovin who developed a theoretical treatment of multiphasic buffer systems (*7*) by extension of classical moving boundary theory (*8*). Figure 1 is a reproduction of a figure of Jovin's paper which is shown here to define the several buffer phases. Jovin used this theory to develop a computer program (*9*) capable of generating any number of multiphasic buffer systems throughout the entire pH range. The generation of systems is limited only by the number of available buffer ions with known pK values and ionic mobilities. The same program provides a comprehensive physical-chemical description of all phases and moving boundaries for each of the generated systems (e.g., Fig. 2). A representative (but by no means exhaustive or final) systems output of 4269 multiphasic buffer systems generated by this program has been published in the form of magnetic tapes and a printed catalog (*10*). Approximately 100 systems of this extensive output have been applied to several thousand protein frac-

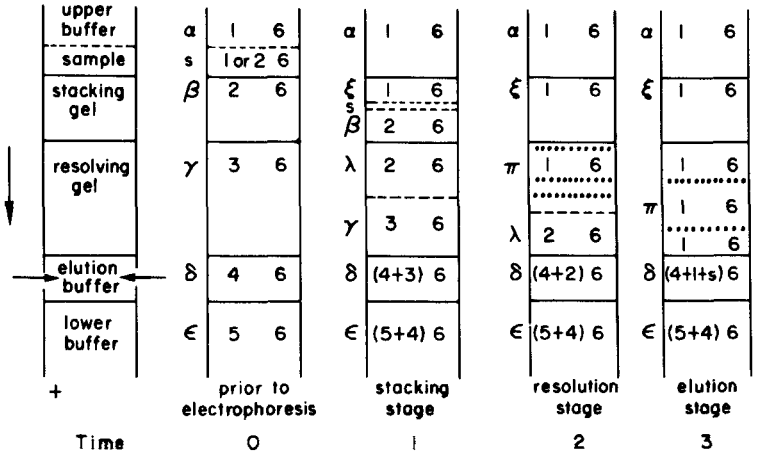

FIG. 1. Definition of the phases of multiphasic buffer systems [reproduced from the work of T. M. Jovin (*7*)]. The numbers (1–6) designate the various buffer constituents corresponding to those in Fig. 2. The phases are designated by Greek letters. Stationary boundaries are shown as lines, moving boundaries as dashed lines, and dotted lines represent bands of sample components. The positions and compositions of the buffer phases in the gel are shown prior to electrophoresis, during stacking, resolution, and elution of the sample.

tionations [including systems generated by earlier versions of the program under different input constraints and reanalysis of systems in the literature (*11*)]. It appears that pH and conductance values of the stacking and separation gel phases, as polymerized and after passage of a moving boundary in electrophoresis, are in reasonable agreement with prediction in many of the systems used to date. Nonetheless, a number of defects (listed below) could remain undetected by a mere test of buffer pH and conductance values:

a. Errors in the theory of multiphasic buffer systems and/or the computer program derived from it (*9*).

b. Errors in the input data for the program (pK and ionic mobility values for the various buffer constituents). Inaccuracies in these values are quite likely since they were obtained by relatively crude methods—the pK values by pH measurement of half-neutralized acids or bases; the mobilities by conductimetry carried out on the same solutions (*12*).

```
PART I
        INPUT DATA

DATE = 01/07/72     COMPUTER SYSTEM NUMBER
POLARITY = - (MIGRATION TOWARD ANODE)      TEMPERATURE =  25  DEG. C.


          SPECIFIED CONSTITUENTS
CONSTITUENT 1 = NO.  29 , GLYCINE
CONSTITUENT 2 = NO.  99 , CHLORIDE -
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 =      0.00490        C6 =      0.03840
PHASE  BETA(2) -   C2 =      0.06000        C6 =      0.06200
PHASE GAMMA(3) -   C3 =      0.06000        C6 =      0.37800

          PHASE DELTA(10) - ELUTION BUFFER
RATIO IONIC STRENGTHS IS(10)/IS(9) =      3.0
MIN PH =       8.5
MAX PH =      10.5

          PHASE EPSILON(11) - LOWER BUFFER
IS =          0.05
PHI(6) =      0.80

          PHASE PSI(5) AND TAU(6) - RESTACKING PARAMETERS
RFMAX =  0.90
MAX ABS(PH(5) - PH(9)) =  2.00
```

FIG. 2. Multiphasic buffer output of the computer program of T. M. Jovin (*9*) analyzing the Ornstein-Davis system. Symbols in the output are as previously defined (*4, 13, 10*). Phase designations correspond to Fig. 1. (Part I) input parameters; (Part II, p. 729) properties and recipes for the main system; (Part III, p. 730) series of related subsystems using the same buffer constituents. Of primary interest for this study are the three measurable physical parameters in Part II of the output: pH, specific conductance (KAPPA), and boundary displacement (NU) for the various phases. Part III of the output lists STACKING AND UNSTACKING RANGES which have been designated as subsystems .1, .2, .3, etc., in descending order.

c. Errors in the displacement of moving boundaries (and thus stacking limits).

d. Errors due to molecular sieving effects imposed by the gel matrix.

e. Error due to the perturbation of pH, ionic strength, and buffer composition by the catalysts used for the polymerization of the gel (*13*).

The present study presents a preliminary attempt to estimate the magnitude of some of the potential errors, using three physical param-

eters which are directly measureable in both the stacking and separation phases of each system: pH, conductance (KAPPA) and boundary displacement (NU). These three parameters were measured as a function of gel concentration in the stacking and separation gels, both as polymerized and in their operative states, after passage of the appropriate moving boundary. Gels were studied under three conditions (a) without purification, (b) after pre-electrophoresis, and (c) after equilibration with gel buffer. Values of pH, conductance, and boundary displacement

PART II

POLARITY = - (MIGRATION TOWARD ANODE) TEMPERATURE = 25 DEG. C.

CONSTITUENT 1 = NO. 29 , GLYCINE
CONSTITUENT 2 = NO. 99 , CHLORIDE -
CONSTITUENT 3 = NO. 99 , CHLORIDE -
CONSTITUENT 6 = NO. 12 , TRIS

	PHASES					
	ALPHA(1)	ZETA(4)	BETA(2)	PI(9)	LAMBDA(8)	GAMMA(3)
C1	0.0049	0.0468		0.0468		
C2			0.0600		0.0600	
C3						0.0600
C6	0.0384	0.0488	0.0620	0.3648	0.3780	0.3780
THETA	7.837	1.043	1.033	7.792	6.300	6.300
PHI(1)	0.328	0.130		0.328		
PHI(2)			1.000		1.000	
PHI(3)						1.000
PHI(6)	0.042	0.125	0.968	0.042	0.159	0.159
RM(1)	-0.236	-0.094		-0.236		
RM(2)			-1.552		-1.552	
RM(3)						-1.552
RM(6)	0.021	0.062	0.484	0.021	0.079	0.079
PH	9.43	8.92	6.59	9.43	8.79	8.79
ION.STR.	0.0016	0.0061	0.0600	0.0153	0.0600	0.0600
SIGMA	0.189	0.718	11.881	1.806	11.881	11.881
KAPPA	95.	351.	5241.	855.	5241.	5241.
NU	-1.248	-0.131	-0.131	-0.131	-0.131	-0.131
BV	0.006	0.025	0.004	0.058	0.116	0.116

RECIPES FOR BUFFERS OF PHASES ZETA(4),BETA(2),GAMMA(3),PI(9)

CONSTITUENT		1X PHASE 4	4X PHASE 2	4X PHASE 3	4X PHASE 9
GLYCINE	GM	3.51			1.41
1N HCL	ML		24.00		
1N HCL	ML			24.00	
TRIS	GM	5.91	3.00	18.31	17.67
H2O TO		1 LITER	100 ML	100 ML	100 ML
AT FINAL CONCENTRATION =					
PH(25 DEG.C.)		8.92	6.59	8.79	9.43
KAPPA(25 DEG.C.)		351.	5241.	5241.	855.

FIG. 2, Part II. See page 728 for legend.

PART III

PHASE DELTA(10) - ELUTION BUFFER

IS = 0.046

0 DEG.C. PH	KAPPA	25 DEG.C. PH	KAPPA	C6	C4
		8.50	4084.	0.1698	0.0460
		9.00	4084.	0.4376	0.0460
		9.50	4084.	1.2844	0.0460
		10.00	4084.	3.9621	0.0460
		10.50	4084.	12.4298	0.0460

PHASE EPSILON(11)-LOWER BUFFER

IS = 0.050

0 DEG.C. PH	KAPPA	25 DEG.C. PH	KAPPA	C6	C5
		7.47	4417.	0.0625	0.0500

STACKING AND UNSTACKING RANGES

PHASE ZETA(4) 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
-0.092	0.128	0.0468	0.0474	8.91	-1.55	1.000	0.0600	0.0606	6.07	0.1531	0.1546	6.07
-0.128	0.178	0.0468	0.0932	9.08	-1.55	1.000	0.0600	0.1063	7.96	0.1102	0.1953	7.96
-0.164	0.228	0.0468	0.1587	9.21	-1.55	1.000	0.0600	0.1719	8.34	0.0861	0.2466	8.34
-0.200	0.278	0.0468	0.2482	9.33	-1.55	1.000	0.0600	0.2614	8.60	0.0706	0.3076	8.60
-0.236	0.328	0.0468	0.3670	9.43	-1.55	1.000	0.0600	0.3801	8.80	0.0599	0.3792	8.80
-0.272	0.378	0.0468	0.5221	9.52	-1.55	1.000	0.0600	0.5353	8.97	0.0519	0.4634	8.97
-0.308	0.428	0.0468	0.7231	9.61	-1.55	1.000	0.0600	0.7362	9.12	0.0459	0.5630	9.12
-0.344	0.478	0.0468	0.9831	9.70	-1.55	1.000	0.0600	0.9963	9.26	0.0411	0.6823	9.26
-0.380	0.528	0.0468	1.3210	9.79	-1.55	1.000	0.0600	1.3342	9.40	0.0372	0.8272	9.40
-0.416	0.578	0.0468	1.7645	9.88	-1.55	1.000	0.0600	1.7777	9.53	0.0340	1.0069	9.53
-0.452	0.628	0.0468	2.3562	9.97	-1.55	1.000	0.0600	2.3694	9.66	0.0313	1.2352	9.66
-0.488	0.678	0.0468	3.1651	10.06	-1.55	1.000	0.0600	3.1783	9.79	0.0290	1.5348	9.79
-0.524	0.728	0.0468	4.3113	10.17	-1.55	1.000	0.0600	4.3245	9.92	0.0270	1.9449	9.92

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
40	0.007	-0.093	0.157	0.0429	0.3609	9.79	0.0429	0.0449	9.31	0.058	130.

FIG. 2, Part III. See page 728 for legend.

were extrapolated to zero gel concentration, and compared with measurements on the buffers in free solution.

The original Tris system (*1*, *3*) of Ornstein and Davis, recalculated and analyzed by Jovin's program (*9*) using pK and mobility values determined in this laboratory (*12*), was arbitrarily selected for this study. This buffer system will be designated as system A-Davis, since it is very similar to the system A described previously (*11*, *14*). This buffer system has been more extensively utilized than any other. Similar studies will be needed to evaluate critically other systems, operative at neutral and acid pH, at 0 and at 25°C, and on *de novo* generated systems before the theory and program of Jovin can be considered fully validated.

In addition to testing a sample of the multiphasic buffer output directly, it seemed of interest to exploit the section of computer output that describes alternative buffer systems made by alteration of the concentrations of some of the buffer constituents of the "main system"

(cf. Fig. 2, Part III). It was suggested to us by Jovin that a pH and concentration gradient encompassing the STACKING AND UNSTACKING RANGES (Fig. 2, Part III, columns 8, 9, 10) in the gel as made (BETA phase) would give rise to a pH gradient in the operative gel during electrophoresis (ZETA phase) (columns 3, 4, and 5). The stability of such pH gradients in the ZETA phase was tested. These pH gradients differ from those previously used (*15*) by their ability to "stack" proteins or other ions.

METHODS

Measurement of Conductance

A Radiometer pH meter No. 25 with expanded scale was used in conjunction with a Metrohm pH microelectrode No. UX or a Radiometer GK 2302B electrode. Specific conductance of solutions was measured with an LKB No. 3216 B conductivity bridge. Samples and conductivity cell (2 ml) were submerged in a constant temperature bath maintained at 25° ± 0.02°C during the measurement (Tamson No. CV 45, Neslab Instruments, Durham, N.H.).

Polymerization of Gels

The polymerization procedure followed that described previously (*11*). Buffer system A-Davis (Fig. 2) was used throughout. Tris (Trizma Base, Sigma) was recrystallized from 77% ethanol at 50°C for preparation of gels purified by diffusion (see below); otherwise the commerical grade was used. Acrylamide and *N,N'*-methylenebisacrylamide No. 19 and 719, respectively (Polysciences, Warrington, Pa.) were used without further purification. Gel concentrations were 5, 10, 15, 20, and 30%T (*11*); cross-linking was maintained constant at 2%C (*11*). For all gel concentrations, 25 μl *N,N,N',N'*-tetramethylethylenediamine (TEMED)/100 ml polymerization mixture were used. Separation (lower) gels contained 7.5 mg potassium persulfate (KP) and 0.5 mg riboflavin (RN)/100 ml polymerization mixture. Stacking (upper) gels contained 5 mg KP and 0.5 mg RN/100 ml polymerization mixture. All gels were made 6 mm in diameter and 2 ml in volume. Photopolymerization was carried out in apparatus described previously (*16*) with a light source consisting of an array of six circular 20-W fluorescent tubes.

Purification of Polyacrylamide Gels by Equilibration (Diffusion) against Buffer

Gels were removed from their glass tubes and equilibrated with the appropriate buffer (BETA or GAMMA phase) for 1 to 2 weeks at 4°C to remove undesired products or reactants of the polymerization reaction. Daily changes of buffer (200–300 ml/ten 2-ml gels) were made.

Purification of Gels by Pre-electrophoresis

Gels (BETA or GAMMA phase) were subjected to electrophoresis at 4 mA/tube for 2 hr or longer, with the electrolyte reservoirs containing the same buffer as the gels. Buffers in the electrolyte chambers were then changed to phases ZETA and EPSILON, respectively, prior to the start of electrophoresis.

Measurement of pH of Polyacrylamide Gels

Gels were sliced transversely using apparatus previously described for 6-mm (*17*) and 18-mm (*16*) diameter gels. Fifteen slices proximal to each end of the gel were placed into a sealed vial containing 3 ml 0.02 *M* KCl overnight. The pH values derived from both sets of fifteen slices were averaged.

To measure the voltage drop across the gel during electrophoresis, gels were subjected to an electric field in an apparatus (Fig. 3) constructed to permit positioning of the electrodes immediately adjacent to the ends of a single gel tube. This was achieved by a movable, circular upper electrode, a stationary circular lower electrode, and a polypropylene tube fitting (Federal Scientific No. H-19268 Connector Tube to M.P.T. drilled out to 8 mm and to 12 mm bore) to facilitate adjustment of the position of the tube. Alternatively, gels which had been removed from their glass tubes for equilibration with buffer (section 3) were placed in destainer tubes, immersed in carbon tetrachloride (for insulation), and subjected to electrophoresis in the destaining apparatus previously described (*18*). Both electrolyte chambers were filled with the buffer contained in the gel. A current of 1, 2, 3, 4, and 5 mA was

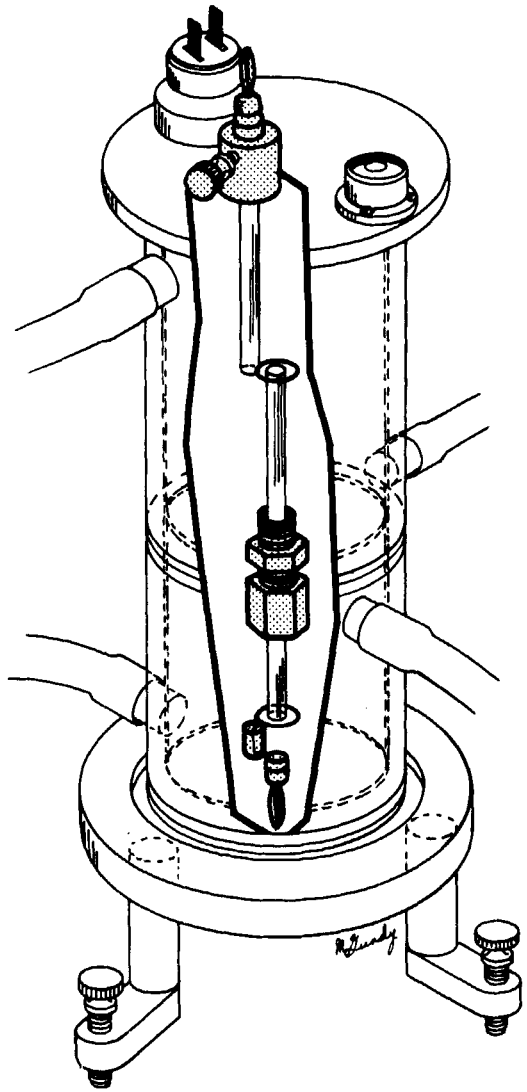

FIG. 3. Apparatus for the measurement of voltage gradient across a single gel. The inner polypropylene tube fitting may be exchanged for use with either 8-mm o.d. tubes or 12-mm o.d. "destainer tubes." The position of the upper platinum electrode can be adjusted to the level of the top of the tube.

applied and the voltage corresponding to each current level was measured by voltmeter (Sencore, Model FE149). The length and radius of the gel were measured. Specific conductance across the gel was calculated as

$$\text{KAPPA} = \frac{i/a}{V/l} \tag{1}$$

where i = current (amperes)
V = potential difference (volts)
l = length (centimeters)
a = cross-sectional surface area (square centimeters)
KAPPA = specific conductance (mhos/centimeter).

Measurement of Boundary Displacement

Measurement of boundary displacement in polyacrylamide gels in glass tubes utilized an apparatus (*16*) providing magnetic stirring of lower buffer to improve heat transfer, hydrostatic equilibrium of gel tubes, temperature control of both upper and lower buffers, and safety interlocks. Millimeter scales were attached to each gel tube by Tygon sleeves. [Again, the destaining tubes and apparatus (*18*) were used for gels purified by diffusion.]

Bromphenol blue (50 μl of a 0.01% solution in 25% sucrose) was applied to each gel. The duration of electrophoresis was recorded by a stopwatch. The migration distance of the bromphenol blue band or "stack" was recorded at 5-min intervals. Electrophoresis was terminated 10 min after the dye band had migrated through the gel. Least-squares linear regression was used to calculate velocity, v (centimeters/second).

Bromphenol blue remained in the stack in stacking gels (ZETA phase) of all gel concentrations used. However, for gel concentrations greater than 17%T, the velocity of bromphenol blue in the PI phase was corrected for its retardation behind the PI/LAMBDA boundary as described previously (Section V, 3 of Ref. *11*). Ferguson plots were obtained for bromphenol blue ($K_R = 0.027$; $Y_0 = 2.86$). The position of the moving boundary between chloride and glycinate was determined after electrophoresis by immersion of the gels in 0.2 M $AgNO_3$ to precipitate the chloride. Then, for each gel concentration, the velocity of the front was calculated as the velocity of bromphenol blue, divided

by the R_f of the dye. The boundary displacement, NU, was then calculated as:

$$\mathrm{NU} = \frac{v}{i/a} \tag{2}$$

where NU = boundary displacement (cubic centimeters/coulomb)
v = velocity (centimeters/second).

Preparation of BETA Phase pH Gradient

A linear gradient of upper gel (BETA phase) buffers (system A-Davis .1 to A-Davis .9) (Fig. 2, Part III) was formed in a 6.7%T polyacrylamide gel of 18 mm diameter (15 ml) by the procedure described previously (*19*). The gradientmaker, adapted for use with polyacrylamide gels by provision of temperature control and protection of the polymerization mixture from light, is described elsewhere (*20*). The pumping rate was 0.3 ml/min. The polymerization mixture containing buffer A-Davis .9 was made 20% in sucrose. After polymerization, the gel was sliced, and the slices were suspended in 0.02 *M* KCl, left to diffuse overnight in sealed vials, and the pH of the diffusates was measured.

pH Gradient Electrophoresis

The buffer gradient gel described in the preceding section was loaded with bromphenol blue and subjected to electrophoresis, using the upper (ZETA phase) and lower (EPSILON phase) buffers of system A-Davis (Fig. 2). The current density was 10 mA/2.54 cm^2. The pH gradient was measured when the stack (indicated by bromphenol blue) traversed the length of the gel (2.5 hr) and after 8 hr in separate experiments.

RESULTS

Validation of Predicted Values of pH and Specific Conductance for Buffer phases BETA, GAMMA, ZETA, PI, and EPSILON

Buffers for the various phases were prepared according to the computer output for the analysis of the Ornstein-Davis system (Fig. 2). The pH and conductance values measured in the various phases are

TABLE 1

Predicted and Experimental Values of pH and Specific Conductance for the Ornstein-Davis Buffer System

Phase	Recrystallized Tris	pH		Specific conductance (μmhos/cm)	
		Theory	Found	Theory	Found
BETA		6.59	7.02	5241	5100
	+	6.59	7.01	5241	5354
ZETA		8.92	9.17	351	404
	+	8.92	8.97	351	385
GAMMA		8.79	9.10	5241	4559
	+	8.79	8.88	5241	4996
PI	+	9.43	9.56	855	855
EPSILON		7.47	7.80	4417	3980
	+	7.47	7.45	4417	4487

summarized in Table 1. Recrystallized Tris was used where indicated. The conductance but not the pH of the BETA phase buffer agrees with theory. The opposite is true for the GAMMA phase (Table 1). This may be due to the relatively large difference between the pH of the BETA phase and the pK of Tris. At the pH of the BETA phase (6.59) a slight perturbation of constituent concentrations will result in a small change of conductance but a large change of pH. Also, the high concentration of un-ionized Tris in the GAMMA PHASE may lower the activity coefficients and, thereby, the conductance.

Validation of Predicted Values of pH and Specific Conductance for Phases BETA and GAMMA in Polyacrylamide Gels of 5 to 30%T

Figure 4 (left column) shows the values of pH and specific conductance for several gel concentrations (%T) under three conditions: (a) gels without purification after polymerization, (b) gels purified by pre-electrophoresis, and (c) gels purified by diffusion against gel buffer. In all three cases, pH values conformed closely to prediction and showed no systematic variation with gel concentration. However, all three types of gel exhibit specific conductances lower than the predicted values. In

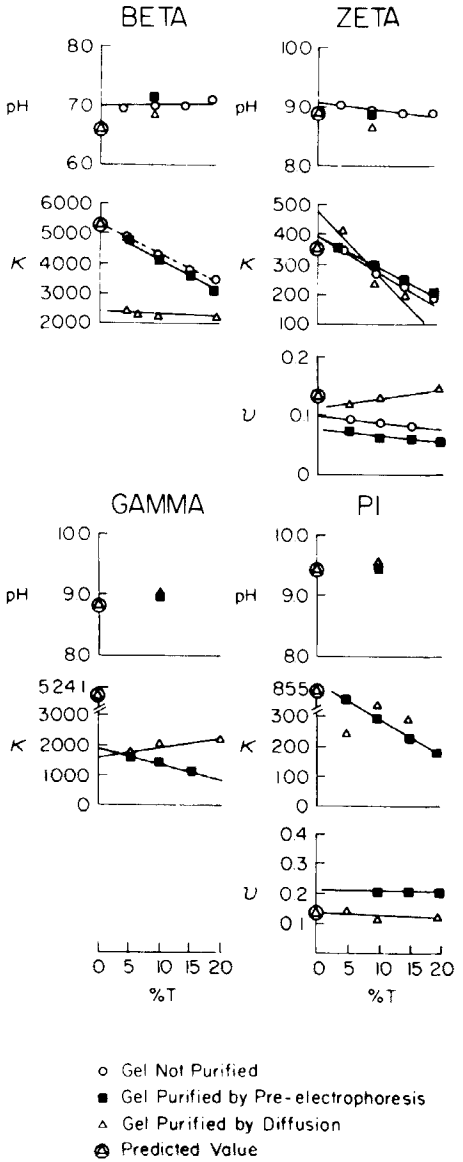

FIG. 4. Values of pH, specific conductance (KAPPA), and boundary displacement (NU) found experimentally at various gel concentrations (%T). Values derived from gels type (a), (b), and (c) are compared with the values predicted by Jovin's theory (Fig. 2). The phase designations BETA, ZETA, GAMMA, and PI correspond to those of Fig. 1 and 2.

types (a) and (b), specific conductance decreases with increasing gel concentration. In the BETA phase, the discrepancy between observed and predicted conductance is maximal for type (c), and specific conductance does not vary with gel concentrations in these gels.

Validation of Predicted Values of pH, Specific Conductance, and Boundary Displacement for Phases ZETA and PI in Polyacrylamide Gels of 5 to 30%T

The ZETA phase is formed when any buffer composed of the constituents of the ALPHA phase (arbitrarily selected at the concentrations of the ZETA phase) is applied above a BETA phase, and an electrical field is applied. The PI phase originates in analogous fashion from the GAMMA phase.

The observed pH values of the ZETA and PI phases (Fig. 4, right column) are in excellent agreement with theory, and are unaffected by gel concentration in all three types of gel.

The specific conductance (KAPPA) in the ZETA phase is in satisfactory agreement with theory, at least when extrapolated to zero gel concentration. As %T increases, KAPPA decreases for all 3 types of gel. In the PI phase, as in the GAMMA phase, the specific conductance is lower than predicted. The specific conductance for gels of type (a) and (b) is a function of gel concentration, but is in good agreement with theory when extrapolated to 0%T. For gels equilibrated with buffer [type (c)], specific conductance is independent of gel concentration.

Values of boundary displacement (NU) (for the BETA–ZETA and GAMMA–PI moving boundaries) are in agreement with prediction, and are independent of gel concentration only for gels purified by diffusion [type (c)]. However, for types (a) and (b), the agreement with theory is not satisfactory. For the BETA–ZETA boundary, NU is lower than expected, and decreases as %T increases. For the GAMMA–PI boundary, NU is anomalously high, and apparently independent of gel concentration for gel types (a) and (b).

Formation of Stable Linear pH Gradient in Polyacrylamide Gel Formed by a Buffer Gradient of the ZETA Phases Computed for Various Tris–Chloride Systems

The computer output for each multiphasic buffer system (9, 10) lists input parameters in Part I, the properties and recipes for the "main"

system in Part II, and a series of closely related subsystems using the same buffer constituents in Part III (cf. Fig. 2). Change of constituent concentrations and pH of either the BETA or GAMMA phase results in a predicted change of the pH of phase ZETA or PI, respectively. Thus, a gel composed of a gradient of the buffers of these subsystems is expected to give rise to a specific pH gradient for pH(ZETA) or pH (PI). Accordingly, a gel with a linear concentration gradient of BETA phase buffers with [Cl^-] constant at 0.06 M, and [Tris] variable from 0.0606 to 1.3342 M was set up and subjected to PAGE. Figure 5 depicts

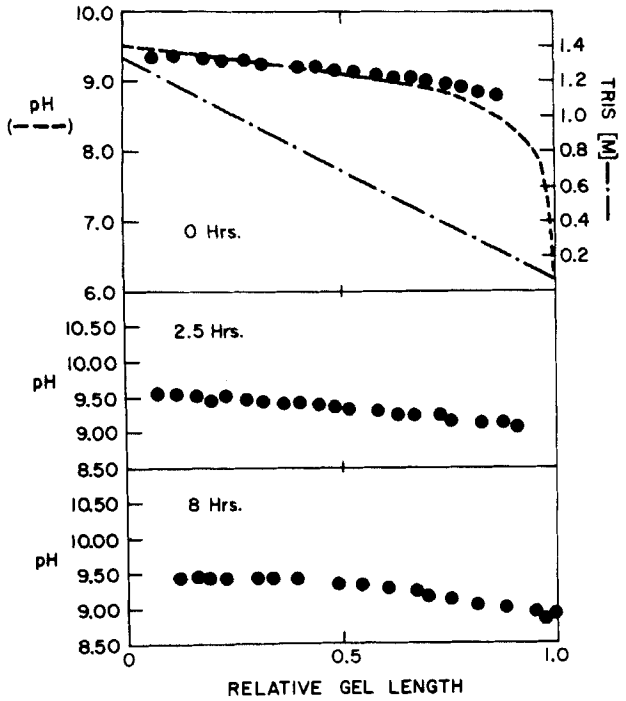

FIG. 5. pH gradients within a 6.7%T gel. Filled circles represent observed pH values. Upper panel: As formed by a linear buffer concentration gradient of the BETA phases between subsystems A-Davis .1 to .9, prior to electrophoresis. The dotted-dashed line represents the predicted linear concentration gradient of Tris. The dashed line represents the predicted nonlinear pH gradient. Center panel: After passage of the moving boundary to the bottom of the gel (2.5 hr). Lower panel: After 8 hr of electrophoresis.

the change of pH along the gel (a) as polymerized in the BETA phase, (b) after electrophoresis of the BETA–ZETA moving boundary to the bottom of the gel, (c) after electrophoresis for 8 hr. It is shown that a stable pH gradient across the gel can be produced for at least 8 hr. The experimental pH values within the linear range of pH agree reasonably with prediction (Part III of the output, Fig. 2, column 5) for the BETA phase.

DISCUSSION

The present study provides a detailed evaluation of the validity of several predictions of Jovin's theory (*7*) of multiphasic buffer systems and the input data which it utilizes [pK and ionic mobility for the various buffer ions (*12*)] in one buffer system, the Tris system of Ornstein and Davis (*1*, *3*). We have measured several of the parameters, summarized in Part II of the systems output (Fig. 2), i.e., pH, specific conductance, and boundary displacement. The tests were carried out on the phases (Fig. 1) corresponding to the stacking gel (phases BETA and ZETA) and to the separation gel (phases GAMMA and PI) in polyacrylamide gel electrophoresis, both as formed in the polymerization mixtures (phases BETA and GAMMA) and as they become "operative," after a moving boundary has migrated through the gels (phases ZETA and PI). The measurements were carried out on these four important buffer phases under four different conditions.

a. Gels as polymerized: these gels contain all components of the polymerization reaction including residual monomer, catalysts, and side reaction products.

b. Gels purified by pre-electrophoresis: these contain uncharged reactants, catalysts, side reaction products and possibly electrolysis products, and are expected to display anomalous ionic strength, due to the initial presence of KP and TEMED.

c. Gels equilibrated by diffusion for 1 to 2 weeks with daily changes of gel buffer (presumably these gels contain no impurities but are significantly altered in their pore structure through extensive swelling, resulting from hydration or water regain.)

d. In buffers in free solution.

Evidently, each of these four test systems is subject to its own limitations. However, the results obtained with the gels after diffusion showed properties which were, overall, closest to the theoretical predictions.

The reasonable agreement between observed and expected values for pH and specific conductance of the buffers in the absence of gel appears to validate the pK and mobility values used as input data for calculations of other parameters.

It may be objected that the buffer system analyzed here was originally derived from Ornstein's and not from Jovin's theoretical treatment and, therefore, does not present an appropriate test for the Jovin theory. However, analysis of the program (*9*) indicates that analysis of a buffer system of specified composition is subject to the same restrictions of the Jovin theory as a *de novo*-generated system. Also, the predicted values of pH, specific conductance, and boundary displacement used here were obtained from Jovin's theory; the two latter parameters are not explicit in the Ornstein treatment.

These results raise the question, whether the customary practice of polyacrylamide gel electrophoresis, i.e., the use of freshly polymerized, unpurified gels is acceptable, or whether new methods should be developed to permit the general use of gels purified by equilibration against buffer.

The procedure used in this work, i.e., surrounding the cylindrical gels by carbon tetrachloride, is too laborious and prone to experimental difficulties to be feasible on a routine basis. Also, it is unlikely that a practical method can be found for application of upper gels and protein samples in this experimental setup.

Morris and Morris (*21*) have previously utilized gel slabs equilibrated with buffer, sealing these slabs between two glass plates. However, this method was used only with a continuous buffer phase. Also, equilibration of the gel with buffer results in hydration (water regain) and a corresponding change in effective pore size, which depends in a complicated fashion on both %T and %C (*21*, *22*). This, in turn, will have marked effects on the relationship between electrophoretic mobility of a protein and gel concentration. It is disappointing that the use of pre-electrophoresis, a much more simple, rapid, and feasible procedure, does not significantly improve the agreement between observed and expected values of KAPPA and NU.

Relationship between KAPPA and Gel Concentration

The present studies indicate that gel concentration has a pronounced effect on KAPPA and, thus, on the mobility of the buffer ions in the gel. It appears that this relationship can be described in a manner

analogous to the effect of gel concentration on the mobility of macroions, i.e., a linear relationship between the log of mobility (or KAPPA) and gel concentration. This would suggest that small ions are "retarded" by the gel in a manner analogous to large ions, in accord with the Ogston model for a gel, as applied to electrophoresis (*11*, *21*, *23*). Due to the finite thickness of the polyacrylamide gel fiber, even molecules with zero radius ($R = 0$) will be retarded. This is also consistent with the findings of White and Dorion (*24*) on the diffusion of small molecules (water, NaCl, urea) in polyacrylamide gels. The present findings (Fig. 4) do not appear consistent with the shape of the relationship between KAPPA and gel concentration observed by Richards and Lecanidou (*25*).

Other factors presumably affecting the relation between gel concentration and conductance may also come into play. Conductance decreases when an increasing proportion of the gel volume is occupied by polyacrylamide fibers. At the same time, conductance is increased by the concentration of gel buffer that occurs in proportion to the occupancy of the gel volume by the fibers. In contrast to KAPPA, the relative conductance, SIGMA, should be independent of gel concentration, since gel effects on buffer and Na^+ ions should be approximately equivalent. It is precisely for that reason that the Jovin theory (*7*) utilizes SIGMA, not KAPPA, in most instances. However, the Ferguson plot for Na^+, needed to evaluate SIGMA, is not available.

Boundary displacement is defined by Eqs. 35, 37, and 38 of Ref. *7* as being dependent only on the mobility ratio of the two constituents in a phase. Since the mobilities of two buffer constituents can be expected to be equally affected by %T, NU would be predicted as constant with gel concentration. This is indeed the case (Fig. 4).

The present findings raise questions with respect to the use of PAGE for calculation of free mobility (*4*, *11*) or molecular net charge (valence, V) (*11*) in multiphasic buffer systems. These calculations assume that RM(1,9) [or RM(1,4)] and the mobility of sodium ion in the gel are known and independent of gel concentration. Evidently, this is not necessarily the case. However, if the product, RM(1,9) Na^+ is related to gel concentration in a manner analogous to the Ferguson plot, then results obtained for M_0 and V will still be valid. One approach to circumvent this problem would be to utilize an experimentally determined value for RM(1,9) (or for NU and the voltage gradient in the gel) rather than the predicted values. This should be readily feasible now, using methods presented above. Also, these difficulties point to an advantage in using a continuous buffer phase, where the voltage gradient can be more readily measured directly (*23*).

Any observed discrepancies in pH, KAPPA, and NU do not affect the validity of estimates of molecular radius or molecular weight obtained by PAGE (*11*, *21*), nor the use of PAGE as a fractionation tool on either the analytical or preparative level. However, values of K_R will be systematically altered by a constant value if the velocity of the "front" is affected by gel concentration.

The method for generation of a pH gradient used here has certain similarities with the pH gradient electrophoresis originally developed by Kolin (*15*). However, several important differences should be noted. The use of a gel matrix, even are latively "minimally restrictive" one, should serve to delay the eventual decomposition of the gradient by diffusion. It appears that the pH gradients generated in this way are subject only to decomposition by diffusion, rather than by electrophoretic effects. The present method allows for stacking of the component of interest within the pH gradient. In the present studies, bromphenol blue remained within the stacking limits provided by each of the Tris subsystems of the gradient. However, if proteins were applied, it would be expected that they would "unstack" as they approached their isoelectric point as soon as their mobilities (relative to sodium) decrease below the value of RM(1,4). Therefore, the proteins should approach their isoelectric point asymptotically, as in isoelectric focusing. However, the utility of this approach compared to isoelectric focusing with Ampholine remains to be tested. The resolution of isoelectric focusing depends on the square root of field strength (*26*), and the field strength obtainable for the present system is significantly smaller than that readily achievable for conventional isoelectric focusing with Ampholine. This defect may be compensated for, in part, by the ability to generate very "shallow" pH gradients (small $d\text{pH}/dx$) over any desired pH range and in any buffer milieu, and by the avoidance of the problem of Ampholine adsorption to proteins.

TEMED Effect

In the studies reported here the TEMED concentration (and also the concentrations of KP and RN) were held constant, irrespective of gel concentration, in order to minimize variability between gels with regard to parameters other than the ones under study. However, in the customary practice of PAGE fractionation, TEMED concentration is varied approximately in inverse proportion to gel concentration. This would be expected to affect the values of pH, KAPPA, and NU, and to

alter their relationship with gel concentration (except in the case where gels were purified by equilibration with buffer). The effect of TEMED on the pH and stacking properties of several buffer systems is discussed elsewhere (*13*).

The present findings suggest that values of pH, NU, KAPPA, RM (1,9), etc., given in the buffer system output must be regarded as provisory, e.g., in the calculation of free mobility and net charge (*11*). Direct experimental validation will be necessary if experimental results are critically dependent on the accuracy of these parameters. In addition to the methods used here, direct measurement of the concentrations of constituents in each phase should provide a generally useful, straightforward method for testing Jovin's theory and validating the buffer systems. This method has been used by Duimel and Cox (*27*) in the study of a modification of Ornstein's Tris–glycine system. However, deviations from ideal behavior of these buffer systems may frequently be insignificant for fractionation of macromolecules. Almost all fractionations with this system have employed reagents of a lower degree of purity than were used in this study, or in the determination of ionic mobilities and pK values for the various constituents (*12*).

The formation of stacks and unstacking was observed. However, the stacking limits [RM(1,4), RM(2,2), RM(1,9)] may be slightly different from the predicted values. Also, some "stacking" systems have been previously developed on a semi-empirical basis by other workers (e.g., *5*) where the operating characteristics were completely unknown. It appears that Jovin's theory provides a much better basis for the interpretation of results.

Acknowledgments

This study was part of a longstanding collaborative effort with T. M. Jovin, on whose theoretical contributions the entire study rests. We thank Dr. Jovin for permission to reproduce Fig. 1. G. Sherman prepared the manuscript on WYLBUR. Mr. H. D. Swank constructed the special apparatus used. Illustrations by M. Grundy, B. Jennings, and S. Mills.

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Received by editor April 24, 1972