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ANALYSIS OF PROTEIN AND PEPTIDE MIXTURES

EVALUATION OF THREE SODIUM DODECYL SULPHATE–POLYACRYLAMIDE GEL ELECTROPHORESIS BUFFER SYSTEMS

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SUMMARY

Three buffer systems were compared for sodium dodecyl sulphate (SDS) electrophoresis of proteins and peptides in gels of 12.5% polyacrylamide. The system based on that of M. Wyckoff, A. Rodbard and A. Chrambach (*Anal. Biochem.*, 78 (1977) 459) gave the best resolution, especially for polypeptides below 10,000 M_r, compared with the other two systems (based on those of D. M. Neville, *J. Biol. Chem.*, 246 (1971) 6328 and U. K. Laemmli, *Nature (London)*, 227 (1970) 680). The low concentration of SDS in the upper buffer (0.03%) suggested by Wyckoff *et al.* was found to be unsatisfactory for the resolution of some proteins and peptides and I have maintained this at 0.1%. The migration velocities of standard proteins and tracking dye in the systems varied in the order predicted by the multiphasic zone electrophoresis (MZE) theory, even in the presence of SDS. Differences of resolution in the systems were rationalized using MZE theory.

INTRODUCTION

Since the publication of the first methods for polyacrylamide gel electrophoresis (PAGE) of proteins using discontinuous buffer systems[1,2] many others have been devised using Jovin and co-workers'[3–7] multiphasic zone electrophoresis (MZE) theory. Several such systems have been adapted for general use with sodium dodecyl sulphate (SDS)[8–10] and it was suggested that minimal amounts only, of SDS in the upper buffer (0.03%) should be used, to minimize the stack width and increase resolution[8].

A useful application of discontinuous SDS–PAGE has been to the characterization of peptides produced by proteolytic degradation of protein substrates[11–14]. However, the choice of which buffer system to use, in the presence of SDS, has been regarded by some as being irrelevant over wide limits[15] or at best empirical[8]. Other workers have suggested that the choice is important with respect to protein migration velocity, separation and resolution[16].

In this paper, I have compared the protein and peptide resolution obtained

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with three discontinuous SDS–gel electrophoresis buffer systems based on those of Wyckoff *et al.*[8] (I), Neville[9] (II) and Laemmli[10] (III), using 12.5% polyacrylamide. The actual and MZE-theoretical results were compared to determine whether SDS affected the relative performance of the three systems, and, whether MZE theory could rationalize their differences in resolution. The minimal requirement suggested for SDS in the upper buffer (0.03%) was evaluated in systems I and III.

EXPERIMENTAL

Acrylamide, methylene-bis-acrylamide (specially pure for electrophoresis), Tris, SDS (specially pure) and boric acid were from BDH (Poole, Great Britain); 2-amino-2-methyl-1,3-propanediol (Ammediol) was from Aldrich (Gillingham, Great Britain); albumin (bovine serum, crystallized), carbonic anhydrase (bovine erythrocyte), phosphorylase *a* (rabbit muscle), cytochrome *c* (horse heart, type IIa) phenylmethane sulphonyl) fluoride (Pms-F), transferrin (human), trypsin (porcine pancreatic, EC 3.4.21.4) and trypsin inhibitor (soybean, type I-s) were from Sigma (London) (Poole, Great Britain). Aprotinin as "Trasylol" was a gift of Dr. E. Phillipp, Bayer (Wuppertal, F.R.G.).

Serum albumin albumin digests

Tryptic digests of denatured bovine serum albumin were prepared for use in the comparison of electrophoresis systems. The albumin (2 mg/ml in 50 m*M* Tris–HCl, pH 7.7, containing 0.5% SDS) was heated at 100°C for 5 min, allowed to cool and mixed with 0.1 volume of trypsin solution (2, 0.2, 0.02 or 0.002 mg/ml of 50% glycerol or phosphate buffered saline). The mixtures were incubated for 10 min at 37°C, then 0.1 volume of 0.1 *M* Pms-F in propan-2-ol was added. As a control, albumin was incubated without trypsin.

Preparation of electrophoresis gels

The procedure was generally as described previously[18]. Slab gels were formed in batches of ten, each between glass plates 81 mm square, and 2.8 mm apart. The lower gels formed in this way contained 12.5% total acrylamides, 2.6% of this as methylene-bis-acrylamide and were about 60 mm long. Three buffer systems were used (see Table I), and polymerization was with ammonium persulphate (0.025%) and N,N,N′,N′-tetramethylethylenediamine (0.04%). To minimize thermal convection during polymerization, linear gradients of sucrose and ammonium persulphate (10–0% and 0.0125–0.025% bottom to top, respectively) were introduced into the gels.

The upper gels (3.2% acrylamide, 6.25% being cross-linker) were formed immediately before the gels were run; they contained the appropriate upper gel buffer (Table I), sucrose (12.5%, w/v), riboflavin phosphate (0.001%), potassium persulphate (0.025%) and tetramethylethylenediamine (0.125%), and were photopolymerized.

Samples for electrophoresis

These were the tryptic digests of denatured bovine serum albumin or a standard protein mixture. Each sample consisted of 8–11 μg of protein in 10 μl. Sample

solutions were prepared by mixing protein solutions with an equal volume of double strength upper gel buffer (Table I), containing glycerol (40%), bromophenol blue (0.01%), 2-mercapto-ethanol (1.0%) and SDS (2%), and were heated for 5 min at 100°C. Thus samples had an SDS:protein ratio of at least 10:1.

Electrophoresis

This was as described[18] in a Pharmacia GE-4 tank, or similar apparatus constructed in the laboratory. Current was 20 mA (per slab gel) until the stack entered the lower gel, and then 40 mA. The run was stopped when the bromophenol blue just reached the bottom of the gel (*i.e.* after about 70 min). The gels were stained for protein as described[18].

Gel measurements

Measurements, taken from the gels or photographs of the gels, were to the nearest 0.1 mm. The time taken for electrophoresis in the separating gel (t) was noted. The length of the gel was measured before staining (l_1) together with the distance moved by the bromophenol blue tracking dye (d_{bb}). Its migration velocity was d_{bb}/t. After staining and destaining, the length of the gel (l_2) and the distance moved by each standard protein (d_p) was measured on a photograph. The migration velocity of the proteins were computed as $d_p\, l_1/t\, l_2$. The mixture of standard proteins contained phosphorylase *a* (M_r 94,000), transferrin (M_r 78,000), bovine serum albumin (M_r 68,000), IgG heavy chain (M_r 50,000), carbonic anhydrase (M_r 29,000), soybean trypsin inhibitor (M_r 21,567), cytochrome *c* (M_r 11,560) and sometimes aprotinin (M_r 6,475).

Theoretical considerations

The equations in the text (1–12) were obtained using the original equations and symbols as derived by Jovin[4–6], the original equation numbers being in parentheses: eqn. 1, $v^{\pi\lambda} = \bar{r}_2^{\lambda}\, I^{\pi}/96.5\, m_2\, \bar{c}_2^{\lambda}\, (r_2 - r_6)$, (eqns. 36, 39, 163); eqn. 2, $v^{\pi\lambda} = \bar{r}_2^{\lambda}\, I^{\pi}\, (1 - r_6/r_2)/96.5\, (r_2 - r_6)\, \bar{c}_3\, m_3\, (1 - r_6/r_3)$, (eqns. 36, 39, 101, 163); eqn. 3, $v_p^{\pi} = I^{\pi}\, \mu_p^{\pi}/96.5\, \bar{c}_1^{\pi} \phi_1^{\pi}\, (r_1 - r_6)\, \mu_{Na^+}{}^{\pi}$, (eqns. 6, 9, 38 and ref. 19); eqn. 4, $\bar{r}_1^{\pi} = r_1 \phi_1^{\pi}$, (eqn. 37); eqn. 5, $\phi_1^{\pi} = \left|\, |a| - a\,(a + b)^{\frac{1}{2}} \right|$, $a = \frac{1}{2}\,(1 + \theta)/(1 - \varrho)$, $\varrho = 10^{-(pK_6 - pK_1)}$, $b = -2\theta/(1 + \theta)$, (eqn. 26); eqn. 6, $\theta = -\bar{c}_6/\bar{c}_1$ (eqn. 44); eqn. 7, $\bar{c}_1^{\pi} = \bar{c}_2^{\lambda}\, m_2\, (1 - r_6/r_2)/(1 - r_6/r_1)$, (eqn. 40); eqn. 8, $\bar{c}_6^{\pi} = \bar{c}_2^{\lambda} + \bar{c}_6^{\lambda} - \bar{c}_1^{\pi}$, (eqn. 42); eqn. 9, $\bar{c}_2^{\lambda} = \bar{c}_3\, m_3\, (1 - r_6/r_3)/m_2\, (1 - r_6/r_2)$, (eqn. 101); eqn. 10, $\bar{c}_6^{\lambda} = \bar{c}_3 + \bar{c}_6 - \bar{c}_2^{\lambda}$, (eqn. 42); eqn. 11, $|\bar{r}_1^{\pi}| \geqslant r_{max.} \geqslant 0.3$, (eqns. 114, 115); eqn. 12, $E^{\pi}/E^{\lambda} = \mu_{Na^+}{}^{\lambda}\, m_2\, \bar{c}_2^{\lambda}\, (r_2 - r_6)/\mu_{Na^+}{}^{\pi}\, \bar{c}_1^{\pi} \phi_1^{\pi}\, (r_1 - r_6)$, (eqns. 38, 39, 6, 9).

Symbols describing upper reservoir (α), stacking gel (β), separating gel (γ) buffer phases, separation phase during electrophoresis (π), phase ahead of π (λ) were also used as superscripts. The subscripts represented the buffer constituents, the ions of which formed the trailing ion of the $\pi\lambda$ boundary, 1; the leading ion of the $\pi\lambda$ boundary (and trailing ion of the $\lambda\gamma$ boundary in system II), 2; leading ion of the $\lambda\gamma$ boundary (system II), 3; common ion, 6. $\bar{c}_j$ was the concentration of constituent j; $c_{j(m)}$, the concentration of subspecies of constituent j with valence m; μ_{Na^+}, the mobility of the sodium ion; μ_p, the average mobility of the SDS-proteins $\left(= \frac{1}{n} \sum_{i=1}^{n} \mu_{p_i} \right)$;

TABLE I

DETAILS OF THE THREE DISCONTINUOUS BUFFER SYSTEMS

Systems I, II and III were based on those of Wyckoff *et al.*[8] (the Ammediol system), Neville[9] and Laemmli[10], respectively. There were slight deviations from the original procedures to facilitate comparison of the system; there was no SDS in any of the lower reservoir or gel buffers (unlike the original system III), and the samples had the same buffer composition as the upper gel (unlike systems I and II).

System	*Buffers*			
(Leading, trailing, common ions)	*Upper reservoir*	*Sample and stacking gel*	*Separating gel*	*Lower reservoir*
System I (Cl^-, glycinate$^-$, Ammediol$^+$)	Ammediol (0.041 *M*)–glycine (0.04 *M*), pH 9.39	Ammediol (0.084 *M*)–HCl (0.062 *M*), pH 8.37	Ammediol (0.11 *M*)–HCl (0.047 *M*), pH 8.96	Ammediol (0.063 *M*)–HCl (0.05 *M*), pH 8.23
System II (SO_4^{2-}, borate$^-$, Tris$^+$)	Boric acid (0.04 *M*)–Tris (0.041 *M*), pH 8.64	Tris (0.0541 *M*)–H_2SO_4 (0.0267 *M*), pH 6.1	Tris (0.424 *M*), HCl (0.031 *M*), pH 9.18	
System III (Cl^-, glycinate$^-$, Tris$^+$)	Tris (0.025 *M*), glycine (0.192 *M*), pH 8.3	Tris (0.125 *M*)–HCl (0.119 *M*), pH 6.8	Tris (0.375 *M*), HCl (0.059 *M*), pH 8.8	Tris (0.025 *M*)–glycine (0.192 *M*), pH 8.3

v_p^{π}, the average SDS–protein migration velocity in phase $\pi \left(= \frac{1}{n} \sum_{i=1}^{n} v_{pi}^{\pi} \right)$. Values of r_j for borate$^-$, glycinate$^-$, Cl^-, SO_4^{2-}, ammediol$^+$ and Tris$^+$ were: -0.62, -0.72, -1.55, -1.42, $+0.5$, $+0.5$ respectively. $\bar{r}_j$, was the relative constituent mobility of constituent j; $v^{\pi\lambda}$ the migration velocity of the moving boundary $\pi\lambda$; r_j, the relative ion mobility of ion subspecies of constituent j; I, the current density; E, the electric field strength; ϕ_j, the fraction of constituent j dissociated into ion subspecies. Values of pK_a for borate$^-$, Tris$^+$, Ammediol$^+$, glycinate$^-$ were 9.2, 8.07, 8.83, 9.74, respectively[7].

RESULTS

SDS concentrations in the upper buffer

I compared the appearance of the gels for systems I and III, where the SDS concentration was 0.03% (Fig. 1), with two more gels where the upper buffer had 0.1% SDS (Fig. 2C and D). With the lower SDS concentration, the standard proteins have run anomalously, with the loss of one protein band (Fig. 1). For the low-molecular-mass peptides in the protein digests, there was a loss of resolution (Fig. 1, lanes b). SDS, used at 0.1% in the upper buffer, gave very good resolution and was adopted for all further work.

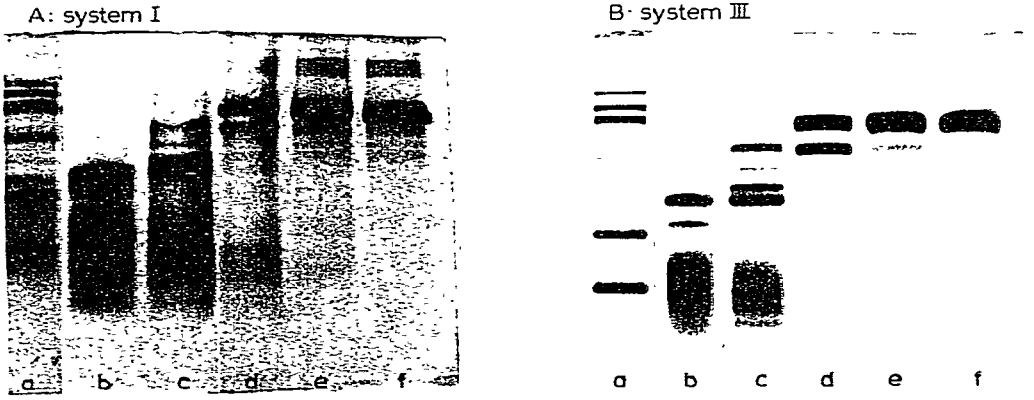

Fig. 1. Effect of 0.03% SDS concentration in the upper buffer of electrophoresis systems I and III. Electrophoresis was carried out using an upper buffer SDS concentration of 0.03%. The samples were from the same batch of trypsin digestions as in Fig. 2C and D. Lanes a–f were as described in Fig. 2C and D. A and B were representative gels from systems I and III respectively.

Practical comparison of the three buffer systems for SDS electrophoresis

Representative results for electrophoresis of tryptic digests and the standard proteins are shown in Fig. 2. The resolution of proteins and peptides of molecular mass less than cytochrome *c* was clearly best in system I. In system III, unstacking of low-molecular-mass peptides was incomplete (Fig. 2D, lane b) and in system II, the low-molecular mass bands, although unstacked, were much more diffuse (Fig. 2B, lane b).

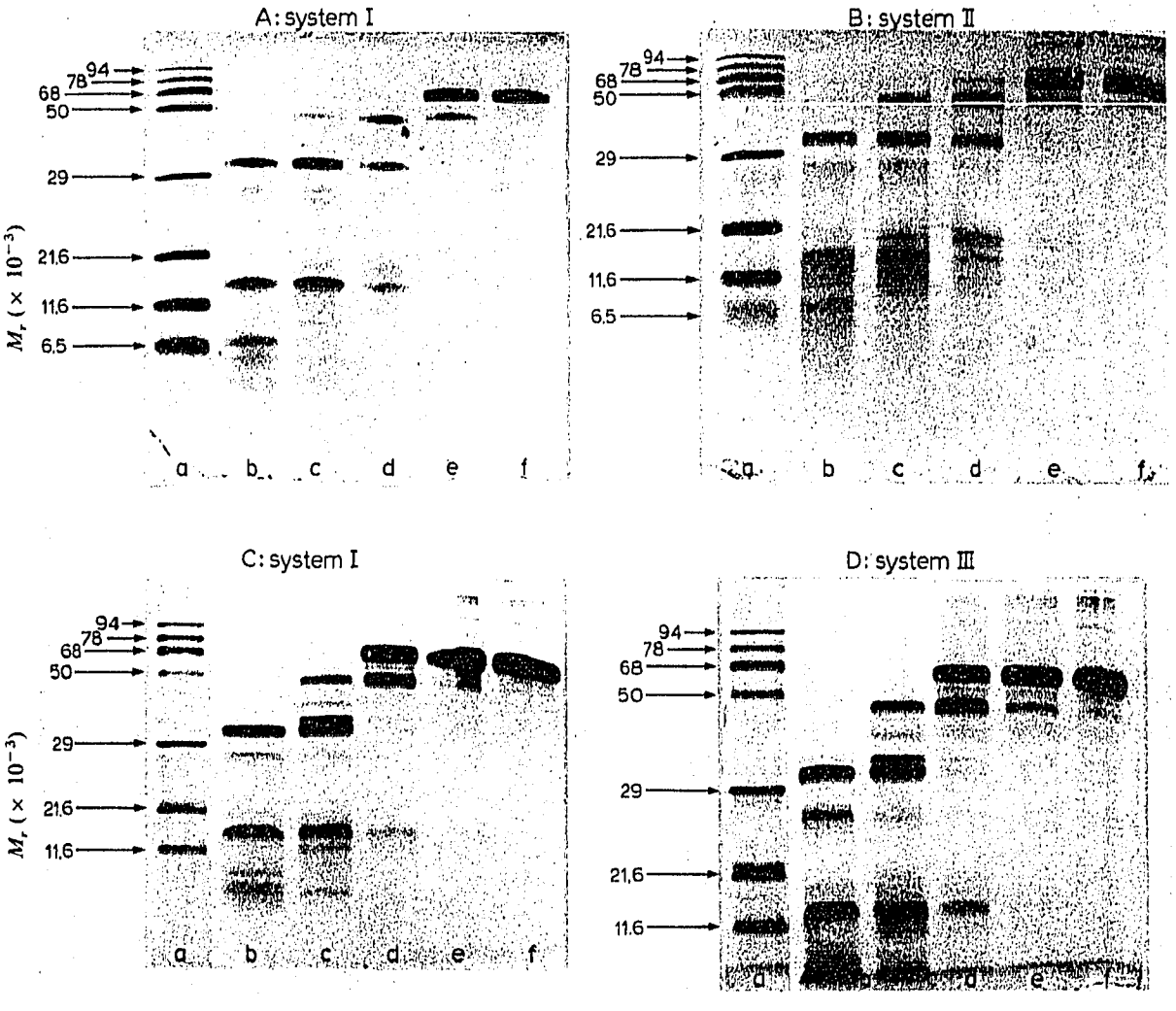

Fig. 2. Electrophoresis of standard proteins and tryptic digests of bovine serum albumin in three electrophoresis systems. A, B, C and D were representative gels from the three systems; similar results have been obtained for gels from at least two batches of each system, and the samples applied to gels A and B were from a different series of digests to those applied to gels C and D. In each part, lane f represents undigested bovine serum albumin; b, c, d, and e were tryptic digests of albumin with substrate/enzyme ratios of 10, 100, 1000 and 10,000 respectively; lanes a show a mixture of seven or eight standard proteins (see the methods section).

Theoretical comparison of three buffer systems for SDS-electrophoresis. Bromophenol blue migration velocity through the separating gel

The migration velocity of the bromophenol blue tracking dye through the separating gel was determined for gels run in systems I (1.34 mm/min, average for four gels, S.D. = 0.061), II (1.833 mm/min, average for three gels, S.D. = 0.090) and III (1.010 mm/min, average for two gels, S.D. = 0.155). The results were obtained for gels made from at least two batches of polymerization mixture for each system. These results were compared with results obtained theoretically (Table II) by substituting known values in $\bar{r}_2^{\lambda}\, I^{\pi}/96.5\, m_2\, \bar{c}_2^{\lambda}\, (r_2 - r_6)$, (eqn. 1) for systems I and III to give $v^{\pi\lambda}$, the migration velocity of the moving boundary $\pi\lambda$. In system II, however, there was an additional moving boundary, $\lambda\gamma$, so $v^{\pi\lambda}$ was given by $\bar{r}_2^{\lambda}\, I^{\pi}\, (1 - r_6/r_2)/96.5\, (r_2 - r_6)\, \bar{c}_3^{\gamma}\, m_3\, (1 - r_6/r_3)$, (eqn. 2). This comparison is considered valid, since, in practice, bromophenol blue dye migrates at the leading edge of the stack at the $\pi\lambda$ boundary in gels of greater than 12.5% acrylamide concentration[8].

TABLE II

COMPARISON OF ELECTROPHORESIS BUFFER SYSTEMS I, II, III FOR VALUES OF MIGRATION VELOCITIES OF TRACKING DYE AND PROTEINS

The systems were compared as ratios of values of dye or average protein migration velocities. Values were determined practically (A) and theoretically (B) for each system as described in the text.

Systems compared (ratio)	*Bromophenol blue dye migration velocity*		*Average protein migration velocity*	
	A	*B*	*A*	*B*
I/II	0.73	0.66	0.84	0.92
III/II	0.55	0.53	0.86	0.92
III/I	0.75	0.80	1.02	1.00

The observed ratio of dye velocities for systems III/I (0.75; Table II) correlated well with the theoretically predicted value (0.8; Table II). Derivation of $v^{\pi\lambda}$ for system III showed it was relatively lower by virtue of a higher value of $|\bar{c}_2^{\lambda}|$ (0.059 *M*, equivalent to the leading ion (Cl^-) concentration in the separating gel buffer). In a similar way, the observed ratio for systems I/II was fairly close to the predicted ratio (0.73 *vs.* 0.66, Table II). Here there was a similar reciprocal dependence of $v^{\pi\lambda}$ on the Cl^- concentration derived from the separating gel ($|\bar{c}_3^{\gamma}|$, 0.031 *M*). In addition, however, there were factors relating to the relative mobility (r_2) and relative constituent mobility ($\bar{r}_2^{\lambda}$) of SO_4^{2-} derived from the stacking gel buffer.

Protein migration velocity in phase II

The average SDS-protein migration velocity in the separating gel was determined in each gel as an average for the seven standard proteins, then further averaged according to the number (as before) of gels run. The results for systems I and III were similar (0.428 and 0.438 mm/min respectively), whilst there was a faster average protein migration velocity in system II (0.512 mm/min).

The results correlated well with theoretical predictions about the average protein migration velocity (v_p^π) in the different systems (Table II) v_p^π is given by $I^\pi \mu_p^\pi/96.5 \bar{c}_1^\pi \phi_1^\pi (r_1 - r_6) \mu_{Na^+}{}^\pi$, (eqn. 3). Since I^π, $\mu_{Na^+}{}^\pi$, $\bar{c}_1^\pi \phi_1^\pi$, r_6 have the same values in systems I, II and III, a larger v_p^π in system II is predicted by virtue of a higher (signed) value of r_1, the relative mobility of the trailing ion, borate (−0.62) in this system, compared with glycinate (−0.72) in systems I and III. An assumption is made that μ_p^π is the same in each gel buffer system, thus the same binding ratio of SDS and degree of polymerization of the gels.

Unstacking of low-molecular-mass peptides in phase π

Some of the low-molecular-mass peptides remain stacked at the moving boundary between phases π and λ during electrophoresis in system III. This is due to the fact that $|\bar{r}_1^\pi|$, the relative constituent mobility of the trailing ion (glycinate$^-$) is not sufficiently greater than the relative constituent mobilities of the SDS–peptides in phase π to allow separation within the geometrical limits of the gel and they remain largely stacked. The theoretical values of $\bar{r}_1^\pi$ for the three systems were compared (Table III) by using $\bar{r}_1^\pi = r_1 \phi_1^\pi$ (eqn. 4). Since r_1 is the same in systems I and III, their differences in unstacking are related to ϕ_1^π, the fraction of constituent 1 ionised in phase π. This itself depends on the nature and composition of buffer phases α, β, γ.

TABLE III

COMPARISON OF THE VALUES OF $\bar{r}_1^\pi$, THE RELATIVE CONSTITUENT MOBILITY OF THE TRAILING ION IN PHASE π; r_1, THE RELATIVE ION MOBILITY OF CONSTITUENT 1 AND ϕ_1^π, THE FRACTION OF CONSTITUENT 1 IONIZED IN PHASE π, FOR THREE ELECTROPHORESIS SYSTEMS

$\bar{r}_1^\pi$ was determined from $r_1 \phi_1^\pi$ (eqn. 4), r_1 being given and ϕ_1^π determined using MZE theory as described in the text.

System	*MZE parameters*		
	r_1	ϕ_1^π	$\bar{r}_1^\pi$
I	−0.720	0.410	−0.295
II	−0.620	0.661	−0.410
III	−0.720	0.330	−0.238

Determination of ϕ_1^π depends on the pK_a values and ratio, $-\bar{c}_6^\pi/\bar{c}_1^\pi$, of the concentrations of constituents 1 and 6 in phase π (eqns. 5 and 6). Substitution of known values in $\bar{c}_2^\lambda m_2 (1 - r_6/r_2)/(1 - r_6/r_1)$, (eqn. 7), gives $\bar{c}_1^\pi$, and in $\bar{c}_2^\lambda + \bar{c}_6^\lambda - \bar{c}_1^\pi$ (eqn. 8), gives $\bar{c}_6^\pi$. Since there is a λγ boundary in system II, $\bar{c}_2^\lambda$ is first obtained as $\bar{c}_3^\gamma m_3 (1 - r_6/r_3)/m_2 (1 - r_6/r_2)$, (eqn. 9), and $\bar{c}_6^\lambda$ as $\bar{c}_3^\gamma + \bar{c}_6^\gamma - \bar{c}_2^\lambda$, (eqn. 10). Thus the value of $|\bar{r}_1^\pi|$ for the system III (Table III) is much less than the 0.3 minimum recommended for most purposes (eqn. 11). The unstacking process for proteins and peptides is less efficient for PAGE in the presence of SDS, since, the migration velocities of SDS–polypeptides are greater[17]. There is evidence for this in Fig. 1, where, under conditions of non-saturation with SDS during electrophoresis (leading to poor resolution) the bands are nevertheless unstacked from the moving boundary.

Zone spreading in phase π

There appeared to be more zone spreading in system II than I, making the low M_r peptides appear more diffuse, particularly at the leading edge (Fig. 2). This may be

due to differences in changes in field strength through the moving boundary[20]. For a buffer system, $E^{\pi}/E^{\lambda} = \mu_{Na^+}{}^{\lambda} m_2 \bar{c}_2^2 (r_2 - r_6)/\mu_{Na^+}{}^{\pi} \bar{c}_1^{\pi} \phi_1^{\pi} (r_1 - r_6)$, (eqn. 12). Considering systems I and II, where the values of $\bar{c}_1^{\pi}\phi_1^{\pi}$ and $\mu_{Na^+}{}^{\lambda}/\mu_{Na^+}{}^{\pi}$ each are the same, it was found that E^{π}/E^{λ} was approximately 1.5 times greater in system I than II.

DISCUSSION

In my hands, 0.03 % SDS in the upper buffer of systems I and III, compared to 0.1 %, resulted in inferior resolution particularly for the low-molecular-mass peptides. It has been shown that saturation by SDS of some proteins *e.g.* ovalbumin, in PAGE is only achieved with at least 0.1 % in the upper buffer[21]. On the other hand, some proteins were quite well resolved using the lower SDS concentration, *e.g.*, phosphorylase *a*, transferrin, bovine serum albumin (Fig. 1), β-lactoglobulin[17], chymotrypsinogen[8]. One explanation for this difference in behaviour may be that some polypeptides do not have a low enough dissociation constant for the binding of SDS to remain saturated with SDS at the lower concentration. It was concluded that 0.1 % SDS in the upper buffer would be sufficient to saturate all the polypeptides in these mixtures, and this procedure was adopted for the rest of the work.

In a comparison of three SDS gel electrophoresis buffer systems using 12.5 % acrylamide concentration, the system based on that of Wyckoff *et al.*[8], in our equipment gave the best resolution over a wide range of M_r SDS-polypeptides. A comparative analysis of the Laemmli and Neville MZE buffer systems was recently carried out by Lanzillo *et al.*[16] but the system of Wyckoff *et al.* was omitted.

The practical performance of the three systems, with respect to the dye and average protein migration velocities, varied in an order predicted by the MZE theory despite the presence of SDS in the sample (1.0 %) and upper buffer (0.1 %). Using MZE theory, the difference in migration velocities of bromophenol blue at the $\pi\lambda$ boundary of the three systems was shown to be dependent on the concentration of the leading ion (Cl^-) in their respective separating gels. Also, the greater average protein migration velocity in phase π of system II was due to the higher (signed) relative mobility for the borate ion (compared to glycinate ion) which originated from the upper buffer phase. Since the theory can be used to rationalize the observed differences in performance, it should be considered relevant for making a choice of buffer system in applications involving SDS. In addition to this general finding, Lanzillo *et al.*[16] found that a few proteins exhibited different degrees of heterogeneity in different buffer systems. These minor differences depended to some extent on the amount of SDS in the sample buffer and on the acrylamide concentration. It is unlikely that this heterogeneity can be explained in terms of MZE theory (an approach not adopted by the authors, ref. 16). This additional source of variability may only be determined by using at least two different buffer systems for any particular application[16].

The incomplete resolution of the low M_r peptides from the tracking dye in system III was, in part, a consequence of the concentration of leading (Cl^-) and common ($Tris^+$) ions in the separating gel buffer and on the pK_a values of the common ion ($Tris^+$) and trailing ion (glycinate) derived from the upper buffer. Resolution may be improved by altering the composition of phase γ (and hence λ and π) to increase the values of ϕ_1^{π} and $|\bar{r}_1^{\pi}|$ (as in system I). Incomplete unstacking of low M_r proteins and peptides was observed in 7 % acrylamide gels by Lanzillo *et al.*[16] using

the Laemmli and Neville systems. Improvement of both systems was achieved by increasing the acrylamide concentration using a gradient of 2.5–27%. This decreased the SDS–peptide migration velocity during electrophoresis[22] and allowed unstacking to occur, although an increased time was needed for electrophoresis. In my experiment, 12.5% acrylamide was sufficient to allow unstacking in system II (based on the Neville system, Fig. 2B). Increasing the time of electrophoresis and length of gel in the Laemmli system (7% acrylamide, ref. 16) did not improve unstacking.

In system II there was more zone spreading of the low M_r peptides than in system I. This correlated with a lesser change in the voltage gradient through the moving boundary of system II (ref. 20). A higher field strength in phase π than λ would tend to sharpen the rear side of protein bands relative to the front during unstacking from the boundary. This occurred to a greater extent in system I and the effect correlated in a complex way with the composition of phases α, β and γ. Zone broadening should be decreased by optimizing the ratio E^{π}/E^{λ} (as in system I) or by increasing the acrylamide concentration[1]. Lanzillo *et al.*[16], on the other hand, showed that the Neville system still gave the least clear band patterns even when the acrylamide concentration was increased.

The superior resolution obtained for system I makes it particularly suitable for the analysis of mixtures containing proteins and peptides ranging widely in M_r such as those produced by the proteolysis of proteins of high M_r. This system provides a good alternative to the other systems (*e.g.* II and III) where a similar resolution may only be achieved in gels of higher linear or gradient acrylamide concentrations.

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