FOUR GEL SYSTEMS FOR ELECTROPHORETIC FRACTIONATION OF MEMBRANE PROTEINS USING IONIC DETERGENTS*

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SUMMARY

Membrane proteins were fractionated electrophoretically in polyacrylamide gels containing either anionic or cationic detergent at either pH 2.4 or 8.3. In all four systems, polypeptides migrated as monomers bearing the charge of the detergent ion and semi-logarithmic molecular weight--mobility relationships pertained. Electrophoresis of erythrocyte membrane proteins in these systems yielded very similar protein staining patterns but revealed significant differences in sialoglycoprotein migration.

The study of membrane proteins has been given great impetus by the development of methods for electrophoretic fractionation of polypeptides in polyacrylamide gels containing SDS+ (1-4). Numerous investigations have been directed at elucidating the basis of the fractionation, introducing

*This work was supported by U.S.P.H.S. Grant No. AM05507 and N.S.F Grant No. GB-8702 to Dr. H. M. Kalckar, and by U.S.P.H.S. Grant No. FR05528 to the Worcester Foundation.

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+Abbreviations: SDS, sodium dodecyl sulfate; CTAB, hexadecyl("cetyl")-trimethylammonium bromide; DTE, dithioerythritol; BIS, N,N'-methylenebis-acrylamide; TEMED, N,N,N',N'-tetramethylethylenediamine; PAS, periodic acid--Schiff; Stains-All, 1-ethyl-2-[3-(1-ethylnaphtho[1,2d]-thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2d]-thiazolium bromide.

For convenience in referring to the four gel systems, we use S(8.3) and C(8.3) to denote electrophoresis at pH 8.3 in SDS and CTAB, respectively; and S(2.4) and C(2.4) for electrophoresis at pH 2.4 with SDS or CTAB.

refinements, and extending the range of applications (5-14). In a study of the Mg^{++}-dependent, Na^{+},K^{+}-stimulated ATPase of the erythrocyte membrane, we wished to apply polyacrylamide gel electrophoresis to characterize the intact phosphorylated intermediate(s) of the enzyme (15). For this purpose we developed methods for fractionating detergent-solubilized membranes at pH 2.4 where the labile, peptide-bound, acyl-phosphate intermediate exhibits its maximum stability. We found that either SDS, an anionic detergent, or CTAB, a cationic detergent, could be incorporated into gels at concentrations of 1%. Both detergents solubilized membranes and both were compatible with electrophoresis at pH 2.4 and 8.3. In this note we describe the four gel systems representing these detergent--pH combinations and compare results of their application to the erythrocyte membrane polypeptides.

METHODS

Sample preparation. Human erythrocyte ghosts were prepared as described previously (9). To suspensions of packed ghosts in 5 mM sodium phosphate (pH 8) were added (final concentrations) 0.25 M sucrose, 1% SDS (Schwarz/Mann No. 2696) or CTAB (Eastman No. P5650), and 25-50 mM DTE (Sigma No. D8255). Samples containing SDS were normally incubated at 37° for 15-30 minutes; those containing CTAB were incubated at room temperature or 100° as indicated. For electrophoresis in the acidic systems, 1 M sodium phosphate (pH 2.4) was added to a final concentration of 50 mM. Pyronin Y was used as tracking dye in SDS systems, amido black in CTAB systems.

Molecular weight markers were similarly prepared, except that they were dissolved in 10 mM Tris, 1 mM EDTA (pH 8) and treated with 100 mM DTE.

Gel polymerization and electrophoresis. The overall procedure was that of Fairbanks et al. (9). Stock solutions were mixed in the order given in Table I. At the same time, an overlay solution was prepared consisting of just catalyst plus detergent at the concentrations in the gel. The gels polymerized in 15-20 minutes and, after 40-60 minutes, the overlay

Table I. Gel Formulas

Solution	Volume[a]			
	1% SDS		1% CTAB	
	pH 8.3	pH 2.4	pH 8.3[b]	pH 2.4
	ml			
acrylamide--BIS (40/1.5)[c]	1.4	1.4	1.4	1.4
20X buffer, pH 8.3[d]	.5	-	.5	-
20X buffer, pH 2.4[e]	-	.5	-	.5
20% (w/w) SDS[f]	.5	.5	-	-
10% (w/w) CTAB	-	-	1.0	1.0
H_2O	6.1	5.2	4.1	5.6
0.5% TEMED	.5	-	-	-
1.5% ammonium persulfate	1.0	-	-	-
0.1% ascorbic acid[g]	-	.8	-	.5
0.003% $FeSO_4 \cdot 7H_2O$[g]	-	.8	-	.5
0.03% H_2O_2[h]	-	.8	-	.5
0.0025% riboflavin	-	-	3.0	-

[a]To make 10 ml of solution containing 5.6% acrylamide.
[b]Photopolymerized.
[c]40% (w/v) acrylamide, 1.5% (w/v) BIS; deionized (9).
[d]1.78 M Tris, 1.78 M boric acid (pH 8.3), 0.05 M EDTA, 0.2 M $(Na_2H+NaH_2)PO_4$ (pH 8.3).
[e]1 M $(H_3+NaH_2)PO_4$ (pH 2.4).
[f]Schwarz/Mann No. 2696 in samples and gels, Matheson, Coleman and Bell No. DX2490 T7003 in the tray buffers.
[g]Freshly prepared.
[h]Fresh dilution from 30% stock solution.

was replaced with tray buffer. All gels were then allowed to "cure" for at least 18 hours at room temperature.

The results obtained with these systems are strongly dependent on the conditions of polymerization. Optimization for SDS gels polymerized with ammonium persulfate--TEMED (as in the S(8.3) system) has been discussed (9). The three "new" systems are more variable in their polymerization. Jordan and Raymond (16) suggested the ascorbic acid--ferrous ion--peroxide catalyst (a modified Fenton's reagent) for acidic gels; success with it requires fresh solutions. The C(8.3) gels had to be photopolymerized because CTAB precipitated ammonium persulfate and the ascorbic acid--ferrous ion--peroxide reagent was non-functional at the higher pH. This system was the least

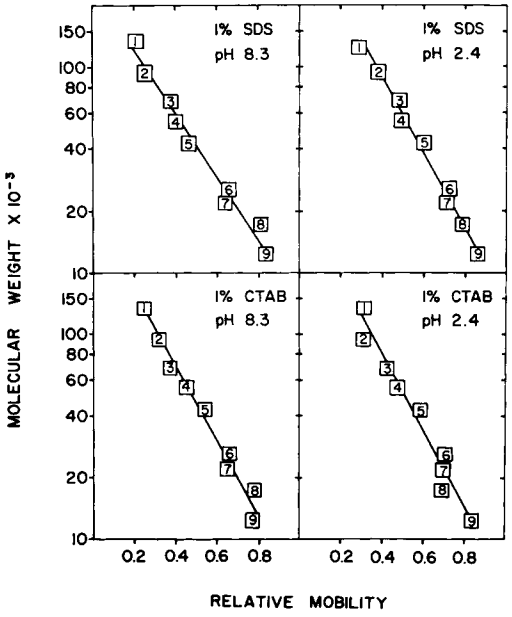

Figure 1. Calibration curves for molecular weight estimation. Gels contained 5.6% acrylamide with 1% SDS or CTAB at pH 2.4 or 8.3, as indicated. The protein standards were highly purified products of Schwarz/Mann and Worthington: (1) β-galactosidase; (2) phosphorylase a; (3) bovine serum albumin; (4) human γ-globulin, heavy chain; (5) ovalbumin; (6) chymotrypsinogen; (7) human γ-globulin, light chain; (8) myoglobin; (9) cytochrome c.

reproducible: In addition to outright failures to polymerize, we encountered irregularities in most gels that caused band distortion.

The tray buffers contained detergent and buffer at the same concentrations as in the gels. Electrophoresis was performed at 80 volts (about 8 volts/cm). The running times for 75 mm tracking dye migration varied from 1.5-3 hours. The CTAB gels were run with reversed polarity (anode at the top). With the C(2.4) system, a yellow electrolysis product formed at the anode; anti-convection baffles prevented it from reaching the gels.

Fixation and staining. SDS gels were stained with coomassie blue in 25% isopropyl alcohol--10% acetic acid (9). This procedure could not be applied directly to CTAB gels because fixation was inadequate and a

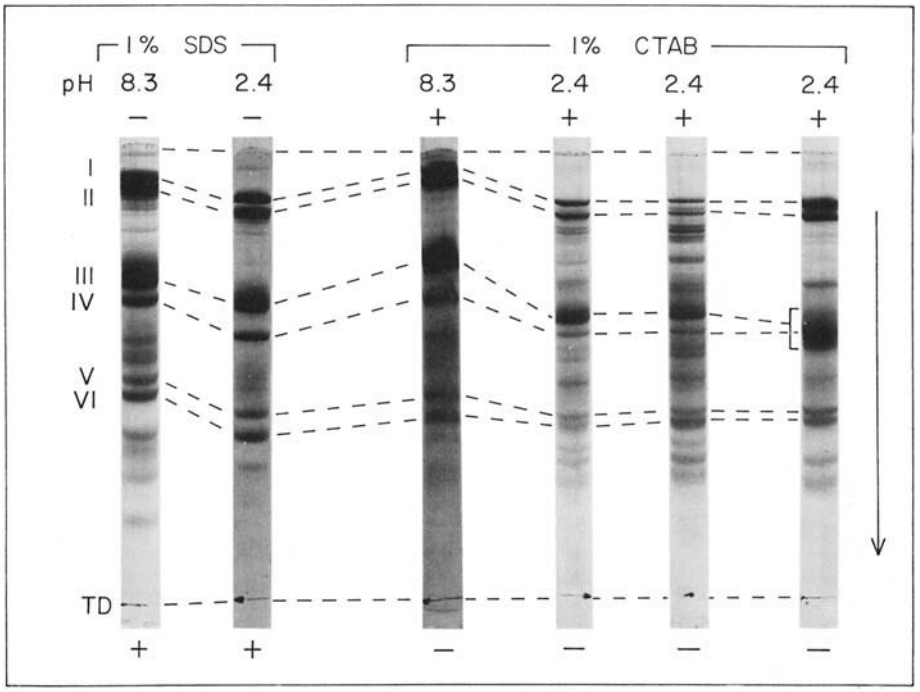

Figure 2. Electrophorograms of human erythrocyte membrane proteins. About 30 μg of protein was applied to each gel. The C(8.3) sample was solubilized at room temperature. The C(2.4) samples were from the same membrane batch but were prepared separately: One (right) was incubated for 1 minute at 100° after addition of DTE and CTAB (pH 8) but before addition of pH 2.4 buffer. Two samples (second and third from the right) were solubilized at room temperature.

persistent detergent--dye complex was formed. CTAB gels were therefore fixed by 30 minute immersion in cold 25% isopropyl alcohol--15% TCA, and were then soaked in 25% isopropyl alcohol--10% acetic acid overnight and in 10% isopropyl alcohol--10% acetic acid for about 6 hours. Subsequently, they were treated as SDS gels, except that the interval in stain was shortened to two hours.

Glycoprotein bands were detected by staining gels by the PAS method (9) or with Stains-All (Eastman 2718--ref. 17).

RESULTS

Electrophoresis of protein molecular weight standards in the four systems yielded the calibration curves shown in Figure 1. In each case, the

Table II. Apparent Molecular Weights of Erythrocyte Membrane Polypeptides in Four Gel Systems.

Component	Molecular Weight[a]			
	1% SDS		1% CTAB	
	pH 8.3	pH 2.4	pH 8.3	pH 2.4
I, II	>200,000	>200,000	>200,000	>200,000
III	95,000	105,000	104,000 (86,000)[b]	104,000 (84,000)[b]
IV	82,000	81,000	78,000	87,000
V	44,000	41,000	38,000	39,000
VI	38,000	34,000	33,000	35,000
SGP[c]	87,000	53,000	62,000	52,000

[a]Estimated from semi-logarithmic approximations to molecular weight--mobility calibration curves, as shown in Figure 1.
[b]Apparent molecular weight of III after boiling in 1% CTAB at pH 8.
[c]The predominant, PAS-positive, sialoglycoprotein band. In the S(8.3) system, SGP corresponds to PAS-1 of Fairbanks et al (9).

standards exhibited mobilities consistent with dissociation of the reduced polypeptides to minimal covalently-bound subunits and the relationship between mobility and the logarithm of molecular weight could be approximated by a straight line in the range 15,000-130,000 daltons.

Electrophorograms of erythrocyte membrane polypeptides in the four gel systems revealed straightforward homologies in the distributions of six major bands (Figure 2). The identification of these components was confirmed by separate electrophoretic analysis, in each system, of fractions prepared by selective elution (9). In the four systems these major components appear in the same order (Figure 2) and exhibit about the same apparent molecular weights (Table II). The patterns show some consistent differences that are reflected in the molecular weight data. For example, it is characteristic of the S(2.4) and C(8.3) systems that they yield greater separation of bands III and IV; and the C(2.4) system always shows relatively poor separation of V and VI.

The glycoprotein pattern in the S(8.3) system was identical to that previously described (9). The predominant sialoglycoprotein band (SGP in Table II) lies between components III and IV, where it is partially obscured. In the other three systems, the proportion of glycoprotein stain in one band is higher and this predominant component migrates well ahead of IV in a region of relatively low coomassie blue staining intensity. Accordingly, the apparent molecular weight of SGP in the S(2.4), C(2.4), and C(8.3) systems is up to 40% lower than the estimate made with the S(8.3) system (Table II). Although the sialoglycoprotein has a uniquely high sialic acid content (18), it exhibits a net positive charge in the presence of CTAB; no detectable protein or PAS-positive material migrated toward the anode in the C(2.4) system.

Figure 2 includes three examples of the fractionation of the membrane polypeptides in the C(2.4) system. These were selected to illustrate varying degrees of attenuation in the intensities of bands I and II coupled with the appearance of numerous faster-moving bands. This picture is characteristic of proteolytic degradation (19). We believe that it takes place after the pH of the CTAB samples is dropped to 2.4. Heating the samples at this stage increases the severity of the artifact. Identical samples held at pH 8 for analysis in C(8.3) gels show no such degradation. Furthermore, when CTAB-solubilized membranes (pH 8) are briefly incubated at 100° before addition of the pH 2.4 buffer, wholesale degradation is prevented (see gel at right, Figure 2).

The 100° treatment alters the mobility of band III, which then overlaps IV (Figure 2, right), as if it had sustained a reduction in size of about 20,000 daltons (Table II). This transformation clearly takes place at the higher pH, because a similar selective band shift is seen in corresponding C(8.3) gels (Table II). In this case, the shift in III is usually accompanied by an increase in the intensity of band VI. This could reflect release of a

large fragment by restricted peptide cleavage. However, no evidence of the products of this hypothetical cleavage can be seen in C(2.4) gels.

Variations in the sample preparation procedure had no effect on sialoglycoprotein mobility in the C(2.4) and C(8.3) systems.

DISCUSSION

The basis for the solubilization and fractionation of membrane proteins in SDS is multiple binding of detergent anions by the proteins. Inter- and intramolecular bonds are extensively disrupted to produce polypeptide monomers bearing a high negative charge (20). In sieving gels these complexes exhibit mobilities that are relatively independent of variations in polypeptide composition but bear a uniform relation to molecular size (4,5,7,8,12). Our results suggest that the principles underlying separation in neutral or alkaline SDS gels are also operative at pH 2.4 and are expressed in a complementary fashion in the cationic detergent.

The divergence of the estimates of sialoglycoprotein molecular weight probably reflects differences in the mode of interaction of the two detergents with the acidic polysaccharide moieties, which constitute two-thirds of the molecule (18). The single band seen in C(2.4) gels had an apparent molecular weight close to published determinations on the purified protein (18,21). Glossmann and Neville (14) reported that several glycoproteins of high carbohydrate content migrated with inappropriately low mobilities in calibrated alkaline SDS gels. A similar effect may be expressed in the S(8.3) system as an overestimation of the sialoglycoprotein molecular weight.

The potential for degradation artifacts in CTAB is great. We have not applied these methods to other membranes, so the scope of the problem is unknown. In the case of the erythrocyte membrane, the artifact probably results from associated proteolytic activities, either intrinsic (22-24) or derived by contamination from other blood elements (9). We have shown that

it can be controlled by boiling in CTAB at pH 8 but is remarkably resistant to heat inactivation at pH 2.4.

The shifts in band III mobility deserve further study. This protein is of particular interest because it spans the membrane (19,25) and is lipophilic (9,26). The band shifts might represent selective proteolysis, non-enzymatic scission of a covalent bond, disaggregation, or conformational change.

Considering the convenience, reliability and high resolution of the neutral and alkaline SDS gel systems, in their various forms, it is well to ask if the three "new" systems we have described offer any advantages that warrant their use. Clearly, the C(8.3) system in its present form is too capricious; results with it were included largely for symmetry in the presentation. On the other hand, we have already demonstrated a requirement for the S(2.4) system in analyzing the unstable phosphopeptide intermediate(s) of the Na^+,K^+ transport ATPase (15). The same system is attractive also because it yields the best separation of major components. The C(2.4) system affords ready isolation of the the sialoglycoprotein as a single electrophoretic species well separated from other major components. In addition, we suggest, for the reasons discussed above, that it may prove to be a more accurate method for determining the molecular weights of acidic glycoproteins in general.

ACKNOWLEDGMENTS

We thank Mrs. Kristina Persson and Miss Carolyn Brown for technical assistance.

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