CHROM. 4499

MOLECULAR WEIGHT DETERMINATIONS AND THE INFLUENCE OF GEL DENSITY, PROTEIN CHARGES AND PROTEIN SHAPE IN POLYACRYLAMIDE GEL ELECTROPHORESIS

D. P. BLATTLER* AND F. J. REITHEL
Department of Chemistry, University of Oregon, Eugene, Ore. 97403 (U.S.A.)

(Received November 3rd, 1969)

SUMMARY

The use of polyacrylamide gels as molecular sieves and electrophoresis as a transport mechanism provides a reliable procedure for estimating the molecular weight of proteins. A series of multimers derived from urease and from bovine serum albumin have been employed as standards. The method described may be used for proteins with a wide range of molecular weights.

INTRODUCTION

It has been pointed out by SMITHIES[1], RAYMOND AND NAKAMICHI[2], TOMBS[3], SHAPIRO *et al.*[4], ZWAAN[5], and BLATTLER[6] that information about the size of protein molecules can be obtained from electrophoresis in polyacrylamide gels. The rationale resembles that for molecular weight determinations by Sephadex chromatography. A complication is the variable charge density characteristic of proteins. ZWAAN[5] and SHAPIRO *et al.*[4] have provided two different approaches to reduce or eliminate the varying influence of charge. The work presented here is an investigation of other factors important to quantitative electrophoresis.

To avoid varying charge effects, a polymerized sample of bovine serum albumin (BSA), or polymerized urease, was used as a standard.

EXPERIMENTAL

Materials and methods

The urease was prepared essentially by the method of SUMNER[7] and DOUNCE[8] as modified by GORIN *et al.*[9]. The extracting solution contained 160 ml acetone, 0.01 *M* β-mercaptoethanol, 0.001 *M* Na_2EDTA and deionized water in a volume of 500 ml. The urease was stored as crystals in the acetone–citrate solution. Under these con-

* Public Health Service Fellow. This work was also supported by U.S. Public Health Service Grant 5 ROI AM11678-03.

ditions urease slowly polymerizes. A significant factor in the success of this work is the utilization of appropriate protein standards and in order to employ proteins of different size but as similar as possible, polymerized proteins were utilized. It is realized that in the proteins chosen, shapes and charges do vary somewhat. Urease and BSA were utilized since these proteins polymerize slowly with age.

CREETH AND NICHOL[10] first studied the associated forms of urease and found evidence for the formation of dimers and trimers of urease by ultracentrifuge studies. We have repeated and confirmed their work and related the ultracentrifuge patterns to the patterns obtained by electrophoresis studies. The greater sensitivity of electrophoresis yielded additional components which were characterized by electrophoresis and found to be the tetramer and pentamer of urease. Higher multimers of urease are evidently insoluble since solutions of polymerized urease often contain precipitates. It is well known that preparations of BSA often contain dimers, trimers and higher multimers of BSA.

We used a commercial sample of BSA which was aggregated to an unusual extent since at least six components were present. This degree of polymerization was probably due, in part, to the age of the sample since it was several years old. A study of the components revealed that they formed an arithmetic polymerization sequence (see Fig. 3 and ref. 11). All components could be reduced to a single band identical with BSA monomer.

The electrophoresis experiments were carried out in a vertical gel apparatus* permitting all the samples to be run in the same gel under identical conditions. The experiments were conducted in a buffer containing Tris, 6 g/l and Na_2EDTA, 0.6 g/l. The gels were formed from cyanogum** and polymerized with the conventional catalyst system of TMED (tetramethylethylenediamine) and ammonium persulfate. To polymerize 150 ml of solution, 0.10 g of $(NH_4)_2S_2O_8$ was used. Sufficient TMED was used to complete polymerization in 15–20 min. This usually required 15–20 μl of TMED solution. The polymerization was carried out in nitrogen. The gels were prepared by adding cyanogum to a fixed volume of water so that, for example, a 5% gel contained 5 g of cyanogum and 100 ml of water. The addition of 1 g of acrylamide to 100 ml of water increased the volume to approximately 101 ml and this relationship was found to be approximately linear up to 15 g of acrylamide per 100 ml. Our experience has shown that cyanogum has too much bisacrylamide for use in making gels denser than 10%. Formulations containing less of the cross linking reagent yield gels that are more supple and less fragile.

The succinylated BSA was prepared as described by CHERRY[12] and HABEEB *et al.*[13]. Succinic anhydride was used at 0° and the pH was maintained at 7.5–8.0 with a pH stat. The reduced succinylated BSA was first succinylated and then reduced in 0.5 *M* β-mercaptoethanol and 6 *M* guanidine hydrochloride. α-Urease (240,000) was prepared as described by BLATTLER *et al.*[14].

RESULTS

Fig. 1 is a plot of the maximum gel "pore" size of polyacrylamide gel *vs.* gel

* This instrument is commercially available from the Biochemical Instrument Co., 881 Oakway Rd., Eugene, Ore. 97401, U.S.A.

** E-C Apparatus Corporation, 222 South 40th Street, University City, Philadelphia, Pa. 19104, U.S.A.

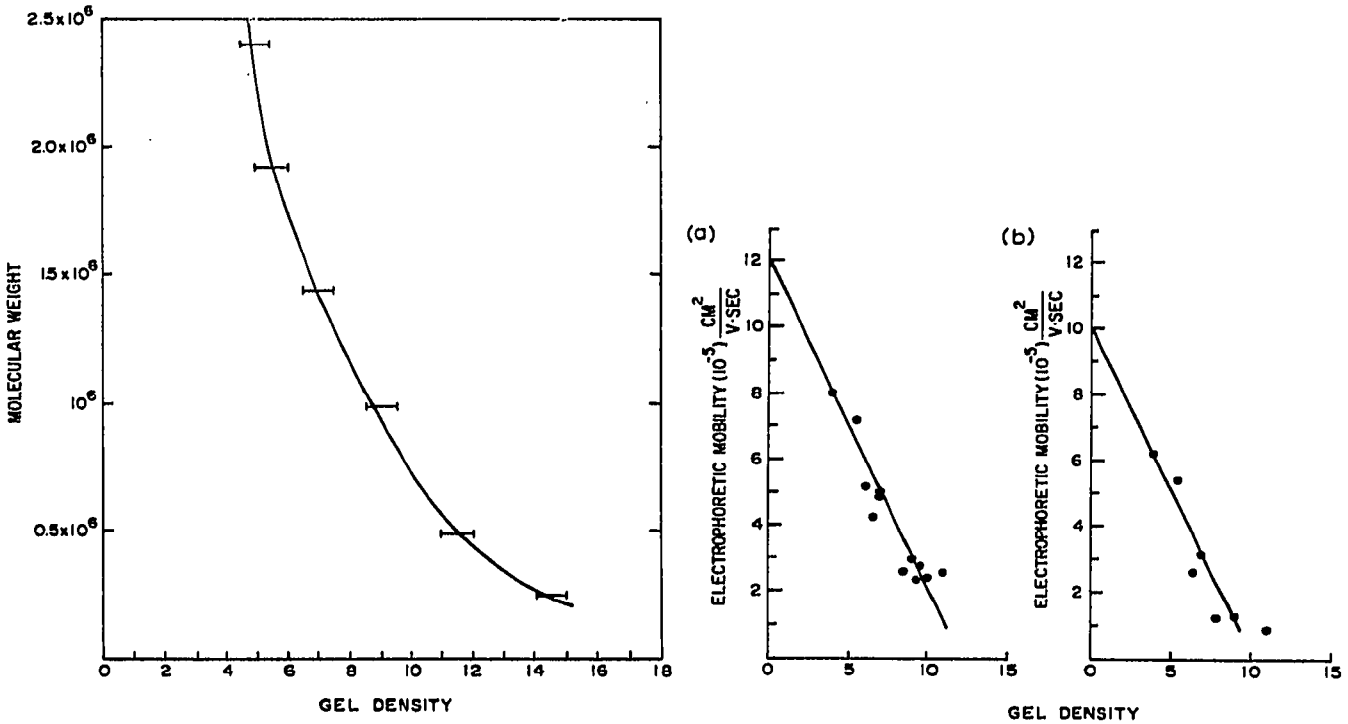

Fig. 1. The exclusion of protein standards from gels of varying density.

Fig. 2. The electrophoretic mobility of BSA at varying gel densities. (a), BSA monomer, zero intercept: $12.0 \cdot 10^{-5}$ cm²/V·sec; (b), BSA dimer, zero intercept: $10.1 \cdot 10^{-5}$ cm²/V·sec.

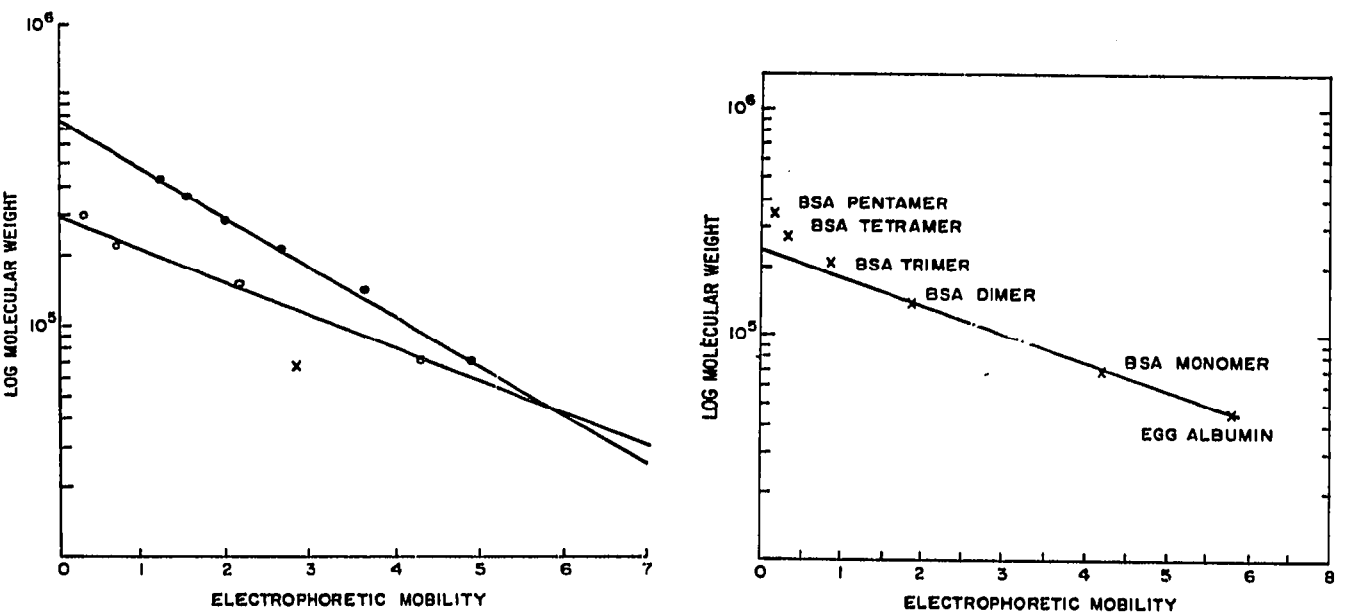

Fig. 3. Relation of molecular weight of BSA and derivatives to electrophoretic mobility in 5% gel. (●—●), native BSA; (○—○), succinylated BSA; × = succinylated reduced BSA.

Fig. 4. Relation of molecular weight to electrophoretic mobility in 9% gel.

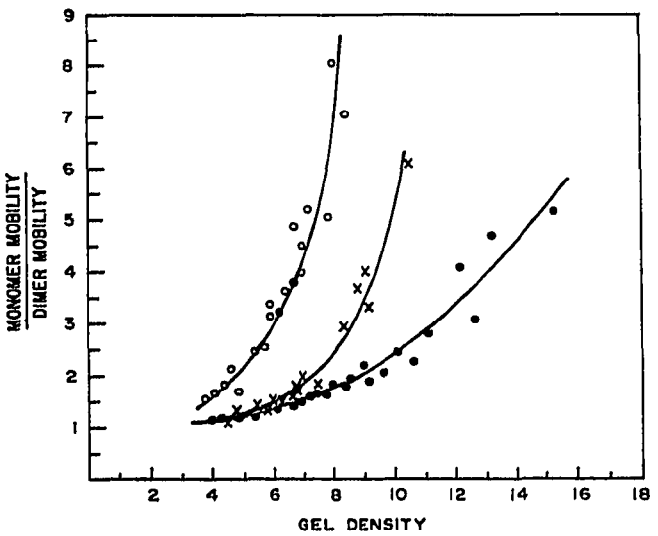

Fig. 5. Ratios of mobilities for monomer–dimer pairs at various gel densities. (○—○), urease monomer/urease dimer; (●—●), BSA monomer/BSA dimer; (×—×), α-urease/native urease.

density. The "pore" size is represented by protein molecular weight. The protein standards used were catalase, urease and the higher multimers of urease. They were subjected to electrophoresis through polyacrylamide gels ranging in density from 4–15% in increments of 0.125%. It was found that urease with a molecular weight of 480,000 was excluded by gels of 11–12%. In 11% gels the urease will enter the gel but the electrophoretic mobility is exceedingly slow. In 12% gels the urease remains at the origin.

In Fig. 2 are graphs of the electrophoretic mobility of BSA monomer and dimer as a function of gel concentration. The curves are approximately linear with indistinguishable slopes but different intercepts.

In Fig. 3 is a graph of the logarithm of the molecular weight of BSA multimers plotted against the electrophoretic mobility in a 5% gel. Mobility is expressed in arbitrary units. The sample of BSA utilized contained multimers up to hexamers.

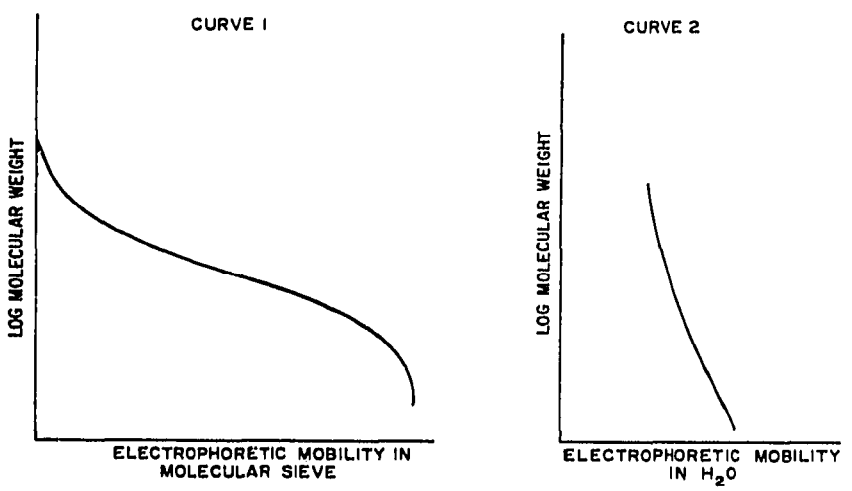

Fig. 6. A comparison of electrophoretic mobilities in gels and in water.

Included in Fig. 3 is a curve for succinylated BSA and BSA multimers in 5% gel. For both sets a linear relationship was obtained. If the same BSA sample was succinylated, and then reduced, only a single component was obtained. Clearly, succinylated BSA has a lower mobility than the native protein and the succinylated, reduced derivative moves even more slowly.

Fig. 4 is a similar plot but represents the course of electrophoresis in a 9% gel. In this denser gel there is a notable deviation from linearity for the higher multimers. In 7% gels there is a slight deviation from linearity for BSA pentamer and higher multimers. There was some indication that small proteins in dilute gels deviate from linearity in the opposite direction.

TABLE I

MIGRATION RATIOS FOR β-LACTOGLOBULIN A AND B

Gel density (%)	*Electrophoretic mobility*		*B/A*
	A	*B*	
6	8.55	7.93	0.929
9	8.25	7.55	0.916
11	8.30	7.73	0.931
12	6.30	5.75	0.922

When the ratios of the mobilities of a protein monomer–dimer pair were measured at various gel concentrations it was noted that characteristic curves were obtained as illustrated in Fig. 5.

In Table I similar data for β-lactoglobulin A and B are presented. The relative mobilities of these two forms are independent of gel densities. This indicates a difference in charge but similarity in size and shape.

DISCUSSION

The exclusion limit for polyacrylamide gels shown in Fig. 1 is useful for experimental design and is analogous to the void volume in gel chromatography. The limits indicated are approximate since the shape of the protein may affect the results. For example, the pentamer of BSA (350,000) cannot enter a 9% gel but urease (480,000) can penetrate the gel although its mobility is low. Presumably the pentamer is less compact than urease. However, the exclusion limit does yield a semi-quantitative estimate of molecular weight.

The electrophoretic mobilities of BSA monomer and dimer are a linear function of gel density. The curves deviate slightly from linearity with dense gels very near the exclusion limit. The intercept is governed by the size and shape of the protein and the gel density in which it has zero mobility. The slope of the line is determined largely by the charge on the protein.

The mobility of the dimer is approximately 80% that of the monomer in aqueous solution. By increasing the gel density from zero to 5% the mobility of the monomer becomes 70% that of the dimer and in a 7.5% gel the dimer mobility is 50% that of the monomer. Such considerations indicate that effective use of poly-

acrylamide gels as molecular sieves requires denser gels than normally used. In order to accentuate the difference in mobility between two similar proteins with a small difference in size it is necessary to use gels approaching the exclusion limit of the larger protein. The mobility of BSA dimer in a 10% gel is approximately 10% that in buffered water. This difference in mobility results in sharp bands and thus dense gels improve band sharpness as well as resolution.

By using a multimeric series of one protein the quantitative relationship between molecular weight and mobility can be explored directly without compensating for charge difference in standards. Succinylation of such a protein series produces a set of proteins with higher electrostatic charge and larger size.

When the same experiment is carried out in a denser gel the relationship may be no longer linear. The larger proteins migrate unexpectedly faster. This effect is even more noticeable for proteins whose size is close to the exclusion limit of the gel. In general there was little deviation from linearity if the protein had a molecular weight smaller than half the exclusion limit.

The general character of electrophoretic mobility is illustrated by curve 1 of Fig. 6. The curve is sigmoid but has a linear region in the middle. Since the frictional coefficient is proportional to $M^{-\frac{1}{3}}$ in aqueous solution the mobility of a monomer should be $2^{\frac{1}{3}}$ or 1.21 times that of the dimer. In Fig. 5 the monomer–dimer ratio for BSA approaches this value of 1.21 in 5% gels. This indicates once again the relative inefficiency of dilute gels as molecular sieves. In aqueous solution the electrophoretic mobility E is proportional to the charge C and frictional coefficient F, so that $E \sim CF$. However, in diffusion $F \sim 1/(M)^{\frac{1}{3}}$ and thus, $E \sim C/M^{\frac{1}{3}}$. Thus an 8-fold difference in mass produces only a 2-fold change in mobility. Curve 2 of Fig. 6 represents this situation. The curve rises steeply as does the right-hand portion of curve 1 because the mobility of small proteins in a dilute gel is little affected by the gel. Larger proteins are strongly influenced by the sieving action of the gel and their mobility is much less than that in aqueous solution. In electrophoresis experiments a 10% difference in charge produces a 10% difference in mobility. Due to the relation $1/M^{\frac{1}{3}}$ a 10% difference in mass produces a negligible effect on mobility. The molecular sieving effect of polyacrylamide gels makes the difference in mass as measurable as a difference in charge.

In this work it was found that, in a 5% gel, proteins with molecular weights between 50,000 and 500,000 tend to fall in the linear region of curve 1. In a 10% gel the upper limit for linearity is approximately 150,000 and may extend as low as 15,000 for the lower limit. In the ZWAAN technique one measures the mobility of a protein in a dense gel and divides by the mobility in a dilute gel. This is equivalent to dividing curve 1 by curve 2 in Fig. 6. Since curve 2 is approximately linear, as is the center portion of curve 1, the result is an approximately linear curve.

It has long been known that proteins could be graded in size by comparing relative mobilities in molecular sieve gels of increasing selectivity. SMITHIES[1] employed starch gels and later RAYMOND[2] used polyacrylamide gels. Nevertheless, this technique has not been widely used in spite of the fact that it is very simple and no compensation need be made for charge difference between proteins. Fig. 5 shows relative mobility ratios for three monomer–dimer protein sets. If only one experiment had been run the low mobility of the dimer might have been attributed to lack of charge instead of larger size. The same approach may be used to show that suspected genetic

variants have the same molecular weight. This is illustrated by the data in Table I for β-lactoglobulin A and B. The relative mobilities of these two components do not change with gel density and hence indicate similar size, but dissimilar charge. The data indicate that the B form has 92% as much charge as the A form. If the difference is due to a single ionizable group this indicates a charge of 13 for the A form and 12 for the B form.

CONCLUSION

Molecular weight determination by molecular sieve electrophoresis is a reliable procedure for the preliminary characterization of minor components.

Variation in charge density can be more reliably compensated for than can variation in shape but the results indicate that shape differences must be rather extreme to affect the results. The technique described is particularly useful for the study of genetic variants or homologous sets of proteins.

REFERENCES

1 O. SMITHIES, *Arch. Biochem. Biophys., Suppl.*, 1 (1962) 125.
2 S. RAYMOND AND M. NAKAMICHI, *Anal. Biochem.*, 7 (1964) 225.
3 W. P. TOMBS, *Anal. Biochem.*, 13 (1955) 121.
4 A. L. SHAPIRO, E. VINUELA AND J. W. MAIZEL, *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.
5 J. ZWAAN, *Anal. Biochem.*, 21 (1967) 155.
6 D. P. BLATTLER, *Ph. D. Thesis*, 1967; *Diss. Abstr. B*, 29, No. 1 (1968) 38.
7 J. B. SUMNER, *J. Biol. Chem.*, 69 (1926) 435.
8 A. L. DOUNCE, *J. Biol. Chem.*, 140 (1941) 306.
9 G. GORIN, M. F. BUTLER, I. M. KATYAL AND J. E. BUCKLEY, *Proc. Oklahoma Acad. Sci.*, 40 (1960) 62.
10 J. M. CREETH AND L. W. NICHOL, *Biochem. J.*, 77 (1960) 230.
11 D. P. BLATTLER, *Anal. Biochem.*, 27 (1969) 73.
12 M. CHERRY, *Ph. D. Thesis*, Yale University, 1964.
13 A. F. S. A. HABEEB, H. G. CASSIDY AND S. J. SINGER, *Biochem. Biophys. Acta*, 29 (1958) 587.
14 D. P. BLATTLER, C. C. CONTAXIS AND F. J. REITHEL, *Nature*, 216 (1967) 274.