Molecular Weight Determination of Protein-Dodecyl Sulfate Complexes by Gel Electrophoresis in a Discontinuous Buffer System

DAVID M. NEVILLE, JR.

From the Section on Physical Chemistry, Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, Maryland 20014

SUMMARY

This report describes methods and results obtained by combining the techniques of sodium dodecyl sulfate (SDS) gel electrophoresis and electrophoresis in discontinuous buffer systems. The SDS gel system utilizes a sulfoborate discontinuity which stacks and unstacks protein-SDS complexes over a range of 2,300 to 320,000 daltons, providing high resolution fractionation. The properties of protein-SDS complexes are investigated by calculating retardation coefficients and apparent free mobilities from Ferguson plots. Apparent free mobilities are approximately constant, establishing a linear relationship between the logarithm of the relative mobility and the retardation coefficient. The retardation coefficient is shown both empirically and theoretically to be a uniform function of molecular weight of protein-SDS complexes over specified ranges, providing a rationale for determining molecular weight from plots of the negative logarithm of relative mobility against molecular weight.

This report describes an electrophoretic system capable of stacking and fractionating protein-dodecyl sulfate complexes over a range of 2,300 to 320,000 daltons. The system combines the advantages of electrophoresing protein-SDS complexes pioneered by Shapiro, Vinuela, and Maize (1) and the advantages of achieving thin starting zones by use of discontinuous buffers discovered by Ornstein (2) and Davis (3). This SDS discontinuous system was developed to fractionate plasma membrane proteins solubilized in SDS and does provide high resolution patterns of solubilized membranes, resolving over 40 discrete bands (4, 5).

In the course of accumulating data with this SDS system, it became apparent that the usual type of calibration curve used in SDS gel electrophoresis was only approximately linear and that the actual curves of log molecular weight (M) versus relative mobility (RF) were sigmoidal in nature. It also became apparent that certain protein markers could not be fitted on linear

log M versus RF plots unless 25% deviations were tolerated. It was not apparent whether this was due to the alkaline nature of the discontinuous system, the lower ionic strength, or the particular proteins used. In order to investigate the sources of these deviations, it became necessary to explore the theoretical foundations of determining molecular weights from relative mobilities in gels. This subject had been investigated for proteins, but at the time of this study only empirical correlations of molecular weight and relative mobility had been reported for protein-SDS complexes. Recently, however, Chrambach and Rodbard (6, 7) have outlined a theoretical treatment for this subject which is similar to the one presented here.

In this report I explore the relationship between relative mobility and molecular weight of protein-SDS complexes by calculating retardation coefficients and free mobilities from gels of differing acrylamide concentrations. The finding that protein-SDS complexes have nearly identical free mobilities provides a theoretical justification for calculating molecular weights from relative mobility values obtained at a single gel concentration. The assumptions behind these calculations illustrate the sources of error inherent in the method, and ways of minimizing these errors are suggested.

MATERIALS AND METHODS

The principle used in obtaining a disc system to operate in SDS was to find a system capable of stacking and unstacking charged, highly mobile polymers. After trying several systems designed for nucleic acids, we discovered that borate-sulfate buffers gave excellent resolution. While attempting to optimize this system, we discovered that this system was one of the 4269 multiphasic buffer systems calculated from theory by Jovin, Dante, and Chrambach (8). The recipe given below comes from their computer output. The only modification consists of the addition of 0.1% SDS to the upper reservoir buffer. Upper reservoir buffer, pH 8.64, 0.04 M borate acid-0.04 M Tris-0.1% SDS; upper gel buffer, pH 6.1, 0.0267 M HSO₄-0.0541 M Tris (running pH 8.64); lower gel buffers, pH 5.77, 0.0954 M HC1-0.0954 M Tris, (running pH 8.64); pH 8.47, 0.0494 M HCI-0.1716 M Tris (running pH 9.05); pH 9.18, 0.0308 M HCI-0.4244 M Tris (running pH 9.50); pH 9.81, 0.0237 M HC1-1.3147 M Tris (running pH 10.01); and lower reservoir buffer, same as lower gel buffer.

The gels are described by the notation of Hjerten (9), in which the first numeral (T) denotes the total weight of monomer
(acylamide plus N, N'-methylenebisacylamide) per 100 ml of solvent, and the second numeral (O) denotes the amount of N, N'-
}

tetramethylenediamine and 0.05% ammonium persulfate at 15°. Several methods insure flat reproducible surfaces. (a) N, N, N-tetramethylenediamine and ammonium per-
sulfate are made up fresh daily in aqueous solutions and are added to
concentrated acrylamide and buffer stock solutions. (b) The
amounts of N, N, N-tetramethylenediamine and ammonium persulfate are the minimal amounts required to produce
polymerization at 10 min. If either catalyst is present in ex-
cess, polymerization time will be increased and distortion of the
surface will occur. (c) Isobutyl alcohol (30 µl) is layered over the
lower gel solution after filling tubes and is removed after polymer-
ization prior to casting the upper gel. (d) If gels 10 cm or
longer are cast in narrow tubes (10 x 0.5 cm), contraction of the
surface will occur. (e) Isobutyl alcohol (30 µl) is layered over the
upper gel solution after filling tubes and is removed after polymer-
ization prior to casting the upper gel.2 (f) If gels 10 cm or
longer are cast in narrow tubes (10 x 0.5 cm), contraction of the
volume on polymerization can cause a downward curvature of the
upper surface. Substituting thin vinyl sheeting tied with dental
floss or rubber bands for rubber stoppers allows the curvature
to take place at the tube bottom thus minimizing the problem.

Gels 5 mm in diameter were run at 1.5 ma per tube at 25° in a
jacketed reservoir with circulation of buffer around the gels by
means of a magnetically driven stirring bar in the lower reservoir.
With large samples (0.2 to 0.4 ml), current was reduced to 0.5
to 0.1 ma until the sample entered the upper gel.

Staining or destaining was done with Coomassie blue as de-
scribed by Weber and Osborn (10), except that staining time was
increased to 15 hours and the dye concentration was reduced to
0.007% to 0.07%.

Relative mobilities were calculated relative to the borate-sul-
fate front which stacked the marker dye bromphenol blue (Ca-
malco, Rockville, Maryland) or a red impurity of high mobility
in the high pH or high gel concentration runs. Dye fronts were
marked by insertion of a section of stainless steel surgical wire.

Proteins used as markers are listed in Table 1 along with their
sources and references for molecular weights. Individual pro-
tein concentrations generally ranged between 0.1 and 5 µg per
sample. When available, chromatographically purified proteins
were purchased. Proteins, except as noted, were dissolved in
0.05 M Na₂CO₃ and exposed to 8 mg of SDS per mg of protein for
1 min before the addition of 10% by volume mercaptoethanol.
They were then dialyzed against upper gel buffer containing
0.1% SDS, 0.05% dithiothreitol, 2% sucrose, and a trace of
bromphenol blue. When the dimers of ß-globulin and ß-galac-
tosidase were desired, these proteins were dissolved in 0.1% SDS
without reduction or heating.

Experiments designed for the calculation of retardation coeffi-
cients utilized a constant ratio of bisacrylamide to acrylamide of
1:90.

RESULTS

Log M Versus Rₚ Plots—The sulfate-borate system stacks pro-
tein-SDS complexes over a molecular weight range of 2,300 to
320,000, providing very sharp bands even when sample column
heights of 2 cm are used. Under conditions of these experiments,
20 ng of protein in the 40,000 to 100,000 molecular weight range
could be resolved by the Coomassie blue stain.

* A. Chambach, personal communication.
FIG. 1. A, molecular weight \((M)\) on a semilog scale is plotted versus relative mobility \((R_F)\) for a variety of protein-SDS complexes subjected to electrophoresis at pH 9.5 on gel \((11.1 \times 0.9)\). In the upper molecular weight region the curve is hyperbolic while in the lower regions the scatter obscures the nature of the relationship. In B, theoretical curves are constructed for \(R_F\) in Fig. 2. This type of plot, first described by Ferguson (16) for starch gels, has been shown by Hedrick and Smith (17) to adequately describe the behavior of 17 globular proteins on acrylamide gels varying in \(M\) from 45,000 to 500,000.

The protein-SDS complexes display two properties not seen with proteins. Complexes of \(M\) greater than 60,000 do not show a linear relationship between \(\log R_F\) and gel concentration. In addition, the molecular weight at which nonlinearity appears is dependent on gel concentration, high gel concentrations augmenting the effect.

The most interesting feature on the Ferguson plot is the fact that the values for the \(Y\) intercept are nearly identical for the 10 different complexes. The \(Y\) intercept value represents the relative mobility at zero gel concentration, and for proteins carrying different charges, different free mobilities are observed (17). Although it has been previously shown that proteins in SDS bind a constant amount of SDS per unit weight of protein (18, 19), this fact alone would not lead to free mobilities independent of \(M\). In order to have free mobilities independent of \(M\), the ratio of the effective charge to frictional coefficient must be independent of \(M\). In other words, the complex must behave under free electrophoresis as a free draining structure so that the mobility of a large molecule is identical with the mobility of a segment or smaller molecule (20). It is interesting to note that the free electrophoretic mobility of DNA, both native and denatured, is independent of \(M\) between one-quarter million and 130 million (20).

Although the \(Y\) intercepts are nearly identical in Fig. 2, the variations are larger than that due to measuring error \((0.02 R_F\) for \(R_F = 0.5\)). In Fig. 3, the slope of the line connecting points \((\log R_F\) and \(T)\) for identical proteins (retardation coefficient) is plotted against \(\log R_F\) to determine whether any trend with molecular weight can be detected. (Plots are done only in regions where \(\log R_F\) versus \(T\) plots are linear.) There is no apparent trend with molecular weight. However, it is interesting that the points for DNase and hemoglobin show a significant deviation from the line. These deviations must be ascribed to an apparent free mobility different from the average free mobility.
The logarithm of the relative mobility for 10 protein-SDS complexes is plotted versus the gel monomer concentration and the plots are extrapolated to $T = 0$. All of the complexes have a nearly identical free mobility. Note that the highest molecular weight complexes show deviations from linear plots at high $T$. Numbers at right refer to protein code in Table I.

**Fig. 2.**

Values of the retardation coefficient ($K_R$) computed from the slopes in Fig. 2 are plotted versus log $R_F$ at four concentrations of $T$ (numbers at right). Points are given for seven proteins (code next to circles). Points 10 (hemoglobin) and 8 (DNase) show significant deviations from the lines. The other points fall on a line giving a linear relationship between $K_R$ and log $R_F$.

It is possible that certain complexes exhibit more complicated interactions with the gel, such as adsorption, resulting in non-linear Ferguson plots as the gel concentration approaches zero. This situation would lead to an apparent free mobility different from the average free mobility when all measurements are made at high gel concentrations.

**Fig. 3.**

Effects of Varying pH—In Fig. 4, the relationship between $-\log R_F$ and $M$ for gels (11.1 x 0.9) at varying pH are shown. Increasing the pH results in ionizing more trailing ion and increasing the apparent velocity of the stack (2, 8). Consequently, some proteins traveling within the stack at low pH are excluded from the stack at high pH. In a gel (11.1 x 0.9) at pH 8.5 (the running pH of the stacking gel), subunits having $M < 50,000$ are in the stack, although at pH 10, $M$ of 5,000 and above are excluded from the stack.

**Discussion**

The results of this study of SDS gel electrophoresis show that the empirical relationship observed between molecular weight and relative mobility in neutral continuous systems is also observed in alkaline discontinuous systems. In addition, these results show that the apparent free relative mobilities ($Y_0$) of protein-SDS complexes are nearly constant. When data obtained with discontinuous systems are plotted in the usual manner, log $M$ versus $R_F$, considerable scatter is observed in the low molecular weight regions. In an attempt to understand the causes of this scatter, it became necessary to investigate the theoretical foundation for obtaining molecular weights from a relative mobility value on a single gel. Previously, it had been shown that molecular weights of proteins could be estimated if relative mobilities were obtained at more than one gel concentration by use of the Ferguson equation (16).

$$\log R_F = -K_R T + \log Y_0$$  

(1)

where $R_F$ is the relative mobility at gel concentration $T$, $Y_0$ is the relative mobility at zero gel concentration (the apparent free relative mobility), and $K_R$ is the retardation coefficient and is a function of molecular size and the percentage of cross-linking. If the percentage of cross-linking is held constant, $K_R$ can be determined from values of $R_F$ taken at various values of $T$. For 17 globular proteins, $K_R$ is directly proportional to $M$ (17).

These results, which show that log $Y_0$ is a constant, establish a linear relationship between log $R_F$ and $K_R$ at any given $T$ (Fig. 3). Once the average value of log $Y_0$ has been determined for a given buffer system and percentage of cross-linking, the retardation coefficient can be calculated from a single log $R_F$ value at
average free mobility. Sider a gel to be made up of spaces or pores and that for any molecule of effective radius, the type of distribution of spaces.

Since \( T \) is proportional to \( k_2 \) (24), this term may be eliminated by division.

and where \( R_e > r \)

Reynolds and Tanford (18) and Fish, Reynolds, and Tanford (19) have shown that the hydrodynamic properties of protein-SDS complexes determined from viscometric and gel filtration data are a unique function of molecular chain length. Their data are expressed in terms of the Stokes radius, \( R_s \).

By assuming that \( R_e \) can be substituted for \( R_s \)

Equation 11 shows that plots of \( \log K_R \) or \( \log K_R' \) against \( \log M \) will be linear with a slope \( ab \) proportional to the relation between \( R_e \) and \( M \). Deviations from linearity will occur when the assumptions underlying Equation 11 break down, for example, when the condition \( K_R > r \) is not satisfied. When \( a = 2 \), deviations in \( ab \) greater than 10% do not occur until \( R_e \leq 15 \) A or \( R_e \leq 25 \) A for \( a = 1 \). The decrease in slope in Figs. 5 and 6 below \( M \) of 15,000 could be due to the influence of the \( r \) term. Equation 11 provides a way of relating relative mobility data to molecular weight by means of a plot which is linear under a set of known assumptions.

When the values of Fish et al. (19) for \( k_4 \) and \( b \) are used with our \( K_R' \) data in Equation 11, we calculate the value of \( a \) as 1. As mentioned previously, the preferred theoretical value is 2.

If it is assumed that \( a = 2 \), then \( b = 0.34 \) and \( R_e \) varies as \( M^{0.34} \) rather than as \( M^{0.71} \), as determined by Reynolds and Tanford (18). This would mean that the effective radius in gel electrophoresis is less than the effective radius in gel filtration. When the data of Hedrick and Smith (17) for globular proteins \( (R_e \) varies as \( M^{0.7} ) \) are plotted according to Equation 11, \( ab \) = 0.3, and again assuming \( a = 2 \), the effective radius is less than the Stokes radius.

Although the values for \( a \) and \( b \) in Equation 11 are in doubt for gel electrophoresis, the equation is useful in that alterations in the relationship of \( R_e \) on \( M \) will be apparent by a change in slope. Such a change occurs below 15,000 daltons and above 70,000 daltons. The decrease at 15,000 daltons was also noted for protein-SDS complexes in gel filtration (19) and may be due to the change in geometry from a prolate ellipsoid to a sphere in this region. The effect of the \( r \) term will also tend to decrease the slope in this region. Above 70,000 (Fig. 7), the slope decreases, indicating a decrease in effective radius. Fisher and Dingman (25) have presented data on electrophoretic behavior of nucleic acids in gels by means of log \( K_R \) versus log \( M \) plots. Rod-shaped nucleic acids\(^3\) show a smaller slope \( ab \) than random coil nucleic acids of the same \( M \). In addition, the \( R_e \) of rod-shaped nucleic acids of the same \( M \).
shaped nucleic acids increased with increasing voltage gradient. These authors speculate that the highly asymmetrical rod-shaped molecules may be capable of orienting in a manner which minimizes their frictional resistance to the gel (25).

If \( K_E \) varied as \( M^{1.8} \) instead of \( M^{0.68} \) as found, \( k_R M \) (see Equation 11) could be substituted directly into Equation 1, giving

\[
-\log R_F \approx k_R M - \log Y_0
\]  

(12)

If within the region of constant dependency of \( K_E' \) on \( M \) any two markers of known \( M \) have their \( R_F \) determined, the constants \( k_R \) and \( \log Y_0 \) are fixed, and \( M \) can be determined directly from values of \(-\log R_F \) for any unknown. This type of plot is shown in Fig. 7. The standard error is \( \pm 3000 \) M. Comparing Figs. 5 and 7 shows that it is not necessary to have the exact relationship of \( K_E' \) on \( M \) for estimating \( M \). Fig. 5 shows chymotrypsinogen and DNase to be anomalous points. However, this fact is not as obvious from the plot in Fig. 7. The commonly used plot, log \( M \) versus \( R_F \), is not as useful since it is sigmoidal in shape when there is a constant dependency of \( K_E \) on \( M \) and when \( Y_0 \) is a constant (see Fig. 1B).

From the foregoing discussion it can be seen that the assumptions involved when \( M \) is calculated from a value of \( R_F \) in SDS gel electrophoresis are (a) a constant value of \( Y_0 \) for marker and unknown protein-SDS complexes, and (b) a constant dependency of \( R_F \) on \( M \) within some range of \( M \). These assumptions are imposed by the use of the marker proteins used to form the calibration line when \( M \) is plotted as some function of \( R_F \). If the markers used have significantly different values of \( Y_0 \) than the unknown protein-SDS complexes, errors in \( M \) will result. Both DNase and hemoglobin are examples of this type of error. Each shows deviations in plots of log \( R_F \) against \( K_E' \) and deviations in log \( R_F \) versus \( M \) plots. That the latter deviations are due to the incorrect estimate of \( Y_0 \) is seen by the fact that the deviations are not present when \( K_E \) rather than \( K_E' \) is plotted against \( M \) (see Table II). Therefore, the Ferguson plot provides a means of detecting this type of error. Similarly, if the markers used to form the calibration line have a different dependency of \( M \) on \( R_F \) than the unknowns, errors will result. Therefore, extrapolations into ranges not covered by markers should be avoided, and multiple markers should be used in transition regions. The deviation of chymotrypsinogen in \(-\log R_F \) versus \( M \) plots appears to reflect the second type of error. The deviation in \( M \) is \( +15\% \) in a \( K_E \) versus \( M \) plot and \( +17\% \) in a \(-\log R_F \) versus \( M \) plot. Cytochrome c and chymotrypsin C chain lie in a region of altered dependency of \( R_F \) on \( M \). An explanation for the scatter of the data is thus provided. Whether or not the conditions of the alkaline buffer system or specific effects of the borate ion aggravate these problems as compared to neutral systems remains to be seen. Gel electrophoresis of protein-SDS complexes with the use of other discontinuous buffer systems is being investigated and those studies may answer some of these questions (6, 26).

**REFERENCES**

1. Shapiro, A. J., Viskup, E., and Maizel, J. V., Jr., Dio- 
chem. Biophys. Res. Commun., 28, 815 (1970).
2. Ornstein, L., Ann. N. Y. Acad. Sci., 121, 321 (1964).
3. Davis, B. J., Ann. N. Y. Acad. Sci., 121, 404 (1964).
4. Neville, D. M., Jr., and Glossmann, H., J. Biol. Chem., 246, 6335 (1971).
5. Glossmann, H., and Neville, D. M., Jr., J. Biol. Chem., 246, 6339 (1971).
6. Chrambach, A., and Rodbard, D., Science, 172, 440 (1971).
7. Rodbard, D., and Chrambach, A., Anal. Biochem., 40, 95 (1971).
8. Jovin, T. K., Dante, M. L., and Chrambach, A., Multiphase buffer systems output, Federal Scientific and Technical Information, United States Department of Commerce, Springfield, Virginia, 1971.
9. Hjerten, S., Arch. Biochem. Biophys., Suppl., 1, 147 (1962).
10. Weber, K., and Osborn, M., J. Biol. Chem., 244, 4406 (1969).
11. Dunker, A. K., and Rueckert, R. R., J. Biol. Chem., 244, 5074 (1969).
12. Klotz, I. M., and Darnall, D. W., Science, 166, 126 (1969).
13. Gazith, J., Himmelheber, S., and Harrington, W. F., J. Biol. Chem., 245, 15 (1970).
14. Lindberg, U., Biochemistry, 6, 335 (1967).
15. Dayoff, M. O., and Eck, R. V., Atlas of protein sequence and structure, National Biomedical Research Foundation, Silver Spring, Maryland, 1967-1968.
16. Ferguson, K. A., Metab. Clin. Exp., 13, 965 (1964).
17. Hedrick, J. L., and Smith, A. J., Arch. Biochem. Biophys., 126, 155 (1968).
18. Reynolds, J. A., and Tanford, C., J. Biol. Chem., 245, 5161 (1970).
19. Fish, W. W., Reynolds, J. A., and Tanford, C., J. Biol. Chem., 245, 5166 (1970).
20. Olivera, B. M., Baine, P., and Davidson, N., Biopolymers, 2, 245 (1964).
21. Ogston, A. G., Faraday Soc. Trans., 54, 1754 (1958).
22. Morris, C. J. O. R., in H. Peeters (Editor), Proteins of the biological fluids, 14th Colloquium, American Elsevier Publishing Company, Inc., New York, 1967, p. 543.
23. Rodbard, D., and Chrambach, A., Proc. Nat. Acad. Sci. U. S. A., 65, 970 (1970).
24. Fawcett, J. S., and Morris, C. J. O. R., Separ. Sci., 1, 9 (1966).
25. Fisher, M. P., and Dinman, C. W., Biochemistry, 10, 1806 (1971).
26. Laemmli, U. K., Nature, 227, 680 (1970).