Observations on Molecular Weight Determinations on Polyacrylamide Gel*

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SUMMARY

1. A simple internal calibration technique was used to construct accurate molecular weight-mobility profiles on polyacrylamide gels with well characterized proteins, and the existence of a "critical point" was observed for small polypeptides. The useful size range limits were determined for several gel concentrations. Apparent molecular weights generally fell within 5 to 6% of literature values, although a few "anomalous" proteins, notably ribonuclease, considerably exceeded this margin of error.

2. The effects of intrinsic molecular charge and conformation on electrophoretic behavior in the presence of sodium dodecyl sulfate, evaluated by studies on a set of model proteins, were found to be small.

3. The results are in accord with a model in which the protein somehow organizes the sodium dodecyl sulfate anions into a micellar complex of definite size. Although the stoichiometry of the complex is apparently governed not only by the size of the molecule, but also by its state of foldedness, the interplay of anion binding and frictional resistance to passage through the gel is such as to produce a relatively constant log size to mobility ratio.

4. The apparent molecular weights of the five polypeptide chains of Mouse-Elberfeld virus were determined.

In 1967, Shapiro, Vinuela, and Maizel (1) pointed out that the molecular sizes of polypeptides could be estimated from the relative electrophoretic mobilities of their sodium dodecyl sulfate complexes on polyacrylamide gels. The method is rapid and versatile, and requires very little sample (often less than 5 μg of each component). In addition, the tools required are economical and demand relatively little laboratory space or specialized training for application. In spite of the apparent utility of the method, there has been relatively little published information about its limits of reliability. A need for caution was suggested by the above authors, who pointed out that two well known proteins, lysozyme and ribonuclease, exhibited "anomalous" behavior in this system. We report here the results of our studies on over 20 proteins, including the above anomalous proteins and a number of viral proteins. The origin of the anomalous behavior of lysozyme was identified, and rough limits of accuracy were estimated for the method. In addition, some ways for detecting anomalous behavior by unknown proteins are suggested.

MATERIALS AND METHODS

Proteins—ME virus was prepared as described previously (2). Carboxypeptidase A, chymotrypsinogen A, lysozyme, and ovalbumin were obtained from Worthington; cytochrome c, ribonuclease A, myoglobin, 7 S γ-globulin, chymotrypsin, trypsin, and pepsin from Mann; β-lactoglobulin, insulin, and bovine serum albumin from Pentex, Inc.; R17-virus, tobacco mosaic virus, and bromegrass mosaic virus were gifts from Paul Kaeberg.

The BSA preparation which had been recrystallized in the laboratory of Professor R. M. Bock in 1964 contained at least five electrophoretically distinct components which evidently represent the monomer and its polymeric forms (3, 4). The mobility of the fastest and most abundant component was that expected for the BSA monomer (mol wt 66,000). The slower components, which travelled as expected for the successive (i.e. dimer, trimer, etc.) oligomers of BSA, largely disappeared from the preparation after treatment with mercaptoethanol. Chymotrypsin B and C chains were obtained from chymotrypsin by reduction and denaturation according to the usual procedures of sample preparation (see below). The A chain (mol wt 1200 daltons (5)), which is theoretically also produced by this treatment, was not detected, evidently because such small polypeptides stain poorly with the procedure used.

Preparation of Samples for Electrophoresis—The proteins, except as noted below, were dissolved at a concentration of about 2 mg per ml in 1% 2-mercaptoethanol, v/v, 4 M urea, and about 1% SDS, and then incubated at 45° for 30 to 60 min. Purified whole virus treated in this way was used without removing the RNA.

In certain cases (i.e. insulin, γ-globulin, and BSA) where the

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The abbreviations used are: BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.
dissociation or reshuffling of disulfide-linked chains was to be
prevented, 2-mercaptoethanol was replaced with 0.002 M iodo-
acetamide. The latter reagent was intended to block sulfhydryl
groups, adventitious or otherwise, which catalyze disulfide inter-
change reactions (6).
Mixed disulfide derivatives of proteins were prepared by incu-
ation with 0.1 M solutions of the appropriate disulfide in the
presence of catalytic amounts of thiol by a modification of the
procedure of Smithies (6). Full details will be described in a
forthcoming communication. Procedures for the preparation
and gel electrophoresis of lysozyme and its mixed disulfide
derivatives in 8 M urea have been described by Rueckert and
Bock.
Electrophoresis was carried out with mixtures prepared by
combining equal volumes of the protein solutions.
Polyacrylamide Gels—Polyacrylamide gels (5, 10, and 15%
fractional total concentrations) were prepared by mixing the
appropriate acrylamide solutions with 0.05 volume of 1% ammonium
persulfate; columns (6 to 7 cm) were cast in soft glass tubing
(inside diameter 6 mm), and the monomer was carefully overlaid
with about 0.3 ml of water. For all three concentrations, the
acrylamide to methylene-bisacrylamide ratio was 30:1 (w/w).
Besides acrylamide, the solutions contained 0.1% SDS, 0.1
m-sodium phosphate, pH 7.2, and 0.10%, v/v, N,N,N',N'-tetra-
methylmethylenediamine (7).
After gelation (10 to 15 min at room temperature) a styrofoam
partition (about 6 mm X 10 mm x 1 mm) was wedged
snugly into each tube against the top of the gel column. Each
column was then overlaid with 0.1 M sodium phosphate, pH 7.2.
Columns prepared in this manner are referred to as “split gels.”
Electrophoresis—The samples, in dense solutions (4 M urea),
were layered under the electrode buffer. Typically, 5 to 30 µl,
containing 5 to 15 µg of each protein, were applied to either side
of the partition. Electrophoresis was carried out at 7 to 9 ma
per tube (roughly 3 volts per cm) for 2 to 3 hours (5% polyacry-
amide gels), 4 to 5 hours (10% gels), or 6 to 10 hours (15% gels).
Staining—After electrophoresis, the SDS was leached out and
the proteins were precipitated by soaking the gels for 18 to 24
hours in 0.02% Coomassie brilliant blue R250, freshly diluted in
12.5% trichloracetic acid (8). Next the gels were immersed for 4 to 6 hours in 0.02% Coomassie brilli-
ant blue R250, freshly diluted in 12.5% trichloracetic acid (8).
After decanting the dye, 10% trichloracetic acid
was added, and
the gels were allowed to stand in faintly blue solutions which
further intensified the staining. Gels of 5% polyacrylamide
usually could be photographed within an hour, but 10%, and
especially 15%, polyacrylamide gels required 48 hours or longer
to develop sufficient contrast for photography.
Measurement of Relative Mobilities—Stained gels were photo-
graphed by transmitted light with a Polaroid MP3 camera and
an orange filter as described by Chernbach et al. (8). From
the photographs, migration distances were measured, and the
conversion to relative mobilities was accomplished by dividing each
migration distance by that of chymotrypsinogen. This proce-
dure of using a reference protein in each gel automatically cor-
certs mobility measurements for changes in gel dimensions due
to swelling during the staining operation.

RESULTS AND DISCUSSION
Method—To estimate the molecular weight of an unknown
polypeptide, its mobility is compared with that of polypeptides
of known size (markers). This may be done on different gels
run in parallel or by coelectrophoresis of markers and unknowns
in the same gel. The latter technique minimizes random errors
caused by asymmetries in the apparatus, small bubbles accumu-
ating at the base of the columns, or slight differences in polyacryl-
amide composition at the tops of the columns, etc., and therefore
appreciably increases the precision with which such comparisons
can be made. The problem of band overlap between markers
and unknowns of similar size is circumvented by using a di-
vided sample application technique (9). An example of an
electrophoretogram prepared in this way is shown in Fig. 1.

Molecular Weight-Mobility Profile and Gel Composition—
Electrophoretograms were prepared on 5, 10, and 15% poly-
acrylamide gels, and the relative mobility of each polypeptide was
plotted against the logarithm of its molecular weight (Fig. 2).
In each case such a plot yielded a straight line over a molecular
weight range which was characteristic of the gel composition.
Thus a linear plot was obtained over a molecular weight range
of 60,000 to 10,000 daltons for an 15% gel (Fig. 2C), 100,000
to 10,000 daltons for 10% gel (Fig. 2B), and 350,000 to 20,000
daltons for a 5% gel (Fig. 2A). The last result is in good agree-
ment with the report of Shapiro et al. (1), who were the first to
note that migration is inversely related to the logarithm of
polypeptide molecular weight in the SDS system. These workers
also reported that ribonuclease A and lysozyme deviated from
this line on 5% gels, but offered no explanation for this anomalous
behavior.

As seen in Fig. 2, the molecular weight-mobility plots undergo
a previously unreported inflection when the molecular weight
falls below a critical size. For 5% gels the critical size is about
20,000 daltons.
Fig. 2. Plot of the logarithm of the molecular weights of a series of proteins versus their relative mobilities on (A) 5%, (B) 10%, and (C) 15% polyacrylamide gels containing 0.1% SDS. Each protein is identified by its number in Table I. The data for each curve were obtained according to the procedure illustrated in Fig. 1 as outlined under "Materials and Methods." The ordinate intercepts obtained by extrapolating to zero mobility are characteristic of gel composition and therefore may be useful for comparing results from different laboratories. Such extrapolations, however, probably do not accurately describe the behavior of polypeptides larger than the ones presented, especially since the slow moving large polypeptides spend an appreciable portion of their time passing through an atypically porous zone, which is formed at the water-polymer interface during gelcasting.

Although no attempt was made to accurately determine the position of this inflection point on the 10 or 15% gels, the mobility of insulin (mol wt 6,000) suggests that the critical size is lowered to between 6,000 and 10,000 daltons at these higher gel concentrations. This inflection to steeper slope, which probably reflects less effective sieving of small molecules, accounts for the reported anomalous behavior of lysozyme on 5% polyacrylamide gels, but fails to account for that of ribonuclease A, which moves more slowly than expected, even after taking into account the change in slope. The apparent linearity below 20,000 daltons on the 5% polyacrylamide gels permits extension of their useful range for molecular weight determinations into the 20,000 to 5,000 dalton region. The steepness of the slope, however, necessitates rather accurate determinations of relative mobility; to this end, long gel columns are useful.

For polypeptides weighing between 50,000 and 10,000 daltons, 10% gels were convenient; they produced sharper and more highly resolved bands than 5% gels at these higher gel concentrations. This inflection to steeper slope, which probably reflects less effective sieving of small molecules, accounts for the reported anomalous behavior of lysozyme on 5% polyacrylamide gels, but fails to account for that of ribonuclease A, which moves more slowly than expected, even after taking into account the change in slope. The apparent linearity below 20,000 daltons on the 5% polyacrylamide gels permits extension of their useful range for molecular weight determinations into the 20,000 to 5,000 dalton region. The steepness of the slope, however, necessitates rather accurate determinations of relative mobility; to this end, long gel columns are useful.

Compiled in Table I are the molecular weight values of the polypeptides used in this study and their apparent molecular weights determined from the plots of Fig. 2. In general, the molecular weights found were independent of the gel composition and agreed within 5% of the literature values. With the notable exception of ribonuclease A, the maximum deviation between literature- and SDS-determined molecular weights was about 11%.

Apparent molecular weights were readily determined with a precision of 2%; it is evident from Table I, however, that discrepancies in the determined values frequently exceeded this uncertainty.

Such discrepancies, therefore, arise not from random experimental errors, but rather from small but significant variations in the log of molecular weight to mobility ratios of different proteins. Such variations might reasonably be due to inherent differences in the conformation or intrinsic charge of different proteins, or to differences in the weight proportion of detergent anions bound to each type of polypeptide.

Effect of Intrinsic Charge and Chain Unfolding—To establish quantitatively the effect of differences in intrinsic charge (i.e. the net charge contributed by the ionizing groups of the polypeptide chain as distinguished from the net charge of the protein-SDS complex) on the mobility of a protein in the SDS system, it would be advantageous to study polypeptide chains differing, insofar as possible, only in charge but not in amino acid sequence.

A model set of proteins closely approximating this requirement was prepared by substituting lysozyme with appropriately charged side chains by the method of disulfide interchange (6). This procedure, which involves reacting the disulfide groups of the protein (I) with a large molar excess of low molecular weight disulfides (II), yields a mixed disulfide product (III) which is stable in the absence of thiol anion. Three homologous mixed disulfide derivatives bearing negative (R is CH₃CH₂CO₂⁻), zero (R is CH₃CH₂OH), or positive (R is CH₃CH₂NH₃⁺) charge groups were examined (Fig. 3A). When fully substituted, the derivatives bear eight additional negative, zero, or positive groups, respectively, in each chain. All three were insoluble in ordinary buffers, but dissolved readily in the presence of 8 M urea.
The electrophoretic mobility of these derivatives on polyacrylamide gels containing 8 M urea (no SDS) was in the order expected from the charge of their added R groups (Fig. 3A). Thus, the most positively charged β-aminoethyl lysozyme derivative moved more rapidly toward the anode than did the homologous β-hydroxyethyl lysozyme derivative bearing an uncharged substituent. The most negatively charged β-carboxyethyl lysozyme derivative, in turn, moved only very slowly toward the anode. Unexpectedly, all three derivatives moved markedly slower than unreacted lysozyme.

The decrease in electrophoretic mobility of lysozyme associated with cleavage and substitution of its disulfide bonds is much too large to be accounted for by the 4 to 5% increase in molecular weight expected from addition of eight R groups to the lysozyme molecule. However, lysozyme is unusually stable and resistant to unfolding even in 8 M urea (14). Viscometric studies in this laboratory have confirmed this result and indicated, in addition, that the molecule becomes extensively unfolded in 8 M urea, as its disulfide bonds are cleaved during the interchange reaction.

Thus, it seems likely that the decreased mobility of the derivatives reflects uncoiling and extension of the lysozyme molecule, as its intrachain disulfide bonds are substituted and cleaved. The unfolded chains, because of their increased Stokes radii, would be expected to encounter greater frictional resistance to passage through the polyacrylamide matrix; furthermore, it is not unlikely that unfolding may expose previously buried ionizing groups, thereby changing the net charge on the molecule. The large effect of such conformational changes on electrophoretic mobility in a gel is most clearly seen by comparing lysozyme with its hydroxyethyl derivative to which no additional charge groups have been added. In this particular example, the effect exceeded.
equivalent weights of detergent anions, and that (b) the 12% difference in mobility of the carboxyl and amino derivatives of lysozyme is determined solely by a charge difference of 16 units, then the amount of SDS bound may be calculated to be of the order of 16/0.12, or about 133 molecules of SDS per lysozyme molecule. This corresponds to 2.5 mg of SDS per mg of protein. Such a value exceeds by almost 2-fold that measured for other denatured proteins which (at about the detergent concentrations, pH, and ionic strength used in these experiments) typically bind about 1.4 mg of SDS per mg of protein (15). Accepting as more reasonable the latter value of 1.4, which corresponds to about 70 SDS molecules per chain, we conclude that the effective charge difference between the derivatives is not 16 but about 8. This discrepancy can be reconciled if the derivatives do not, in fact, bind equivalent amounts of SDS, but rather, if the $\beta$-aminoethyl lysozyme derivative binds about 8 fewer molecules than does $\beta$-aminoethyl lysozyme.

The model set of lysozyme derivatives suggests that the intrinsic charge of a polypeptide may slightly modify its SDS-binding capacity; specifically, a negatively charged polypeptide may tend to bind slightly less SDS and a positively charged polypeptide slightly more SDS than a comparable neutral polypeptide. Further support for this notion comes from comparing electrophoretic mobilities and intrinsic charges. For example, titration studies indicate that denatured pepsin should have a charge of about $-35$ at pH 7.2 (10). Assuming that pepsin binds a typical complement of SDS (about 170 molecules based on 1.4 mg of SDS bound per mg of protein (15)), it would be expected to migrate about (170 + 35)/170, or about 1.2 times faster than a neutral protein of the same size. Such a mobility should lead to an apparent molecular weight of about 30,000 to 32,000 by the SDS-gel procedure. The 37,000 to 38,000 actually found (Table I) can be explained if less than a typical amount of SDS is bound by pepsin. Differential SDS binding can be similarly invoked to explain the unexpectedly small effects of intrinsic charge on the mobilities of several other polypeptides, including for example, lysozyme, $\beta$-lactoglobulin, and BSA.

Although the state of foldedness can have a relatively large effect on the electrophoretic mobility of a protein in non-SDS gels, it has a surprisingly small effect in SDS-containing gels. This is illustrated by comparing the mobilities of lysozyme and its $\beta$-hydroxethyl derivative in urea gels (Fig. 3A) and in SDS gels (Fig. 3B). All other polypeptides tested for the consequences of chain unfolding, with the notable exception of ribonuclease, showed little or no change in apparent molecular weight following disruption of intramolecular disulfide bonds (Table I). Again, as in the preceding discussion of intrinsic charge, differential SDS binding may be invoked to explain the unexpectedly small effect of chain unfolding on mobility in SDS gels; in the
apparent size would then be increased in proportion to the fraction of time the molecules exist in the aggregated state. The expression of a rapid reversible dimerization in solution; its is not borne out by the binding measurement in free solution. The unfolded forms differs appreciably from that of other proteins, modified by the presence of the polyacrylamide matrix. Alterative, the presence of the polyacrylamide matrix. Alterative, it is possible that the low mobility of ribonuclease is the expression of a rapid reversible dimerization in solution; its apparent size would then be increased in proportion to the fraction of time the molecules exist in the aggregated state.

The wide variation in apparent molecular weights of the various RNase derivatives suggests that comparative electrophoresis of several different derivatives may be a useful diagnostic procedure for detecting anomalous behavior in uncharacterized polypeptides.

Molecular Weight of Virus Capsid Proteins—One of the primary motives for initiating this study was interest in evaluating and using SDS gels to determine the molecular weights of the five polypeptide components of ME virus, a small RNA-containing animal virus. For this purpose, whole virus was dissociated in urea-SDS solutions into its polypeptide and ribonucleate components, and the entire solution was subjected to electrophoresis. Similar studies on three well characterized, RNA-containing viruses, tobacco mosaic virus, bromegrass mosaic virus, and R17 virus showed that their protein subunits behaved typically on SDS gels, and its molecular weight is therefore subject to greater uncertainty than that of the other chains. However, experiments on 20-cm long, 5% gels (cf. Fig. 2A) confirmed the molecular weight value obtained on 10% gels.

In the present case it is necessary to suppose that a substantially greater amount of SDS is bound by unfolded as compared with folded polypeptides. The resulting greater negative charge could then counteract the increased frictional resistance expected for the unfolded state. That such a substantial increase in SDS binding actually occurs is supported by equilibrium dialysis studies, which demonstrate a large (40 to 50%) increase in SDS binding accompanying the disruption and blockade of structure-stabilizing disulfide bonds (15, 16). The nearly exact balance between the consequences of chain unfolding and enhanced SDS binding must be regarded as fortuitous.

Of all the proteins examined in the study, the most anomalous was pancreatic ribonuclease, which migrated more slowly than expected in the SDS gels. The reason for this behavior remains unclear. It was aggravated by reduction, but improved by alkylolation or by disulfide interchange (Table II). One possibility, that the SDS-binding capacity of either the folded or the unfolded forms differs appreciably from that of other proteins, is not borne out by the binding measurement in free solution (15); however, it remains possible that this situation is somehow modified by the presence of the polyacrylamide matrix. Alternatively, it is possible that the low mobility of ribonuclease is the expression of a rapid reversible dimerization in solution; its apparent size would then be increased in proportion to the fraction of time the molecules exist in the aggregated state.

The wide variation in apparent molecular weights of the various RNase derivatives suggests that comparative electrophoresis of several different derivatives may be a useful diagnostic procedure for detecting anomalous behavior in uncharacterized polypeptides.

**Fig. 4.** Determination of the molecular weights of the polypeptide components of ME virus using a 10% polyacrylamide gel; ME virus proteins were enriched in the minor components, 6 and 8, by recovering the precipitate formed during incubation for 10 min at 37° in 0.1 M sodium chloride and 0.05 M sodium citrate, pH 5.7. The relative mobility of each known (O) was plotted against its apparent molecular weight. The apparent molecular weight of each ME virus polypeptide was then determined from its mobility with the aid of this plot.

### Table III

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<th>Chain</th>
<th>Reduced</th>
<th>Mixed disulfide*</th>
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<tr>
<td>α</td>
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<td>32,800 (5)</td>
</tr>
<tr>
<td>β</td>
<td>30,500 (5)</td>
<td>30,500 (5)</td>
</tr>
<tr>
<td>γ</td>
<td>25,000 (5)</td>
<td>26,500 (5)</td>
</tr>
<tr>
<td>δ</td>
<td>10,500 (2)c</td>
<td>N.D.</td>
</tr>
<tr>
<td>ε</td>
<td>41,000 (2)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Corrected for mass contribution of the substituted acetyl-cystaminyl mixed disulfide derivative of the viral polypeptide chains of ME virus.

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