The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*

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SUMMARY

Forty proteins with polypeptide chains of well characterized molecular weights have been studied by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate following the procedure of Shapiro, Vinuela, and Maizel (Biochem. Biophys. Res. Commun., 28, 815 (1967)). When the electrophoretic mobilities were plotted against the logarithm of the known polypeptide chain molecular weights, a smooth curve was obtained. The results show that the method can be used with great confidence to determine the molecular weights of polypeptide chains for a wide variety of proteins.

The proteins used were obtained from Boehringer Mannheim: L-amino acid oxidase (Crotalus terrificus), D-amino acid oxidase (pig kidney), fumarase (pig heart), and glutamate dehydrogenase (bovine liver); from Calbiochem: cytochrome c (horse heart), enolase (rabbit muscle), glyceroldehyde phosphate dehydrogenase (rabbit muscle), β-lactoglobulin, and pyruvate kinase (rabbit muscle); from Mann: myoglobin (horse heart), lysozyme (egg white), pepsin (hog stomach), ribonuclease (bovine pancreas), serum albumin (bovine), and subtilisin (Bacillus subtilis); from Sigma: α-chymotrypsin (bovine pancreas), α-chymotrypsinogen A (bovine pancreas), hemoglobin (bovine), lactate dehydrogenase (bovine heart), liver alcohol dehydrogenase (horse liver), and yeast alcohol dehydrogenase; from Worthington: aldolase (rabbit muscle), carbonic anhydrase B (human blood), creatine kinase (chicken muscle), β-galactosidase (E. coli), γ-globulin (rabbit), leucine aminopeptidase (bovine eye balls), paranymosin, tropomyosin, and myosin (rabbit muscle) were gifts from Drs. Edsall, Quiocho, and Bell, was recrystallized from ethanol. Dialysis tubing 8/32 and 23/32, obtained from Union Carbide, was cleaned by standard procedures. Distilled water was used in all experiments.

Analytical grade NaH₂PO₄·H₂O and Na₂HPO₄·7H₂O were obtained from Mallinckrodt Chemical Works, New York. Glacial acetic acid and methanol were reagent grade chemicals. Acrylamide (for electrophoresis), methylenebisacrylamide, N,N',N,N'-tetramethylethylenediamine and β-mercaptoethanol were obtained from Eastman. Ammonium persulfate, analytical grade, was a Fisher product. Bromphenol blue and Coomassie brilliant blue (R-250) were obtained from Mann. Sodium dodecyl sulfate (95%), obtained from Matheson Coleman and Bell, was recrystallized from ethanol. Dialysis tubing 8/32 and 23/32, obtained from Union Carbide, was cleaned by standard procedures. Distilled water was used in all experiments.

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Preparation of Protein Solutions

The proteins were incubated at 37° for 2 hours in 0.01 M sodium phosphate buffer, pH 7.0, 1% in SDS, and 1% in β-mercaptoethanol. Tropomyosin, paramyosin, and myosin were dissolved in this buffer in the presence of 8 M urea. The protein concentration was normally between 0.2 and 0.6 mg per ml. After incubation the protein solutions were dialyzed for several hours at room temperature against 500 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% β-mercaptoethanol. In most cases the dialysis step may be omitted and the protein dissolved directly in dialysis buffer.

Preparation of Gels

Gel buffer contained 7.8 g NaH2PO4·H2O, 38.6 g of Na2HPO4·7H2O, 2 g of SDS per liter. For the 10% acrylamide solution, 22.2 g of acrylamide and 0.6 g of methylenebisacrylamide were dissolved in water to give 100 ml of solution. Insoluble material was removed by filtration through Whatman No. 1 filter paper. The solution was kept at 4° in a dark bottle. Gels with normal amount of cross-linker remained clear, those with doubled cross-linker turn opaque. *Just before use they were soaked in cleaning solution, rinsed, and oven-dried. For a typical run of 12 gels, 15 ml of gel buffer were deaerated and mixed with 13.5 ml of acrylamide solution. After further deaeration, 1.5 ml of freshly made ammonium persulfate solution (15 mg per ml) and 0.005 ml of A, N, A', N'-tetramethylethylenediamine were added. After mixing, each tube was filled with 2 ml of the solution. Before the gel hardened a few drops of water were layered on top of the gel solution. After 10 to 20 min an interface could be seen indicating that the gel had solidified. Gels with normal amount of cross-linker remain clear, those with doubled cross-linker turn opaque. Just before use the water layer was sucked off, and the tubes were placed in the electrophoresis apparatus.

Preparation of Samples

For each gel, 3 μl of tracking dye (0.05% Bromphenol blue in water), 1 drop of glycerol, 5 μl of mercaptoethanol, and 50 μl of dialysis buffer was mixed in a small test tube. Then 10 to 50 μl of the protein solution were added. After mixing, the solutions were applied on the gels. Gel buffer, diluted 1:1 with water, was carefully layered on top of each sample to fill the tubes. The two compartments of the electrophoresis apparatus were filled with gel buffer, diluted 1:1 with water. Electrophoresis was performed at a constant current of 12 mA per gel with the positive electrode in the lower chamber. Under these conditions the marker dye moved three-quarters through the gel in approximately 4 hours. The time taken to run the gel may be decreased by decreasing the molarity of the gel buffer. After electrophoresis, the gels were removed from the tubes by squirting water from a syringe between gel and glass wall and by using a pipette bulb to exert pressure. The length of the gel and the distance moved by the dye were measured.

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Staining and Destaining—The gels were placed in small tubes filled with staining solution prepared by dissolving 1.25 g of Coomassie brilliant blue in a mixture of 454 ml of 30% methanol and 46 ml of glacial acetic acid, and removing insoluble material by filtration through Whatman No. 1 filter paper. Staining was at room temperature. The time varied from 2 to 10 hours. The gels were removed from the staining solution, rinsed with distilled water, and placed in destaining solution (75 ml of acetic acid, 50 ml of methanol, and 875 ml of water) for a minimum of 30 min. The gels were then further destained electrophoretically for 2 hours in a gel electrophoresis apparatus using destaining solution. The length of the gels after destaining and the positions of the blue protein zones were recorded. The gels were stored in 7.5% acetic acid solution.

The gels swell some 5% in the acidic solution used for staining and destaining. Gels with lower amount of cross-linker show more swelling. Therefore the calculation of the mobility has to include the length of the gel before and after staining as well as the mobility of the protein and of the marker dye. Assuming even swelling of the gels, the mobility was calculated as

\[
\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length before destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}
\]

The mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale.

Time Required for Molecular Weight Determination—It is possible to obtain the molecular weight of a particular protein within a day: preparation of gels and samples, 2 hours; electrophoresis, 3 to 4 hours, removing the gels, ½ hour; staining, 2 to 3 hours.

Amount of Protein

Usually 0.01 mg of protein was applied per gel. The amount could be lowered if the gel was stained for a longer period. If 0.1 mg was applied although the trailing edge of the band was diffuse, the leading edge was still very sharp and molecular weights could still be found very accurately.

Elution of Proteins from Gels

On each of two gels, 0.1 mg or more was run. One gel was stored wrapped in Saran Wrap at 4°. The other gel was stained and destained to localize the protein band. The corresponding volume of the first gel was cut into smaller pieces. The gel material was suspended in a small amount of 0.1% SDS solution and kept for several hours at 37°. The solution was withdrawn and a second elution was performed. The combined eluents were lyophilized in a conical centrifuge tube. Distilled water was added to obtain a 1% SDS solution (usually about 50 to 100 μl). Then nine parts of ice-cold acetone were added for one part of solution. The protein precipitated and could be centrifuged off, whereas the detergent stayed in solution. The precipitate could be transferred to a hydrolysis tube by dissolving it in 5% piperidine solution or in 12 N HCl.

RESULTS

Two procedures were used to examine the relationship between electrophoretic mobilities and molecular weights of various proteins. Either several SDS-denatured proteins were mixed
and run together on one gel or the proteins were run in a parallel manner on different gels. Identical results were obtained by both methods.

Fig. 1, A, B, and C, shows examples of the separation of several different polypeptide chains on a single gel. Remarkably good separation is obtained for the polypeptide chains of catalase, fumarase, aldolase, glyceraldehyde phosphate dehydrogenase, carboxyl anhydrase, and myoglobin (Fig. 1A). The identity of each band was established by running each protein on a different gel. In each case a single protein band with an electrophoretic mobility corresponding to the one assigned to it in the mixture was observed. Fig. 1B shows a similar mixture which was run on a different occasion, but without myoglobin. In Fig. 1C the same proteins were run as in Fig. 1A, but substituting liver alcohol dehydrogenase for aldolase. Again, separation of all six polypeptide chains was observed. The control gel for liver alcohol dehydrogenase indicated only one band with a mobility corresponding to that assigned to it in the mixture. The molecular weights for the different polypeptide chains are taken from Table I, in which the proteins we studied by SDS gel electrophoresis are listed. The pictures illustrate the dependence of the mobility on the logarithm of the molecular weight of the polypeptide chains (1). The separation in the molecular weight range of 30,000 to 60,000 is excellent. It can be improved further either by increasing the length of the gel or by decreasing the amount of protein applied.

As an example of the method in which each protein is run on a different gel, Fig. 2 illustrates the determination of the molecular weight of the polypeptide chains of different proteins in gels with the normal amount of cross-linker. The proteins are listed from top to bottom, and the molecular weights given for the different polypeptide chains are taken from Table I. A, catalase (60,000), fumarase (49,000), aldolase (40,000), glyceraldehyde phosphate dehydrogenase (36,000), carboxyl anhydrase (29,000), and myoglobin (17,200); B, same as A, but omitting the myoglobin. This gel was run on a different occasion; C, catalase, fumarase, liver alcohol dehydrogenase (41,000), glyceraldehyde phosphate dehydrogenase, carboxyl anhydrase, and myoglobin.

Fig. 1. Separation of the polypeptide chains of different proteins in gels with the normal amount of cross-linker. The proteins are listed from top to bottom, and the molecular weights given for the different polypeptide chains are taken from Table I. A, catalase (60,000), fumarase (49,000), aldolase (40,000), glyceraldehyde phosphate dehydrogenase (36,000), carboxyl anhydrase (29,000), and myoglobin (17,200); B, same as A, but omitting the myoglobin. This gel was run on a different occasion; C, catalase, fumarase, liver alcohol dehydrogenase (41,000), glyceraldehyde phosphate dehydrogenase, carboxyl anhydrase, and myoglobin.

<table>
<thead>
<tr>
<th>Table I</th>
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<tr>
<td>Proteins studied by SDS-electrophoresis</td>
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<tr>
<td>Myosin*</td>
</tr>
<tr>
<td>β-Galactosidase*</td>
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<tr>
<td>Paramyosin*</td>
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<tr>
<td>Phosphorylase a*</td>
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<tr>
<td>Serum albumin</td>
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<tr>
<td>l-Amino acid oxidase</td>
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<tr>
<td>Catalase*</td>
</tr>
<tr>
<td>Pyruvate kinase*</td>
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<tr>
<td>Glutamate dehydrogenase*</td>
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<tr>
<td>Leucine amino peptidase</td>
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<tr>
<td>γ-Globulin, H chain*</td>
</tr>
<tr>
<td>Fumarase*</td>
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<tr>
<td>Ovalbumin</td>
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<tr>
<td>Alcohol dehydrogenase (liver)*</td>
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<tr>
<td>Elolase*</td>
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<tr>
<td>Aldolase*</td>
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<tr>
<td>Creatine kinase*</td>
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<tr>
<td>β-Amino acid oxidase*</td>
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<tr>
<td>Alcohol dehydrogenase (yeast)*</td>
</tr>
<tr>
<td>Glyceraldehyde phosphate dehydrogenase*</td>
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<tr>
<td>Tropomyosin*</td>
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<tr>
<td>Lactate dehydrogenase*</td>
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<tr>
<td>Pepsin</td>
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<tr>
<td>Aspartate transcarbamylase, C chain*</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
</tr>
<tr>
<td>Carboxyl anhydrase</td>
</tr>
<tr>
<td>Subtilisin*</td>
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<tr>
<td>γ-Globulin, L chain*</td>
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<tr>
<td>Chymotrypsinogen</td>
</tr>
<tr>
<td>Trypsin</td>
</tr>
<tr>
<td>Papain (carboxymethyl)</td>
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<tr>
<td>β-Lactoglobulin*</td>
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<tr>
<td>Myoglobin</td>
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<tr>
<td>Aspartate transcarbamylase, R chain*</td>
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<tr>
<td>Hemoglobin*</td>
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<tr>
<td>Q3 coat protein</td>
</tr>
<tr>
<td>Lysozyme</td>
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<tr>
<td>R17 coat protein</td>
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<tr>
<td>Ribonuclease</td>
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<tr>
<td>Cytochrome c</td>
</tr>
<tr>
<td>Chymotrypsin*</td>
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* Corrected according to the X-ray structure (J. Drenth, personal communication).
* W. Konigsberg, personal communication.
weight of D-amino acid oxidase. Again the electrophoretic mobilities for marker polypeptide chains are plotted against the log of their molecular weights. From the electrophoretic mobility of D-amino acid oxidase, a molecular weight of 37,000 is found.

The reproducibility of the system can be illustrated by the following experiment. Aspartate transcarbamylase from E. coli was run in a parallel manner on 12 individual gels. Two protein bands were obtained which correspond to the two different polypeptide chains of this enzyme (2). The electrophoretic mobilities were 0.44 to 0.46 for the C Band and 0.74 to 0.76 for the R Band. The average mobilities calculated from these 12 gels were 0.445 ± 3% and 0.745 ± 3%, respectively. From these results the reproducibility appears better than 5%. However, the absolute values of the mobility of the same polypeptide chain run on separate occasions sometimes show a deviation of 5 to 10%. This slight deviation seems to occur with new batches of polyacrylamide. However, in all cases, the plot of the mobilities for a given set of standards gives the same value for the molecular weight of a particular protein.

The identity of a particular protein band has been repeatedly established by the elution procedure given in the previous section, followed by amino acid analysis. Greater than 75% recovery may be obtained by this method. The method allows easy separation and recovery of small quantities of proteins that are sufficiently different in molecular weight.

The procedure may be adapted to study different molecular weight ranges. This can be seen in Fig. 3. Six different polypeptide chains were run using 10% acrylamide solution, but changing the amount of methylenebisacrylamide. A drastic change in the mobilities occurs. For example, the mobility of fumarase with a molecular weight of 50,000 changes from 0.28 to 0.63 if the amount of cross-linker is reduced from the normal amount to half of this value. This suggests that gels with half the normal amounts of cross-linker would be useful to study proteins over a wide range of molecular weights (see below).

The results of our measurements on close to 40 different proteins are shown in Figs. 4 and 5 where the mobilities are plotted against the log of the molecular weight taken from Table I. Each protein has been run on at least four different occasions. Fig. 4 shows results for 10 different polypeptide chains in the molecular weight range from 10,000 to 70,000. In no case has a serious deviation from the molecular weights given in Table I been observed. The maximum deviation from the predicted values in this range is less than 10% and for most proteins the agreement is better. This accuracy is expected because, as shown in Fig. 1, polypeptide chains differing by only 10% in weight can be separated on the same gel.

Fig. 5 shows the results for 10 different polypeptide chains in the higher molecular weight range from 50,000 to 200,000. Gels with half the normal amount of cross-linker were used in these experiments. In spite of the hyperbolic curve obtained (see also Shapiro et al. (1) and Fig. 3), the mobilities follow the known molecular weights. Although fewer standards are available for this range, an accuracy of ±10% still seems possible.

Our results show that meaningful molecular weights can be obtained by SDS gel electrophoresis for the three muscle proteins tropomyosin, paramyosin, and myosin. We were interested in these proteins because they are known to have a high helical content. Tropomyosin yields a molecular weight of 36,000 in good agreement with the values given in the literature (13, 34). Using very low protein concentrations we found that the protein actually did not give a single band, but a doublet that was hardly separated. At the present it is unclear whether this is an artifact with the preparation we have available or whether the polypeptide chains of tropomyosin are not identical. Paramyosin yielded the expected polypeptide chain of molecular weight 100,000 (13). For myosin we obtained a polypeptide chain...
FIG. 4. Comparison of the molecular weights of 37 different polypeptide chains in the molecular weight range from 11,000 to 70,000 with their electrophoretic mobilities on gels with the normal amount of cross-linker. The references to the molecular weights are given in Table I.

with a molecular weight close to 200,000, in agreement with the reported values for this protein (9, 10). However, we also detected three minor components of lower molecular weight (16,000 to 23,000). At this time, we don't know if these components are impurities in the preparation we had available or if they are inherent components of myosin. The latter assumption is supported by recent results obtained in different laboratories.3

In a few cases we studied the proteins after performic acid oxidation or carboxymethylation. The electrophoretic mobilities of papain and ribonuclease after performic acid oxidation were identical with the ones obtained before. No differences were seen after carboxymethylation for lysozyme, aspartate transcarbamylase, tropomyosin, and paramyosin. This indicates that the β-mercaptoethanol prevents the formation of interchain cystine bonds. This assumption is supported by the fact that we did not find dimers of polypeptide chains. The possibility that some rare protein will not be denatured by the SDS treatment in the presence of β-mercaptoethanol suggests that a control should be performed using an alkylated or oxidized derivative.

3 S. Lowey, personal communication.

FIG. 5. Comparison of the molecular weights of different polypeptide chains (see Table I) in the molecular weight range from 40,000 to 200,000 with their electrophoretic mobilities on gels with half the amount of cross-linker.

DISCUSSION

Separation of native proteins on polyacrylamide gels was shown by Ornstein (7) and Davis (8) to be dependent not only on the charge but very strongly on the size of the molecules. The binding of dodecyl sulfate ions to proteins has been shown for several protein molecules (see Tanford (41) for a recent review), and was assumed to be the basis of the separation of the denatured proteins upon SDS electrophoresis on polyacrylamide (1). If so, one must assume that the individual charge pattern of each protein is totally changed by the binding of SDS anions, rendering all molecules negatively charged. At present it is difficult to see why proteins that differ widely in amino acid composition and isoelectric points should all follow the general pattern. It is possible that the sieving effect, which is an exponential function, overcomes the charge effect which may be of minor importance. Because of these theoretical difficulties we will discuss the results only from a practical point of view.

Two questions were raised during this study. How reproducible are the results obtained by SDS gel electrophoresis and how reliable are the molecular weights obtained? The results presented above quite clearly show that a high degree of reproducibility in the determination of the electrophoretic mobility can be obtained whether the proteins are run on different gels or on the same gel.

We have shown that close to 40 different proteins have electrophoretic mobilities which are independent of the isoelectric point and the amino acid composition and seem governed solely by the molecular weights of their polypeptide chains. Since the majority of the polypeptide chains used are either characterized by molecular weights known from amino acid sequence analysis,
or X-ray crystallography, or by very careful studies in guanidine-
HCl with similar results obtained in different laboratories, we
feel confident that this study is a very stringent test for the
technique of Shapiro et al. (1). All the proteins studied yielded
molecular weights within the experimental error of the known
values. We have not studied proteins containing large amounts
of carbohydrate or lipid material. It is possible that such pro-
cteins, because of their large nonprotein part, will behave dif-
f erently on gel electrophoresis. It is, however, noteworthy that
SDS gel electrophoresis yields not only excellent results for pro-
cteins, which are globular in the native state, but also for the
highly helical, rod-shaped molecules, myosin, paramyosin, and
tropomyosin. For all three proteins we obtained polypeptide
chains with molecular weights in agreement with the values
from the literature (see “Results”).

With these results one is inclined to assume that the technique
of Shapiro et al. (1) yields molecular weights with an accuracy
of better than ±10% for polypeptide chains with molecular weights
between 15,000 and 100,000. This range can be covered with
numerous commercially available proteins as standards. The
difficulty with higher molecular weights is probably only the
fact that fewer markers are available for the range 90,000 to
200,000. Polypeptide chain markers commercially available
for this range are phosphorylase with a molecular weight of 94,000
(12, 14) and thyroglobulin with a molecular weight of 180,000
(42). The preparation of β-galactosidase from E. coli can be
easily accomplished (43) and yields a marker band with 135,000
molecular weight (12). Myosin and paramyosin can usually be
obtained from the numerous laboratories working on these pro-
cteins and yield additional markers with molecular weights of
200,000 and 100,000. With enough markers an accuracy of
about 10% in the determination of the molecular weight of an
unknown protein falling in this molecular weight range may be
possible.

Four examples will be used to illustrate these points further.
Phosphorylase a gave a single polypeptide chain of molecular
weight 94,000 without any indication of heterogeneity. This is
in agreement with the results of Ullmann et al. (12), using the
approach to equilibrium centrifugation in the presence of 6 M
guanidine-HCl. Scery, Fischer, and Teller (14) found a similar
value by equilibrium centrifugation in the same solvent only
after accounting for some heterogeneity and preferential hydra-
tion. The latter, however, is an unlikely phenomenon in view of
the results obtained by Kirby Hade and Tanford (44), Reithol
and Sakura (45) and Ullmann et al. (12).

A further example is liver alcohol dehydrogenase. The tech-
nique of Shapiro et al. (1) yields a value of 40,000 in agreement
with results obtained by osmotic pressure measurements in
guanidine-HCl (24), X-ray analysis (25), and preliminary se-
quence analysis5, but contrary to prior sedimentation analysis
studies (46).

The separation of the polypeptide chains of fumarase, aldolase,
and glyceraldehyde phosphate dehydrogenase on one gel is very
striking. The molecular weights of these polypeptide chains are
today well established. Aldolase was for some time assumed to
have a molecular weight of 50,000. This value was mainly
based on preferential hydration in guanidine-HCl (47). How-
ever by using glyceraldehyde phosphate dehydrogenase with a
known amino acid sequence as one marker and fumarase, which

4 Jörnvall, H., AND Harris, J I., Abstracts of the Federation of
European Biochemical Sciences, Praha, 1968, abstract 750.
is well characterized, as the other, it can be concluded that the
molecular weight of the aldolase polypeptide chain is 40,000.
Meanwhile, this is the value accepted for careful measurements
by ultracentrifugation (27) and osmotic pressure (24) in guani-
dine-HCl, as well as from chemical studies (28).

Conflicting values for the size of the polypeptide chain of
D-amino acid oxidase are found in the literature and have been
summarized recently by Henn and Ackers (30). They showed a
value of 35,000 to 40,000 by gel filtration. The SDS electroph-
oresis yields 37,000. From all the evidence presented for the
other proteins we think that the two values indicating 37,000
are much better than the one of 50,000 reported by Fonda and
Andersson (48). Their value is based mainly on fingerprint
analysis and a gel filtration study, which is less extensive than
the one by Henn and Ackers (30).

Since the method appears to yield values in agreement with the
best current estimates for all the proteins studied, it seems fair
to compare SDS gel electrophoresis with other methods. The
excellent resolving power of the gels over a wide range of
molecular weights has been illustrated. In this respect the
method is much superior to gel filtration patterns on Sephadex in a
denaturing solvent like guanidine-HCl (49). The good resolution
and the fact that an estimate of the molecular weight can be
obtained within a day, together with the small amount of protein
needed, makes the method strongly competitive with others
commonly employed. The theoretically fully developed meth-
ods of osmotic pressure and sedimentation equilibrium in 6 M
guanidine-HCl are still superior. However, osmotic pressure
measurements suffer from the large amount of protein needed
and the fact that extremely accurate protein determination is
necessary. Both limitations do not apply for sedimentation
equilibrium using interference optics. However this method is
very demanding experimentally if good results are to be obtained.
But even in the optimal case the calculation depends on the
value chosen for the partial specific volume v. An uncertainty
of 0.02 in this value introduces a deviation of up to 10%, even
if all the centrifuge measurements are performed well. Also,
in some cases, in spite of the fact that there is good evidence for
no major change in v in guanidine-HCl (12, 44, 45), the assump-
tion of preferential hydration is still quite often used (14, 47) to
account for deviations.

It is by no means intended to assume that the accuracy of
SDS gel electrophoresis will be comparable with the well
developed physicochemical methods. Also, certain theoretical
aspects of SDS gel electrophoresis are still not clearly under-
stood. In spite of these limitations, however, the ease with
which the method can be applied, together with the results
presented above, encourage its wider use, especially in case of
conflicting data obtained by other methods.

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