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LIMITATIONS OF THE DETERGENT–POLYACRYLAMIDE GEL ELECTROPHORESIS METHOD FOR MOLECULAR WEIGHT DETERMINATION OF PROTEINS

J. G. WILLIAMS* AND W. B. GRATZER
Medical Research Council Biophysics Unit, King's College, Drury Lane, London W.C.2 (Great Britain)
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SUMMARY

The relation between molecular weight and electrophoretic mobility in sodium dodecyl sulphate–polyacrylamide gels breaks down for proteins with molecular weights lower than about 15,000. Below about 6,000 all species migrate with the same mobility. These effects are essentially independent of acrylamide concentration, and are interpreted in terms of the loss of asymmetry of the protein–sodium dodecyl sulphate complexes in this range, as predicted from the results of REYNOLD AND TANFORD. It is shown that the sodium dodecyl sulphate–acrylamide method also fails for proteins of highly negative or positive charge. The anomalous mobilities of two acidic proteins were normalised on esterification of carboxyl groups. The procedure for molecular weight determination also works well when a cationic detergent is substituted for sodium dodecyl sulphate. Some advantages result from the reaction of samples with dansyl chloride prior to electrophoresis to give strongly fluorescent zones.

INTRODUCTION

The observation, originally made by SHAPIRO *et al.*[1], that the mobility of proteins in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) is simply related to their molecular weights, has become the basis of a widely applied method of molecular weight determination. Since SDS is also an effective denaturing and disaggregating agent it appears to be the monomer molecular weight that is obtained in the case of subunit proteins. Since its introduction the method has been widely explored, and evidently works very well with a wide range of common proteins[2–4]. At the same time it appears now to be regarded by many workers as an infallible means of measuring molecular weights, and it has been used somewhat indiscriminately even on very small proteins. We have attempted a systematic study of the method in the low-molecular-weight range, and we give the results, together with a consideration of a number of points of techniques.

* Present address: Imperial Cancer Research Fund Laboratories, London, W.C.2, Great Britain.

MATERIALS AND METHODS

Proteins

The following samples were obtained from Sigma Chemical Co.: horse heart cytochrome *c*, hen egg lysozyme, bovine α-chymotrypsin, chymotrypsinogen, serum albumin and insulin, glucagon, creatine phosphokinase, γ-globulin, carbonic anhydrase, ovalbumin, polymixin and bacitracin; subtilin was from K and K Chemicals; rabbit muscle tropomyosin and myosin light chains were a gift from R. L. STARR and ferredoxins from spinach and from Chloropseudomonas from Dr. M. EVANS.

Mixed insulin A and B chains were prepared both by performic acid oxidation[5], and by reduction with β-mercaptoethanol followed by carboxymethylation with iodoacetamide (0.3 *M* for 30 min at 45°). This preparation was screened prior to use by electrophoresis in polyacrylamide gels in 8 *M* urea, pH 8.5, in the absence of detergent. All other proteins were incubated before use at a concentration of 2 mg/ml in 1 % SDS, 1 % β-mercaptoethanol, 4 *M* urea at 45° for 45 min. For esterification of carboxyl groups 1 mg of protein was suspended in 1 ml methanol, containing 1 *N* HCl. After three days the protein was collected by centrifugation, washed several times with methanol and dried by rotary evaporation.

Electrophoresis

The electrophoretic method essentially followed that of WEBER AND OSBORN[3]. Gels of 5–27.5 % acrylamide were all 0.5 % in methylenebisacrylamide. Gels were prepared with tetramethylethylenediamine and ammonium persulphate to initiate polymerisation. Tubes of 0.5 × 7 cm were used. The composition of the buffers were those suggested by WEBER AND OSBORN[3]. 10-μl samples of each protein were applied to the gels, together in general with 10 μl of 0.1 % Bromphenol Blue. Running buffer was then carefully layered on the sample. Electrophoresis was performed at a constant current of 5 mA per tube. The marker dye was allowed to migrate some 4 cm, which required not more than 5 h, depending on the acrylamide concentration. With buffer compartments of about 0.5 l capacity, it was found desirable to change the buffer several times in the course of a run to avoid migration of electrode products into the gel.

With samples labelled with dimethylaminonaphthalenesulphonyl chloride (dansyl chloride) the tubes were viewed under UV light from a low-pressure mercury arc with a Woods glass filter. In other cases the gels were rimmed with the needle of a syringe containing a dilute "Teepol" solution, which was injected around the wall to lubricate the expulsion of the gel. This was found expedient particularly with gels of high concentration. Gels were stained overnight in a solution containing 0.25 % Coomassie Blue and 1 % cupric chloride in acetic acid–methanol (1:10). The gels were rinsed and destained electrophoretically in a medium containing 75 ml acetic acid and 50 ml methanol per l. Densitometry was performed on a Joyce–Loebl microdensitometer, using a red filter.

RESULTS AND DISCUSSION

Molecular weight limit of SDS gel method

The relations between mobility of proteins in SDS–acrylamide gels under standard conditions and molecular weight are shown in Fig. 1 for gels of high and low

acrylamide concentrations. On a logarithmic basis it is apparent that the plot has two limbs, one at high the other at low molecular weights. In the latter range, the mobility is independent of molecular weight. The two lines intersect at a molecular weight of 10^4, irrespective of gel concentration. The method is thus inapplicable below this critical value. It is clear from the indistinguishable mobilities of the low-molecular-weight species, and the fact that they separate readily in consequence of differences in their intrinsic charge in polyacrylamide gels containing no detergent, that they bind SDS, and there is no reason to believe that the constant binding ratio of 1.4 g SDS per g polypeptide, established for a range of proteins by REYNOLDS AND TANFORD[6], does not extend to short chains.

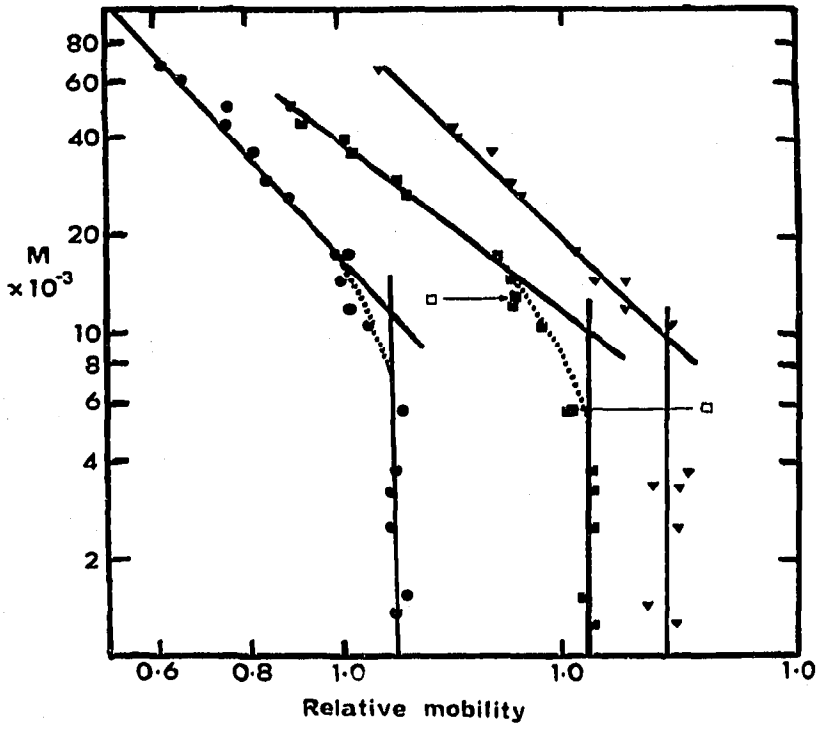

Fig. 1. Relation between molecular weight and electrophoretic mobility of proteins and peptides in SDS-polyacrylamide gels. The materials are given in MATERIALS AND METHODS. Mobilities are expressed relative to the Bromphenol Blue markers. The abscissa scale is displaced as indicated for the two higher gel concentration curves. ●, 5 % acrylamide; ■, 10 % acrylamide; ▼, 5 % acrylamide; □, highly acidic ferredoxins from spinach and Chloropseudomonas. After esterification of carboxyl groups the mobilities were displaced to the positions indicated.

The results are consistent with expectations from hydrodynamic data. REYNOLDS AND TANFORD[7] have observed that the exponent in the Mark–Houwink equation for protein–SDS complexes is 1.3, indicating that the particles are highly asymmetric. Assuming prolate ellipsoids, they have derived the length of the major and minor semi-axes, a and b, for a series of protein–SDS complexes. It was found that b is constant, at about 17–18 Å, so that a varies linearly with the molecular weight. REYNOLDS AND TANFORD[6,7] note that a simple relation between mobility and molecular weight in SDS gels requires that two conditions be fulfilled: Firstly the charge/mass ratio of the particles must be constant. This condition is embodied in the fixed binding ratio of SDS to protein, the electrophoretic charge being determined by the

double layer on the outside of the particle, *i.e.* the coat of SDS. Secondly the particles must be hydrodynamically homologous, which the viscosity results show to be the case. The latter condition will break down as a approaches b. From the dimensions given by REYNOLDS AND TANFORD[7], $a = b$ when the molecular weight of the protein is 5000. At this point the frictional coefficient will become essentially independent of molecular weight, and at some point above this — evidently about 15000 — the curve of mobility against molecular weight will deviate from the law followed for larger molecules. As expected, this point is effectively independent of acrylamide concentration.

Cationic detergent in place of SDS

As will be noted, the SDS method may fail with strongly acidic proteins. In such cases, and with materials prepared by procedures involving cationic rather than anionic detergents, it may be advantageous to replace SDS by a species such as cetyltrimethylammonium bromide. Using in all other respects the same procedure as for SDS gels, we find that the cationic detergent gives very similar results, except for rather steep curvature towards higher molecular weights (Fig. 2).

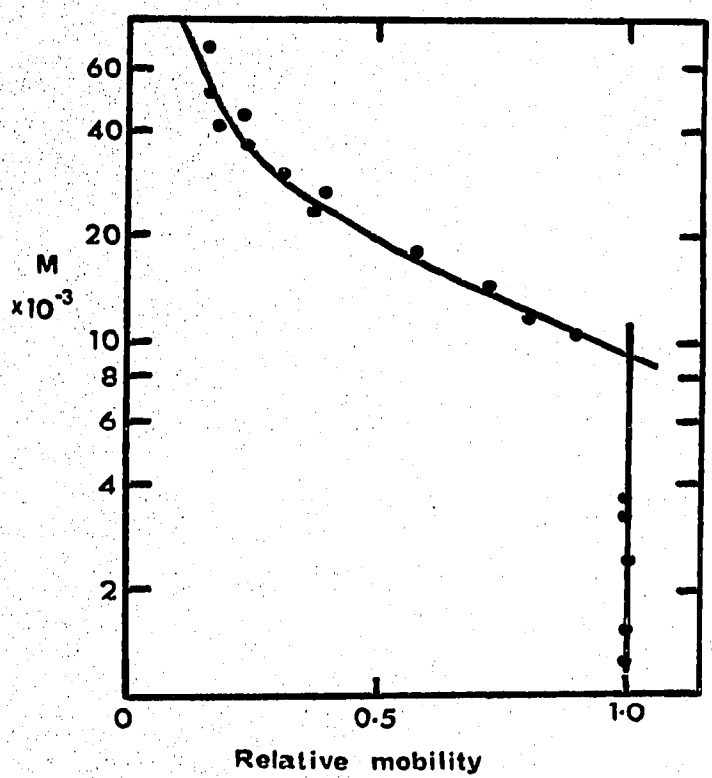

Fig. 2. Relation between molecular weight and electrophoretic mobility of proteins and peptides in polyacrylamide gel, containing the cationic detergent cetyltrimethylammonium bromide. Acrylamide concentration, 10%.

Highly charged proteins

In SDS very basic species, such as protamines, precipitate and cannot be examined. The ferredoxins are highly acidic proteins, and behave anomalously on SDS–acrylamide gels. Chloropseudomonas ferredoxin, which may be presumed to have a

molecular weight of about 6,000, like other bacterial ferredoxins[8,9], has a much higher mobility than other chains of similar size, and spinach ferredoxin, which is a chain of 97 residues, 20 of which are negatively charged[10], migrates at 0.8 of the expected mobility, indicating that little or no SDS is bound. When these proteins were esterified to reduce the negative charge, their mobility in both cases fell on the calibration curve (Fig. 1b). It may be noted that the intensity of staining with Coomassie Blue also greatly increased after this treatment.

Staining

Difficulties with staining were encountered in proteins containing few lysine or arginine residues. This could be partly overcome by introducing a cupric salt into the staining solution: the stain perhaps binds to the complex of copper ion and the peptide group[11].

With the anionic detergent, the Coomassie Blue gave a precipitate and the same would be expected with other common protein stains, which are all negatively charged. To overcome this problem the proteins were treated with dansyl chloride before electrophoresis. This procedure is also useful when gels of very high acrylamide concentration are used, which are difficult to remove intact from the tubes. The reagent combines with amino and phenolic groups[12] (*e.g.* in oxytocin, which contains tyrosine, but no amino groups). The dansylated proteins are strongly fluorescent, and the zones are readily observed under UV light without removal from the tubes.

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