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On the Determination of Molecular Weight of Proteins and Protein Subunits in the Presence of 6 M Guanidine Hydrochloride*

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ABSTRACT: For six proteins of well-established molecular structure, the apparent molecular weights have been measured in 6 M guanidine hydrochloride. It is shown that this apparent value is proportional to the true molecular weight of the protein subunits. The proportionality constant can be computed and may be used for the determination of molecular weights of polypeptide chains in 6 M guanidine hydrochloride.

Guanidine hydrochloride is now widely used as a denaturing and solubilizing agent for proteins, because of its property of abolishing noncovalent inter- and intramolecular interactions of polypeptide chains. Therefore it should be a valuable tool for determining the molecular weight of the subunits (protomers) of oligomeric proteins. However because of uncertainties regarding the partial specific volume and the degrees of hydration and solvation of proteins in the presence of high concentrations of guanidine hydrochloride, centrifugation experiments have not so far allowed molecular weight determinations in the presence of guanidine hydrochloride. However, to the extent that in the presence of guanidine hydrochloride the secondary, tertiary, and quaternary structures of proteins are completely abolished, one would presume that the relevant physical properties mentioned above should depend only on the amino acid composition of the polypeptide chain, regardless of its actual sequence. Then, for most natural polypeptides, the amino acid composition of which do not differ widely, the partial specific volume, degrees of hydration and solvation in guanidine should be practically identical. Therefore and to the extent that this assumption is correct, the apparent molecular weight of a polypeptide chain in guanidine hydrochloride should be proportional to its molecular weight.

In order to test this assumption, six proteins, whose molecular weights and subunit structures seemed well established, have been studied. Their apparent molecular weights have been determined, using the Archibald method, in the presence of 6 M guanidine hydrochloride. The experiments which we report in the present paper appear to justify the above assumptions allowing a direct determination of the molecular weight of proteins in 6 M guanidine hydrochloride solutions.

Materials and Methods

Protein Sources. All proteins used during this work were highly purified or crystalline preparations. Lysozyme, rabbit muscle lactic dehydrogenase, *Escherichia coli* alkaline phosphatase, beef liver glutamic dehydrogenase, and bovine serum albumin were commercial preparations. Rabbit muscle phosphorylase *b* was prepared according to Fischer and Krebs (1958) and the twice-recrystallized preparation was freed from 5′-AMP[1] by passing it through a Norit A column. β-Galactosidase from *E. coli* was prepared according to Perrin (1965) and was a twice-crystallized preparation.

Guanidine Treatment. The guanidine hydrochloride

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[1] Abbreviation used: 5′-AMP, adenosine 5′-monophosphate.

used during this work was purchased from Carlo Erba. Guanidine hydrochloride solutions (6 M) were prepared in the following buffer: Tris (2.10^{-2} M), EDTA (10^{-2} M), NaCl (10^{-2} M), and β-mercaptoethanol(10^{-1} M). The pH of the buffer was brought to 7.4 before addition of guanidine.

The crystals or ammonium sulfate precipitates of the different proteins were centrifuged and the precipitate was dissolved in the guanidine hydrochloride solution in order to obtain about 15–20 mg/ml of protein, measured by absorption at 280 mμ. This solution was dialyzed against 6 M guanidine hydrochloride for at least 36 hr at 25°. After dialysis the protein solution was diluted with 6 M guanidine hydrochloride solution in order to obtain 9, 6, and 4 mg/ml of protein. The diluted protein solutions were dialyzed again for 24–48 hr against the 6 M guanidine hydrochloride solution.

Archibald Measurements. The Archibald method was used. After dialysis of the protein, the sectors of a double-sector centrifugation cell (12 mm, 2° sectors) were equally filled, using a graduated Hamilton syringe, one with the protein solution and the other one with the guanidine hydrochloride–buffer solution.

Centrifugation was performed in a Spinco Model E analytical ultracentrifuge, using the schlieren optics. The speed and time of centrifugation were chosen according to LaBar (1966) and will be described for each protein in Table I. After the proper Archibald diagram was obtained, the rotor was accelerated to high speed (52,740–59,780 rpm) and the protein sedimented away from the meniscus. The area of the boundary, after correction for radial dilution, gives the value of the initial protein concentration. For lysozyme the initial concentration was determined with a boundary-forming cell (double sector, capillary type). The temperature was 25°. For each protein, measurements were made at three or more different concentrations and the result was extrapolated to zero protein concentration.

TABLE I: Physical Properties of Different Protein Subunits.

Protein	Rotor Speed (rpm)	Subunit Mol Wt	$\lim_{c\to 0} M_{app}\cdot(1-\bar{v}^*\rho)$
Lysozyme	31,410	14,500[a]	2,560
Lactic dehydrogenase	24,630	32,000[b]	5,450
Alkaline phosphatase	21,740	41,000[c]	6,850
Bovine serum albumin	21,740	65,000[d]	10,400
Phosphorylase *b*	19,780	94,000[e]	14,800
β-Galactosidase	19,780	135,000[f]	22,200

[a] Blake *et al.* (1965). [b] Pesce *et al.* (1964); Markert and Appella (1961). [c] Garen and Levinthal (1960). [d] Scatchard and Pigliacampi (1962). [e] H. Buc *et al.*, manuscript in preparation. [f] Craven *et al.* (1965); Steers *et al.* (1965).

Results

The Archibald method allows the direct measurement of

$$M_{app}(1-\bar{v}^*\rho) = \frac{\left(\frac{dc}{dr}\right)_m}{c_m}\frac{RT}{\omega^2 r_m} \qquad (1)$$

where M_{app} is the apparent molecular weight of the polypeptide chain, $\bar{v}^*$ its apparent partial specific volume, ρ the density of the solution, $(dc/dr)_m$ the protein concentration gradient at the meniscus, c_m the protein concentration at the meniscus, R the gas constant, T the absolute temperature, ω the angular speed of centrifugation, and r_m the distance from the axis of rotation to the meniscus.

Figure 1 shows the diagram obtained by plotting $1/M_{app}(1-\bar{v}^*\rho)$ *vs.* protein concentration at the meniscus. For each protein the experimental points can be interpolated by a straight line, the slope of which is virtually the same for all six. This will be interpreted in the discussion. Assuming the extrapolation to zero protein concentration to be correct, $\lim_{c\to 0} M_{app}(1-\bar{v}^*\rho)$ is obtained as the reciprocal of the intercept on the ordinate. The values of $\lim_{c\to 0} M_{app}(1-\bar{v}^*\rho)$ for the six polypeptide chains are shown on Table I. It will be noted that the value given for the protomer molecular weight of phosphorylase *b* is not the one commonly referred to in the literature. It is the value derived from a new set of measurements on the native protein, made in our laboratory, using several independent methods (H. Buc *et al.*, manuscript in preparation).[2]

Figure 2 shows the variation of $\lim_{c\to 0} M_{app}(1-\bar{v}^*\rho)$ as a function of the polypeptide chain molecular weight. Within the precision of the measurements, the points fall on a straight line passing through the origin. These results show that, under the conditions used in this study, the value of $\lim_{c\to 0} M_{app}(1-\bar{v}^*\rho)$ for the six proteins are proportional to the polypeptide chain molecular weight. This can be written as

$$\lim_{c\to 0} M_{app}(1-\bar{v}^*\rho) = 0.162M$$

where M is the polypeptide chain molecular weight.

Application of the Method to the Measurement of the Glutamic Dehydrogenase Protomer. The $\lim_{c\to 0} M_{app}(1-\bar{v}^*\rho)$ for the glutamic dehydrogenase protomer has been measured at three protein concentrations. The Archibald runs were performed at 21,740 rpm. The result is

[2] Also E. H. Fischer, personal communication.

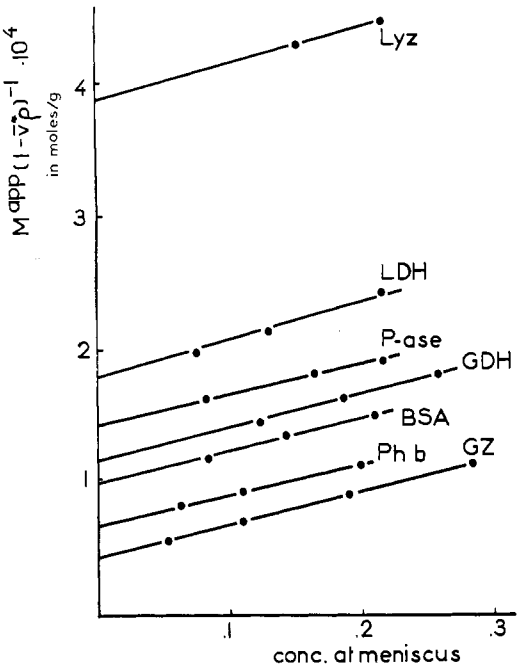

FIGURE 1: $1/M_{app}(1 - \bar{v}^*\rho)$ values as a function of protein concentration. Concentrations are expressed in centimeters squared measured on the photographic plates. The conversion of the areas into protein concentrations (milligrams per milliliter) can be only approximate, because the specific extinction coefficients of the various proteins in 6 M guanidine hydrochloride are not known. Assuming that the extinction coefficients reported for aqueous solutions do not differ widely from those in guanidine hydrochloride, 0.1 cm^2 would correspond to 3 mg/ml of protein. Abbreviations: Lyz, lysozyme; LDH, lactic dehydrogenase; P-ase, alkaline phosphatase; GDH, glutamic dehydrogenase; BSA, bovine serum albumin; Ph *b*, phosphorylase *b;* and GZ, β-galactosidase.

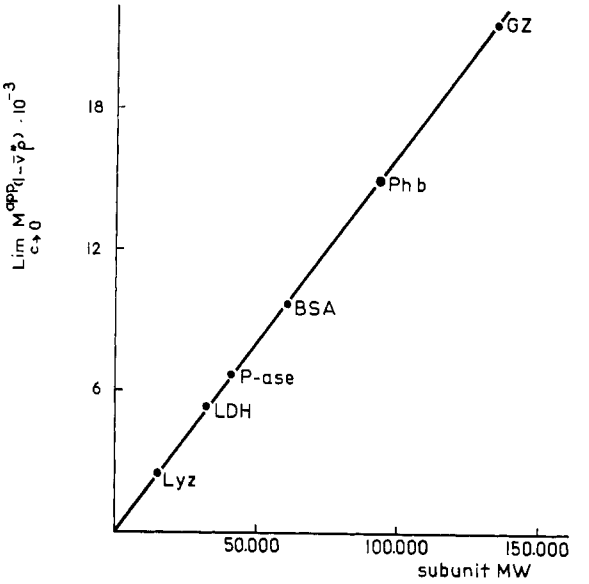

FIGURE 2: Plot of $\lim_{c\to 0} M_{app}(1 - \bar{v}^*\rho)$ *vs.* subunit molecular weight. The coordinates of each point correspond to the values reported in Table I and are expressed in grams per mole. The abbreviations used are the same as for Figure 1.

shown in Figure 1. Extrapolation to zero protein concentration yields

$$\lim_{c\to 0} M_{app}(1 - \bar{v}^*\rho) = 8450 \text{ g}$$

From eq 1 one obtains $M = 52{,}500$.

The molecular weight of the glutamic dehydrogenase protomer was estimated by Marler and Tanford (1964) (using sedimentation equilibrium in 6 M guanidine hydrochloride) to lie between 44,000 and 53,000 depending on the value assumed for $\bar{v}$. Assuming $\bar{v} = 0.73$, they calculate a molecular weight of 53,000, in excellent agreement with our determination.

Discussion

Within the range of experimental errors, six different polypeptide chains from various biological sources and ranging from 15,000 to 135,000 in molecular weight behave as if their partial specific volumes, degrees of hydration, and degrees of solvation were the same in 6 M guanidine hydrochloride. However, the amino acid compositions of these proteins do not vary so much as to cause important variations of the partial specific volumes $\bar{v}_2$ of the proteins in dilute buffer (0.720–0.737 cc/g; see below). For some proteins, $\bar{v}_2$ can vary in quite a wide range: between 0.69 and 0.75 cc/g according to McMeekin and Marshall (1952). Such extreme values for $\bar{v}_2$ are likely to affect the proportionality between $\lim_{c\to 0} M_{app}(1 - \bar{v}^*\rho)$ and M. It now will be shown how the variations of $\bar{v}_2$ can be taken into account.

The solution which is centrifuged must be considered as a multicomponent system; therefore the direct measurement of the transport coefficients (*i.e.*, sedimentation and diffusion coefficients) of each component becomes very complex. However, the use of the Archibald method introduces an important simplification. Instead of measuring the ratio of the transport coefficients, one measures an equilibrium property of the system as proven by Williams *et al.* (1958; p 781), eq 122–124. Then, for the mathematical description of the system, Archibald and equilibrium experiments are equivalent.

If one considers that the solution is a three-component system (component 1, water; component 2, protein; and component 3, guanidinium hydrochloride) one can use the derivation of Williams *et al.* (1958; pp 728–730) leading to their eq 46.

$$\lim_{c_2\to 0} M_{2app} = M_2\left(1 + \gamma'\frac{1 - \bar{v}_3\rho}{1 - \bar{v}_2\rho}\right)\left(1 + \gamma'\frac{\phi_3}{\phi_2}\right) \quad (2)$$

where γ' is the solvation coefficient and ϕ_i the differential refractive index increment of the ith component; $\bar{v}_i$ is the partial specific volume in dilute buffer of the ith component which, for the protein, can be calculated from the amino acid composition, according to McMeekin and Marshall (1952).

Equation 2 is the basic equation which we shall use for further discussion. As a first approximation one may assume that

$$\alpha = \left(1 + \gamma' \frac{1 - \bar{v}_3\rho}{1 - \bar{v}_2\rho}\right)\left(1 + \gamma' \frac{\phi_3}{\phi_2}\right)$$

is the same for all proteins once unfolded by guanidine hydrochloride.

It then becomes possible to correct the experimental value of $\lim_{c\to 0} M_{app}(1 - \bar{v}^*\rho)$ by writing the following equation

$$\lim_{c_2\to 0} M_{2app} = \frac{\lim_{c\to 0} M_{app}(1 - \bar{v}^*\rho)}{(1 - \bar{v}_2\rho)}$$

where $\bar{v}_2$ is the partial specific volume of the protein computed from the amino acid composition, and ρ is the density of the guanidine hydrochloride solution.

The results of the experiments described above have been reexamined in that way and are shown in Table II. When plotted *vs.* the molecular weight of the protomers these values fit very nicely a straight line with a maximum deviation of less than 2%. This result strongly supports the assumption that α does not vary significantly from protein to protein.

The expression of α shows that it will depend on $\bar{v}_2$, ϕ_2, and γ', $\bar{v}_3$, ϕ_3, and ρ being the same for a guanidine solution of given concentration.

TABLE II: Physical Properties of Different Protein Subunits.

Protein[a]	$\bar{v}_2$	$\lim_{c_2\to 0} M_{2app}$
Lysozyme	0.720	14,600
Alkaline phosphatase	0.731	42,400
Serum albumin	0.734	65,500
Phosphorylase	0.737	96,800
β-Galactosidase	0.727	134,000

[a] The partial specific volume of rabbit muscle lactico dehydrogenase has not been calculated because the amino acid composition of this protein was not available. The $\bar{v}$ of serum albumin was taken from McMeekin and Marshall (1952). The $\bar{v}$ of the other proteins was calculated from the amino acid compositions described in the following references: lysozyme, Jollès *et al.* (1963); alkaline phosphatase, Rothman and Byrne (1963); phosphorylase, E. Fischer (private communication); and β-galactosidase, Craven *et al.* (1965).

Since the slope of the plot of $\lim_{c_2\to 0} M_{2app}$ *vs.* M_2 is virtually equal to 1, we must conclude that γ' is close to zero indicating very little preferential binding of either guanidine or water onto the protein. This is in good agreement with the results of Kirby Hade and Tanford (1967). Therefore, for the present purpose, the partial specific volume of the polypeptide must be identical whether in water or guanidine hydrochloride.

That these considerations may apply to proteins having partial specific volumes very different of those of the six proteins examined above appears justified by the results of Reithel *et al.* (1964). In conditions roughly similar to ours, they find that the apparent molecular weight of ribonuclease measured in 6 M guanidine hydrochloride is identical with its real molecular weight provided one uses as $\bar{v}$ the value 0.700 found in dilute buffer solutions. This shows that, even for a protein having such an unusually low partial specific volume

$$M_2 = \frac{\lim_{c\to 0} M_{app}(1 - \bar{v}^*\rho)}{1 - \bar{v}_2\rho}$$

Moreover, the slope of the plot of $1/M_{2app}$ *vs.* protein concentration found by Reithel *et al.* is again close to those shown in Figure 1 of the present paper, which lie around $1.5.10^{-3}$ cc/g^2.

The interpretation of these slopes is not straightforward. If one considers that they represent twice the second virial coefficient B, the experimental value we obtain for B is $B = 7.5 \times 10^{-4}$ mole cc/g^2.

According to Tanford (1963; Table 12-1, p 210), this is about the value expected for a flexible polymer in a good solvent. The same author (pp 220–221) shows that for a flexible polymer B should vary with the molecular weight M of the polymer, roughly as $1/M^x$, with $0.05 < x < 0.25$, the value of x depending on the assumptions made for the polymer. All we can say is that, taking into account the experimental imprecision in the determination of the slope, our results are compatible with a low value of x.

From the above discussion it appears that it is possible to determine experimentally the molecular weight of a polypeptide chain in 6 M guanidine hydrochloride. This is extremely useful, mainly for measuring the number of protomers contained in an oligomeric enzyme.

However, for the measurement to be correct, the protein solution in guanidine hydrochloride has to be homogeneous in molecular weight; it can therefore not be used for proteins made of different kinds of subunits, unless the different types of polypeptide chains are previously separated from each other.

Summary

Using six different proteins of well-established molecular weights and subunit structures whose partial specific volumes range between 0.720 and 0.737 cc/g, it has been shown that as a first approximation the molecular weights of the polypeptide chains can be measured in

6 M guanidine hydrochloride by using the simple formula

$$M = 6.17 \frac{\lim_{c \to 0} M_{app}(1 - \bar{v}^* \rho)}{1 - \bar{v}_2 \rho}$$

where $\bar{v}_2$ is the partial specific volume of the protein computed from the amino acid composition. The applicability of this method to proteins whose $\bar{v}_2$ values are more extreme is discussed.

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