DETERMINATION OF MOLECULAR WEIGHTS AND FRICTIONAL RATIOS OF MACROMOLECULES IN IMPURE SYSTEMS: AGGREGATION OF UREASE*

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The chromatography of macromolecules on column beds composed of porous gel material, a process most commonly termed gel filtration, has for several years provided a convenient method for separation of solutes by means of relative molecular size. Because the proteins commonly used in studies of the mechanism of gel filtration possess quite similar frictional ratios and partial specific volumes, it has been difficult up to this time to ascertain whether the elution positions of the solutes could be correlated with parameters more clearly defined than molecular "size". Some workers have presented empirical correlations of elution volume with molecular weight (Andrews, 1962, 1964; Whitaker, 1963; Andrews, et al, 1964), while others have found the data to correlate best with Stokes radius (Ackers, 1964; Laurent and Killander, 1964).

Using a pair of proteins of widely different frictional ratios, fibrinogen and ferritin (iron complex), we have been able to demonstrate that elution position on columns of the dextran gel Sephadex G-200 (Pharmacia) does not correlate with molecular weight. Thus, fibrinogen, which has the larger molecular radius, is eluted first from the column, while ferritin, which has the larger molecular weight, is eluted later. Moreover, using a series of seven proteins, we have found an excellent correlation between elution position and Stokes radius. The equations relating elution volume to molecular radius proposed on various theoretical grounds by Porath (1963), Laurent and Killander (1964), and Ackers (1964) all accomodate the

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experimental data with a high degree of accuracy. The correlation appears to be valid for molecules with Stokes radius at least as large as that of fibrinogen (107 Å). The data upon which these conclusions are based will be published elsewhere.

Since the equation of Ackers (1964) possesses a single arbitrary constant, the chromatography of a single macromolecule of known Stokes radius, together with a substance which is completely excluded from the gel grains, is all that is necessary to calibrate a given column. A calibrated column may be used for estimation of the molecular radius of a macromolecule present in impure form, even in unfractionated tissue extracts, provided a specific assay for that protein is available. When the Stokes radius, $\underline{a}$, of such a molecule is combined with the sedimentation coefficient, $s_{20,w}$ (which may be obtained for enzymes in impure systems by means of density gradient centrifugation (Martin and Ames, 1961)), and the partial specific volume, $\bar{v}$ (for which a value may usually be assumed), one may obtain both the molecular weight, M, and the frictional ratio, f/f_o, of the macromolecule by means of equations (1) and (2):

$$M = 6\pi\eta\, \underline{a}/(1-\bar{v}\rho) \qquad (1)$$

$$f/f_o = \underline{a} \Big/ \left(\frac{3\bar{v}M}{4\pi N}\right)^{1/3} \qquad (2)$$

where η = viscosity of water at 20°, ρ = density of water at 20°, N = Avagadro's number.

In the case of enzymes, the technique of gel filtration permits the determination of diffusion constants ($D_{20,w} = kT/6\pi\eta\, \underline{a}$, where $\underline{a}$ is calculated from the elution volume, $\underline{k}$ is the Boltzman constant, $\underline{T}$ is the absolute temperature, η is the viscosity of water at 20°C.) at very low protein concentrations, so that extrapolation to zero concentration is probably unnecessary. Sucrose density gradient centrifugation provides a similar advantage for the determination of $s_{20,w}$ values at low concentration. By the combination of these techniques, we have obtained values for the molecular parameters for the enzyme sulfite reductase in a 4-fold purified preparation from Salmonella typhimurium and for the three hydroxylamine reductases found in crude extracts of Neurospora crassa (Siegel, et al, 1965). Rogers, et al, (1965) have used a combination of gel filtration and ultracentrifugation using a fixed partition cell to determine the molecular weight and f/f_o of glutamic dehydrogenase in dilute solution.

An additional use of gel filtration is in the analysis of polydisperse

enzymes. Creeth and Nichol (1960) have reported, from ultracentrifuge studies of crystalline urease, the presence of three components possessing $s_{20,w}$ values of 19, 28, and 36s. The distribution of protein among these components was not affected by dilution, temperature change, or standing, indicating that the species involved are not in rapid equilibrium. In the presence of sulfite, samples which previously gave three ultracentrifugal peaks exhibited only the one at 19s, indicating that the species are aggregates held together by intermolecular disulfide bonds. Creeth and Nichol found, as would be expected, that diffusion experiments gave much lower values for D_{20} of urease in the absence of sulfite than in its presence. By the use of the deviation plot of Creeth (1958) it was

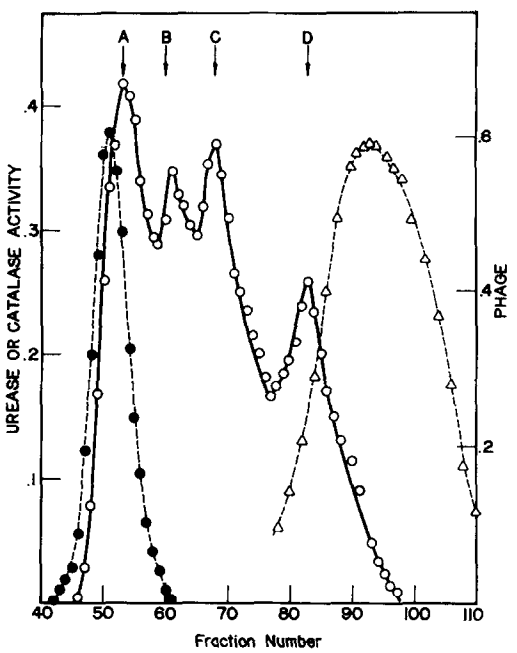

Figure 1. Chromatography of Crystalline Urease on Sephadex G-200. A 1.5 ml. sample containing 0.1 mg. of crystalline urease was applied to a 48 x 3 cm. column of Sephadex G-200 equilibrated with 0.04 M Phosphate-0.005 M EDTA buffer, pH 8.0. Elution was performed with the same buffer and fractions of 1.59 ml. each were collected. In a previous experiment, a 1.5 ml. sample containing 3 x 10^{11} P-22 (mutant H-5) bacteriophage and 0.1 ml. of crystalline catalase (Worthington) were chromatographed on the same column. Urease activity in the eluted fractions was measured in 0.02 ml. aliquots by the method of Gorin, et al (1962). Ammonia was determined using the Berthelot reaction (Brown, et al (1957)), rather than by Nesslerization, and the absorbancy at 630 mμ resulting from incubation of substrate in the absence of protein was subtracted from all readings. Catalase was determined by the method of Beers and Sizer (1952). Symbols and units are as follows: P-22 phage •– –•, absorbancy at 260 mμ; catalase △– –△, ΔE_{240}/min./0.01 ml.; and urease o——o, ΔE_{630}/15 min./0.02 ml.

possible to detect the presence of great heterogeneity in the absence of sulfite, but no estimate of the D_{20} values for the individual components was possible by means of existing techniques. Thus it was not possible with existing techniques to define precisely the degree of aggregation represented by each of the ultracentrifugal species of urease observed by Creeth and Nichol.

The technique of gel filtration has provided the means for determining the Stokes radii for the component species of urease. A sample of crystalline urease (0.1 mg., Sigma Chemical Co., Type C-1) was applied to a column of Sephadex G-200 which had previously been calibrated using catalase and the Salmonella phage P-22. The latter marker served to establish the void volume of the column. The equivalent pore size of the gel (the arbitrary constant in the equation of Ackers (1964)) was 199 Å by reference to catalase (K_d = 0.264).

The elution pattern of crystalline urease is presented in Figure 1. Four peaks of activity may be seen at eluted volumes corresponding to K_d values of 0.016, 0.063, 0.106, and 0.201. These peaks have been termed "A", "B", "C", and "D", respectively, in order of elution. The Stokes radius corresponding to each peak activity was calculated by the method of Ackers (1964). Employing equation (1), these new data were combined with $s_{20,w}$ and $\bar{v}$ values obtained from the literature (Creeth and Nichol, 1960; Sumner, et al., 1938) assuming peak "D" to be the monomeric form of urease. The values of M and f/f_o obtained in this way are presented in Table 1.

Table I

Molecular Parameters of Urease

Peak	Stokes Radius (Å)	$s_{20,w}$ (x 10^{13} sec.)	Calculated Molecular Weight	Calc. f/f_o	Mol. Wgt. 483,000
A	122	—	———	——	——
B	92	36	1,395,000	1.25	2.9
C	79	28	932,000	1.23	1.9
D	61	19	487,000	1.18	1.0

Chromatography of urease was performed as described in Figure 1. Stokes radii were determined by the method of Ackers (1964), with catalase as calibration standard. $S_{20,w}$ values are those reported by Creeth and Nichol (1960). Molecular weights and frictional ratios were calculated by means of equations (1) and (2) in the text, using $\bar{v}$ = 0.73 (Sumner, et al, 1938; Creeth and Nichol, 1960) for each species.

The calculated molecular weight for the peak "D" species of 487,000 agrees quite well with the molecular weight of 483,000 reported for urease by Sumner, et al., (1938). Furthermore, it is evident that peaks "C" and "B" represent dimer and trimer molecules, respectively. The discrepancy between the calculated and theoretical molecular weights based on this model is less than 4% in each case. The frictional ratio calculated for the urease monomer compares quite favorably with the value of 1.19 reported previously. It is apparent that no great change in shape occurs as the urease monomer aggregates.

Even though no sedimentation data are available, the Stokes radius value corresponding to peak "A" urease is too large to be reasonably that of either a tetramer or pentamer. If a hexamer is assumed, then by means of equation (2), one may calculate a frictional ratio of 1.29 for this component, a value which is in reasonable agreement with those found for the three other species. It is quite possible, of course, that peak "A" represents a mixture of polymeric species which were unresolved on the column.

The high proportion of aggregated species present in the crystalline urease preparation used in these experiments is in contrast to the decreasing proportion of the more rapidly sedimenting species reported by Creeth and Nichol (1960). Our results may be correlated with the high degree of insolubility of the commercial urease preparation employed in these studies. Only 10% of a 1 mg. sample of urease added to 2 ml. of 0.04 M phosphate-0.005 M EDTA buffer, pH 8.0, would dissolve on standing overnight at 5°.

In conclusion, we feel that the results obtained with urease strongly support the utility of gel filtration in the determination of molecular radii of macromolecules present in impure systems. By obtaining in addition an estimate of the sedimentation coefficient and of partial specific volume of such molecules, one may determine molecular weight and frictional ratio in the absence of extensive purification. The utility of such techniques in the study of macromolecules which cannot be obtained in sufficient purity or quantity for the application of classical methods, of genetic effects on protein structure, of conformational changes or aggregation, to mention only a few applications, should be quite evident.

REFERENCES

Ackers, G. K. (1964) Biochemistry 3: 723.
Andrews, P. (1962) Nature 196: 36.

Andrews, P. (1964) Biochem. J. 71: 222.
Andrews, P., R. C. Bray, P. Edwards, and K. V. Shooter (1964) Biochem. J. 92: 630.
Beers, R. F., and I. W. Sizer (1952) J. Biol. Chem. 195: 133.
Brown, R. H., G. O. Duda, S. Korkes, and P. Handler (1957) Arch. Biochem. Biophys. 66: 301.
Creeth, J. M. (1958) J. Phys. Chem. 62: 66.
Creeth, J. M., and L. W. Nichol (1960) Biochem. J. 77: 230.
Gorin, G., E. Fuchs, L. G. Butler, S. L. Chopra, and R. T. Hersh (1962) Biochemistry 1: 911.
Laurent, T. C., and J. Killander (1964) J. Chromatog. 14: 317.
Martin, R. G., and B. N. Ames (1961) J. Biol. Chem. 236: 1372.
Porath, J. (1963) Pure Appl. Chem. 6: 233.
Rogers, K. S., L. Hellerman, and T. E. Thompson (1965) J. Biol. Chem. 240: 198.
Siegel, L. M., F.-J. Leinweber, and K. J. Monty (1965) Fed. Proc. 24: 218.
Sumner, J. B., N. Gralen, and I. Eriksson-Quensel (1938) J. Biol. Chem. 125: 37.
Whitaker, J. R. (1963) Anal. Chem. 35: 1950.