Williams, J. A., and Wolff, J. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1901.
Williams, J. A., and Wolff, J. (1972), *J. Cell Biol. 54*, 157.
Wilson, L. (1970), *Biochemistry 9*, 4999.
Wilson, L., Bryan, J., Ruby, A., and Mazia, D. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 807.
Wolff, J., and Williams, J. A. (1973), *Recent Progr. Horm. Res. 29*, 229.

Molecular Characterization of Proteins in Detergent Solutions†

Charles Tanford,*‡ Yasuhiko Nozaki, Jacqueline A. Reynolds, and Shio Makino§

ABSTRACT: The molecular weight and Stokes radius of a protein in a detergent solution can be determined unambiguously by measurement of sedimentation equilibrium and sedimentation velocity, with analysis of the data by equations appropriate for multicomponent systems. The procedure can be simplified without much loss of accuracy by use of a calculated buoyant density factor, and detergent partial specific volumes required for this calculation have been measured. The Stokes radius can be measured independently by gel exclusion chromatography and the molecular weight can then be determined by use of sedimentation velocity alone, which is of considerable advantage since gel chromatography and sedimentation velocity do not require as high a degree of protein purity as sedimentation equilibrium. A simplified procedure for calibration of gel chromatography columns is described, and the significance and interpretation of the Stokes radius are discussed. The most important procedures have been verified by experimental measurements using the AI apoprotein of human high density serum lipoprotein in several detergent solutions.

This paper describes procedures suitable for the determination of the molecular weight and Stokes radius of proteins in detergent solutions. The procedures are intended primarily for the study of proteins that are only sparingly soluble in simple aqueous media, such as membrane proteins, and for detergent solutions in which these proteins are not grossly denatured. They are of course equally applicable to strong denaturing detergents, such as sodium dodecyl sulfate (SDS),[1] and can be used, for example, for the determination of the molecular weight of glycopolypeptides, which behave anomalously in SDS polyacrylamide gel electrophoresis.

With application to membrane proteins in mind, allowance is made for possible inclusion of bound lipids with detergent-extracted proteins, and methods for obtaining the desired information for incompletely purified proteins are considered. All of the procedures are adapted to minimize the amount of protein required: considerably less than 1 mg is needed for most of the measurements, and the same sample can often be used for more than one measurement. The emphasis on economy of material dictates a modest goal as regards accuracy, and we consider an accuracy of $\pm 5\%$ in molecular weight and Stokes radius as satisfactory.

Materials and Methods

Materials. SDS used for density measurements and for chromatography was synthesized by the method of Emerson and Holtzer (1967). "Lauryl sodium sulfate" (Schwarz/Mann, Orangeburg, N. Y.) was used for sedimentation studies, and it has been our experience that this product is generally similar to pure synthetic SDS. However, the same manufacturer provides a product named "sodium lauryl sulfate," which we have found to be of inferior quality and unsuitable for use in molecular characterization.

Tetradecyltrimethylammonium chloride (TTAC) was purchased from Lachat Chemicals Inc., Chicago Heights, Ill., and twice recrystallized from methanol–ether; sodium deoxycholate and Triton X-100 were the same preparations used previously (Makino *et al.*, 1973); Triton N-101 was provided by Rohm and Haas Co.; other nonionic detergents were purchased from Sigma Chemical Corp., St. Louis, Mo. The commercial detergents were dried in a vacuum desiccator over P_2O_5, and used without further purification.

The preparation of the AI polypeptide chain of human high density lipoprotein is described elsewhere (Reynolds and Simon, 1974).

Methods. Density measurements were carried out using an Anton Paar precision densimeter, Model DMA 02C, at 25.00°, maintained constant to within ±0.006°. Density measurements with this instrument have a precision of 2×10^{-6} g/cm^3, and this is essential if measurements are to be made at sufficiently low concentrations to permit an accurate evaluation of $\bar{v}$ for detergents both below and above the cmc.

Sedimentation equilibrium and velocity measurements were made using a Beckman Model E analytical ultracentrifuge, equipped with photoelectric scanner. A wavelength of 280 nm was employed. Use of the photoelectric scanner makes it possible to use very low protein concentrations: initial protein concentrations for the experiments of Table II were about 0.2 mg/ml. Sedimentation equilibrium measurements are routinely made at several different rotor speeds, and the results are discarded if self-consistent data are not obtained.

Gel exclusion chromatography measurements were made at

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received December 28, 1973.* This work was supported by Research Grants GB-14844 from the National Science Foundation and AM-04576 from the U. S. Public Health Service.

‡ Research Career Awardee, National Institutes of Health, U. S. Public Health Service.

§ On leave of absence from the Faculty of Science, Hokkaido University, Sapporo, Japan.

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; TTAC, tetradecyltrimethylammonium chloride; cmc critical micelle concentration; DOC, sodium deoxycholate.

room temperature, as previously described (Fish *et al.*, 1970).

Procedures and Results

Polypeptide Chain Analysis. It is assumed that prior knowledge of the constituent polypeptide chains of the protein and their approximate molecular weights is available. Ability to account for the molecular weight of a detergent-solubilized protein in terms of an integral number of constituent polypeptide chains is an essential test for self-consistency of the results.

The simplest procedures for polypeptide chain analysis are gel electrophoresis in SDS (Shapiro *et al.*, 1967; Weber and Osborn, 1969; Williams and Gratzer, 1971) and gel chromatography in guanidine hydrochloride (Fish *et al.*, 1969). Both methods require that disulfide bonds be reduced. Both methods are empirical, do not give correct results for glycoproteins with a large carbohydrate content, and may fail on other occasions. If any doubt exists, we use gel chromatography in SDS as an auxiliary tool (Fish *et al.*, 1970) and employ appropriate fractions of the eluate for sedimentation equilibrium and other procedures described in this paper.

Lipid Content. For lipoproteins or membrane proteins it is essential to know whether lipids remain associated with the detergent-solubilized protein. Depending on the composition of the system from which the protein is derived, an analysis for organic phosphorus may suffice to demonstrate the absence of lipids and in some cases even as a measure of bound lipid. When the lipid content is large the distribution among major types is desirable.

Detergent Binding. Measurement of precise binding isotherms over a wide range of detergent concentration is a time-consuming process that is not part of the gross characterization procedures described in this paper. The amount of bound detergent under the experimental conditions is, however, required for some of the procedures, and an approximate figure is needed for interpretation of the Stokes radius for evaluation of the contribution of bound detergent to the effective molecular volume. Some idea of the dependence of binding on detergent concentration is essential even when explicit binding data are not needed. The reason for this is that detergent binding isotherms frequently contain steep cooperative portions where the binding can be greatly altered by very small changes in the concentration of unbound detergent, such as would result, for example, from concentration gradients set up in the course of ultracentrifugation. It is generally desirable to carry out molecular characterization procedures in a region of the binding isotherm where small changes in detergent concentration have little effect.

From this point of view there is an advantage to working above the cmc of the detergent, as the detergent micelles then present act to buffer the concentration of monomeric detergent, and thereby tend to prevent changes in the extent of binding (Reynolds and Tanford, 1970a; Makino *et al.*, 1973; Nozaki *et al.*, 1974). Preliminary data we have obtained with membrane proteins and lipoproteins indicate, however, that this principle cannot be assumed to be universally applicable. For proteins of this type we have observed cooperative increments in binding above the cmc, and such processes may be accompanied by changes in the state of aggregation of the protein as well.

For the purposes of this paper, detergent binding is most conveniently expressed on a weight basis rather than a molar basis: we shall use δ_D to designate g of detergent/g of protein. This quantity should be measured as a function of the free detergent concentration in equilibrium with the protein, over a range sufficient to encompass conditions encountered in all subsequent experiments. Equilibrium dialysis is the most convenient procedure for pure proteins (Steinhardt and Reynolds, 1969), but binding can also be determined as an adjunct of separation methods such as gel chromatography (Makino *et al.*, 1973; Nozaki *et al.*, 1974). Such procedures are usually faster in determining binding above the cmc because the rate of dialysis across a membrane is very slow when the monomeric detergent concentration is nearly the same on the two sides. Purity requirements are also reduced in chromatographic procedures in that only the elution peak containing the protein of interest has to be pure, *i.e.*, binding can be measured as part of a step in a purification procedure.

Binding measurements by any method require a precise analytical method for total detergent (bound plus unbound), and this is easiest if the detergent is available in radioactive form. The protein concentration has to be sufficiently high to allow measurement of bound detergent above the unbound detergent that will always be present in the same sample, and, if a chromatographic procedure is used, the concentration of protein may need to be much above the concentration required to detect the elution position of the protein.

Rigorous Determination of Molecular Weight by Sedimentation Equilibrium. The molecular weight of a protein in any solution can be determined unambiguously by sedimentation equilibrium if the experimental data are treated in accord with the equations appropriate for multicomponent systems (Casassa and Eisenberg, 1964). The protein must be in a high state of purity, and we shall assume that measurements can be made at sufficiently low concentrations to avoid problems from self-aggregation or thermodynamic nonideality. (Linearity of data when plotted according to eq 1 indicates that this condition is fulfilled.) The molecular weight (M) that is measured is the molecular weight of pure protein, *excluding bound detergent and other solvent components*, and it is related to the experimentally determined protein concentration (c) as a function of radial position (r) by the relation

$$(2RT/\omega^2)(\mathrm{d}\ln c/\mathrm{d}r^2) = M(1 - \phi'\rho) \qquad (1)$$

where ω is the radial velocity of rotation, ρ is the density of the solvent, and ϕ' is the *effective partial specific volume*, which includes both the true partial specific volume and the effects of interaction with detergent and other solvent components.

The quantity ϕ' in eq 1 can be measured directly by determining the density of protein solutions that have been dialyzed to equilibrium against a detergent solution of the desired concentration. This method requires great care (*e.g.*, *precise* concentration measurements are required) and demands relatively large amounts of protein.

Use of a Calculated Buoyant Density Factor. A more convenient procedure than measurement of ϕ' is to use a calculated buoyant density factor, as was first done for protein–detergent complexes by Hersh and Schachman (1958). To do this, the factor $M(1 - \phi'\rho)$ of eq 1 is replaced by (Casassa and Eisenberg, 1964)

$$M(1 - \phi'\rho) = M[(1 - \bar{v}_P\rho) + \delta_D(1 - \bar{v}_D\rho) + \Sigma\delta_i(1 - \bar{v}_i\rho)] \qquad (2)$$

where $\bar{v}_P$ is the true partial specific volume of the protein and $\bar{v}_D$ the partial specific volume of detergent *when bound to the protein*. The last term in the equation allows for binding of other substances, bound lipids being the only likely contrib-

TABLE I: Partial Specific Volumes of Detergents in Aqueous Solution at 25°.[a]

	$\bar{v}_D$ (cm³/g)	
	Below cmc	Above cmc
SDS	0.815	0.870
$C_{14}H_{29}NMe_3^+Cl^-$	1.075	1.110
$C_{12}H_{25}NMe_3^+Br^-$		0.97[b]
$C_{16}H_{33}NMe_3^+Br^-$		0.995[b]
DOC	0.778	0.778
Triton X-100		0.908
Triton N-101		0.922
Brij-56[c]		0.955
Brij-58		0.919
Brij-96		0.973
Tween-20		0.869
Tween-80		0.896

[a] Measurements for SDS were made for the buffer solution used for most of the ultracentrifugal data; all other results are for water. The $\bar{v}_D$ value for $C_{14}H_{29}NMe_3^+Cl^-$ above the cmc was also determined in a variety of buffer solutions, and always agreed with the value in H_2O to within ±0.001 cm³/g. [b] Calculated from the experimental result for TTAC by using the experimental difference between the molar volumes of Br^- and Cl^- ions (Noyes, 1964) and Traube's rule for the CH_2 group. [c] This detergent formed hazy solutions.

utors. Since δ_D and any important δ_i are known parameters, eq 2 permits the determination of molecular weight without measurement of ϕ', provided that the $\bar{v}$ values are known. In our experience it is a good approximation to calculate $\bar{v}_P$ on the basis of amino acid composition (Cohn and Edsall, 1943; McMeekin and Marshall, 1952), with allowance for sugar content in the case of glycoproteins (Gibbons, 1972), and to assume that $\bar{v}_D$ and $\bar{v}_i$ are not altered significantly by association with the protein, so that experimental values determined in the absence of protein can be used.

The value of $\bar{v}_D$ for any detergent in water or in a dilute aqueous buffer is readily determined from measurement of the solution density ρ as a function of detergent concentration c, expressed in g/cm³. The $\bar{v}_D$ value for an isolated detergent molecule can be obtained from the limiting slope at $c = 0$

$$\mathfrak{L}(d\rho/dc) = 1 - \bar{v}_D\rho_0 \quad (3)$$

where ρ_0 is the density of pure solvent. If $\bar{v}_D$ is independent of detergent concentration, the effect of increasing concentration is given by the relation[2]

$$d\rho/dc = (1 - \bar{v}_D\rho)/(1 - \bar{v}_D c) \quad (4)$$

The curvature in a plot of ρ *vs.* c that is predicted by this equation is small, and observation of significant curvature therefore indicates that $\bar{v}_D$ depends significantly on detergent concentration.

Typical experimental results are shown in Figure 1, and indicate that ionic detergents undergo a distinct change in slope at the cmc, such that $\bar{v}_D$ in the micellar state is several per cent larger than it is below the cmc. This result agrees with previous work (Corkill *et al.*, 1967), though there are significant differences in the absolute values of $\bar{v}_D$. (Precision densimeters of the type used here were not available when the previous work was done.) We did not detect any change in $\bar{v}_D$ at the cmc for sodium deoxycholate. The cmc values for the commercial nonionic detergents are too low to permit accurate measurement below the cmc, but small changes in $\bar{v}_D$ upon micellization have been observed for nonionic detergents with shorter hydrocarbon chains and correspondingly larger cmc's (Corkill *et al.*, 1967).

[2] Equation 4 follows directly from the definition of ρ and c, with no assumption other than that $\bar{v}_D$ is constant.

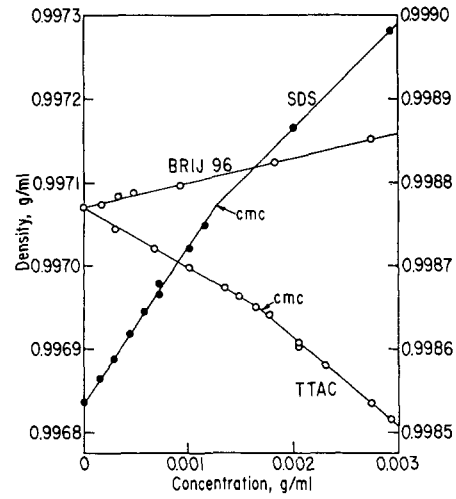

FIGURE 1: Typical data for the determination of partial specific volume. The solvent for TTAC and for Brij-96 was water (densities on left ordinate), and the solvent for SDS was sodium phosphate buffer (pH 8.2), with 0.01 M NaN_3 to a total ionic strength of 0.033 (densities on right ordinate). Most points represent averages of duplicate determinations; the duplicates are shown individually where they differ significantly. Additional points at higher concentrations do not alter the slopes of the lines and have been omitted.

All $\bar{v}_D$ values determined in the course of this study are given in Table I. Since commercial detergents are not homogeneous products, some variation between different lots of the same detergent may be expected. However, we have calculated $\bar{v}_D$ for each of these detergents on the basis of the nominal compositions given by the manufacturer, using Traube's volume additivity rule (Traube, 1899; Cohn and Edsall, 1943). The difference between calculated and observed $\bar{v}_D$ is 0.01 cm³/g or less in each case, suggesting that the commercial products adhere closely to nominal formulas and that Traube's method can be used to calculate their partial specific volumes when precision densimeters for an experimental determination are not available. Since the experimental volumes for these detergents refer to the micellar state, whereas the calculated values are based on volume measurements for unassociated organic molecules, the result suggests that $\bar{v}_D$ for the nonionic detergents listed in Table I does not change significantly upon micellization. This is not unreasonable, since the long polyoxyethylene chains of these detergents (which do not enter the micelle core) represent more than 50% of the molar volume.

There are two possible reasons for the observed change in $\bar{v}_D$ on micelle formation. One arises from the hydrophobic effect: it is generally believed that the arrangement of water molecules around exposed aliphatic groups is accompanied by a constriction (Kauzmann, 1959; Friedman and Scheraga, 1965; Corkill *et al.*, 1967) so that expulsion from the aqueous medium, such as occurs in micelle formation, would be accompanied by an increase in volume. Recent data of Boje and Hvidt (1972), however, question the validity of this belief. For ionic detergents (the only ones for which the change in $\bar{v}_D$ has been unambiguously demonstrated) there is an alternative ex-

TABLE II: Molecular Weight of the AI Apoprotein of Human High Density Lipoprotein, Determined with the Aid of a Calculated Buoyant Density Factor.

Bound Detergent, g/g of Protein	$M(1 - \phi'\rho)$ Exptl	Mol Wt by Eq 2[a] with $\bar{v}_D$ Value: Above cmc	Below cmc
From Sedimentation Equilibrium[b]			
None[c]	7,565	28,400	28,400
SDS, 0.24	8,445	28,400	27,300
SDS, 1.4	12,700	28,300	24,200
TTAC, 1.17	4,290	28,200	23,900
DOC, 0.40	9,510	27,000	
From Sedimentation Velocity and an R_s Value Determined by Gel Chromatography[d]			
SDS, 1.4[e]	12,630	28,200	
SDS, 1.4[f]	12,050	27,200	
DOC, 0.40[f]	9,345	26,600	

[a] $\bar{v}_P$ = 0.734 cm³/g on the basis of amino acid analysis (Baker *et al.*, 1973). [b] $M(1 - \phi'\rho)$ obtained from equilibrium distributions by eq 1. [c] Values between 28,000 and 29,000 have been obtained in this laboratory and elsewhere, in dilute aqueous buffers and in 6 M guanidine hydrochloride. [d] $M(1 - \phi'\rho)$ obtained by eq 7. [e] From s values at several concentrations, extrapolated to zero concentration, giving s^0 = 2.83 S at 25°. Sodium phosphate buffer (pH 8.2) with 0.01 M NaN_3 to a total ionic strength of 0.033. [f] $NaHCO_3$–Na_2CO_3 buffer (pH 9.2), ionic strength 0.10, 24°. Measured s values (not extrapolated) at 0.6 mg/cm³ were 2.54 S in SDS and 2.38 S in DOC.

planation, namely that the detergents are dissociated into their constituent ions below the cmc, whereas the high surface potential in the micellar state leads to extensive association of counterions with the micelle (Mukerjee, 1967) and consequent release of ion-bound water into the solution. The large change in $\bar{v}_D$ for SDS, for example, corresponds to a change in molar volume of 17 cm³, which is of the same order of magnitude as the volume change for formation of H_2O from H^+ and OH^- or for formation of undissociated carboxylic acids from their constituent ions (Edsall and Wyman, 1958). The difference in molar volume between dipolar ions and their uncharged isomers is also of similar magnitude (Cohn and Edsall, 1943). Whichever reason is applicable, it is likely that protein-bound detergents will have $\bar{v}_D$ values close to those in the micellar state when δ_D is large and the detergent molecules or ions are consequently densely concentrated at the protein surface, and this is confirmed by the results of Table II. Since the difference between the $\bar{v}_D$ values below and above the cmc becomes significant in eq 2 only when δ_D is large, we need not be concerned about this problem for proteins associated with only a small number of detergent molecules.

The validity of eq 2 for the calculation of the buoyant density factor has been tested by using the apoprotein AI of human high density lipoprotein. Though highly lipophilic, this protein is soluble in aqueous media without detergents or lipids and its anhydrous molecular weight has been established not only by numerous direct determinations, but also on the basis of the amino acid composition (Baker *et al.*, 1973). The value of $\bar{v}_D$ based on that composition is 0.734 cm³/g. The results are shown in Table II, and it should be noted that they represent a very stringent test of the validity of eq 2 because SDS and TTAC are both bound to this protein in large amount. Moreover, they affect $M(1 - \phi'\rho)$ in opposite directions, one having $\bar{v}_D < 1$, the other $\bar{v}_D > 1$. If apparent molecular weights (ignoring detergent binding) were calculated for the highest levels of binding of these two detergents, they would differ by a factor of 3. Correction for the binding by eq 2 not only abolishes this difference but leads to perfect agreement between the calculated values of M and the best value derived from data in the absence of detergents.

It has not been possible to extend these measurements to the nonionic detergents listed in Table I because (apart from Triton X-100) we do not have them available in radioactive form, so that the determination of δ_D becomes difficult. Triton X-100 contains an aromatic ring and absorbs light at 280 nm, thereby interfering with the use of the uv optics of the ultracentrifuge.

Partial specific volumes of phospholipids depend both on the head group and the hydrocarbon chains attached to them. A value of 0.981 cm³/g has been determined for egg yolk phosphatidylcholine, containing a mixture of saturated and unsaturated hydrocarbon chains (Huang and Charlton, 1971). Estimates of $\bar{v}$ for other phospholipids can be made by using Traube's rule to estimate *differences* in molar volume between phosphatidylcholine and other phospholipids. Such calculations suggest that appropriate $\bar{v}$ values would be 1.015 cm³/g for phosphatidylglycerol, 1.005 cm³/g for sphingomyelin, 0.965 cm³/g for phosphatidylethanolamine, and 0.93 cm³/g for phosphatidylserine. The composition of hydrocarbon chains derived from the constituent fatty acids should not have a significant effect unless there is an exceptionally high content of polyunsaturated or short-chain saturated chains, in which case $\bar{v}$ might be reduced by at most 0.01 cm³/g. Cholesterol has $\bar{v}$ = 0.95 cm³/g in aggregated form. Values calculated for nonaggregated molecules in water and in organic solvents are 0.99 and 1.02 cm³/g, respectively (Haberland and Reynolds, 1973).

Sedimentation Equilibria in Solvents of Different Density. A procedure sometimes used in the absence of detergents, when ϕ' can be replaced by the partial specific volume of the protein, is to obtain sedimentation equilibrium data in two or more solvents of different density (Edelstein and Schachman, 1967). Both M and $\bar{v}_P$ can then be determined if the latter is independent of the solvent medium. This procedure cannot be used in conjunction with eq 1 in the presence of detergents, because, as shown by eq 2, ϕ' cannot be independent of solvent density when detergent is bound, even if δ_D is independent of the solvent.

If the amounts of bound detergent and lipid are the same in media of different density and if there are no other preferential interactions, the procedure can in principle be used to obtain the molecular weight M^* of the entire complex, defined as

$$M^* = M(1 + \delta_D + \Sigma\delta_i) \tag{5}$$

and the corresponding partial specific volume

$$\bar{v}^* = \frac{\bar{v}_P + \delta_D\bar{v}_D + \Sigma\delta_i\bar{v}_i}{1 + \delta_D + \Sigma\delta_i} \tag{6}$$

This procedure offers no advantage in general, but it probably becomes the procedure of choice in the study of proteins combined with very large amounts of lipid, or with detergents of low density. Under these circumstances $\phi'\rho$ and $\bar{v}^*\rho$ can both become close to unity in an aqueous buffer, and the approxi-

mate determination of ϕ' by eq 2 becomes inadequate. On the other hand, these are circumstances under which large effects on the sedimentation equilibrium distribution can be obtained by changing the concentration of innocuous salts, so that the present method gains accuracy and reliability. An example is provided by the determination of the molecular weight of a low density lipoprotein containing 20% protein and 80% lipid (Fisher *et al.*, 1971). Sedimentation velocity and diffusion measurements were used, but the principle is the same.

Sedimentation Velocity in the Analytical Ultracentrifuge. The sedimentation coefficient determined in the analytical ultracentrifuge, measured at very low protein concentration or extrapolated to zero concentration, is given by

$$s = \frac{M(1 - \phi'\rho)}{Nf} = \frac{M(1 - \phi'\rho)}{6\pi\eta NR_s} \quad (7)$$

where N is Avogadro's number, η the viscosity of the solvent (including detergent), and f the frictional coefficient. The Stokes radius R_s of the hydrodynamic particle formed by the protein molecule is defined as the radius of a sphere that would have the same frictional coefficient as the particle (Tanford, 1961), *i.e.*

$$f = 6\pi\eta R_s \quad (8)$$

It is important to note that R_s is a property of the whole macromolecular particle, including bound detergent and other solvent components. Unlike molecular weight, it cannot be considered as composed of additive contributions from the anhydrous protein and other thermodynamic components.

Equation 7 can be used to determine R_s from measurement of sedimentation equilibrium and sedimentation velocity under identical conditions, and it should be noted that this does not involve knowledge of the buoyant density factor ϕ'. Equation 7 can also be combined with R_s values obtained by gel chromatography to obtain a molecular weight value. Since s values may under favorable circumstances be measurable for incompletely purified proteins, this method may sometimes be usable when unambiguous results cannot be obtained by equilibrium ultracentrifugation.

Table II illustrates the application of eq 7 to the calculation of the molecular weight of the AI apoprotein of serum high density lipoprotein. The sedimentation velocity of the protein–SDS complex was found to be extremely sensitive to protein concentration: at an ionic strength of 0.033, the observed s at a protein concentration of only 1 mg/ml differed from the extrapolated value at zero concentration by 13%. At an ionic strength of 0.10 the concentration dependence is less, but even here a measurement at 1 mg/ml would not have been adequate. The R_s values determined by gel chromatography were 44 Å in the SDS solutions (same value at both ionic strengths) and 36 Å in DOC.

Zone Centrifugation in Sucrose Gradients. It would be advantageous to be able to determine sedimentation coefficients by following the effective rate of sedimentation in a sucrose gradient in a preparative ultracentrifuge (Martin and Ames, 1961). If the protein under study can be assayed in the presence of other proteins, s values could be obtained with a very small amount of protein and without extensive purification. Since the existence of a specific assay would also permit R_s to be determined by gel chromatography in the presence of other proteins, one might hope to arrive at a molecular weight by eq 7, *i.e.*, the two principal parameters of interest to us might both be obtained with an unpurified extract containing the protein of interest. Attempts to do this, albeit without awareness of the difficulties that are involved, have been made, for example, for the cholinergic receptor protein of the electric eel in solutions of Triton X-100 (Meunier *et al.*, 1972).

In actuality there are formidable difficulties in this procedure. It is, for example, not valid to obtain s values by interpolation, based on the rates of sedimentation of standard proteins with known s values, run simultaneously with the protein of interest. This procedure would be valid only if the buoyant density factors for all proteins used remained in the same ratio to each other at all densities, which is an unattainable condition in a mixture of proteins with different affinities for the detergent. One could employ the tables of integrals provided by McEwen (1967), or some suitable modification thereof, to obtain absolute values of s. The requirement for validity of this procedure can be seen by expressing s in terms of the particle mass and volume given by eq 5 and 6. Equation 7 would then be replaced by

$$s = M^*(1 - \bar{v}^*\rho)/6\pi\eta NR_s \quad (9)$$

and the requirement would be that M^*, $\bar{v}^*$, and R_s are all independent of sucrose concentration. This condition is not necessarily satisfied: a dependence of δ_D on sucrose concentration has been reported for a virus protein in Triton X-100 solution (Simons *et al.*, 1973). Even if the requirement is met, the interpretation of the results would require binding data and these could not be obtained without purification, at least to the extent that the chromatographic or centrifugation fractions containing the protein of interest must be free of contaminating proteins.

Measurement of Stokes Radius by Gel Exclusion Chromatography. The value of R_s can be conveniently determined from the partition coefficient (K_d) of the protein between the pores of a gel column and the mobile fluid in equilibrium with them. The column is first equilibrated with the appropriate detergent-containing solvent and calibrated with standard proteins whose R_s values in that solvent have been measured directly, using sedimentation velocity or viscosity measurements. We have found that R_s is generally a linear function of $\text{erf}^{-1}(1 - K_d)$, as proposed by Ackers (1970),[3] and use this as a basis for a calibration plot. We determine elution position by weight rather than volume for better accuracy. Since detergent micelles are often present in the solutions, the internal volume marker should be a substance not likely to be soluble in the micelles: we have found some DNP-amino acids to be unsuitable.

It is shown in a previous paper (Fish *et al.*, 1970) that the relation between K_d and R_s for two samples of agarose appeared to be independent of the solvent system employed, implying that the solvent has no significant effect on the pore size distribution. We have confirmed this observation for other resins and solvents, as shown for example by the data of Figure 2. Column calibration is thereby greatly facilitated, since it allows the calibration curve obtained in one detergent (or other solvent) to be applied to the determination of R_s in other detergents. Calibration and measurement need not even be carried out on the same column, as parallel columns made from the same lot of resin and similarly packed generally behave identically in terms of K_d values, though column length and internal and void volume markers may vary. On the other hand, column calibration curves may shift when columns are used over a long period, especially if subjected to relatively

[3] Ackers uses the symbol $\text{erfc}^{-1}K_d$ for this function. We believe that $\text{erf}^{-1}(1 - K_d)$ expresses the operational definition of the function more clearly.

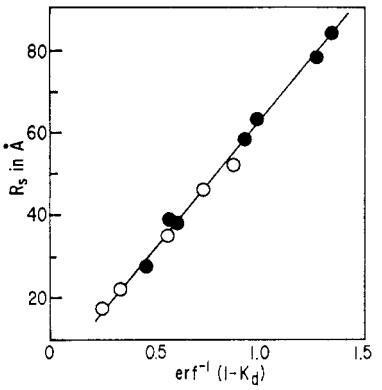

FIGURE 2: Calibration of Sephadex G-200 column with native proteins (○) and with reduced and carboxymethylated polypeptide chains in SDS (●). The proteins used were selected from those listed in Table III and may be identified on the basis of their assigned R_s values.

high pressures, so that periodic checks on the calibration are necessary: it is thus desirable to have one or two standard proteins of known R_s for each detergent employed so as to be able to verify the calibration curve.

Table III lists some proteins suitable for calibration in aqueous buffers and in SDS solution.

Interpretation of Stokes Radius. As noted previously, the Stokes radius is a property of the whole macromolecular particle, including bound detergent, hydration, etc. It is a manifestation of the "shape" of this particle, which obviously cannot be rigorously specified in terms of a single parameter, so that, when a single parameter such as the Stokes radius is used, ambiguities are bound to result, as illustrated by the fact that R_s values determined from viscosity measurements differ from those determined from diffusion or sedimentation velocity by eq 8 (Tanford, 1961). For randomly coiled polymers the difference is 15%; for very long thin fibrous molecules an even larger difference is predicted theoretically[4] and differences up to a factor of 2 have been observed experimentally for linear double-stranded DNA (Eigner and Doty, 1965). Observed differences for native proteins, even when they do not have globular conformations, are usually not larger than 10%. Even thin fibrous proteins never have differences as large as are observed in DNA because proteins do not attain great length: the largest reported difference is 30%, for collagen (Tanford, 1961).

The R_s values determined by gel chromatography depend on the method of calibration: no systematic studies using a mixture of globular and highly asymmetric molecules has been carried out to determine whether partition in the gel is consistently responsive to the R_s value based on either frictional coefficient or intrinsic viscosity. Warshaw and Ackers (1971) have shown that the "partition radius" seen by gel chromatography is not quite consistent with R_s determined from diffusion, but they employed only globular proteins and point out that the small inconsistencies they observed could result from inaccurate measurement of diffusion coefficients. A special problem in the gel chromatography of native proteins is that they may possess affinity for carbohydrates and thus may be retarded by complexing with the resin. Lysozyme is an example, and cannot be used as a calibration substance in aqueous buffer.

On the basis of these considerations it can be expected that the Stokes radius of a protein–detergent complex is a parameter with an inherent ambiguity of less than 10%. Only for highly asymmetric particles, such as are formed by reduced proteins in SDS, is it likely that the ambiguity may exceed this limit. For example, if serum albumin at a binding level of 1.4 g of SDS/g of protein is a rigid prolate ellopsoid (and there is no evidence to suggest that this is necessarily the correct model), it would have an axial ratio of 12.5:1 (Reynolds and Tanford, 1970b), and this would correspond theoretically[4] to a difference of 15% between R_s values from frictional coefficients and viscosity.

As is well known, hydrodynamic measurements alone can

TABLE III: Proteins Suitable for Calibration of Gel Chromatography Columns.

	Stokes Radius, Å	
	Native Proteins in Aqueous Buffers	Reduced Polypeptide Chains in SDS[a]
Cytochrome *c*	17	26
Ribonuclease	17.5	
Lysozyme	*b*	27.5
Myoglobin	19	
Chymotrypsinogen	22	39
Ovalbumin	(30)[c]	58
Hemoglobin	32	27.5
Serum albumin	35	78
Transferrin	36	84
Alk. phosphatase (*E. coli*)	39	58
Aldolase	46	
Immunoglobulin G	51	38[e] 63[e]
Catalase	52	
f_2 Bacteriophage	105[d]	

[a] These R_s values apply to SDS concentrations at which maximal binding (typically 1.4 g/g of protein) is observed. The SDS–protein complexes are asymmetric and R_s values based on viscosity measurements therefore differ from those based on frictional coefficient. It is not known which one of these is closer to the R_s value that determines partition on the gel, and the tabulated values represent averages between the two. They were obtained by taking the viscosity-based R_s values of Fish *et al.* (1970) and *calculating* the R_s to be expected from frictional coefficients by assuming rigid ellipsoid behavior (footnote 4) and using the ellipsoid dimensions given by Reynolds and Tanford (1970b). The R_s values for transferrin and immunoglobulin light chain are interpolated values. [b] Native lysozyme has chemical affinity for the resin polymer and cannot be used. [c] Ovalbumin should have R_s = 27 Å on the basis of its diffusion coefficient, but we have consistently found a higher value by chromatography. Warshaw and Ackers (1971) have reported a similar observation. [d] This radius was determined by electron microscopy. We are grateful to Dr. R. E. Webster for making the virus available to us. [e] The two values are for the light and heavy chain, respectively.

[4] The ratio of R_s (viscosity) to R_s (s or D) is numerically equal to the ratio of the parameter β (or $\phi^{1/3}/P$) of the equation of Mandelkern and Flory (1952) to the value of the same parameter for a perfect unsolvated sphere. Values of β for ellipsoids of revolution have been calculated by Scheraga and Mandelkern (1953).

unequivocally distinguish between particles that are globular (compact and sparingly solvated) and particles that are not globular, but they cannot without auxiliary measurements define the shape of a nonglobular particle (Tanford, 1961). Thus the interpretation of R_s values for proteins in detergent solution is inherently limited to a comparison with the expected R_s value for a globular particle, and for this purpose a 10% ambiguity in the value of R_s is unimportant. To evaluate the expected R_s value for a globular particle we first determine the minimum possible radius of the particle, which would be the radius corresponding to a perfect sphere, containing the known bound detergent and lipid, but no bound water, *i.e.*, in terms of the parameters of eq 2

$$\frac{4}{3}\pi N R_{min}^3 = M(\bar{v}_P + \delta_D\bar{v}_D + \Sigma\delta_i\bar{v}_i) \qquad (10)$$

Since both proteins and detergent micelles normally contain bound water, and since a perfect spherical shape is improbable, R_s values as small as R_{min} are not possible. Thus observed ratios of R_s/R_{min} up to about 1.25 would define a globular particle, as exemplified by globular native proteins or globular detergent micelles.

If $R_s/R_{min} > 1.25$ the protein particle in detergent solution cannot be considered to be globular and a variety of more extended structures are then possible. Examples would be an asymmetric rigid particle, such as has been suggested for reduced proteins in SDS solution (Reynolds and Tanford, 1970b), a complex consisting of two or more globular domains joined by flexible links as in native immunoglobulins in aqueous solutions (Noelken *et al.*, 1965), or a single globular domain joined to a thin rod, as in myosin (Lowey *et al.*, 1969), or to a flexible segment of polypeptide chain. A protein–detergent complex could never exist as a structureless random coil because the apolar part of the detergent would not be stable unless shielded from contact with water.

Other measurements are required to distinguish between the various possibilities that could be compatible with relatively large R_s values. Some simple methods that have been employed for this purpose are considered below.

Auxiliary Methods. Proteolytic enzymes constitute a simple tool of quite general utility to detect the presence of unstructured or exposed links between structured domains. They were crucial to the determination of the gross structures of native myosin and immunoglobulin, to which reference was made in the preceding paragraph. The technique has been used to demonstrate the existence of two-domain proteins in membranes (Spatz and Strittmatter, 1971, 1973) and current work in progress in this laboratory indicates that one of these proteins, cytochrome b_5, retains its two-domain structure when solubilized in pure form in appropriate detergents, the detergent combining exclusively with the hydrophobic domain.

Electron microscopy has proved to be a powerful auxiliary tool on at least one occasion (Laver and Valentine, 1969), and spectral methods are of course as applicable here as they are to other problems in protein chemistry. Interpretation of the results of spectral measurements may, however, be more complicated. The grossly denatured state of proteins in SDS and some cationic detergents is characterized by a high content of α-helical polypeptide chain, and the circular dichroism or optical rotatory dispersion spectra in the peptide absorption region are therefore similar to those of many native proteins (Nozaki *et al.*, 1974). Similarly, the transfer of an aromatic side chain from the interior of a globular protein structure to an exposed position, which in the absence of detergents involves a change in the refractive index of the immediate environment, may not do so in detergent solution since the exposed group may be coated with detergent. Relatively small changes in the circular dichroism associated with the peptide chromophore or relatively small shifts in the aromatic absorption spectrum cannot be used in detergent solutions as strong evidence for relatively small alterations in structure.

Discussion

The procedures described in this paper are intended primarily for application to the study of membrane proteins, many of which are insoluble in aqueous buffer solutions. Even if they can be solubilized in simple aqueous solutions, they may then not be in their native conformations, since the native state is likely to involve intimate interaction with membrane lipids.

Many membrane proteins can be solubilized in biologically active form by use of mild detergents. The detergents may completely replace the lipids with which a protein was originally associated, or the solubilized particle may retain some tightly bound lipid. Sometimes the amount of detergent added allows a choice between these possibilities (Garland and Cori, 1972; Helenius and Söderlund, 1973). Membrane proteins solubilized in this way clearly have a relatively high probability of retaining their native conformations, and this probability can be increased by using detergents that share some of the structural features of natural lipids or by incorporating selected lipids in the protein–detergent complex.

Detergent solutions are thus the most promising medium for the study of membrane proteins in isolation in a state resembling the native state. The methods described in this paper can thus be used to determine the tendency for association between like or unlike polypeptide chains of membrane proteins. Measurements of the Stokes radius and auxiliary data used for its interpretation can assess the likelihood that a given protein may penetrate all the way through a membrane or that it may consist of separate external and membrane-bound domains.

References

Ackers, G. K. (1970), *Advan. Protein Chem. 24*, 343.

Baker, H. N., Jackson, R. L., and Gotto, A. M. (1973), *Biochemistry 12*, 3866.

Boje, L., and Hvidt, A. (1972), *Biopolymers 11*, 2357.

Casassa, E. F., and Eisenberg, H. (1964), *Advan. Protein Chem. 19*, 287.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, pp 157–161, 370–375.

Corkill, J. M., Goodman, J. F., and Walker, T. (1967), *Trans. Faraday Soc. 63*, 768.

Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, New York, N. Y., Academic Press, p 456.

Eigner, J., and Doty, P. (1965), *J. Mol. Biol. 12*, 549.

Emerson, M. F., and Holtzer, A. (1967), *J. Phys. Chem. 71*, 1898.

Fish, W. W., Mann, K. G., and Tanford, C. (1969), *J. Biol. Chem. 244*, 4989.

Fish, W. W., Reynolds, J. A., and Tanford, C. (1970), *J. Biol. Chem. 245*, 5166.

Fisher, W. R., Granade, M. E., and Mauldin, J. L. (1971), *Biochemistry 10*, 1622.

Friedman, M. E., and Scheraga, H. A. (1965), *J. Phys. Chem. 69*, 3795.
Garland, R. C., and Cori, C. F. (1972), *Biochemistry 11*, 4712.
Gibbons, R. A. (1972), *in* Glycoproteins, 2nd ed, Part A, Gottschalk, A., Ed., Amsterdam, Elsevier, p 78.
Haberland, M. E., and Reynolds, J. A. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 2313.
Helenius, A., and Söderlund, H. (1973), *Biochim. Biophys. Acta 307*, 287.
Hersh, R. T., and Schachman, H. K. (1958), *Virology 6*, 234.
Huang, C., and Charlton, J. P. (1971), *J. Biol. Chem. 246*, 2555.
Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.
Laver, W. G., and Valentine, R. C. (1969), *Virology 38*, 105.
Lowey, S., Slayter, H. S., Weeds, A. G., and Baker, H. (1969), *J. Mol. Biol. 42*, 1.
Makino, S., Reynolds, J. A., and Tanford, C. (1973), *J. Biol. Chem. 248*, 4926.
Mandelkern, L., and Flory, P. J. (1952), *J. Chem. Phys. 20*, 212.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
McEwen, C. R. (1967), *Anal. Biochem. 20*, 114.
McMeekin, T. L., and Marshall, K. (1952), *Science 116*, 142.
Meunier, J. C., Olsen, R. W., and Changeux, J. P. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 24*, 63.
Mukerjee, P. (1967), *Advan. Colloid Interface Sci. 1*, 241.
Noelken, M. E., Nelson, C. A., Buckley, C. E., III, and Tanford, C. (1965), *J. Biol. Chem. 240*, 218.
Noyes, R. M. (1964), *J. Amer. Chem. Soc. 86*, 971.
Nozaki, Y., Reynolds, J. A., and Tanford, C. (1974), *J. Biol. Chem.* (in press).
Reynolds, J. A., and Simon, R. (1974), *J. Biol. Chem.* (in press).
Reynolds, J. A., and Tanford, C. (1970a), *Proc. Nat Acad. Sci. U. S. 66*, 1002.
Reynolds, J. A., and Tanford, C. (1970b), *J. Biol. Chem. 245*, 5161.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Amer. Chem. Soc. 75*, 179.
Shapiro, A. L., Vinuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Simons, K., Helenius, A., and Garoff, H. (1973), *J. Mol. Biol. 80*, 119.
Spatz, L., and Strittmatter, P. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1042.
Spatz, L., and Strittmatter, P. (1973), *J. Biol. Chem. 248*, 793.
Steinhardt, J., and Reynolds, J. A. (1969), Multiple Equilibria in Proteins, New York, N. Y., Academic Press, pp 45–50.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, Chapter 6.
Traube, J. (1899), *Samml. Chem. Chem. Tech. Vortr. 4*, 255.
Warshaw, H. S., and Ackers, G. K. (1971), *Anal. Biochem. 42*, 405.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Williams, J. G., and Gratzer, W. B. (1971), *J. Chromatogr. 57*, 121.

Calorimetric Determination of the Heat of Oxygenation of Human Hemoglobin as a Function of pH and the Extent of Reaction†

Donald H. Atha‡ and Gary K. Ackers*

ABSTRACT: Oxygenation of human hemoglobin has been studied by calorimetry at various pH values and buffering conditions. The intrinsic heat of oxygenation was found to be essentially independent of pH in the region of the alkaline Bohr effect, between pH 7.5 and 9.5. A value of -14.1 ± 0.4 kcal/mol of heme was obtained for the intrinsic enthalpy pertaining to total oxygenation in distilled water at 20.0°. Combining this value with the unitary free energy of oxygenation of -7.55 kcal/mol of heme (Imai, K. (1973), *Biochemistry 12*, 798) yields an overall entropy change of -22 eu/heme. Experiments carried out in the presence of phosphate or Tris buffers yielded results indicating the existence of pronounced heat effects, in addition to those associated with the release and buffering of oxygen-linked Bohr protons and the binding of oxygen. These uncorrected heat terms are probably attributable to the endothermic release upon oxygenation of exothermically bound buffer ions. Measurements were also made of the fraction of total heat produced as a function of the fraction of total oxygenation. The results were compared with simulated curves in order to place constraints on the heats of binding to the individual sites of the hemoglobin molecule. It was found that a model in which the heats are evenly distributed could not be distinguished from other distributions representing certain limits of variation in individual site heats but that a number of more widely varying models could be eliminated.

Reaction of tetrameric human hemoglobin with oxygen leads to a sequence of events in which approximately 25–30 kcal/mol of heme of free energy is expended upon binding cooperatively to the four heme sites (Roughton and Lyster, 1965; Imai, 1973). Associated alterations in tertiary and quaternary structure lead to a decrease in free energy of interaction between pairs of $\alpha\beta$ dimers amounting to some 8 kcal/mol of heme (Noble, 1969; Thomas and Edelstein, 1972), while release of oxygen-linked Bohr protons occurs with an increase in free energy of about 6 kcal/mol of tetramer

† From the Department of Biochemistry, University of Virginia Charlottesville, Virginia 22901. *Received January 31, 1974.* Support was provided by Grant GM-14493 from the National Institutes of Health.

‡ This work constitutes part of the dissertation presented by D. H. A. in partial fulfillment of the requirements for the Ph.D. degree.