CHAPTER 5

# Determination of Macromolecular Homogeneity, Shape, and Interactions Using Sedimentation Velocity Analytical Ultracentrifugation

*Stephen E. Harding*

## 1. Introduction

Since its inception by Svedberg and coworkers in the 1920s, the analytical ultracentrifuge has provided a powerful tool in biochemistry and molecular biology, with applications ranging from simple purity checks and particle shape determinations from sedimentation velocity, isolation and purification of macromolecules using density gradient techniques right through to the evaluation of mol wt and mol-wt distributions, thermodynamic second virial coefficients, and association constants using sedimentation equilibrium, *without* the need for calibration standards. By the 1960s, the familiar "Beckman Model E" had become commonplace in biochemical laboratories worldwide.

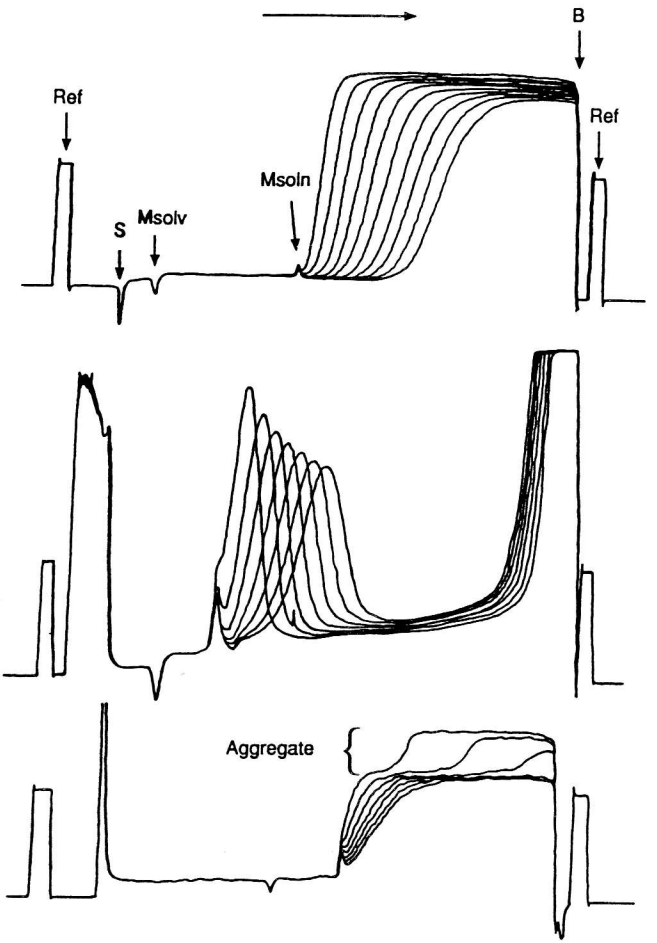
With the advent of the "high-resolution" techniques of X-ray crystallography and nuclear magnetic resonance (NMR) for structural analysis and the relatively straightforward techniques of gel electrophoresis and gel permeation chromatography for purity and mol-wt analysis, the technique suffered a serious decline in popularity in biochemistry and molecular biology with the result that, by 1980, relatively few laboratories had this facility. However, for both structural and mol-wt analysis, the technique is now undergoing a form of renaissance, culminating in the launch of the new Optima XLA

From: *Methods in Molecular Biology, Vol. 22: Microscopy, Optical Spectroscopy, and Macroscopic Techniques* Edited by: C. Jones, B. Mulloy, and A. H. Thomas

Analytical Ultracentrifuge from Beckman Instruments (Palo Alto, CA) *(1)*. The reasons for the revival of analytical ultracentrifugation for mol-wt analysis are outlined in Chapter 6 in relation to the sedimentation equilibrium technique. For *conformational* analysis, this revival is probably the result of a realization among molecular biologists that: (1) not all biological macromolecules can be crystallized; many are available in too small a quantity (e.g., some newly engineered proteins) or are not presently amenable to full structural analysis (for example, intact, immunologically active antibodies). (2) The requirement of very high concentrations for NMR analysis of biological macromolecules—particularly those with a mol wt, $M > 10{,}000$—can lead to serious difficulties in data interpretation. Solution techniques like sedimentation analysis or X-ray scattering (*see* Chapter 4), although of low resolution, may therefore represent for many systems the only realistic "handle" on macromolecular conformation in solution. This chapter is concerned with *sedimentation velocity* analytical ultracentrifugation (i.e., where the speeds are sufficiently high to cause the macromolecule to sediment, and the nature of the form and movement of the boundary between solution and solvent is used to deduce useful information about the macromolecular system).

The basic principle of the technique is as follows: A solution of the macromolecule is placed in a specially designed sector shaped cell with transparent end windows. A mercury arc or similar light source positioned below the rotor transmits light via a monochromator or filter through the solution and then other appropriate optical components. The moving boundary can then be recorded either on photographic film, on chart paper (Fig. 1), or as digital output. For further details of the method, we still refer the reader to the classical texts of Tanford *(2)* and Schachman *(3)*. A useful introductory text is Bowen *(6)*. Introductory chapters are to be found in Price and Dwek *(7)*, van Holde *(8)*, and Rowe *(9)*.

Fig. 1. *(opposite page)* Optical records of sedimentation velocity in the analytical ultracentrifuge obtained from the MSE Centriscan. The direction of sedimentation in each case is from left to right. Top: Sedimenting boundary for a protein (methylmalonyl mutase) recorded using scanning absorption optics. Monochromator wavelength = 295 nm; scan interval = 9 min; rotor speed = 44,000 rpm; temperature = 20.0°C; Loading concentration, c° ~0.7 mg/mL; $s_{20} = (7.10 \pm 0.04)$ *S*.




Ref: Reference marks, allowing calibration of abscissa positions in terms of actual radial displacements from the center of the rotor; S: start of cell position; B: cell base; $M_{solv}$: meniscus position in the solvent cell; $M_{soln}$: meniscus position in the solution cell. Redrawn from ref. *4*. Middle: Sedimenting boundary for a polysaccharide (heat-treated sodium alginate) recorded using scanning schlieren optics. Monochromator wavelength = 546 nm; scan interval = 30 min; rotor speed = 49,000 rpm; temperature = 20.0°C; c°~5.0 mg/mL; $s_{20}$ = (1.22 ± 0.05) S. Lower: Sedimenting boundaries for a DNA binding protein (Gene 5) recorded using scanning absorption optics. Monochromator wavelength = 278 nm; scan interval = 8 min; rotor speed = 40,000 rpm; temperature = 20.0°C; c° ~0.7 mg/mL; $s^{\circ}_{20,w}$ = (35.5 ± 1.4) S (faster boundary) and (2.6 ± 0.1) S (slower boundary). Redrawn from ref. *5*.

## 2. Summary of Information Available

### 2.1. Homogeneity of the Macromolecular Solute

For homogeneity checks, sedimentation velocity can be used to detect the presence of impurities/polydispersity (i.e., components of different mol wt or density not in chemical equilibrium with each other) and the presence of self-association phenomena for a range of concentrations (for example, for a protein, from typically 0.2 mg/mL up to the solubility limit). It is a useful tool for assaying whether the protein solutions are still homogeneous in the very high concentrations often used for NMR.

### 2.2. Sedimentation Coefficient Evaluation

For the determination of sedimentation coefficients, $s^\circ_{20,w}$ we measure the rate of movement of the sedimenting boundary, recorded using refractometric (classical "schlieren" optics) or scanning absorption optics. The $s^\circ_{20,w}$ value by itself is of little interest these days, as is the traditional practice of quoting "frictional ratios" and corresponding equivalent ellipsoid of revolution "axial ratios." The necessary hydrodynamic theories have now been developed, so that the structures of complex macromolecules can be modeled using the sedimentation coefficient and related parameters *(10)*. Sedimentation coefficients have recently been used to distinguish between possible solution models for the immunological complement system *(11)* and *intact, immunologically active* antibodies for which no high-resolution structural information from X-ray crystallography or NMR is available *(12)*.

### 2.3. Frictional Ratio and Derived Parameters

The frictional ratio, $f/f_o$, can be calculated from the sedimentation coefficient and associated parameters *(see* Note 6*)*. Theoretical representations are available linking this with the axial ratios of ellipsoids (both ellipsoids of revolution and general triaxial ellipsoids) and also "bead model" representations for representing the solution conformation of relatively rigid complex structures (if combined with other solution techniques).

### 2.4. Flexibility Parameters

Flexibility parameters include the contour length, $L$, the persistence length, $a$, and the characteristic ratio, $C_\infty$. These, particularly the ratio

of *L/a*, can be useful for representing the conformations of linear biopolymers, such as nucleic acids and polysaccharides *(13)*.

### *2.5. Molecular Weight, M*

The sedimentation coefficient can be used to give an estimate for M directly, after assumptions concerning the conformation. Alternatively, an absolute estimate can be obtained by using the sedimentation coefficient with the translational diffusion coefficient or (to a good approximation) the intrinsic viscosity, to eliminate the effects of particle conformation.

### *2.6. Assay for Self-Association Behavior*

For self-associating or other interacting systems, simple qualitative assays (i.e., detecting whether a system is self-associating or not) and also more complicated quantitative representations in terms of interaction parameters are possible. (For details of all of Sections 2.1.–2.6., the reader is referred to ref. *13)*.

## 3. Availability of Instrumentation

The instrument that is still almost synonymous with analytical ultracentrifugation is the Beckman Model E, although others, such as the MSE (Crawley, UK) Centriscan, have proven popular. Many interesting adaptations of these "classical" machines have been described *(see,* for example, ref. *13)*, and many of these ideas are being incorporated into the Beckman Optima XLA Analytical Ultracentrifuge with the facility for full "on-line" analysis of the data *(1)*.

The Beckman Model E is no longer commercially available, nor is the MSE Centriscan. Second-hand instruments are usually available, but because of the complexity of the instrumentation, the molecular biologist, unless he or she has direct access to the new Beckman Optima XLA ultracentrifuge, is probably advised to consult centers where the expertise/instrumentation is available (e.g., in the US, the National Ultracentrifuge Facility at Storrs, CT, and in the UK, the Nottingham/Leicester Joint Centre for Macromolecular Hydrodynamics).

As far as the author is aware, the Model 3180 Analytical Ultracentrifuge, from the Hungarian Optical Works MOM (Budapest) is also still commercially available, at the time of going to press.

## 4. Materials

### 4.1. Choice of Solvent

It is usual to perform measurements on solutions of biological macromolecules in the presence of a low-mol-wt electrolyte at an appropriate ionic strength (typically 0.1*M* for a protein) to suppress charge effects.

### 4.2. Concentration/Volume Requirements of the Macromolecular Solute

If schlieren optics are to be used with ~10 mm optical path length cells, a minimum amount of 2.0 mg/mL (and ~0.4 mL) is required (lower if long [≥20 mm] optical path length cells are available); for nucleic acids and some proteins, reasonable estimates for the sedimentation coefficient may be possible for concentrations as low as 0.1 mg/mL using the UV absorption optical system. The reader may appreciate here that, compared to sodium dodecyl sulfate (SDS) gel electrophoresis or gel permeation high-pressure liquid chromatography (HPLC), the technique usually requires more material (of the order of milligrams), but much smaller amounts than, for example, NMR or X-ray crystallography. This can represent an important factor when deciding on an appropriate method for the characterization of a newly engineered protein not available in large quantities.

## 5. Methods

### 5.1. Choice of Optical System

The appropriate optical system has to be chosen, either absorption optics at an appropriate wavelength, if your macromolecule has a suitable chromophore (for proteins, conventionally ~280 nm, nucleic acids, 256 nm), or schlieren (refractive index gradient) optics, if concentrations are sufficient (usually ≥2.0 mg/mL) and if no suitable chromophore is present.

### 5.2. Choice of Appropriate Speed

For a globular protein of sedimentation coefficient ~2 Svedbergs (S, where $1S = 10^{-13}$ *s*), a rotor speed of 50,000 rpm will give a measurable set of optical records after some hours. For larger macromolecular systems (e.g., 12S globulins or 30S ribosomes), speeds of <30,000 rpm are appropriate.

### *5.3. Choice of Appropriate Temperature*

The standard temperature at which sedimentation coefficients are quoted is now 20.0°C (sometimes 25.0°C). If the macromolecule is thermally unstable, and depending on the nature of the macromolecule and the conditions chosen (a sedimentation velocity run can take between 1 and 12 h), temperatures down to ~4°C can be used without difficulty.

### *5.4. Sedimentation Coefficient Measurement*

If a sedimentation coefficient (symbol "s") is desired, repeat for several concentrations, *c*, unless a low enough concentration can be used so that concentration effects are negligible (usually ≤0.5 mg/mL for a protein, but care has to be expressed if there is the possibility of association/dissociation phenomena).

### *5.5. Sedimentation Coefficient Calculation*

The *s* value is obtained as the rate of movement of the boundary per unit gravitional field: $s = (dr/dt)/\omega^2 r$, where *r* is the radial position of the "2nd moment" of the boundary (effectively the boundary center for most applications) and ω the angular velocity in rad/s.

### *5.6. Correlation to Standard Conditions*

For each concentration, correct the sedimentation coefficient to standard conditions (water at 20°C): symbol "$s_{20,w}$" using formulae given in, e.g., Tanford *(2)*. Knowledge of a parameter known as the "partial specific volume" (essentially the reciprocal of the anhydrous macromolecular density) is needed; this can usually be obtained from standard tables or, for proteins, can be calculated from amino acid composition data *(14)*.

### *5.7. Extrapolation to Zero Concentration*

Plot $s_{20,w}$ vs *c* (the latter corrected for "average radial dilution" because of the sector design of the cell centerpiece) and extrapolate (usually linearly) to zero concentration (Fig. 2) to give a parameter, $s^\circ_{20,w}$, which can be directly related to the frictional properties of the macromolecule (the so-called "frictional ratio") and from which size and shape information may be inferred. If the macromolecule is very asymmetric or solvated, plotting $1/s_{20,w}$ vs *c* generally gives a more useful extrapolation *(9)*.

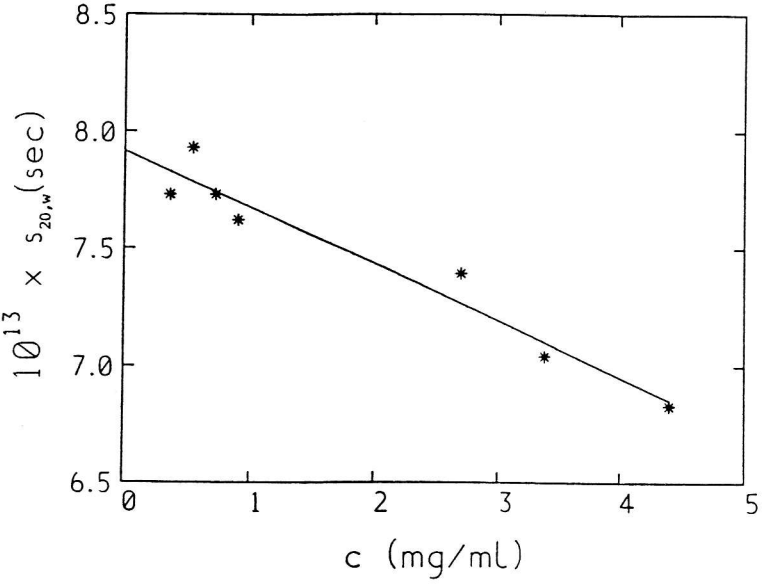



Fig. 2. Sedimentation coefficient $s_{20,w}$ vs concentration plot for an antibody (Rat IgE). $s°_{20,w}$ = (7.92 ± 0.06) S.

## 6. Notes

1. How do we infer homogeneity? The presence of more than one sedimenting boundary (Fig. 1, bottom) can demonstrate either polydispersity or self-association phenomena. However, the converse is not necessarily true: A single sedimenting boundary is not in itself *conclusive* proof of sample homogeneity *(15)*.
2. The downward slope in a plot of $s_{20,w}$ vs concentration is a result of nonideality behavior and is characterized by the parameter $k_s$ in the equation:

$$s_{20,w} = s°_{20,w}(1 - k_s c) \quad (1)$$

3. $k_s$ (mL/g) itself is quite a useful parameter. The ratio $k_s/[\eta]$ (where $[\eta]$ is the intrinsic viscosity [mL/g] of the macromolecule), the so-called "Wales-van Holde ratio," is a function of macromolecular conformation having a value of ~1.6 for spheres and random coils, and somewhat less for extended conformations *(see*, e.g., *16)*.
4. $s°_{20,w}$ can be combined with the translational diffusion coefficient (again corrected to standard conditions), $D°_{20,w}$—the latter usually obtained from quasi-elastic light scattering (QLS) measurements *(see* Chapter 8)—to give an absolute value for the mol wt, M, via the Svedberg equation *(2)*.

5. For a polydisperse material, if $s^\circ_{20,w}$ is a weight average and $D^\circ_{20,w}$ from QLS a $z$-average, the M will be a *weight average* ($M_w$). It is also possible to evaluate a sedimentation coefficient distribution and, if a reasonable assumption about the conformation can be made, a mol-wt distribution *(17)*.
6. If M is known (e.g., for a protein from amino acid sequence data), together with a good idea of the "hydration" of the macromolecule (i.e., a measure of the amount of solvent—both chemically bound and physically entrapped—associated with the macromolecule), $s^\circ_{20,w}$ can provide a useful handle on macromolecular conformation.

   Specifically, $s^\circ_{20,w}$ can be used to evaluate the "frictional ratio," $f/f_o$ (where $f$ is the frictional coefficient and $f_o$ is the frictional coefficient of an anhydrous* spherical particle having the same mass and $\bar{v}$ as the macromolecule under consideration):

$$[(f/f_o)] = \{M(1 - \bar{v}p)]/[N_A \cdot (6\pi\eta_o s^\circ_{20,w})]\}[(4\pi N_A/3\bar{v}M)]^{1/3} \quad (2)$$

   $N_A$ is Avogadro's number, and $\eta_o$ is the solvent viscosity (in this case water at 20.0°C); $f/f_o$, in turn, is a function of the hydration of the macromolecule, $w$, and the conformation via a particle shape factor known as the "Perrin function," $P$ (or "frictional ratio owing to shape"):

$$[(f/f_o)] = P \cdot [(w/\bar{v}\rho_o) + 1]^{1/3} \quad (3)$$

   If the macromolecule is fairly rigid, $P$ can be related directly to the axial dimensions of the macromolecule using ellipsoid of revolution or general triaxial ellipsoid representations of the data *(18)*, or perhaps more usefully, by representing the solution structure of the macromolecule in terms of arrays of spherical beads, its measurement enables complex molecules like antibodies or bacteriophages to be modeled *(10)*. An example of this is shown for two forms of a T-even bacteriophage in Fig 3. It should be stressed that, realistically, for complex modeling of this sort, a good starting estimate for the solution conformation has to be known (from, e.g., electron microscopy or X-ray crystallography) because of uniqueness problems, and for *any* type of modeling using $s^\circ_{20,w}$, a reasonable assumption about the degree of "hydration" of the protein has to be made *(10)*. A survey of 21 proteins *(19)* has shown that, for proteins, values for the hydration can vary quite markedly (for this range, $w = (0.53 \pm 0.26)$ g $H_2O$/g protein). For some glycoproteins,

*In the literature, the convention is sometimes chosen so that $f_o$ refers to the *hydrated* spherical particle: In this case Eqs. (2) and (3) have to be modified accordingly, but both treatments are of course equivalent.

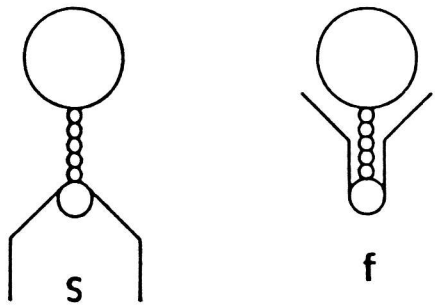



Fig. 3 Hydrodynamic bead model for a T-even bacteriophage based on sedimentation coefficient and translational diffusion coefficient data on slow (s) and fast (f) forms ($s°_{20,w}$ ~710 S and 1020 S, respectively). Only two of the six tailfibers (each of which is modeled by 64 small beads) are shown. The calculated frictional ratios based on these models are in agreement with the measured frictional ratios determined from the sedimentation coefficient or translational diffusion coefficient, after allowance for hydration. (From ref. *10* and refs. cited therein).

this can be an order of magnitude higher. In this respect, the more complicated the models chosen, the more essential it is to have supporting information from further hydrodynamic, scattering, or thermodynamic data (e.g., intrinsic viscosity, rotational diffusion coefficients, radius of gyration, or the thermodynamic second virial coefficient *[18]*).

For polydisperse macromolecular systems, such as polysaccharides, proteoglycans, glycoproteins, and nucleic acids, the dependence of $s°_{20,w}$ with M can give a useful guide to the general conformation (between the extremes of compact sphere, rigid rod, and random coil) and also provide flexibility information as indicated earlier *(see*, e.g., ref. *20)*.

7. A plot of $s_{20,w}$ vs concentration may show an initial upward tendency before dropping. The initial upward tendency if present is symptomatic of self-association/dissociation behavior. It is possible to model this behavior to obtain an association constant($s$) *(21)*.
8. The technique can be used in a very simple way for investigating interactions in mixed solute systems (Fig. 4).

## Glossary of Symbols

M, Mol wt (g/mol); $M_w$, Weight average mol wt; S, Svedberg unit ($1S = 1 \times 10^{-13}$ s); $s$, Sedimentation coefficient measured at a finite sedimenting (i.e., corrected for radial dilution) concentration. Unit: S or $s$; $s_{20,w}$, Sedimentation coefficient at a finite sedimenting (i.e., cor-

Fig. 4. Cosedimentation diagram for methyl-malonyl mutase and its cofactor (offset toward the top) scanned within 2 min of each other. The center of the sedimenting boundary is virtually the same for both, confirming that under the solvent conditions used (50 m*M*-Tris/HCl, pH 7.5 + 5 m*M* EDTA) the cofactor is bound to the protein. These scans were obtained from an MSE Centriscan, monochromator wavelength = 295 nm (bottom), 608 nm (top); rotor speed = 44,000 rpm; temperature = 20.0°C, $c^\circ$ = 0.7 mg/mL.

rected for radial dilution) concentration, $c$, and corrected to standard solvent conditions (i.e., water as solvent at a temperature of 20.0°C); $s^\circ_{20,w}$, Infinite dilution (i.e., $c = 0$) sedimentation coefficient; $D^\circ_{20,w}$, Infinite dilution translational diffusion coefficient ($cm^2/s$); $k_s$, Sedimentation concentration dependence regression parameter (mL/g); $(f/f_o)$, Frictional ratio; $P$, "Perrin function" or "frictional ratio owing to shape"; $L$, Contour length (nm or cm); $a$, Persistence length (nm or cm); $C_\infty$, Characteristic ratio; I, Ionic strength (mol/L or mol/mL); $[\eta]$, Intrinsic viscosity (mL/g); $\bar{v}$, Partial specific volume (mL/g); $w$, Hydration (g $H_2O$/g macromolecule); $\omega$, Angular velocity (rad/$s$); $r$, Radial displacement from the center of the rotor (cm).

## References

1. Schachman, H. K. (1989) Analytical ultracentrifugation reborn. *Nature* **341**, 259–260.
2. Tanford, C. (1961) *Physical Chemistry of Macromolecules*. Wiley, New York.
3. Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry*. Academic, New York.

4. Marsh, E. N., Harding, S. E., and Leadley, P. F. (1989) Subunit interactions in *Propionibacterium shermanii* methylmalonyl-CoA mutase studied by analytical ultracentrifugation. *Biochem. J.* **260,** 353–358.
5. Kneale, G. G. (1989) Hydrodynamic and fluorescence analysis of a DNA binding protein and its interaction with DNA, in *Dynamic Properties of Biomolec-ular Assemblies* (Harding, S. E. and Rowe, A. J., eds.), Royal Society of Chemistry, Cambridge, UK, pp. 171–178.
6. Bowen, T. J. (1970) *An Introduction to Ultracentrifugation.* Wiley-Interscience, London.
7. Price, N. C. and Dwek, R. A. (1978) *Principles and Problems in Physical Chemistry for Biochemists.* Clarendon, Oxford.
8. Van Holde, K. E. (1985) *Physical Biochemistry.* Prentice Hall, Englewood Cliffs, NJ.
9. Rowe, A. J. (1984) Techniques for determining mol wt. *Protein Enzyme Biochem.* **BS106,** 1–37.
10. Garcia de la Torre, J. (1989) Hydrodynamic properties of macromolecular assemblies, in *Dynamic Properties of Biomolecular Assemblies* (Harding, S. E. and Rowe, A. J., eds.), Royal Society of Chemistry, Cambridge, UK, pp. 3–31.
11. Perkins, S. J. (1989) Hydrodynamic modelling of complement, in *Dynamic Properties of Biomolecular Assemblies* (Harding, S. E. and Rowe, A. J., eds.) Royal Society of Chemistry, Cambridge, UK, pp. 226–245.
12. Gregory, L., Davis, K. G, Sheth, B., Boyd, J., Jefferis, R., Nave, C., and Burton, D. R. (1987) The solution conformations of the subclasses of IgG deduced from sedimentation and small angle x-ray scattering studies. *J. Mol. Immunol.* **24,** 821–829.
13. Harding, S. E., Rowe, A. J., and Horton, J. C. (eds.) (1992) *Analytical Ultracentrifugation in Biochemistry & Polymer Science.* Royal Society of Chemistry, Cambridge, UK.
14. Perkins, S. J. (1986) Protein volumes and hydration effects. The calculations of partial specific volumes, neutron scattering matchpoints and 280-nm absorption coefficients for proteins and glycoproteins fram amino acid sequences. *Eur. J. Biochem.* **157,** 169–180.
15. Gilbert, G. A. and Gilbert, L. (1980) Detection in the ultracentrifuge of protein heterogeneity by computer modeling, illustrated by pyruvate dehydrogenase multienzyme complex. *J. Mol. Biol.* **144,** 405–408.
16. Creeth, J. M. and Knight, C. G. (1965) On the estimation of the shape of macromolecules from sedimentation and viscosity measurements. *Biochim. Biophys. Acta* **102,** 549–558.
17. Fujita, H. (1975) *Foundations of Ultracentrifuge Analysis.* Wiley, New York.
18. Harding, S. E. (1989) Modelling the gross conformation of assemblies using hydrodynamics: The whole body approach, in *Dynamic Properties of Biomolecular Assemblies* (Harding, S. E. and Rowe, A. J., eds.), Royal Society of Chemistry, Cambridge, UK, pp. 32–56.
19. Squire, P. G. and Himmel, M. E. (1979) Hydrodynamics and protein hydration. *Arch. Biochem. Biophys.* **196,** 165–177.

20. Harding, S. E., Berth, G., Ball, A., Mitchell, J. R., and Garcia de la Torre, J. (1991) The mol wt distribution and conformation of citrus pectins studied by hydrodynamics. *Carbohydrate Polymers* **16,** 1–15.
21. Gilbert, L. M. and Gilbert, G. A. (1973) Sedimentation velocity measurement of protein association. *Methods Enzymol.* **27D,** 273–296.