Chapter 5

Ultracentrifugation of Food Biopolymers

S.E. Harding
Department of Applied Biochemistry and Food Science, University of Nottingham, UK

5.1 Introduction and historical comment

Ultracentrifugation – whether it be preparative or analytical – is hardly a new technique. It was in the early 1920s that T. Svedberg, on sabbatical leave from the University of Uppsala, working with J.B. Nichols at Madison, Wisconsin, produced the first ultracentrifuge, known as the optical centrifuge. Svedberg then designed and produced the oil-turbine ultracentrifuge (the basis of his Nobel lecture in 1926) capable of speeds of 40 000 rev./min (producing gravitational fields close to 100 000 that of gravity). At these speeds it was possible to sediment *biopolymers* and two classes of experiment rapidly developed: preparative ultracentrifugation for the separation and purification of biopolymers (including density gradient methods), and analytical ultracentrifugation, where, by replacing the plastic rotor tubes with precision cells with windows at top and bottom, and constructing an optical system around these, the distribution of solute in these cells could be assayed, and from this information on the sizes, shapes and interaction properties of biopolymers could be obtained. Details of all this earlier history can be found in Svedberg's early papers such as Svedberg and Nichols (1923), Svedberg and Rinde (1923), and Svedberg and Fåhraeus (1926) – describing the first use of sedimentation equilibrium analytical ultracentrifugation to measure the molecular weight of a protein – and his Nobel Prize lecture (Svedberg, 1927).

Insofar as food biopolymers are concerned, Svedberg himself published several papers on using both sedimentation velocity and sedimentation equilibrium. This includes studies on egg albumin (Sjörgen and Svedberg, 1930; Svedberg and Erikkson, 1934), and gelatin solutions (Krishnamurti and Svedberg, 1930). Although the bulk of Svedberg's polysaccharide work was on cellulose and its derivatives (Jullander, 1985), work was published on polyuronides (i.e. polysaccharides with carboxyl groups) such as pectins, alginates and gum arabic. In these papers the necessity of working in salt solutions to suppress electric charge effects (polyelectrolyte

behaviour) was clearly demonstrated. In another important contribution to our understanding of the behaviour of food biopolymers, Siholta and Svedberg (1948) gave a new ultracentrifuge method for determining the solvation (an important functional property) of food macromolecules. It would be, of course, wrong to assume that it was only Svedberg and his co-workers who provided all the early input into our knowledge of the solution properties of food macromolecules, although it is true to say that, without his contribution, the field now would be still rather primitive.

The great boom period of the ultracentrifuge was 1950–1970, with almost every Biochemistry Department and most Chemistry Departments in the UK possessing *and running* the famous Beckman Model E analytical ultracentrifuge or its MSE (Crawley, UK) equivalent. Although the vast bulk of the work during this period was on macromolecules (in particular proteins) other than from food, this period was not devoid of food activity. It was during this period, for example, that Johnson and co-workers, using sedimentation velocity, developed our understanding of the structure of gelatin gels (Johnson, 1964; Johnson and Metcalfe, 1967) and that the self-assembly properties of milk proteins became largely understood (McKenzie, 1970).

So why is a chapter on the ultracentrifuge present in a volume on *new* physico-chemical techniques? The reason is that there has been, over the last five years, a rebirth of interest in analytical ultracentrifugation, coupled with significant advances in instrumentation and analysis, all of which can, and indeed is, being applied to food polymer research. Preparative ultracentrifugation has always remained an important separation tool, but interest in *analytical* ultracentrifugation, after the boom period 1950–1970, had dramatically declined so that, up to 1987, only a handful of workers were active in this field worldwide. Protein biochemists had largely switched to gel filtration and gel electrophoresis for their purity and molecular weight work (together with sequence analysis and mass spectrometry for the latter) and to NMR and X-ray crystallography for their conformation work. Polysaccharide chemists and the synthetic polymer community also switched to gel filtration, and also to light scattering methods for absolute molecular weight and size determinations. Towards the end of the 1980s, however, scientists began to realize the limitations of these other procedures; and this coincided with a growing demand for physico-chemical types of measurement, not only from food scientists, but also from molecular biologists desperate for relatively inexpensive ways (in terms of quantities of material required) of characterizing their gene products.

The result of this rebirth has been twofold: the emergence of national centres (in the UK and USA) for this type of measurement, and the launch of the first commercially available analytical ultracentrifuge (with full on-line data capture and analysis facilities) for almost two decades.

The aim of this chapter is to show just why modern analytical ultracentrifugation is worthy of consideration by food scientists and technologists interested in characterizing and understanding the behaviour of the macromolecular components of food products. The main focus of attention will be on food proteins and food polysaccharides (the latter proving quite a challenge for most physical methods because of their heterogeneity and high non-ideality in solution), in both dilute and concentrated solution form, and how the modern analytical ultracentrifuge can provide information on food biopolymers about in terms of:

(i) the size (in terms of either the molecular weight or dimensions);
(ii) the size distribution (for a polydisperse solution of macromolecules);
(iii) the solution conformation (in terms of the overall or gross conformation, and flexibility);
(iv) the purity of a macromolecular preparation (in terms of a sedimentation velocity diagram or density spectrum);
(v) the interactions between macromolecules (including self-association behaviour, complex formation);
(vi) the behaviour of gels; and
(vii) interfacial diffusion phenomena, and diffusion through gels and matrices.

The four analytical ultracentrifuge techniques used to provide this information are: sedimentation velocity analysis; sedimentation equilibrium analysis; density gradient equilibrium analysis; and diffusion analysis. It should be borne in mind, however, at the outset that ultracentrifugation is at its most powerful when used with complementary techniques such as (for conformation analysis) X-ray scattering, viscometry, rotational diffusional techniques and electron microscopy, or (for protein subunit composition work) gel electrophoresis, or (for molecular weight distribution studies) gel filtration and light scattering.

5.2 Types of analytical ultracentrifuge measurement

What sort of information can we get from sedimentation analysis in the analytical ultracentrifuge? It depends on the type of ultracentrifuge technique we apply – all are possible with the same instrumentation. *Sedimentation velocity* can provide us with information on the sample heterogeneity, shape information – in some cases to surprising detail – and also interaction information by, for example, assaying for what we call co-sedimentation phenomena (i.e. species sedimenting at the same rate). At lower rotor speeds, *sedimentation equilibrium* can provide absolute size and size distribution information (in terms of molecular weight averages and molecular weight distributions). There are two other important types of analytical ultracentrifuge measurement – namely

isopycnic (= constant density) *density gradient analysis*, important for assaying the composition (and hence purity) of a macromolecular preparation, and finally *diffusion analysis*. Although dynamic light scattering is now the method of choice for the measurement of translational diffusion coefficients, the optical system on the analytical ultracentrifuge is proving very useful for investigating the diffusion of molecules through matrices, as well as towards and through interfaces in two-phase systems.

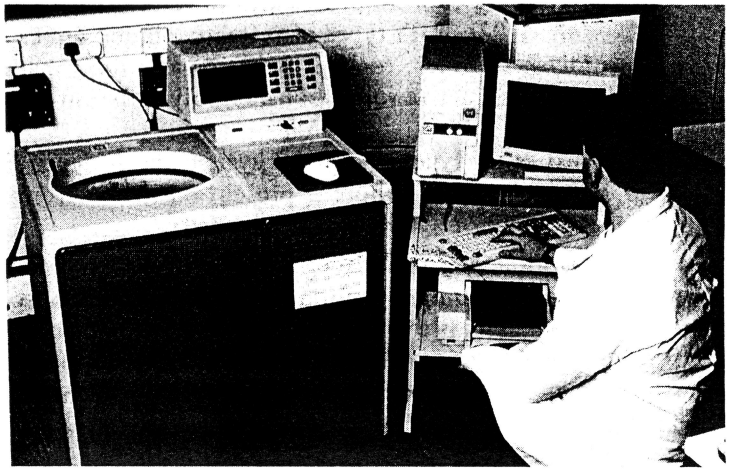
5.3 Instrumentation/optical systems

An analytical ultracentrifuge is simply a high-speed ultracentrifuge (up to 60 000 rev./min) with an appropriate optical system for observing and recording solute distributions during and at the end of the sedimentation process. There are three types of analytical ultracentrifuge now in common use. Firstly there are increasing numbers of the new Beckman Optima XLA (Schachman, 1989; Giebeler, 1992) analytical ultracentrifuge (Fig. 5.1(a)) being installed, the initial model with just scanning absorption optics, and also a more recent version with scanning Rayleigh interference optics. Although the Beckman Model E ultracentrifuge (with refractive index optics and absorption optics, although the latter is very noisy) has not been commercially available now for almost 20 years, there are still many in use (many adapted and modified, Fig. 5.1(b)) – a testimony to the reliability of this instrument. In East European countries, analytical ultracentrifuges made by the MOM optical works at Budapest (with refractive index and absorption optics) are also still in use (Görnitz and Linow, 1992).

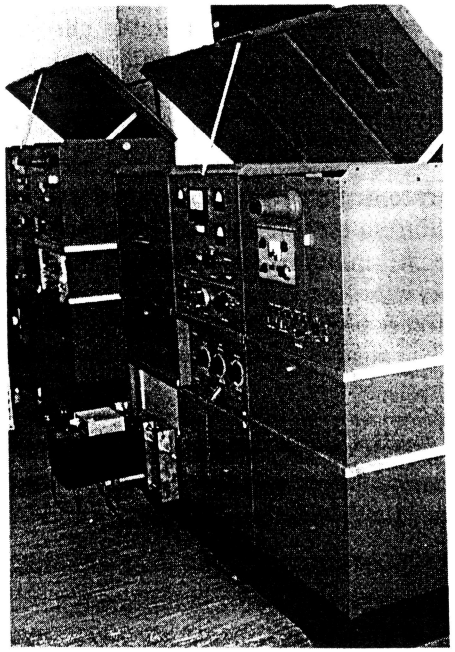
Concentration distributions of a macromolecular solution (whether the macromolecule be a food protein, food polysaccharide or whatever) during ultracentrifugation can be recorded using refractive index optics. There are two types: Interference and Schlieren.

Interference: This gives a record of solute concentration as a function of radial distance, and is popular for sedimentation equilibrium measurements. Extraction of molecular weight parameters is quite laborious, but it gives the most accurate results for weight-average molecular weight data. Although in the past the fringe patterns were unwieldy to analyse, computers have now made this a realistic possibility, particularly for the analysis of distributions of sedimentation coefficient and molecular weight (Stafford, 1992).

Schlieren: This is another name for 'refractive index gradient' optics. It gives a direct record of the concentration gradient as a function of radial position, and is ideal for sedimentation velocity (where a sedimenting boundary appears as a peak) or isopycnic density gradient equilibrium (where a band appears as a positive peak followed by a negative peak). It can also be applied to solute distributions at sedimentation equilibrium, where it gives the z-average molecular weight directly.



(a)



(b)

Fig. 5.1 (a) Beckman Optima XLA analytical ultracentrifuge. (b) Beckman Model E analytical ultracentrifuge (with modifications).

Interference optics requires a minimum solute concentration of ~0.2 mg/ml, whereas Schlieren optics normally requires at least ~ 0.8 mg/ml, although with modern data capture procedures lower concentrations are possible (Rowe *et al.*, 1992). Both types of refractive index optics can be applied to food polysaccharides, food proteins or any food macromolecules, since all have a refractive index considerably different from that of water or any aqueous solvent.

There are also two other types of optical system, both depending on whether or not a suitable chromophore is present on the macromolecule (either naturally occurring or synthetically attached).

Absorption optics: Insofar as food macromolecules are concerned, until recently this technique was limited to food proteins using UV absorption at 278 nm, or for coloured proteins like haemoglobin and myoglobin, absorption in the visible region. With the new Beckman Optima XLA ultracentrifuge and its stable light source down to 220 nm, some polysaccharides can also be detected. For proteins, concentrations down to ~0.2 mg/ml can be detected and fluorescein labelled macromolecules (λ_{max} ~ 480 nm) can be detected to lower concentrations. Details can be found in Lewis (1992).

Fluorescence optics: This is a variant of the above, in which a fluorescing wavelength of fluorescein or another suitable chromophore is used. It needs a specially adapted instrument (Schmidt and Riesner, 1992) although work is currently underway in producing a commercially available variant of the XLA ultracentrifuge with this facility (T. Laue, private communication)

A preparative ultracentrifuge can also be used as an analytical tool for sedimentation equilibrium measurements, if the macromolecule is labelled with an appropriate chromophore (absorption, fluorescent or radio-label) and the final equilibrium distribution is pooled and assayed immediately at the end of the ultracentrifuge run (Howlett, 1992), although this is only a substitute for proper analytical ultracentrifugation.

5.4 Sedimentation velocity: shape analysis and homogeneity

A typical analytical ultracentrifuge cell contains two sector-shape channels (one for the solution, one for the reference solvent) which can take between 0.1 and 0.8 ml. With an *analytical* ultracentrifuge, using the appropriate optical system one can record the position and rate of movement of the sedimenting boundary within the solution channel. (For a simple description of the method and what it can do, see Harding (1994a).) Figure 5.2 compares patterns for a monodisperse food protein (β-lactoglobulin) recorded using absorption optics at 278 nm, a food polysaccharide (a heat degraded alginate) recorded using Schlieren optics, and another food protein (lipase), containing some contaminating material, again recorded using Schlieren optics. The rate of movement of a sedimenting boundary from these patterns per unit centrifugal field gives the sedimentation coefficient *s*. By adjusting this (using simple formulae) to standard conditions (water as solvent at 20°C), and extrapolating it

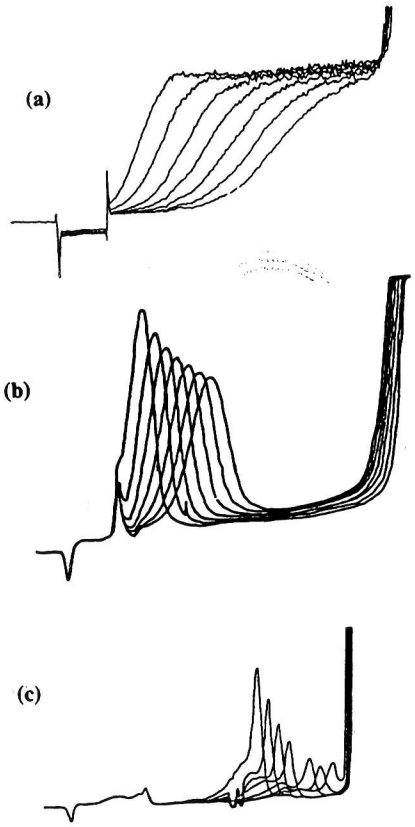


Fig. 5.2 Optical records of sedimentation velocity of food biopolymers in the analytical ultracentrifuge. The direction of sedimentation in each case is from left to right. (a) Sedimenting boundary for a monodisperse β-lactoglobulin B preparation recorded using scanning absorption optics in Optima XLA ultracentrifuge. Monochromator wavelength = 279 nm; scan interval = 20 min; rotor speed = 50 000 rev./min; temperature = 25.0°C; loading concentration, c° ~ 1.0 mg/ml; s_{25} = (2.97 ± .03) S (from Harding and Advani, 1995). (b) Sedimenting boundary for a food polysaccharide (heat-treated sodium alginate) recorded using scanning Schlieren optics on an MSE Centriscan ultracentrifuge. Monochromator wavelength = 546 nm; scan interval = 30 min; rotor speed = 49 000 rev./min; temperature = 20.0°C, c° ~ 5.0 mg/ml; s_{20} = (1.22 ± 0.05) S. (c) Sedimenting boundaries for *Chromobacter viscosum* lipase using scanning Schlieren optics on the MSE Centriscan. Monochromator wavelength = 546 nm; scan interval = 10 min; rotor speed = 49 000 rev./min; temperature = 20.0°C; two clear sedimenting boundaries are s_{20} = (3.1 ± 0.1) S (faster peak) and s_{20} ~ 1.0 S (slower peak) (from Simpkin *et al.*, 1991).

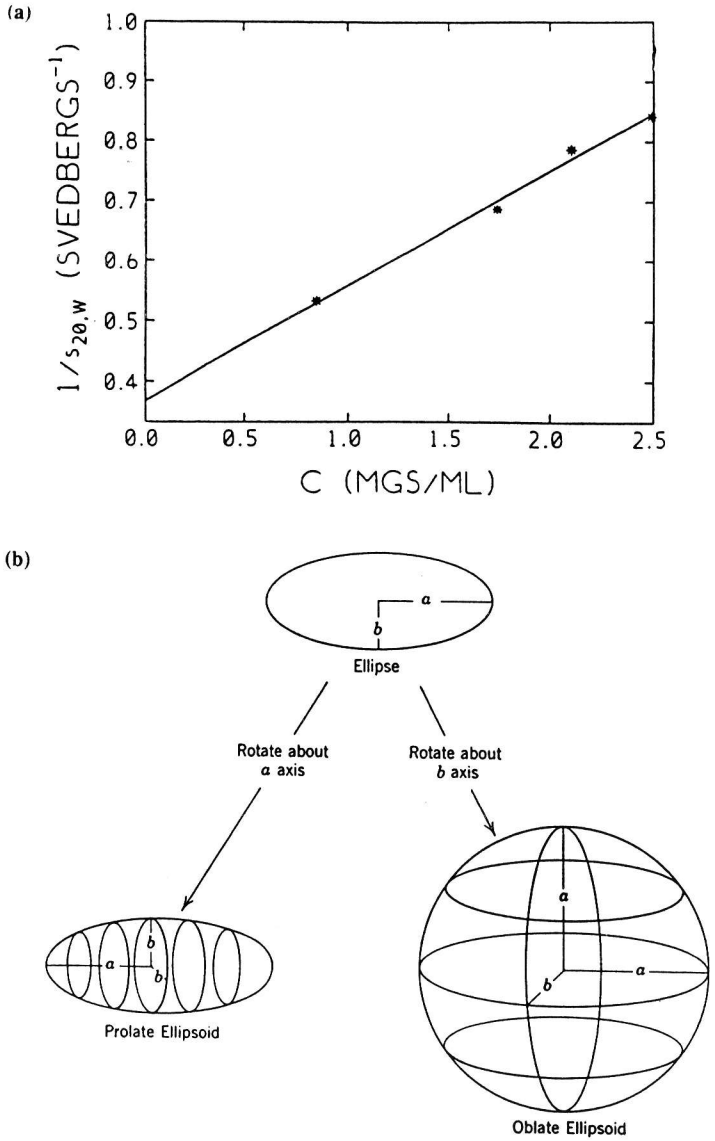


Fig. 5.3 (a) Plot of the reciprocal of the sedimentation coefficient $s_{20,w}$ versus concentration c for sodium carboxymethyl cellulose in phosphate-chloride buffer (pH 6.8, $I = 0.10$). $s^{\circ}_{20,w} = (2.7 \pm 0.2)$ S; $k_s = (521 \pm 34)$ ml/g (from Anderson, 1991). N.B. For food polysaccharides the reciprocal plot usually gives the best linear fit; for food proteins, a plot of $s_{20,w}$ against c is usually better (Rowe, 1977). (b) Ellipsoids of revolution models (from Tanford, 1961). The shape is given by the axial ratio a/b in both cases.

(or the reciprocal thereof) to zero concentration, we can get from the intercept the corrected sedimentation coefficient, $s^{\circ}_{20,w}$ (unit = seconds or Svedbergs, S, where $1\ S = 10^{-13}$ s), and from the slope we can get the sedimentation concentration regression coefficient, k_s (unit = ml/g). Then from both parameters, shape information can be extracted (Fig. 5.3a). Table 5.1 gives some s and k_s values for a range of food biopolymers.

Besides providing shape information, for each scan or sedimentation diagram, the form and number of the sedimenting boundaries can provide a rapid indication of the heterogeneity of a sample, with impurities, etc., showing up as separate boundaries. Figure 5.2(c) shows such a case for a preparation of lipase (the pattern scanned seven times at 10-min intervals) containing a slower moving impurity.

For shape analysis there are two general stategies. One strategy, which is applied to *relatively* rigid structures (for example, many food protein systems), is to use ellipsoid or bead models to represent the overall structure of these molecules in *solution*. The sort of information that can reasonably be sought with this approach is the overall or gross conformation of the food protein in solution: its axial dimensions, how its

Table 5.1 Sedimentation velocity parameters for some food macromolecules

Macromolecule	M[a]	$s^{\circ}_{20,w}$ (S)	k_s (ml/g)	Reference
Ovalbumin	45000	3.42[b]	6.2	Miller and Golder (1952); Holt (1970); Harding (1981b)
Bovine serum albumin	67000	4.31	5.4	Shulman (1953); Baldwin (1957)
β-Lactoglobulin (B) (Dimer)	36000	2.83	4.6	Cecil and Ogston (1949); Advani and Harding (1995)
Myosin	490000	5.6	94	Emes and Rowe (1978)
Collagen α-1 chain		3.16	56.9	Nishihara and Doty (1958)
Lipase (*Chromobacter viscosun*)	35000	3.17	18.7	Simpkin *et al.* (1991); Simpkin (1994)
Lipase (*Pseudomonas* spp.)	38000	2.99	7.0	Simpkin *et al.* (1991); Simpkin (1994)
Lipase (*Aspergillus* spp.)		3.01	9.5	Simpkin *et al.* (1991); Simpkin (1994).
Alginate	130000	2.4	449	Anderson (1991)
Carboxymethylcellulose		2.7	522	Anderson (1991)
Xylan	177000	2.4	270	Elizabeth *et al.* (1995)
Xylinan	275000	9.4	273	Berth *et al.* (1995)
κ-Carrageenan	284000	4.2	591	Day *et al.* (1995)
Pectin (citrus)	90000	1.9	128	Harding *et al.* (1991b)
Pectin (lime)	130000	2.3	196	Harding *et al.* (1995)

$s^{\circ}_{20,w}$ values for proteins normally correct to ± 1% or better; for polysaccharides to ± 5%. k_s values normally correct to ± 2% for proteins, ± 20% for polysaccharides. Some k_s values have been corrected for 'radial dilution' and to 'solution density' (Rowe, 1977). Most determinations done in (aqueous) solvents of ionic strength 0.1 M for proteins, 0.1–0.3 M for polysaccharides, and proteins and most polysaccharides buffered to pH between 6 and 7.

[a]Weight-average values for polydisperse polysaccharide samples.

[b]After correction from 25°C to 20°C.

subunits are arranged etc. The second strategy is for those molecules like food polysaccharides where it is unreasonable to assume, at least *a priori*, a rigid conformation; here it is necessary to perform more general modelling. The approach here is firstly to identify the conformation type (random coil, rigid rod or compact sphere, or something in between these extremes), and once this is established then to make further deductions about the conformation. For example, if the conformation is that between a random coil and rigid rod, we ask what is the flexibility of the molecule in terms of parameters like the persistence length a and the contour length L? If the conformation is between that of rigid rod and compact sphere, then, and only then, can ellipsoid or bead modelling be applied. It has to be borne in mind that, whatever strategy is actually followed, sedimentation data are best used in conjunction with other hydrodynamic data.

5.4.1 *Ellipsoid and bead modelling*

This can be done via calculation of the so-called frictional ratio, the ratio of the friction coefficient, f, of the macromolecule to the frictional coefficient of a spherical particle, f_o, with the same mass and anhydrous volume. The ratio f/f_o is related to the sedimentation coefficient by

$$(f/f_o) = \{M(1 - \bar{v}\rho)]/[N_A \times (6\pi\eta_o s^o_{20,w})]\}[(4\pi N_A/3\bar{v}M)]^{1/3}, \quad (5.1)$$

where M is the molar mass (g/mol), $\bar{v}$ the partial specific volume (ml/g) (typically ~0.73 ml/g for proteins, 0.45–0.65 ml/g for polysaccharides), ρ is the density (g/ml) of the solution (which can be approximated as the density of the solvent (Harding and Johnson, 1985)), η_o (poise) is the solvent viscosity (in this case, after the correction referred to above, the density of water at 20.0°C) and N_A is Avogadro's number (mol^{-1}). The quantity f/f_o, in turn, is a function of the hydration of the macromolecule, w (mass H_2O bound/mass of macromolecule) and the conformation via a particle shape factor known as the 'Perrin shape factor' P (also known as the frictional ratio due to shape), in recognition of Perrin (1936) who developed the theory for the frictional coefficients of ellipsoids:

$$P = (f/f_o)[(w/\bar{v}\rho) + 1]^{-1/3}. \quad (5.2)$$

The factor $[(w/\bar{v}\rho) + 1]$ is sometimes referred to as the swelling of the macromolecule due to hydration, i.e. $v_s/\bar{v}$ with v_s the swollen specific volume (ml/g). For a food protein the value of $v_s/\bar{v}$ is typically ~1.4. For a food polysaccharide it can be as high as ~100.

If the macromolecule is fairly rigid, P can be related directly to the axial dimensions of the macromolecule using ellipsoid of revolution models. Ellipsoids of revolution are so-called because they are the

Table 5.2 Axial dimensions of some proteins from X-ray crystallography

Protein	Dimensions (Å)	Reference
Myoglobin	43 × 35 × 23	Kendrew *et al.* (1958)
Haemoglobin	64 × 55 × 50	Perutz *et al.* (1960)
Lysosyme	45 × 30 × 30	Blake *et al.* (1965)
Cytochrome *c*	35 × 25 × 25	Dickerson and Geiss (1969)
Ribonuclease	38 × 28 × 22	Kartha *et al.* (1967)
Carboxypeptidase	50 × 42 × 38	Lipscomb *et al.* (1971)
Pre-albumin	70 × 55 × 50	Blake *et al.* (1978)

structures formed by rotating an ellipse, of semi-axes a and b ($a > b$), about either its major or a-axis (to give a three-dimensional ellipsoid of semi-axes a, b and b, known as a prolate ellipsoid) or rotated about its minor axis b (to give a three-dimensional ellipsoid of semi axes a, a and b (Fig. 5.3(b)). Table 5.2 gives the dimensions of some protein molecules from their X-ray crystal structures, and it can be seen that some of these can be reasonably approximated by two equal axes (especially two equal minor axes, namely the prolate case). The axial ratio (a/b) characterizing such ellipsoids is related to P by

$$P = \frac{(1 - b^2/a^2)^{1/2}}{(b/a)^{2/3} \ln \dfrac{1 + (1 - b^2/a^2)^{1/2}}{b/a}} \quad (5.3a)$$

(prolate ellipsoid), or for an oblate ellipsoid from

$$P = \frac{(a^2/b^2 - 1)^{1/2}}{(a/b)^{2/3} \tan^{-1}(a^2/b^2 - 1)^{1/2}}. \quad (5.3b)$$

Table 5.3 gives a/b as a function of P in the range $1 \leqslant (a/b) \leqslant 100$. A simple analytical inversion of these formulae giving a/b directly in terms of P is not possible. However, simple polynomial expansions are possible (Laue *et al.*, 1992) giving a/b values correct to better than ~0.1%:

$$(a/b) = 1 + 2.346X^{1/2} + 8.297X + 8.4X^2 - 0.4589X^3 + 0.0314X^4 \quad (5.4a)$$

for the prolate case, and

$$(a/b) = 1 + 2.975X^{1/2} + 8.48X + 13.295X^2 - 3.219X^2 + 0.0996X^4 \quad (5.4b)$$

for the oblate case, and for both cases $X = P - 1$.

A problem with this treatment is that f/f_o (and hence P and the sedimentation coefficient) is a relatively insensitive function of shape. A more serious problem is that an assumption has to be made concerning the hydration w (or equivalently $v_s/\bar{v}$). A value of 0.35 has in the past been popularly taken for proteins (Tanford, 1961) although a survey of 21 proteins by Squire and Himmel (1979) has suggested w values in the range (0.53 ± 0.26) (see also Pessen and Kumosinski (1985) and Laue

Table 5.3 Values of the ellipsoid of revolution shape parameters P, R, Π as a function of axial ratio for prolate and oblate ellipsoids of revolution

Axial ratio (a/b)	P (prolate)	R (prolate)	Π (prolate)	P (oblate)	R (oblate)	Π (oblate)
1	1.000	1.600	3.200	1.000	1.600	3.200
2	1.044	1.471	3.122	1.042	1.494	3.180
3	1.113	1.291	2.960	1.105	1.369	3.179
4	1.183	1.138	2.778	1.166	1.274	3.192
5	1.250	1.017	2.601	1.224	1.203	3.208
6	1.314	0.921	2.438	1.277	1.148	3.225
7	1.375	0.844	2.291	1.327	1.105	3.241
8	1.434	0.782	2.159	1.373	1.071	3.255
9	1.490	0.730	2.041	1.417	1.043	3.268
10	1.543	0.686	1.935	1.458	1.019	3.280
20	1.996	0.465	1.288	1.783	0.901	3.352
30	2.359	0.379	0.980	2.020	0.856	3.384
40	2.671	0.332	0.799	2.212	0.833	3.403
50	2.950	0.302	0.679	2.375	0.819	3.415
60	3.205	0.280	0.593	2.519	0.810	3.423
70	3.442	0.264	0.527	2.648	0.803	3.429
80	3.664	0.251	0.476	2.765	0.798	3.434
90	3.874	0.240	0.435	2.873	0.793	3.437
100	4.074	0.231	0.400	2.974	0.790	3.441

To measure P, the sedimentation coefficient and an assumed value for the hydration of the molecule are required. To measure R, the sedimentation concentration dependence parameter and the intrinsic viscosity [η] are required. To measure Π, the second virial coefficient B (from sedimentation equilibrium measurements) and [η] are required.

et al. (1992) for further discussions on this). For polysaccharides, hydration levels are usually much higher and it is almost impossible to give a reasonable estimate for them (although it should be borne in mind that most polysaccharides cannot be represented by these rigid models in any case).

An alternative approach is to use the alternative shape function known as the Wales–van Holde ratio $k_s/[\eta]$ (Wales and van Holde, 1954), which is often referred to by the symbol R (Harding and Rowe, 1982). This combination of the concentration dependence parameter k_s with the intrinsic viscosity [η] gives, after certain assumptions and approximations, a shape function which is not dependent on any knowledge of w or υ_s, and is a more sensitive function of axial ratio a/b (Table 5.3). The full functional dependence of R on a/b can be found in Rowe (1977). Table 5.4 gives estimates for the shapes of some food proteins determined in this way. Another shape function not requiring knowledge of the hydration level is the Π function (Harding, 1981a). This comes from measurements of the thermodynamic second virial coefficient B, [η], and the molar mass M:

$$\Pi = \{2BM/[\eta]\} - \{f(Z)/[\eta]M\}. \qquad (5.5)$$

Here $f(Z)$ is a function of the net charge Z on the macromolecule. This function is zero at the isoelectric point of proteins and tends to zero at

Table 5.4 Axial ratios of food proteins from k_s and intrinsic viscosity [η] measurements

Protein	k_s (ml/g)	[η] (ml/g)	R ($= k_s/[\eta]$)	axial ratio (a/b)[a]	Reference
Ovalbumin	5.45	3.49	1.56	1.5	Miller and Golder (1952); Holt (1970); Harding (1981b)
Bovine serum albumin	5.4	3.9	1.38	2.3	Baldwin (1957); Tanford and Buzzell (1956)
β-Lactoglobulin (B) (dimer)	4.6	2.86	1.61	1.0	Advani and Harding (1995); Townend (1960)
Collagen (seven fractions sonically degraded to varying extents)	250–142	1075–245	0.23–0.58	100–15	Nishihara and Doty (1958); see also Creeth and Knight (1965)

[a]Value for the equivalent prolate ellipsoid. The k_s values have been corrected for radial dilution and to solution density (Rowe, 1977).

Table 5.5 Axial ratios of food proteins from the Π function

Protein	Π	Axial ratio (a/b)[a]	Reference
Haemoglobin	3.20	1.0	Harding (1981a)
Ovalbumin	3.18	1.0–2.0	Harding (1981b)
Myosin[b]	0.47	80	Harding (1987)

[a]Value of the equivalent prolate ellipsoid.
[b]An ellipsoid is a poor approximation here because of the flexibility of the molecule; only a very approximate estimate of a/b is possible.

high ionic strengths. If not negligible, it can be measured by, for example, titration (see Jeffrey *et al.* (1977) who have done this for ovalbumin). The second thermodynamic virial coefficient B (A_2 in some texts) can be measured by sedimentation equilibrium (see below) or from osmotic pressure or light scattering measurements. Table 5.3 also includes Π as a function of a/b and Table 5.5 gives an example of its application to three well-known food proteins.

Another combined function involving s and [η] (referred to as the Scheraga and Mandelkern (1953) β-function), the first published hydration independent shape parameter, is also independent of w or υ_s but is extremely insensitive to a/b, and it is really only of use as a quasi-constant parameter for evaluating the molar mass from $s^{\circ}_{20,w}$ and [η] measurements.

In many cases a crude ellipsoid of revolution, with two axes necessarily equal, gives a poor approximation to the true overall (or gross) conformation of proteins in solution. Furthermore, a decision has to be made *a priori* as to which ellipsoid of revolution model to apply: oblate or prolate? (Usually it is the latter which gives the better representation.) For cases like these the much more general tri-axial ellipsoid, with three unequal semi-axes ($a \geq b \geq c$) and hence two characteristic axial ratios (a/b, b/c), is much more appropriate. Although this model is considerably more complicated to apply (it involves the use of *two* hydration independent shape functions and computer-graphical intersection procedures), the

necessary theory and computational procedures have been developed. By using a combination of Π with a radius of gyration function (from light scattering), the overall rod conformation and axial ratio of myosin is successfully predicted (a/b, b/c) = (80, 1) *without assuming* a priori *a prolate ellipsoid* (Harding, 1987).

Even with the extra degree of freedom the general tri-axial ellipsoid gives, there are many structures which cannot be reasonably represented by a symmetric ellipsoidal structure. The classic example from the non-food protein world is the antibody molecule. The basic idea is that the frictional ratio f/f_o (and hence P and $s^o_{20,w}$) can be calculated for a given array of spherical beads which do not have to be equal in size. This is done for various possible models for a given macromolecule, and the one which gives the predicted f/f_o (or P or $s_{20,w}$) in closest agreement with the experimentally measured value is chosen as the best model. Because of uniqueness problems (i.e. the possibility of other equally complex but quite different models giving similar agreement), it is necessary to rely on (i) other hydrodynamic or scattering data (such as [η], rotational diffusion coefficients or the radius of gyration) and (ii) a close starting estimate (from electron microscopy, X-ray crystallography, etc.) to the true conformation. This method has had extensive application to several protein systems (Garcia de la Torre, 1989), especially immunological ones like immunoglobulins (Byron, 1992) and complement (Perkins, 1989), and has successfully been applied to model the segmental flexibility of myosin (Iniesta *et al.*, 1988; Garcia de la Torre, 1989). Surprisingly, so far as the author is aware, it has not been applied to the study of seed or milk proteins, where it would appear to have potential application in terms of modelling the arrays of subunits, although similar approaches using light or low-angle X-ray scattering have been applied (Plietz *et al.*, 1983).

5.4.2 *General modelling*

For polydisperse and not-so-rigid macromolecules, like many polysaccharides, we have to use shape analysis by sedimentation velocity in a general way, first of all to indicate what is the conformation *type* of the macromolecule (random coil, rigid rod or compact sphere, or whatever). For this purpose relations, known as the Mark–Houwink–Kuhn–Sakurada (MHKS) relations and their corresponding (exponential) coefficients have proved vital. One of these is the b coefficient which comes from the relation between $s^o_{20,w}$ and the molecular weight M:

$$s^o_{20,w} \propto M^b. \tag{5.6}$$

(Similar coefficients exist for the relationships of the intrinsic viscosity, the diffusion coefficient, and radius of gyration with M (Yamakawa, 1971; Smidsrød and Andresen, 1979). The MHKS-b coefficient is usually

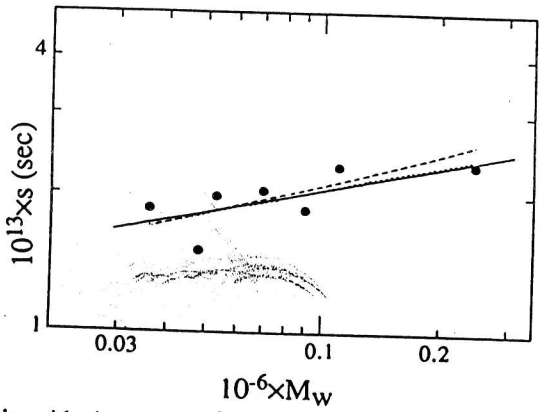


Fig. 5.4 Double-logarithmic plot of $s^o_{20,w}$ versus weight-average molar mass M^o_w (from low-speed sedimentation equilibrium) for citrus pectin fractions. Solid line corresponds to a fit of the data to the MHKS equation. Dashed line corresponds to the fit to equations representing a worm-like coil with persistence length 60 nm and mass per unit length 420 g mol^{-1} nm^{-1}, values which are only consistent with a rigid rod model (from Harding *et al.*, 1991b).

obtained by preparing a homologous series (i.e. the same polymer but different molecular weights) of the polymer (by chromatographic separation, ultrasonication or heat degradation) and then taking the slope of a double logarithmic plot of log $s^o_{20,w}$ *versus* log M (Fig. 5.4). The characteristic values of b for spheres, random coils and rods are respectively 0.667, 0.4–0.5 and 0.15 (Smidsrød and Andresen, 1979). The Wales–van Holde parameter $k_s/[\eta]$ ($\equiv R$) is also useful in this context, having a values of ~1.6 for spheres and random coils, and <<1 for rods. Some examples of applications of both these parameters to food polysaccharides are given in Table 5.6.

The MHKS-b coefficient can also be used to model the flexibility of a polymer in terms of wormlike-coil models (Hearst and Stockmayer, 1962; Yamakawa, 1971) from the ratio of the contour length L to the persistence length a (see Harding *et al.*, 1991a).

Table 5.6 Polysaccharide shapes from sedimentation velocity

Polysaccharides[a]	$k_s/[\eta]$	b	Conformation
Dextran fractions		0.44	Random coil
DIT-dextrans		0.56	Semi-flexible coil
Pullulans	1.4	0.45	Random coil
β-Glucans	0.4		Extended
Amylopectin	1.45		Spheroidal
Alginates	0.6		Extended
Citrus pectins	0.2	0.17	Rigid rod

From Harding (1992) and references cited therein.
[a]All in aqueous solvents apart from amylopectin (90% DMSO, 10% H_2O).

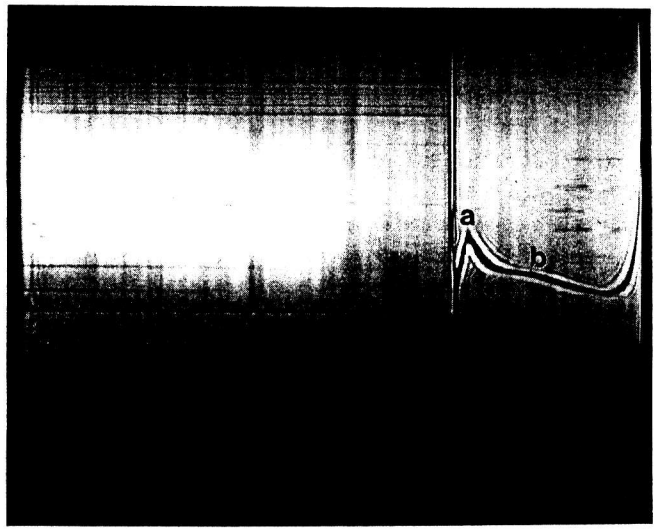


Fig. 5.5 Sedimentation velocity diagram for a solution containing 1 mg/ml bovine serum albumin and 1 mg/ml of an alginate (Pronova, Protan inc., Drammen, Norway) in a phosphate-chloride buffer (pH 6.8, $I = 0.10$ M). Rotor speed = 34 790 rev./min; temperature = 25.0°C. Peak (a) is unbound alginate ($s_{20} = 1.5$ S); peak (b) is BSA–alginate complex sedimenting at ~10.6 S. The BSA control sediments at ~3.9 S (from Harding *et al.*, 1993).

Sedimentation velocity can also be used to assay for interaction in a mixed solute system, by using the principle of co-sedimentation. Figure 5.5 shows such an example, showing interaction between bovine serum albumin and alginate. If the species in a mixture have chromophores absorbing in different regions of the UV or visible spectrum, the absorption optical system can be used to optimal effect (Marsh and Harding, 1993).

5.5 Sedimentation equilibrium: molecular weight distribution analysis

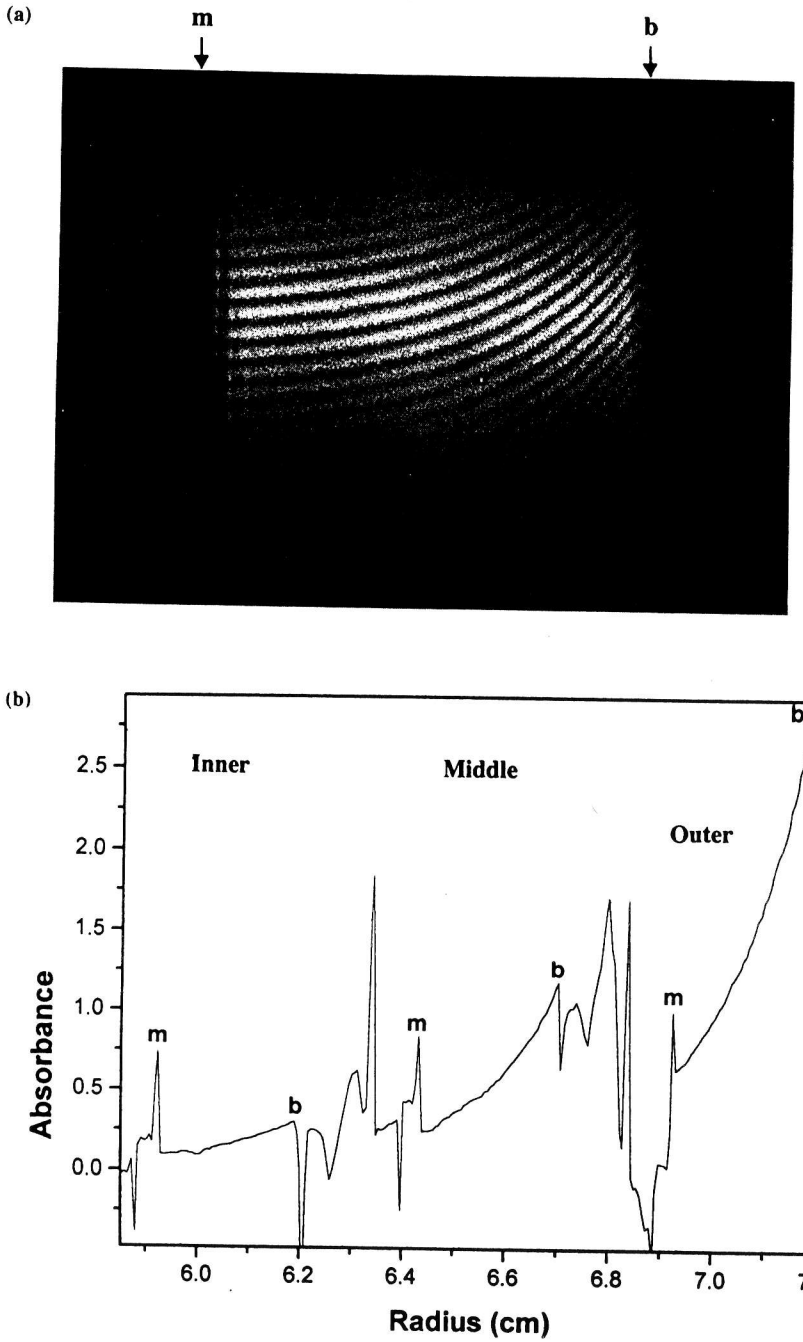
Whereas in a sedimentation velocity experiment at relatively high rotor speeds – for a globular protein or linear polysaccharide, say, 40 000–50 000 rev./min – the sedimentation rate and hence the sedimentation coefficient is a measure of the size and shape of the molecule, at much lower speeds (say 10 000 rev./min or less) in a sedimentation *equilibrium* experiment the forces of sedimentation and diffusion on the macromolecule become comparable. Now, instead of getting a sedimenting boundary, one gets, after a period of time (from a few hours to a few days depending on the nature of the solute) a steady state equilibrium

distribution of macromolecules with a low concentration at the meniscus building up to a high concentration at the cell base. This final steady state pattern is a function *only* of molecular weight and related parameters (virial coefficients and association constants where appropriate) and not on molecular shape, since, at equilibrium, there is no further movement of the macromolecules; thus frictional effects through shape variation are not involved. (For a simple description of this method and what it can do, see Harding (1994b).) So, like 'static' (as opposed to 'dynamic') light scattering, sedimentation equilibrium is an absolute way of obtaining the molecular weight or 'molar mass' M (g/mol) of a food biopolymer, and Table 5.7 gives examples for a range of polysaccharides and seed proteins. Although a sedimentation equilibrium experiment is longer to perform compared to a light scattering measurement (it can take up to 3 days), it is usually less prone to problems (such as clarification, etc.) With the ability to run samples multiply (up to 9 at a time in the Beckman Optima XLA), it may become the main method of choice once again.

Table 5.7 Molecular weights of food polysaccharides and some 12S seed proteins (weight averages from sedimentation equilibrium analysis)

Biopolymer	M_W	Reference
Pullulan P100	95000	Kawahawa *et al.* (1984)
Pullulan P800	76000	Kawahawa *et al.* (1984)
Pullulan P1200	124000	Kawahawa *et al.* (1984)
Dextran T500	500000	Edmond *et al.* (1968)
Guar	800000	Gaisford *et al.* 1986
Locust bean gum (cold water soluble fraction)	340000	Gaisford *et al.* (1986)
Locust bean gum (hot water soluble fraction)	330000	Gaisford *et al.* (1986)
Glucomannan	280000	Winwood (1986)
β-Glucan (barley)	160000	Woodward *et al.* 1983
β-Glucan (wheat)	230000	Harding *et al.* (1991a)
Chitosan (Protan Sea Cure)	162000	Errington *et al.* (1993)
Chitosan (Trondheim KN50-1)	64100	Errington *et al.* (1993)
Chitosan (Trondheim KN50-4)	4300	Errington *et al.* (1993)
Xanthan (Kelco RD)	5900000	Dhami *et al.* (1995)
Alginate (Kelco 'Pro-nova')	210000	Horton *et al.* (1991)
Pectin (Koch-Light, citrus)	90000	Harding *et al.* (1991b)
Pectin (green tomato)	160000	Seymour and Harding (1987)
Pectin (red tomato)	96000	Seymour and Harding (1987)
Pectin (red tomato, antisense polygalacturonase)	135000	Smith *et al.* (1990)
Carmin (from safflower seed)	260000	Prakash (1992) and refs. therein
α-Globulin (from sesame seed)	270000	Prakash (1992) and refs. therein
Arachin (from peanut)	330000[a]	Prakash (1992) and refs. therein
Brassin (from rapeseed)	300000	Harding *et al.* (1987)
Helianthin (from sunflower seed)	300000[a]	Prakash (1992) and refs. therein

These values are normally precise to ± 5–15%.
[a]From combining the sedimentation coefficient with the diffusion coefficient via the Svedberg equation (Harding, 1994a).



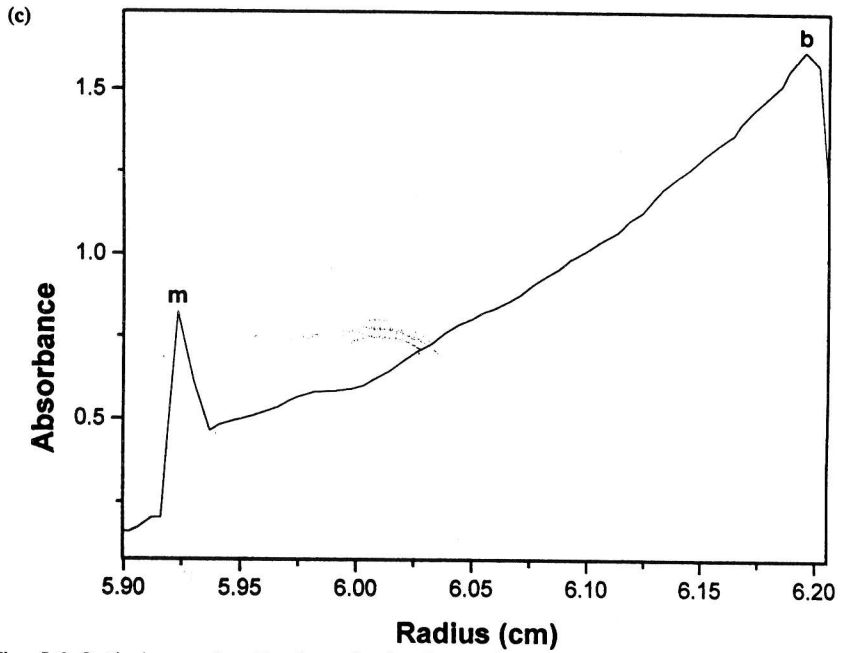


Fig. 5.6 Optical records of solute distributions of food biopolymers at sedimentation equilibrium. The direction of the centrifugal field is from left to right. (a) Rayleigh interference profiles for ovalbumin (M = (45 000 ± 2000) g/mol). Phosphate-chloride buffer, pH 6.8, I = 0.10 M). Each fringe profile represents a plot of concentration of the protein (relative to the solution/air meniscus 'm') against distance from the centre of the ultracentrifuge rotor; 'b' represents the base of the cell. Rotor speed = 17 250 rev./min; temperature = 20.0°C. Loading concentration $c^\circ \sim 0.5$ mg/ml. (b) Absorption optics profiles (recorded at a wavelength λ of 280 nm) for β-lactoglobulin B. A special multichannel cell was used permitting three samples: inner (c° = 0.1 mg/ml); middle (c° = 0.2 mg/ml) outer: (c° = 0.3 mg/ml). The same buffer as (a) was used. Rotor speed = 15 000 rev./min. Because of restrictions of the Lambert–Beer law, with the outer channel only absorbances < 1.5 could be used. (c) Rescan of the inner channel of (b) but using a wavelength of 230 nm to enhance the signal; this picks up the peptide bond in the protein which usually gives a higher absorbance than the aromatic amino acid absorbances at 280 nm.

The most accurate way of recording these final steady state concentration distributions is using Rayleigh interference optics (Fig 5.6a). More appropriately for proteins, we can take advantage of absorption of the aromatic amino acids or the peptide, bond to use the much more convenient absorption optics set for the ultraviolet (Fig. 5.6(b,c)): if absorption optics are used patterns can be routinely captured automatically on-line and analysed (Giebler, 1992; Lewis, 1992). With the Rayleigh interference system, it is much more complicated, and indeed expensive, to perform on-line data capture (Laue, 1992). Whatever the system, these patterns are usually now captured and read automatically, either directly into a computer, or via photography or chart recorder output first (Rowe *et al.*, 1992).

How do we get molecular weight (or molar mass) information? The interfaced computer converts the digitized information of Fig. 5.6 into an accurate record of log concentration (expressed in terms of absorbance units, A, or fringe displacement units, J) versus radial distance squared. For fairly ideal monodisperse systems (e.g. dilute solutions of some small proteins) such plots are linear, but for non-ideal or heterogeneous systems they are not (Fig. 5.7). The average slope of these plots, linear or otherwise, gives what we call the whole distribution weight average molar mass, or whole cell weight average molar mass, $M^{\circ}_{w,\,app}$. (We use a procedure known as 'MSTAR' for this purpose (Harding *et al.*, 1992).) These are apparent values, hence the subscript 'app'. This is because of the thermodynamic non-ideality of the system arising from excluded volume effects (related to the effective volume, after hydration, of the macromolecular species) and from charge effects if the macromolecule is highly charged. These effects are usually represented by the virial term B (A_2 in some texts) in the relation

$$(1/M^{\circ}_{w,\,app}) = (1/M^{\circ}_{w})(1 + 2BM^{\circ}_{w}c). \qquad (5.7)$$

So the quantity $(1 + 2BM^{\circ}_{w}c)$ is the factor by which, at a given concentration c, the measured molar mass, $M^{\circ}_{w,\,app}$, differs from the true or

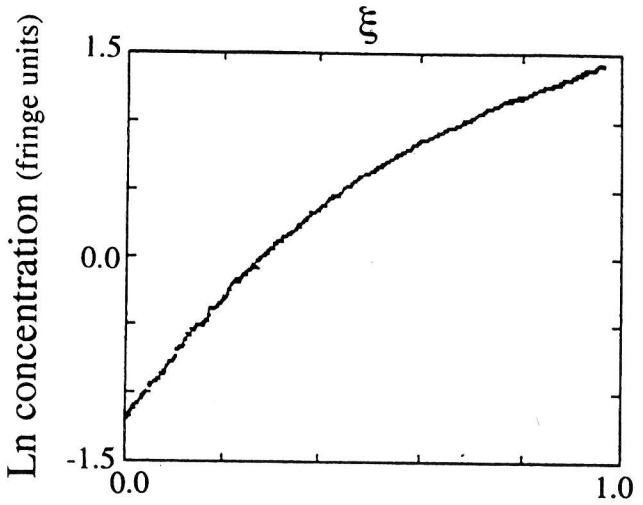


Fig. 5.7 Plot of log concentration (expressed in Rayleigh fringe units J) versus the normalized radial displacement squared parameter ξ for κ-carrageenan, where $\xi = (r^2 - a^2)/(b^2 - a^2)$, r is the radial displacement at a given point in the distribution, and a and b are the corresponding positions of the meniscus and cell base, respectively. Rotor speed = 6419 rev./min; temperature = 21.0°C (pH 6.5, I = 0.10 M). The downward curvature (negative $d^2 \ln J/d\xi^2$) is caused by the large degree of non-ideality of this macromolecule at the loading concentration employed (~ 1.4 mg/ml). $M^{\circ}_{w,\,app}$ = 77 000 g/mol at this concentration compared with the ideal M°_{w} of 284 000 g/mol. ($B \sim 3.4 \times 10^{-3}$ ml mol g^{-2}) (from Day *et al.*, 1995).

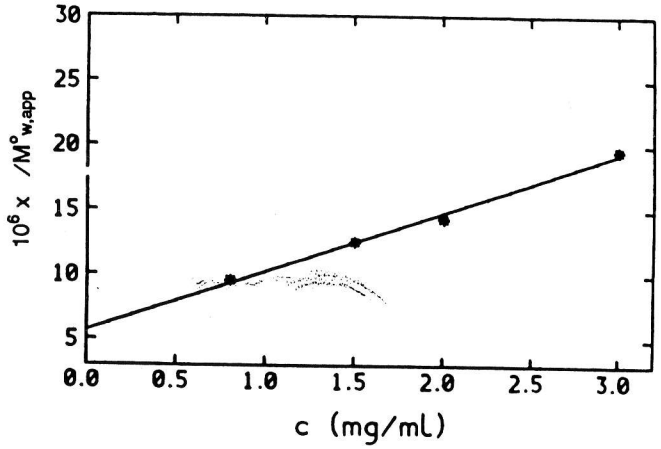


Fig. 5.8 Plot of $1/M^{\circ}_{w,\,app}$ versus loading concentration c for the fibre polysaccharide xylan. M°_{w} = 177 000 g/mol; $B = 2.3 \times 10^{-3}$ ml mol g^{-2} (from Elizabeth *et al.*, 1995).

'ideal' molar mass, M°_{w}. The minimum concentration required for a sedimentation equilibrium run is in the range ~0.1–0.3 mg/ml, if 'long' (i.e. 20–30 mm) path length cells are used. At these concentrations, for food proteins and many food polysaccharides, this factor is relatively small (5% or less) and so it is reasonable to approximate M°_{w} as $M^{\circ}_{w,\,app}$ (Harding *et al.*, 1991a). For other food polysaccharides – especially the polyanions such as alginates and xanthan – where $(1 + 2BM^{\circ}_{w}c)$ is > 1.05, or where working at low concentrations is not possible or may lead to loss of structural integrity of the molecule (possible dissociation of a multi-subunit protein), a range of loading concentrations must be used and an extrapolation to zero concentration is necessary (Fig. 5.8). But a word of warning is required: although the fitting of reciprocal $M^{\circ}_{w,\,app}$ as a function of concentration gives reliable B values, depending on the form of the extrapolation an extrapolation of $1/M^{\circ}_{w,\,app}$ can give poor estimates of M°_{w} and often it is safer to extrapolate $M^{\circ}_{w,\,app}$ values directly (and manually). This is specially true for highly concentration dependent systems where other virial terms (C, D or A_3, A_4, etc.) may be necessary (Horton *et al.*, 1991).

Local slopes along the curves of Fig. 5.7 can also be taken to give point or 'local' average molecular weights ($M_{w,\,app}$) at a given radial position in the ultracentrifuge cell, and these can be particularly useful for assaying interacting systems, especially when given as a function of the local concentration in the cell. One of the classic examples of this, which has been extensively studied (see Visser *et al.*, 1972), is the effect of temperature on the association/dissociation equilibrium of β-lactoglobulin

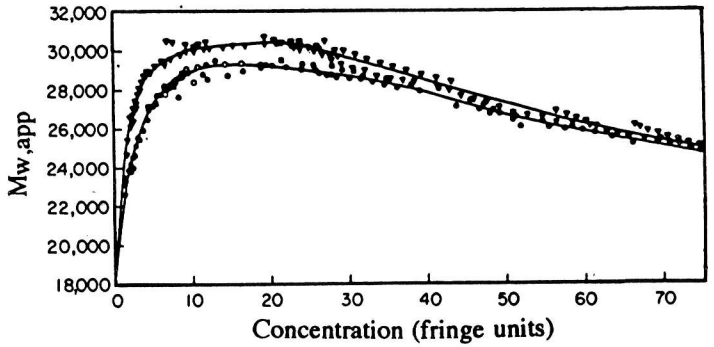


Fig. 5.9 Association–dissociation plot of apparent weight average molecular weight as a function of concentration (expressed in fringe numbers) for β-lactoglobulin B at low pH (pH 2.64, $I = 0.16$ M). Upper curve, temperature = 15°C, lower curve, temperature = 25°C (from Visser *et al.*, 1972). The several kinds of points which provide the information for the two interpolation curves (open and closed circles and triangles) represent different cell loading concentrations. The fact that for a given temperature they overlap is diagnostic of genuine self-association behaviour (Roark and Yphantis, 1969). The fits give values for the association (dimerization) constant 'k_2' of (3900 ± 1010) ml/g at 15°C and (2310 ± 50) ml/g at 25°C.

B at low pH (Fig. 5.9). Further, if $M^{\circ}_{w,app}$ is given as a function of the square of the radial displacement, then the value of $M^{\circ}_{z,app}$ can be estimated from.

$$M^{\circ}_{z,app} = \{M_{w,app}(b)\cdot c(b) - M_{w,app}(a)\cdot c(a)\}/\{c(b) - c(a)\}, \qquad (5.8)$$

where a is the radial position of the cell meniscus and b the cell base. (In some special cases the number average molecular weight can also be obtained from Rayleigh optical records.) $M_{z,app}$ values can also be obtained directly by using the Schlieren optical system mentioned earlier in the context of sedimentation velocity (see Rowe *et al.*, 1992). The ratio of the z-average to the weight-average molecular weight is often used as an 'index of polydispersity' of polymeric samples, as it can be related to the breadth of the distribution. For example, for a preparation of the polysaccharide xylan (which provides fibre in some food products), a value of $M^{\circ}_{z,app}/M^{\circ}_{w,app}$ of ~1.2 has been obtained (Elizabeth *et al.*, 1995) using this procedure.

There are three other ways of using sedimentation equilibrium data to get molecular weight distributions (Harding, 1994c). Perhaps the simplest in principle and most useful in practice is to combine it with gel filtration, giving the latter an 'absolute' basis (i.e. not subject to assumptions concerning the conformation of calibration standards). Gel filtration is a very simple but powerful way of separating macromolecules of different sizes in a polydisperse solution. It can also give molecular

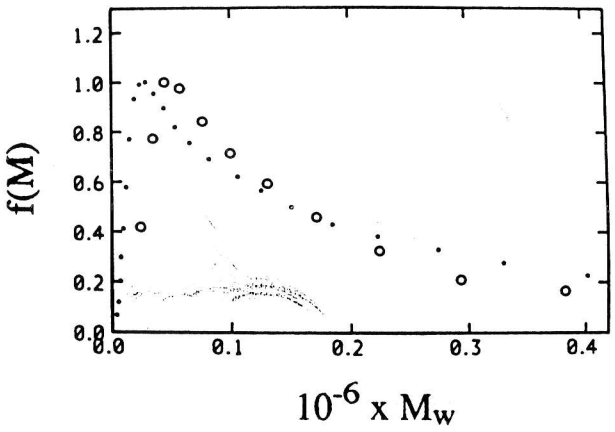


Fig. 5.10 Distribution of molecular weight $f(M)$ for a citrus pectin determined by gel permeation chromatography with columns calibrated by absolute molecular weight measurements on pectin fractions by low speed sedimentation equilibrium (open circles). The results agree well with a similar calibration procedure using classical light scattering measurements (small filled circles). Reproduced, with permission, from Harding *et al.* (1991).

weight distributions by comparing elution profiles with those of standard molecules of known molecular weight and the same conformation as the molecules being studied. Although the latter requirement is usually satisfied if globular proteins are being studied and globular protein standards are used, it fails for synthetic polymer and polysaccharide systems where so-called 'polystyrene' or 'pullulan' standards are often used. These problems can be avoided by taking fractions from the column and separately determining their molecular weights by sedimentation equilibrium (Fig. 5.10).

5.6 Analytical density gradient sedimentation equilibrium

5.6.1 Composition analysis

Density gradient sedimentation equilibrium procedures using *preparative* ultracentrifuges are routinely used in biochemical science for purifying macromolecular systems on the basis of their density; the method is sometimes called *isopycnic* density gradient equilibrium. The analogous situation in an analytical ultracentrifuge permits us to assay for purity. (This was the classical method used by Meselson *et al.* (1957) to show that the replication of DNA was semi-conservative.) The idea is to have as a solvent a dense material which redistributes so that there is a

distribution or 'gradient' of density throughout the sedimentation cell. Any dissolved macromolecule will move until at equilibrium it reaches its isodensity point, i.e., the position in the cell where its own density matches that of the solvent. In aqueous solvents caesium salts are often used to produce the gradient (in the range 1.2–1.7 g/ml). Proteins, polysaccharides and nucleic acids have their isodensity points at ~1.3 g/ml, ~1.6 g/ml and ~1.7 g/ml, respectively, and hence this method is very useful for checking the purity of food polysaccharide or food protein preparations (see Harding, 1992).

5.6.2 Gels

The technique of sedimentation equilibrium can also be applied to the study of the structure of gels, especially if the Schlieren optical system is used, and it can provide complementary information to conventional rheological methods. For example, because the network concentration will vary in the gel as a function of radial position, it is possible to monitor the swelling pressure and other thermodynamic properties of the gel as a function of concentration. These procedures are outside the scope of this introductory text; for further details the interested reader should consult Borchard (1991), Cölfen and Borchard (1991) and Holtus *et al.* (1991). Using the absorption optical system, and after selection of an appropriate wavelength (i.e. in which the gel matrix is invisible), it is possible in principle to follow the diffusion of small molecules – including small proteins – through the gel, as a probe into gel structure.

5.6.3 Diffusion analysis

Historically, the ultracentrifuge was used as a tool for measuring the diffusion coefficients of macromolecules in solution using a procedure known as 'boundary spreading'. That is, how fast a sedimenting boundary broadened out was a function of the diffusion coefficient; then by combining the diffusion coefficient with the sedimentation coefficient, the molecular weight could be obtained via a relation known as the 'Svedberg equation'. Nowadays, the more rapid technique of dynamic light scattering is the method of choice for diffusion measurements, but for certain systems the analytical ultracentrifuge has clear advantages. One of these is for the analysis of the diffusion of small molecules or macromolecules through concentrated media, including gels and two-phase systems. With the latter, it is possible to model membrane phenomena and the diffusion of small molecules through food gels/matrices (a low rotor speed is chosen simply to minimize convection effects). Applied in this way, the ultracentrifuge is not being used as a sedimentation tool as such, but rather the optical system alone is being

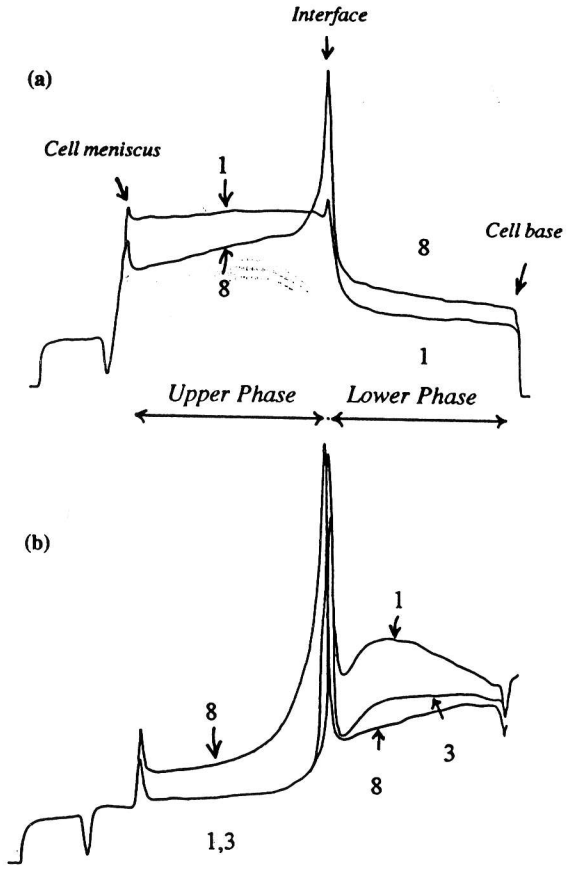


Fig. 5.11 Diffusion of lipase (*Chromobacter viscosum*) through an interface separating two aqueous phases, monitored using the absorption optics (at 280 nm) on an analytical ultracentrifuge. The phases are formed by mixing dextran and polyethylene glycol in the appropriate ratio. The numbers (1,3,8) correspond to various scan times. (a) Lipase initially in the top (i.e. less dense) phase in the ultracentrifuge cell. (b) Lipase initially in the 'bottom' phase. Note in both cases the massive accumulation of protein at the interface, and also (in (b) only) the irregularities near the interface on the lower phase side (from Simpkin, 1994; see also Harding and Tombs, 1989).

used (Fig. 5.11). Nonetheless relatively detailed information can be drawn concerning the diffusion through and towards interfaces between two phase systems (Tombs and Harding, 1988; Harding and Tombs, 1989). This has proved highly valuable in the study of lipases which are being increasingly considered for use in oil and fat processing (Macrae and Hammond, 1985), and their structure and interfacial behaviour has recently been studied in some depth by this technique (Simpkin, 1994).

References

Advani, M. and Harding, S.E. (1995) Unpublished results.

Anderson, M.T. (1991) *The Interaction of Mucous Glycoproteins with Polymeric Materials*, Ph.D. Dissertation, University of Nottingham, UK.

Baldwin, R.L. (1957) Boundary spreading in sedimentation velocity experiments. *Biochem. J.*, **65**, 503–512.

Berth, G., Jumel, K. and Harding, S.E. (1995) Unpublished results.

Blake, C.C.F., Loenig, D.F., Mair, G.A., North, A.C.T., Phillips, D.C. and Sarma, V.R. (1965) Structure of hen egg-white lysosyme. *Nature (London)*, **206**, 757–761.

Blake, C.C.F., Geisow, M.J. and Oatley, S.J. (1978) Structure of pre-albumin: secondary, tertiary and quaternary interactions determined by Fourier refinement at 1–8 Å. *J. Mol. Biol.*, **121**, 335–356.

Borchard, W. (1991) Swelling pressure equilibrium of swollen crosslinked systems in an external field. I: Theory. *Prog. Colloid Polym. Sci.*, **86**, 84–91.

Byron, O.D. (1992) *Solution Studies on the Conformation and Assembly of the Monoclonal Antibody B72.3*, Ph.D. Dissertation, University of Nottingham, UK.

Cecil, R. and Ogston, A.G. (1949) The sedimentation constant, diffusion constant and molecular weight of β-lactoglobulin. *Biochem. J.*, **44**, 33–35.

Cölfen, H. and Borchard, W. (1991) Swelling pressure equilibrium of swollen crosslinked systems in an external field. III: Unsolved problems concerning the systems gelatin/water and κ-carageenan/water. *Prog. Colloid Polym. Sci.*, **86**, 102–110.

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.

Day, K.M., Dhami, R. and Harding, S.E. (1995) Unpublished results.

Dhami, R., Harding, S.E., Mitchell, J.R., Hunt, T. and Jones, T. (1995) Unpublished results.

Dickerson, R. and Geiss, I. (1969) *The Structure and Action of Proteins*, Benjamin, California.

Edmond, E., Farquhar, S., Dunstone, J.R. and Ogston, A.G. (1968) The osmotic behaviour of Sephadex and its effects on chromatography. *Biochem. J.*, **108**, 755–763.

Elizabeth, N.J., Dhami, R. and Harding, S.E. (1995) Unpublished results.

Emes, C.H. and Rowe, A.J. (1978) Hydrodynamic studies on the self association of vertebrate skeletal muscle. *Biochim. Biophys. Acta*, **537**, 110–124.

Errington, N., Harding, S.E., Vårum, K.M. and Illum, L. (1993) Hydrodynamic characterization of chitosans of differing degrees of acetylation. *Int. J. Biol. Macromol.*, **15**, 113–117.

Gaisford, S.E., Harding, S.E., Mitchell, J.R. and Bradley, T.D. (1986) A comparison between the hot and cold water soluble fractions of two locust bean gum samples. *Carbohydr. Polym.*, **6**, 423–442.

Garcia de la Torre, J. (1989) Hydrodynamic properties of macromolecular assemblies, in *Dynamic Properties of Biomolecular Assemblies*, Royal Society of Chemistry Special Publication no. 74, Cambridge, U.K. pp. 3–31.

Giebeler, R. (1992) The Optima XL-A: a new analytical ultracentrifuge with a novel precision absorption optical system, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. S.E. Harding, A.J. Rowe and J.C. Horton) Royal Society of Chemistry, Cambridge, chap. 2.

Görnitz, E. and Linow, K.-J. (1992) Experience with the MOM 3180 analytical ultracentrifuge, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. S.E. Harding, A.J. Rowe and J.C. Horton) Royal Society of Chemistry, Cambridge, chap. 3.

Harding, S.E. (1981a) A compound hydrodynamic shape function derived from viscosity and molecular covolume measurements. *Int. J. Biol. Macromol.*, **3**, 340–341.

Harding, S.E. (1981b) Could egg albumin be egg shaped? *Int. J. Biol. Macromol.*, **3**, 398–399.

Harding, S.E. (1987) A general method for modelling macromolecular shape in solution. A graphic (P–G) intersection procedure for triaxial ellipsoids. *Biophys. J.*, **51**, 673–680.

Harding, S.E. (1989) Modelling the gross conformation of assemblies using hydrodynamics: the whole body approach, in *Dynamic Properties of Biomolecular Assemblies*, Royal Society of Chemistry, Special Publication No. 74, Cambridge, pp. 32–56.

Harding, S.E. (1992) Sedimentation analysis of polysaccharides, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. S.E. Harding, A.J. Rowe and J.C. Horton) Royal Society of Chemistry, Cambridge, chap. 27.

Harding S.E. (1994a) Determination of macromolecular homogeneity, shape and interactions using sedimentation velocity analytical ultracentrifugation, *Methods Mol. Biol.*, **22**, 61–73.

Harding, S.E. (1994b) Determination of absolute molecular weights using sedimentation equilibrium analytical ultracentrifugation. *Methods Mol. Biol.*, **22**, 75–84.

Harding S.E. (1994c) The analytical ultracentrifuge spins again. *Trends Anal. Chem.*, **99**, 439–446.

Harding S.E. and Johnson, P. (1985) The concentration dependence of macromolecular parameters. *Biochem. J.*, **231**, 543–547.

Harding S.E. and Rowe, A.J. (1982) Modelling biological macromolecules in solution. I. The ellipsoid of revolution, *Int. J. Biol. Macromol.*, **4**, 160–164.

Harding S.E. and Tombs, M.P. (1989) Protein diffusion through interfaces, *Interactions in Protein Systems* (eds. K.D. Schewnke and B. Raab), Abhandlungen der Akademie der Wissenschaften der DDR, Akademi-Verlag, Berlin, pp. 65–72.

Harding S.E., Kyles, P., West, G. and Norton, G. (1987) The relative molecular mass, heterogeneity and subunit composition of the 12S globulin from oil seed rape. *Biochem. Soc. Trans.*, **15**, 684.

Harding S.E., Vårum, K.-M., Stokke, B.-T. and Smidrsød, O. (1991a) Molecular weight determination of polysaccharides. *Adv. Carbohydr. Anal.*, **1**, 63–144.

Harding S.E., Berth, G., Ball, A., Mitchell, J.R. and Garcia de la Torre, J. (1991b) The molecular weight distribution and conformation of citrus pectins in solution studied by hydrodynamics. *Carbohydr. Polym.*, **16**, 1–15.

Harding S.E., Horton, J.C. and Morgan, P.J. (1992) MSTAR: A FORTRAN program for the model independent molecular weight analysis of macromolecules using low speed or high speed sedimentation equilibrium, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. Harding S.E., A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 15.

Harding S.E., Jumel, K., Kelly, R., Gudo, E., Horton, J.C. and Mitchell, J.R. (1993) The structure and nature of protein-polysaccharide complexes, in *Food Proteins: Structure and Functionality*, (eds. K.-D. Schwenke and R. Mothes), VCH Verlagsgesellschaft, Weinheim, Germany, pp. 216–226.

Harding S.E., Horton, J.C. and Berth, G. (1995) *Ultracentrifuge Studies on Lime Pectin.* Manuscript in preparation.

Hearst, J.E. and Stockmayer, W.H. (1962) Sedimentation constants of broken chains and wormlike coils. *J. Chem. Phys.*, **37**, 1425–1433.

Holt, J.C. (1970) *A Study of the Reversibility of Denaturation of Ovalbumin*, Ph.D. Dissertation, University of London, UK.

Holtus, G., Cölfen, H. and Borchard, W. (1991) Swelling pressure equilibrium of swollen crosslinked systems in an external field. II: The determination of molecular parameters of gelatin/water gels from the swelling pressure-concentration curves. *Prog. Colloid Polym. Sci.*, **86**, 92–101.

Horton, J.C., Harding S.E., Mitchell, J.R. and Morton-Holmes, D.F. (1991) Thermodynamic non-ideality of dilute solutions of sodium alginate studied by sedimentation equilibrium ultracentrifugation. *Food Hydrocolloids*, **5**, 125–127.

Howlett, G.J. (1992) The preparative ultracentrifuge as an analytical tool, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. S.E. Harding, A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 4.

Iniesta, A., Diaz, F.G. and Garcia de la Torre, J. (1988) Transport properties of rigid bent-rod macromolecules and of semi-flexible broken rods in the rigid body treatment. Analysis of the flexibility of myosin rod. *Biophys. J.*, **54**, 269–275.

Jeffrey, P.D., Nichol, L.W., Turner, D.R. and Winzor, D.J. (1977) The combination of molecular covolume and frictional coefficient to determine the shape and axial ratio of a rigid macromolecule. Studies on ovalbumin. *J. Phys. Chem.*, **81**, 776–781.

Johnson, P. (1964) A sedimentation study on gel systems. *Proc. R. Soc. London Ser. A* 278, 527–542.

Johnson, P. and Metcalfe, J.C. (1967) Physico-chemical studies on gelatin gels from soluble and insoluble collagens. *Eur. Polym. J.*, **3**, 423–447.

Jullander, I. (1985) Svedberg and the Polysaccharides, in *Physical Chemistry of Colloids and Macromolecules (The Svedberg Symposium)*, (ed. B. Rånby), Blackwell Scientific, Oxford, UK, pp. 29–36.

Kartha, G., Bellow, J. and Harker, D. (1967) Tertiary structure of ribonuclease. *Nature, (London)*, **213**, 862–865.

Kawahawa, K., Ohta, K., Miyamoto, H. and Nakamura, S. (1984) Preparation and solution properties of pullulan fractions as standard samples for water-soluble polymers. *Carbohydr. Polym.*, **4**, 335–356.

Kendrew, J.C., Bodo, G., Dintzis, H.M., Parish, H.M., Wycoff, H. and Phillips, D.C. (1958) A three-dimensional model of the myoglobin molecule obtained by X-ray analysis. *Nature (London)*, **181**, 662–666.

Krishnamurti, K. and Svedberg, T. (1930) The ultracentrifuge study of gelatin solutions. *J. Am. Chem. Soc.*, **52**, 2897–2906.

Laue, T.M. (1992) On-line data aquisition and analysis from the Rayleigh interferometer, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. S.E. Harding, A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 6.

Laue, T.M., Shah, B.D., Ridgeway, T.M. and Pelletier, S.L. (1992) Computer aided interpretation of analytical sedimentation data for proteins, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. Harding S.E., A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 7.

Lewis, M.S. (1992) Data acquisition and analysis systems for the absorption optical system of the analytical ultracentrifuge, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. Harding S.E., A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 8.

Macrae, A.R. and Hammond, R.C. (1985) Present and future applications of lipases. *Biotechnol. Gent. Eng. Rev.*, **3**, 193–217.

Marsh, E.N. and Harding, S.E. (1993) Methylmalonyl-CoA mutase from *Propionibacterium shermanii:* characterization of the cobalamin-inhibited form and subuniy-cofactor interactions using analytical ultracentrifugation. *Biochem. J.*, **290**, 551–555.

Meselson, M., Stahl, F.W. and Vinograd, J. (1957) Equilibrium sedimentation of macromolecules in density gradients, *Proc. Natl. Acad. Sci. USA*, **43**, 581–583.

McKenzie, H.A. (ed.) (1970) *Milk Proteins, Chemistry and Molecular Biology*, Academic Press, New York. Vol. I.

Miller, G.L. and Golder, R.H. (1952) Sedimentation studies with the Spinco ultracentrifuge. *Arch. Biochem. Biophys.*, **36**, 249–258.

Nishihara, T. and Doty, P. (1958) The sonic fragmentation of collagen macromolecules. *Proc. Natl. Acad. Sci. USA*, **44**, 411–417.

Pessen, H. and Kumosinski, T.F. (1985) Measurements of protein hydration by various techniques. *Methods Enzymol.*, **117**, 219–255.

Perkins, S.J. (1989) Hydrodynamic modelling of complement, in *Dynamic Properties of Biomolecular Assemblies*, Royal Society of Chemistry Special Publication no. 74, Cambridge, U.K. pp. 226–245.

Perrin, F. (1936) (Translated title) Brownian motion of an ellipsoid II. Free rotation and depolarization of fluorescence. Translation and diffusion of ellipsoidal molecules. *J. Phys. Radium*, **7**, 1–11.

Perutz, M.F., Rossmann, M.G., Cullis, A.F., Muirhead, H., Will, G. and North, A.C.T. (1960) Structure of haemoglobin-A. Three-dimensional Fourier synthesis at 5.5 Å resolution, obtained by X-ray analysis. *Nature (London)*, **185**, 416–422.

Plietz, P., Damaschun, G., Müller, J.J. and Schwenke, K.-D. (1983) The structure of the 11-S globulins from sunflower and rape seed. A small-angle X-ray scattering study. *Eur. J. Biochem.*, **130**, 315–320.

Prakash, V. (1992) Sedimentation analysis of seed proteins, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. Harding S.E., A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 24.

Roark, D.E. and Yphantis, D.A. (1969) Studies of self-associating systems by equilibrium ultracentrifugation. *Ann. N.Y. Acad. Sci.*, **164**, 245–278.

Rowe, A.J. (1977) The concentration dependence of transport processes. A general description applicable to the sedimentation, translational diffusion and viscosity coefficients of macromolecular solutes. *Biopolymers*, **16**, 2595–2611.

Rowe, A.J., Wyne-Jones, S., Thomas, D.G. and Harding, S.E. (1992) Methods for off-line analysis of sedimentation velocity and sedimentation equilibrium patterns, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. Harding S.E., A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 5.

Schachman, H.K. (1989) The analytical ultracentrifuge reborn. *Nature (London)*, **941**, 259–260.

Scheraga, H.A. and Mandlekern, L. (1953) Consideration of hydrodynamic properties of proteins. *J. Am. Chem. Soc.*, **75**, 179–184.

Schmidt, B. and Riesner, D. (1992) A fluorescence detection system for the analytical ultracentrifuge and its application to proteins nucleic acids, viroids and viruses, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. S.E., Harding, A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 11.

Seymour, G.B. and Harding, S.E. (1987) Analysis of the molecular size of tomato (*Lycopersicon esculentum* Mill) fruit polyuronides by gel filtration and low-speed sedimentation equilibrium. *Biochem. J.*, **245**, 463–466.

Shulman, S. (1953) The determination of sedimentation constants with the oil-turbine and Spinco ultracentrifuges. *Arch. Biochem. Biophys*, **44**, 230–239.

Siholta, H. and Svedberg, T. (1948) A new method to determine the degree of solvation of macromolecules. *Acta Chem. Scand.*, **2**, 474–485.

Simpkin, N.J. (1991) *Solution Behaviour and Phase-Phase Diffusion of Microbial Lipases*, Ph.D. Dissertation, University of Nottingham, UK.

Simpkin, N.J., Harding, S.E. and Tombs, M.P. (1991) Solution behaviour of *Chromobacter viscosum* and *Pseudomonas* sp. lipases: no evidence of self-association. *Biochem. J.*, **273**, 611–613.

Smith, C.J., Watson, C.F., Morris, P.C., Bird, C.R., Seymour, G.B., Gray, J.E., Arnold, C., Tucker, G.A., Schuch, W., Harding, S.E. and Grierson, D. (1990) Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. *Plant Mol. Biol.*, **14**, 369–379.

Squire, P.G. and Himmel, M. (1979) Hydrodynamics and protein hydration. *Arch. Biochem. Biophys.*, **196**, 165–177.

Sjögren, B. and Svedberg, T. (1930) The pH stability region of egg albumin. *J. Am. Chem. Soc.*, **52**, 5187–5192.

Smidsrød, O. and Andresen, I.L. (1979) *Biopolymerkjemi*, Tapir, Trondheim, Norway.

Stafford, W.F. (1992) Methods for obtaining sedimentation coefficient distributions, in *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, (eds. S.E. Harding, A.J. Rowe and J.C. Horton), Royal Society of Chemistry, Cambridge, chap. 20.

Svedberg, T. (1927) *Prix Nobel 1926*, Stockholm; see also Farber, E. (ed.) (1953) *Nobel Prize Winners in Chemistry 1901–1961*, Abelard-Schuman, London, pp. 99–102.

Svedberg, T. and Erikkson. I.-B. (1934) Ultracentrifugal study of the action of papain on ovalbumin. *Biol. Bull.*, **66**, 191–223.

Svedberg, T. and Fåhraeus, R. (1926) A new method for determination of the molecular weight of the protein. *J. Am. Chem. Soc.*, **48**, 430–438.

Svedberg, T. and Nichols, J.B. (1923) Determination of size and distribution of size of particles by centrifugal methods. *J. Am. Chem. Soc.*, **45**, 2910–2917.

Svedberg, T. and Rinde, H. (1923) The determination of the distribution of size of particles in disperse systems. *J. Am. Chem. Soc.*, **45**, 943–954.

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York.

Tanford, C. and Buzzell, J. G. (1956) The viscosity of aqueous solutions of bovine serum albumin between pH 4.3 and 10.5. *J. Phys. Chem.*, **60**, 225–231.

Tombs, M.P. and Harding, S.E. (1988) Protein transport processes in the water–water interface in incompatible two-phase systems, in *Advances in Separations using Aqueous Phase Systems in Cell Biology and Biotechnology*, (eds. D. Fisher and I.A. Sutherland) Plenum, New York, pp. 229–232.

Townend, R., Weinberger, L. and Timasheff, S.N. (1960) Molecular interactions in β-lacto-

globulin. IV. The dissociation of β-lactoglobulin below pH 3.5. *J. Am. Chem. Soc.*, **82**, 3175–3179.

Visser, J., Deonier, R.C., Adams, E.T. Jr. and Williams, J.W. (1972) Self-association of β-lactoglobulin B in acid solution and its variation with temperature. *Biochemistry*, **11**, 2634–2643.

Wales, M. and van Holde, K.E. (1954) The concentration dependence of the sedimentation constants of flexible macromolecules. *J. Polym. Sci.*, **14**, 81–86.

Winwood, R. (1986) *Functional Properties of a Novel Food Gelling System*, Ph.D. Dissertation, University of Nottingham, UK

Woodward, J.R., Phillips, D.R. and Fincher, G.B. (1983) Water-soluble (1–3),(1–4)–β–D–glucans from barley (*Hordeum vulgare*) endosperm. I. Physicochemical properties. *Carbohydr. Polym.*, **3**, 143–156.

Yamakawa, H. (1971) *Modern Theory of Polymer Solutions*, Harper and Row, New York.