THE SEDIMENTATION EQUILIBRIUM ANALYSIS OF POLYSACCHARIDES AND MUCINS:

A GUIDED TOUR OF PROBLEM SOLVING FOR DIFFICULT HETEROGENEOUS SYSTEMS

Stephen E. Harding

INTRODUCTION

For the last 12 years we have been applying hydrodynamic methods to study a particularly difficult heterogeneous class of biological macromolecule - the polysaccharides - which possibly present the ultracentrifuge with one of its biggest challenges (see, e.g. Harding, 1992). In this paper I am going to describe some of the many problems we've encountered in trying to use sedimentation equilibrium to study the size, size distribution and interactions of these molecules and, in particular, a particular class of polysaccharide-protein conjugate known as the "mucopolysaccharides" or "mucins" (Harding, 1989).

Although the vast majority of analytical ultracentrifuge users have no direct interest in these molecules in particular, they might possibly pick up a few tips or clues as to how to apply sedimentation equilibrium procedures to difficult heterogeneous macromolecules

in general, of which the polysaccharides are only one small - but nontheless interesting - class.

WHY ARE POLYSACCHARIDES SO DIFFICULT?

A typical polysaccharide, for example a pectin molecule from Mrs. G. Berths laboratory at Potsdam (Fig.1a), can have a poorly defined conformation in solution with a large capacity to trap and entrain surrounding solvent molecules. This results in a high exclusion volume (sometimes >100x in excess of the anhydrous volume) and hence high thermodynamic non-ideality. Another contribution to thermodynamic non-ideality can arise from polyelectrolyte behaviour (Fig. 1b), particularly if a molecule has a high unsuppressed charge in solution, such as a pectin in a low ionic strength solvent. Many "food grade" polysaccharides for example are polyannionic. Mucins are polyanionic because of their sialic acid content. To add to the difficulty, polysaccharide preparations are usually polydisperse (Fig. 1c), that is, they contain species of different molecular weight (either in a "discrete" - viz. paucidisperse - or quasi-continuous sense). Finally, some can have the ability to self-associate in solution (Fig. 1d), such as guar. The net result is that these molecules place a considerable strain on the available ultracentrifuge methodology, and in many cases satisfactory information can only be achieved by combining results with other techniques, notably light scattering, gel permeation chromatography or electron microscopy (see, e.g. Harding et al, 1991).

BASIC INFORMATION SOUGHT

Leaving aside sedimentation velocity and analytical density gradient analyses, what is the sort of basic information we're after using sedimentation equilibrium analysis on these molecules? Clearly its in terms of molecular weight analysis in the form of either molecular weight averages (both "point" and "whole cell"), molecular weight distributions and, if the system performs in complexation phenomena or self-association reactions, in terms of stoichiometries. But there are a number of problems we have to overcome or "troubleshoot".

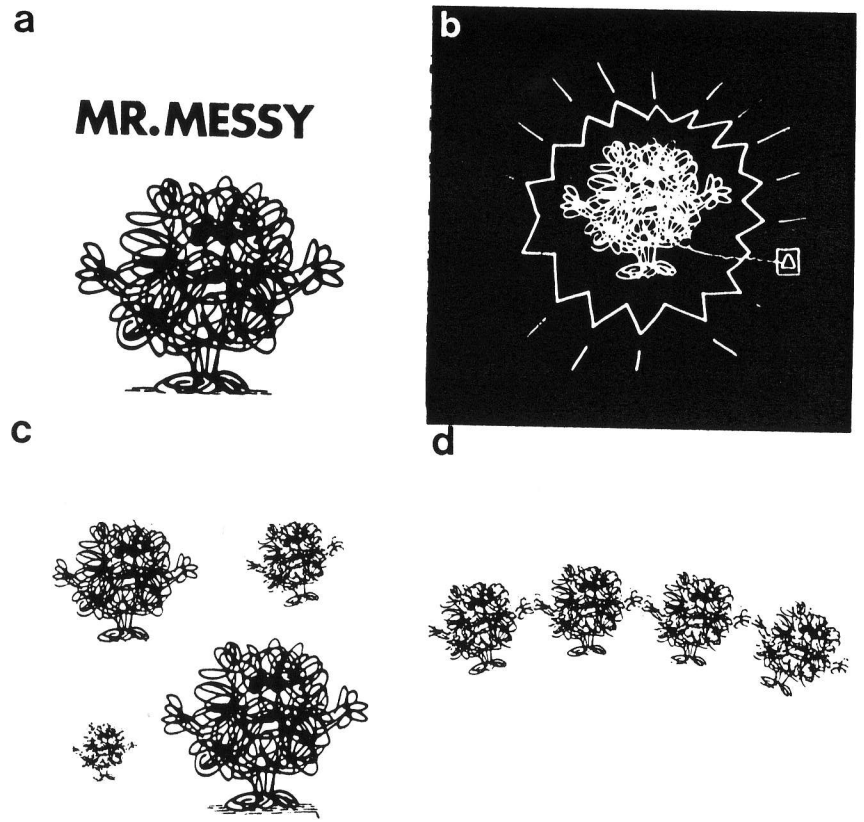


Fig. 1. A typical polysaccharide and its properties.

THE FIRST PROBLEM: LIMITED CHOICE OF OPTICAL SYSTEM

Most polysaccharides do not contain a chromophore in the visible or near (i.e. "useable") part of the ultraviolet, so in general we cannot use the absorption optical system, but must rely instead on either of two refractometric methods: schlieren optics or Rayleigh interference. The latter is preferable because of its greater sensitivity at lower concentration, but the optical record is one of solute concentration *relative* to the meniscus, rather than absolute concentration directly. For simple experiments on proteins, a popular way of avoiding this problem is to use the meniscus depletion method (Yphantis, 1964)

where the ultracentrifuge is run at a high enough speed so the meniscus is effectively depleted of macromolecular solute - the optical record is then one of absolute concentration - in fringe number or weight terms - versus radius.

It is worth pointing out also that, in the future, application of the absorption optical system *may* be possible: the new XLA ultracentrifuge from Beckman instruments (Giebler, 1992) appears to have stable optics in the far ultraviolet (210-230nm). All mucins and many polysaccharides absorb significantly in this region and we are exploring the use of "far-uv" detection, with appropriate baselines, for both sedimentation equilibrium and sedimentation velocity.

THE SECOND PROBLEM: INAPPLICABILITY OF THE "HIGH SPEED" OR "MENISCUS DEPLETION" METHOD

Fig. 2 illustrates the next problem any sedimentation equilibrium analysis on these materials needs to overcome: that is we cannot use the "meniscus depletion" (see, e.g., Yphantis, 1964), method, widely used in protein biochemistry. Fig. 2 in fact shows the solution Rayleigh fringes from a low-speed sedimentation equilibrium experiment on a mucin of weight average molecular weight ~6 million, and at low loading concentration (~0.4 mg/ml), run in three separate solvents Because of the polydispersity of these materials it is generally impossible (except in cases of pseudo-non-ideality) to choose run conditions to get proper meniscus depletion without losing optical registration of the fringes near the cell base: one can observe clearly in Fig. 2 the steep rising fringes at the cell base but finite slope of the fringes near the meniscus.

There is a further problem with the high speed meniscus depletion method in terms of a speed dependent enhancement of the effective thermodynamic second virial coefficient, B_{eff}, as the following equation shows (Fujita, 1975)

$$B_{eff} = B\{1 + \frac{\lambda^2 M_z^2}{12} + \ldots\} \qquad (1)$$

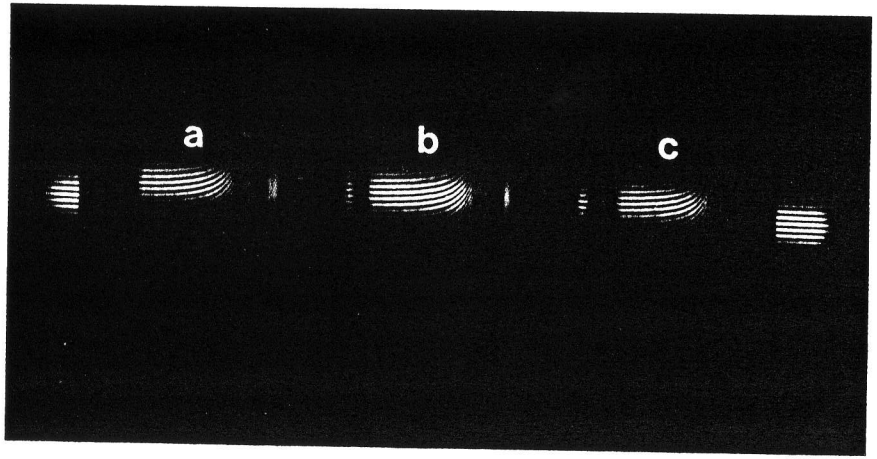


Fig. 2. Rayleigh equilibrium interference patterns for a mucin (bronchial mucin BM GRE) in three different solvents (a) a phosphate/chloride buffer containing 0.4M CsCl; (b) a phosphate/chloride buffer containing 5mg/ml fucose; (c) a phosphate/chloride buffer containing 5 mg/ml N-acetylglucosamine. The initial mucin cell loading concentration in each case was ~0.4mg/ml (30mm path length cell). The rotor speed was 1967 rev/min. From Harding (1984).

where λ is a function of the square of the rotor speed {$\lambda = (1-v\rho_o)\omega^2(b^2-a^2)/2RT$, $\bar{v}$ being the partial specific volume, ρ_o the solvent density, ω the angular velocity and a and b the radial positions at the cell meniscus and base respectively}. The collective result is that with polysaccharides we have to use the low or intermediate speed method with the requirement of a method for evaluating the concentration at the meniscus, either in terms of g/ml or in terms of fringe numbers. The next problem is thus: how do we get meniscii concentrations out?

EVALUATION OF THE MENISCUS CONCENTRATION

My old mentor J.M. Creeth produced in the late 60's with R. Pain (Creeth & Pain, 1967) a very useful review of all the methods for getting meniscii concentrations {denoted "C_a" in weight concentra-

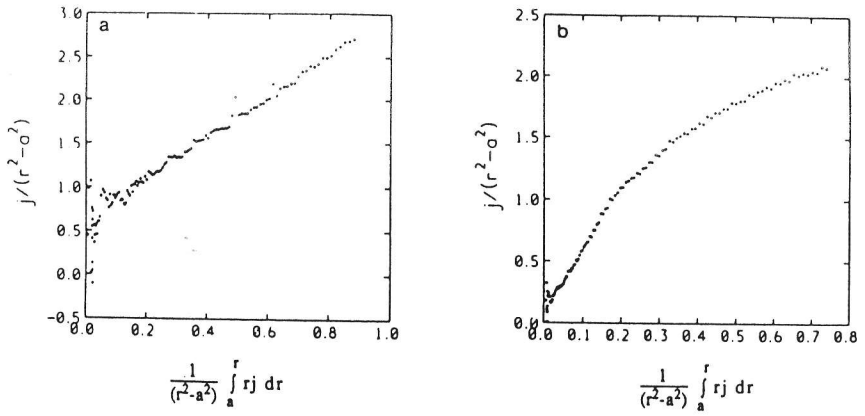


Fig. 3. Extraction of meniscus concentration using the method of ref. 9 for (a) a fairly homogeneous/ideal solution of colonic mucin "T-domains" {J_a ~0.58±0.05}; (b) a highly non-ideal solution of xanthan ("RD") {J_a ~0.01±0.01, i.e. near depletion conditions}. From Harding et al (1992).

tion terms or, more usually "J_a" in the equivalent fringe number terms where the subscript a represents the radial position at the meniscus} out; however, we found the most useful method was one where we get J_a by some simple graphical manipulation of the basic fringe data (Creeth & Harding, 1982a). We can get J_a usually to an accuracy of a few percent from the ratio of twice the intercept to the limiting slope of a plot of $j/(r^2-a^2)$ against $\{1/(r^2-a^2)\}\int_a^r rjdr$ (Fig. 3), where j is the concentration in fringe numbers ("fringe concentration") *relative to the meniscus* and r is the radial displacement. The method usually gives J_a to an accuracy of the order of 0.1 fringe. We find an adapted sliding strip procedure useful for this purpose (Harding et al, 1992), especially where the plots are strongly curved as in Fig.3b. Strong curvature is a symptom of either non-ideality or heterogeneity (the sense of the curve depends on which is the stronger effect)

MULTIPLE DATA CAPTURE AND ANALYSIS

For getting out J_a using this method multiple data collection and averaging is very important for strongly curving systems such as shown in Fig. 3b, and for this purpose the use of automatic multiple data capture and analysis is of extreme value here. We capture our data automatically but not directly *on-line*, as described by for example T. Laue (Laue, 1992) but *off-line*. That is to say we take a photograph and digitise it using a laser densitometer of the sort you can find in many Biochemistry departments (Fig. 4) - these things are normally used for scanning SDS gels, and we use a simple Fourier cosine series algorithm to average over the fringe data set to give our sedimentation equilibrium concentration distribution, to an accuracy comparable with the on-line set-up that T. Laue has described (see, e.g. Rowe et al, 1992).

EXTRACTION OF AVERAGE MOLECULAR WEIGHTS

Whole cell weight average

The next problem is in getting molecular weights out - even getting whole cell or whole distribution weight average values can be much more tricky compared with simple well behaved protein systems. Plots of the logarithm of the concentration against radial displacement squared are often strongly curved because of either heterogeneity (Fig. 5a) or non-ideality (unless by a lucky coincidence the results of the two effects cancel to give a "pseudo-ideal" profile, as in Fig. 5b) Whatever, to get the whole distribution weight average you need to estimate the concentration or the logarithm of the concentration not only at the meniscus, but also *at the cell base*, and this can be very tricky, especially in the case of strong curvature (e.g. Fig.5a) or if the base is not well defined. One way of minimising this problem is to use a function known as "M*" defined by (Creeth & Harding, 1982a)

$$M^*(r) = \frac{j}{kJ_a(r^2-a^2)+2k\int_a^r rjdr} \qquad (2)$$

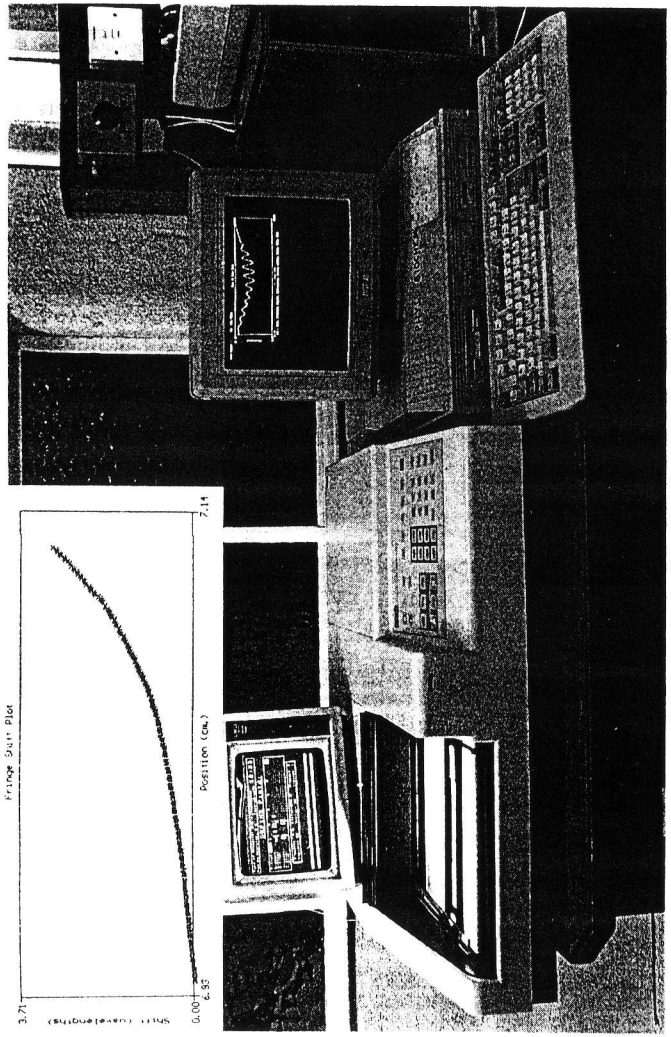


Fig. 4. LKB (Bromma, Sweden) Laser Densitometer set up at Nottingham. This is used to capture (into the Amstrad PC) automatically off-line from photographic film our Rayleigh interference data. The Fourier cosine series algorithm "ANALYSER" produces a 100-200 pt concentration versus distance dataset {as shown in the inset for a low speed sedimentation equilibrium experiment on a commercial guar sample }which is transferred to the FORTRAN programme MSTAR (Harding et al, 1992) on the mainframe IBM 3081/Q for full molecular weight analysis.

where a and b are the radial positions at the solution meniscus and cell base respectively and k is the usual constant (see e.g., Harding, 1992) in terms of rotor speed, ω, solvent density,ρ_o, (the solvent density should be used, not the solution density - see e.g., ref. 1) and partial specific volume v:

$$k = \frac{(1 - \bar{v}\rho_0)\omega^2}{2\,RT} \tag{3}$$

J.M. Creeth and myself (Creeth & Harding, 1982a) found M* defined in such a way to have some useful properties: the most important of these is that its value extrapolated to the cell base equals the weight average over the whole solute distribution "$M^o_{w,app}$", {where the "o" signifies its over the whole cell and the "app" signifies an apparent value at a finite cell loading concentration} and as we found by extensive simulations (Harding, 1992; Creeth & Harding, 1982a) for five different types of systems the M* method appeared to represent a considerable improvement for extracting $M^o_{w,app}$ compared to the conventional "average slope" or log concentration extrapolation methods (Creeth & Pain, 1967).

Point weight average molecular weights ("$M_{w,app}$")

These are relatively straightforward to produce from the fringe concentration data as $d\ln J/dr^2$ times(1/k), (where $J=j + J_a$ and k is as defined in eq. (3)), but again this depends on a reasonable estimate for J_a. J (i.e. the difference in fringe concentration between the meniscus and cell base) needs to be at least 4 fringes for the $M_{w,app}$ data to be reliable without heavy smoothing. We use sliding strip procedures along the lines discussed by Teller (1965, 1973), with an 11pt sliding strip for a total data set of 100-200 radial positions.

Number and z- whole cell averages

The number point average molecular weight $M_{n,app}$ you can, in my opinion, forget about for these substances (unless, for pseudo-ideal systems, you can use the meniscus depletion method): besides J_a you

also need to estimate $M_n(a)$ (Teller, 1965, 1973): the same problem applies to the whole-cell number average. The situation is not quite so bad for z-averages. If Rayleigh optics are used the point z-average is independent of errors in J_a, although it depends on the ratio of a double differential to a single differential viz, data of very high precision is necessary; the whole cell z-average requires accurate estimates of not only J_a and J_b but also the point weight averages at the meniscus and base (see e.g., Teller, 1973; Harding et al, 1992) If reliable z-averages are required then Schlieren optics should be used, which yield $M^{\circ}_{z,app}$ and $M_{z,app}$ directly via the Lamm equation (see, e.g., Creeth & Pain, 1967), and at concentrations now claimed as low (using the Fresnel fringes - Rowe et al, 1992) as can be achieved from Rayleigh fringes (say ~0.3 mg/ml): this would therefore be my method of choice. Indeed, at Nottingham, although we have two Model E's with laser light sources dedicated to Rayleigh optics, we have another dedicated to the Schlieren system producing M_z information.

THE NON-IDEALITY PROBLEM

Focussing on the problem of polysaccharide non-ideality, the first feature which I'm sure many are aware of is that the symptoms of heterogeneity, which tend to produce upward curvature in the log concentration. versus distance squared plots -as in Fig. 5a - can sometimes apparently cancel the symptoms of non-ideality which produce downward curvature to give a pseudo-ideal monodisperse linear plot - as shown in Fig. 5b for a mucin -which can be very misleading. The "pseudo-ideal" system corresponding to Fig. 5b is in fact is both very polydisprese and very non-ideal, and this feature illustrates a statement made long ago by D.Teller (1965) that a linear plot of log c versus distance squared from a single sedimentation equilibrium expt. is not by itself sufficient evidence for monodispersity or ideality.

And, as the Gilberts (G. A. Gilbert & L.M. Gilbert, 1980) and others have shown, since a single symmetric boundary from a sedimentation velocity experiment is also insufficient criterion for solute homogeneity, to this end J.M. Creeth and myself developed a simple assay for solute homogeneity using two cells of different optical path

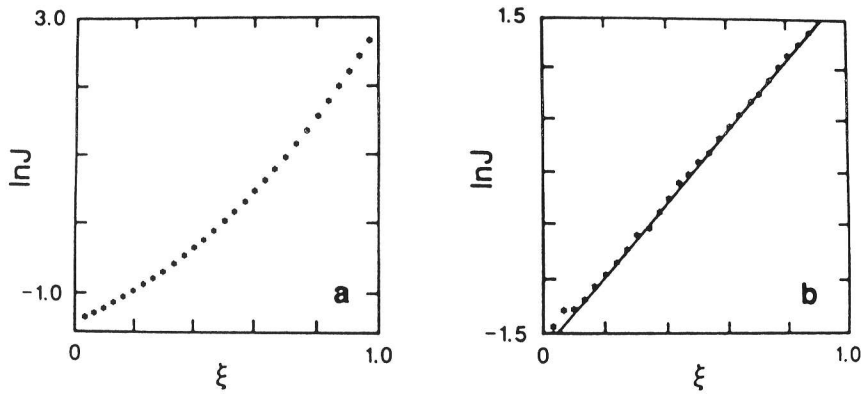


Fig. 5. Log concentration versus distance squared data evaluated from Rayleigh equilibrium optical records for 2 mucins (a) bronchial mucin (from a chronic bronchitis patient) BM GRE (b) bronchial mucin (from a cystic fibrosis patient) CF PHI. ξ is a normalised radial displacement squared parameter, normalised so it has a value of 0 at the meniscus and 1 at the cell base: $\xi = \{r^2-a^2\}/\{b^2-a^2\}$. Note the strong curvature of (a) and the pseudo-ideality of (b). {see, e.g. Harding et al, 1992}

length in a multihole rotor (Creeth & Harding, 1982b). The idea is to compare solution interference fringes of the same initial loading concentration if expressed on a *fringe number* basis but different loading concentrations expressed on a *weight* basis. This test can be performed in a single sedimentation equilibrium experiement if a multi-hole rotor is used together with a suitable combination of two cells (double sector, one with wedge window if the instrument does not have a multiplexing system, or multichannel). One cell or pair of interference channels has say a 30mm optical path length, the other only a 12mm path length but 2 and a half times higher weight concentration to compensate: fringe patterns, and corresponding average molecular weights will be identical *only* for a homogeneous ideal system, and not otherwise. In this way the mucopolysaccharide CFPHI, which *appeared* homogeneous and ideal from the linear log concentration versus distance squared plot of Fig. 5b can clearly be shown to be otherwise (Fig. 6). These observations clearly illustrate the dangers of inferring solute homogeneity or ideality from

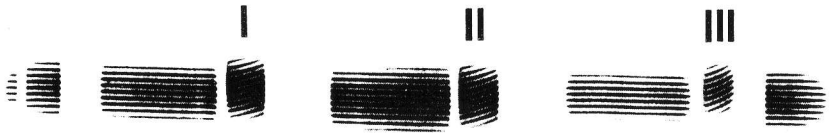


Fig. 6. Sedimentation equilibrium sample homogeneity test. Comparison of Rayleigh equilibrium patterns of the same initial loading concentration on a fringe number basis but differing initial loading concentration on a weight (mg/ml) basis, c^o. Patterns I and II: c^o = 3.0 mg/ml, 12mm path length multi-channel cell (inner two pairs of channels used, outer pair masked) pattern III: c^o = 1.2 mg/ml, 30 mm path-length multi-channel cell (outer pair of channels used, the inner two pairs masked off). Note the different curvature of III confirming the system is neither heterogeneous nor ideal. The identical nature of I and II confirms that the differing radial positions of each does not affect their concentration distribution. The solution column of II appears shorter because (i) the apparent width of the meniscus is much greater in the cell of longer optical path length and (ii) the fringes at the cell base are steeper and accordingly less intense and so are partially lost on photographic reduction (from Creeth & Harding, 1982b).

a single log concentration versus distance squared plot. Some (non-polysaccharide) systems do "pass the test" however, such as a dilute solution of the small virus TYMV (Harding & Johnson, 1985).

Moving back to the non-ideality problem, this can be very severe for polysaccharides as Table 1 shows. The interesting column is the "1 + 2BMc" one and represents the factor by which an apparent molecular weight, measured at a concentration as low as 0.2 mg/ml, underestimates the true or infinite dilution value. For many of course, the effect is not too bad - less than a few percent, but for some like alginates, serious error can result - an underestimate of over 40% for example. And for cases like these its necessary to measure the apparent molecular weight -whether it be weight or z-average, at a number of concentrations and extrapolate to zero in the standard way (see, e.g. Harding, Rowe & Creeth, 1983). In extreme cases - such

TABLE 1. Comparative non-ideality of polysaccharides

	10^{-6}xM g/mol	10^{4}xB ml.mol/g^2	BM ml/g	a_1+2BMc	Ref.
Pullulan P5	0.0053	10.3	5.5	1.002	b
Pullulan P50	0.047	5.5	25.9	1.010	c
Xanthan (fraction)	0.36	2.4	86	1.035	b
β-glucan	0.17	6.1	104	1.042	d
Chitosan (KN-50-1)	0.064	1.7	109	1.044	e
Dextran T500	0.42	3.4	143	1.057	f
Pullulan P800	0.76	2.3	175	1.070	b
Chitosan (Protan 203)	0.44	5.1	224	1.090	g
Pullulan P1200	1.24	2.2	273	1.109	b
Bronchial mucin CFPHI	2.0	1.5	300	1.120	h
Chitosan (Protan SeaCure)	0.16	27.5	445.5	1.178	e
Pectin (citrus fraction)	0.045	50.0	450	1.180	i
Scleroglucan	5.7	0.50	570	1.228	j
Alginate	0.35	29.0	1015	1.406	k

a: At a loading concentration, c of 0.2 mg/ml; b: Kawahara et al (1984); c: Sato et al (1984); d: Woodward et al (1983); e: Errington et al (1993); f: Edmond et al (1968); g: Muzzarelli et al (1987); h: Harding et al (1983); i: Berth et al (1990); j: Lecacheux et al (1986); k: Horton et al (1991)

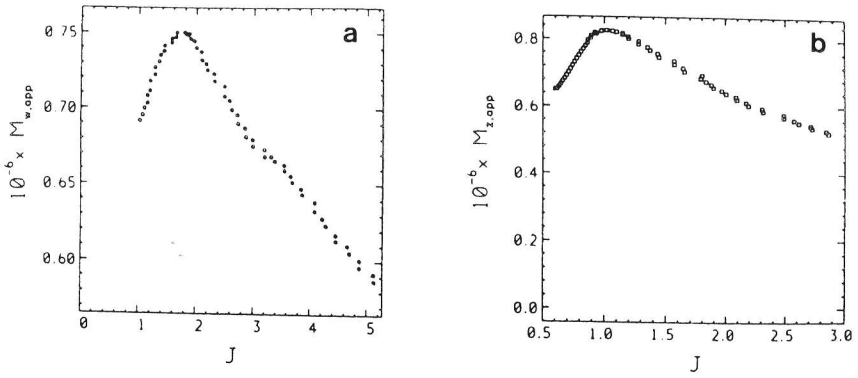


Fig. 7. Plots of (apparent) point M_w, M_z versus fringe number concentration J from a low speed sedimentation equilibrium experiment on a purified guar preparation. Rotor speed = 5200 rev/min, loading concentration $c^o \sim 0.7$ mg/ml. Rayleigh interference optics. From Jumel et al (1993).

as some alginate preparations, even under conditions of ionic strength where polyelectrolyte behaviour should be largely suppressed, the two virial coefficients (1/M and B) are not sufficient to account for the concentration behaviour, even under dilute solution conditions (Horton et al, 1991).

Non-ideality also reveals itself in *point average* representations of the data as shown in Fig. 7 for dilute solutions of a neutral but tricky polysaccharide know as guar gum. For many polysaccharides like this we observe a maximum in the point average versus fringe concentration data, both in terms of weight average (Fig. 7a) and in terms of the point z-average (Fig. 7b) and the existence of these maxima is symptomatic of a heterogeneous but highly non-ideal system, with the effects of polydispersity dominating at low radial positions and non-ideality effects dominating at the higher positions.

POLYDISPERSITY OR SELF-ASSOCIATION OR BOTH?

Fig. 8 (Creeth & Cooper, 1984) shows another point weight average versus concentration plot for a mucopolysaccharide, again with a

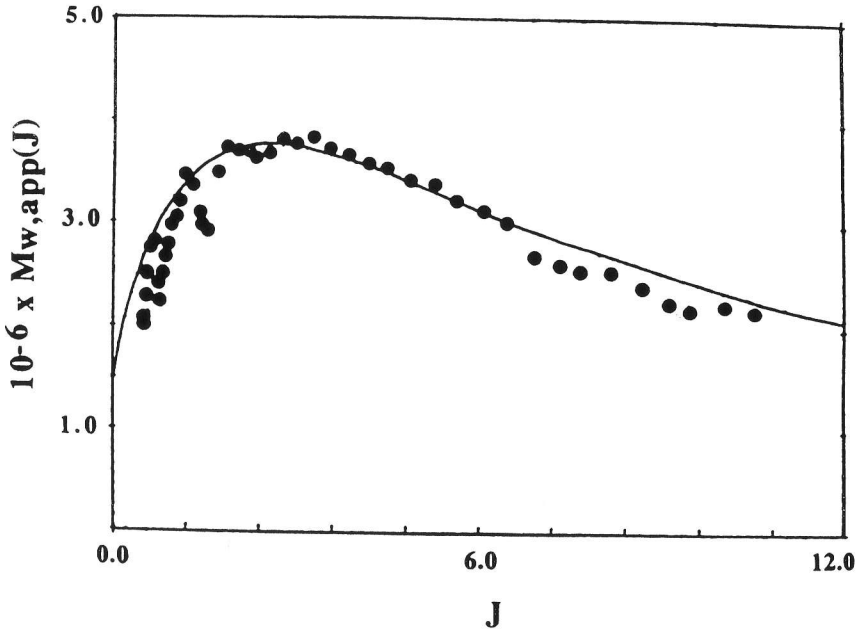


Fig. 8. Plot of (apparent) point M_w versus fringe concentration (with respect to a *12mm* optical path length cell) from a low speed sedimentation equilibrium experiment on bronchial mucin BM GRE (loading concentration, c^o = 1.0mg/ml, solution column length = 3mm) The line fitted corresponds to an effective non-ideal isodesmic self-association with monomer molecular weight $M_1 = 1.5 \times 10^6$ g/mol, second virial coefficient, B = 0.033×10^{-6} mol.ml^{-1}.fringe^{-1}, k=1.2 fringe^{-1} (see, e.g. Creeth & Cooper, 1984, where similar fits are given but with respect to a 30mm path length cell).

maximum, again characteristic of a heterogeneous but highly non-ideal system. The question of interest is what is the prime source of the heterogeneity? Is it because of the presence of components of different molecular weight that are not-interacting (i..e not in chemical equilibrium) , a phenomenon we call "polydispersity", or is self-association behaviour the main contributor?: Many polysaccharides, such as guar and mucopolysaccharides are thought to self-associate, and there is now increasing interest in the food and pharmaceutical fields of possible interactions in mixed polysaccharide systems

(Mannion et al, 1992). Both effects produce upward curvature (i.e. positive second differential) in plots of log concentration versus distance squared or point average molecular weight versus concentration. Initially when we worked on mucopolysaccharides or mucins we thought that the prime cause was self association (Harding & Creeth, 1982). Indeed, we get an excellent fit to the observed point average data if as a first approximation we ignore polydispersity and assume a self-association {Creeth & Cooper (1984) give two good examples example and another is given in Fig. 8 for the bronchial mucopolysaccharide "BM GRE"}. The fit given in Fig. 8 corresponds to a non-ideal isodesmic (i.e. each monomer is added on with constant free energy increment) self-association with plausible values for the isodesmic association constant, k, the "monomer" molecular weight , M_1 and the second virial coefficient B. We can also model the log concentration versus distance squared data directly {the line fitted in Fig. 5b for the mucin "CFPHI" corresponds to an indefinite isodesmic asociation with $M_1 = 2.15 \times 10^6$, k = 260 ml/g and $B = 1.5 \times 10^{-4}$ ml.mol.g^{-2} }. Despite the good fits, we know from other measurements that both BM GRE and CF PHI *are not interacting at all,* illustrating another pitfall we can easily fall into, viz. the effects of polydispersity of these types of system cannot be ignored. There are diagnostic procedures available to assay whether polydispersity effects *can* be ignored. D. Roark & D. Yphantis have shown (Roark & Yphantis, 1969) that for a purely non-ideal self-associating system plots of point weight average molecular weight versus concentration for differing cell loading concentrations should superimpose. So for a simple self-association such as lysosyme they do (Howlett et al, 1972) but for mucins for example they don't as the three examples in Fig. 9 show. In fact, further experiments comparing fringe profiles and corresponding molecular weights for these molecules in nondissociative and dissociative solvents (in the case of mucins by adding 6M GuHCl or swamping concentrations of fucose, galactose or N-acetyl glucosamine) and observing the lack of any effect of the latter (Fig. 2) (Harding, 1984, 1989) have shown that for mucopolysaccharides in dilute solution, self-association phenomena is negligible - the observed heterogeity is due virtually entirely to heterogeneity of components not in chemical equilibriium - i.e., polydispersity.

DISTRIBUTIONS OF MOLECULAR WEIGHT

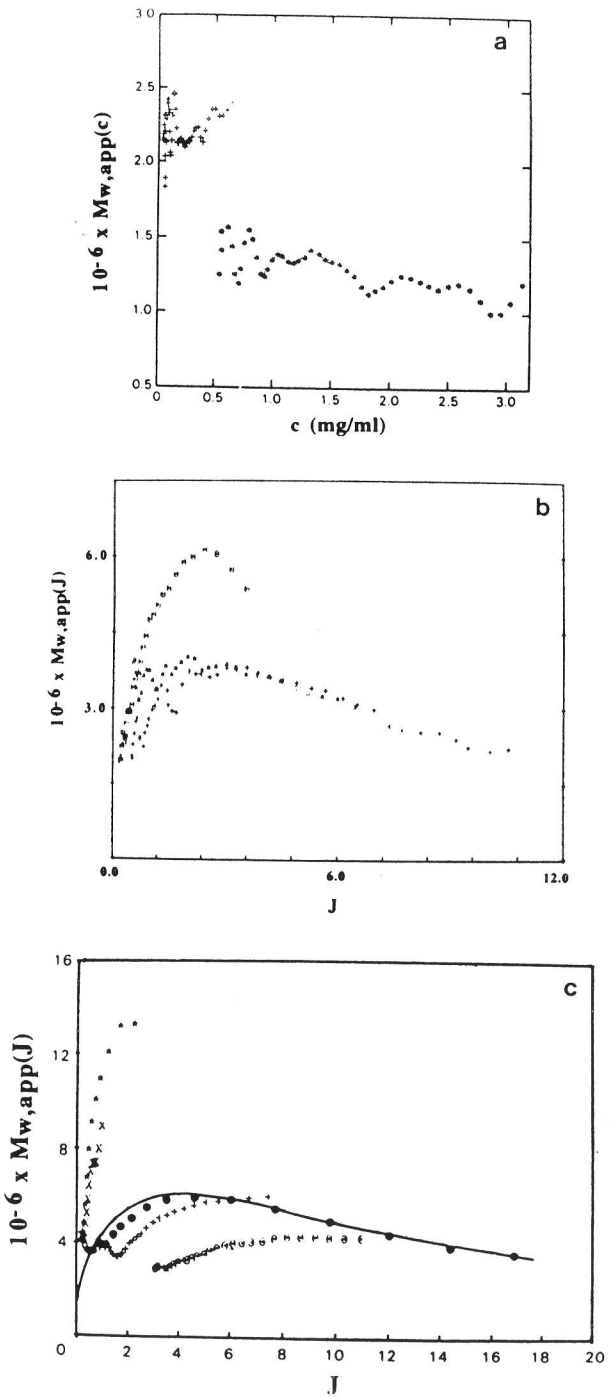
The profiles in Figs. 7-9 are not molecular weight distributions, and in trying to get this sort of information, once again the main stumbling block we have to overcome is that of thermodynamic non-ideality. There are four possible routes open to us here (Table 2).

Method I. Polydispersity indices The simplest way is by using the ratios of whole cell averages, or polydispersity indices, which is OK so long as you can measure numbers or z-averages to a reasonable precision *after* correction for non-ideality. Number averages can only be extracted with ease from Rayleigh patterns if the high speed method is used (Yphantis, 1964) - thereby effectively ruling them out for polysaccharides for the reasons given above. Z-averages can be obtained with relatively high precision using Schlieren optics and now at concentrations as low as can be obtained using

TABLE 2. Molecular weight distribution analysis by low speed sedimentation equilibrium

Method	Type of Analyis	Ref
I	Polydispersity indices (M_Z/M_W etc)	a
II	Non-ideal-polydisperse modelling of log concentration versus distance squared data	b,c
III	Equivalent self-association fit	d
IV	Off-line coupling to gel permeation chromatography	e

a: Herdan (1949); b: Harding (1985); c: Lechner (1992); d: Creeth & Cooper, 1984; e: Harding et al (1988)



Rayleigh optics (Rowe et al, 1992) {or from Rayleigh records themselves but at much lower precision - see above}. These ratios can either be used directly, as so-called "polydispersity indices", or related to the standard deviation of a distribution (whatever form this may take) via special relations known as the "Herdan relations" (Herdan, 1949 - see also Harding, 1989 and Creeth & Pain, 1967).

Method II. Non-ideal-polydisperse modelling of log concentration versus distance squared data The more direct way is to model directly the log concentration versus radial displacement plots by fitting the parameters of a non-ideal polydisperse system. Although this method came out in the Biophysical Journal over 8 years ago now (Harding, 1985), because of the particularly complex interdependent nature of the non-linear equations involved *for the low speed case* - largely caused by the non-ideality term, it takes a great toll on computer resources, even on the fastest computers around such as the IBM 3081 at Cambridge. So, we've presently been unable to apply it to quasi-continuous distributions of molecular weight, that are, for example, the hallmark of polysaccharides, but nontheless succesfully applied to discrete distributions of molecular weight,

---

Fig. 9. Sedimentation Equilibrium Polydispersity assay. Different loading concentration "non-overlap" plots of point weight average apparent M_w versus concentration for three mucins:
(a) Bronchial mucin CF PHI +: c^o (initial loading concentration in mg/ml) ~0.2 mg/ml, 30mm cell; *: c^o~2.0 mg/ml, 12 mm cell Both with 3mm solution columns. From Harding (1984).
(b) Bronchial mucin BMGRE ⊙:c^o~0.4 mg/ml, 30mm cell (fringe concentrations corrected to the equivalent values in a 12mm cell) *: c^o~0.7 mg/ml, 12 mm cell, +: c^o~1.0 mg/ml, 12mm cell. All three data sets correspond to 3mm solution columns. Adapted from Creeth & Cooper, 1984.
(c) Pig gastric mucin *: J^o (initial loading concentration in fringe numbers) 0.32, column length 3mm; x: J^o~0.42, 1.5mm; +: J^o~3.19, 1.5mm; ●:J^o~4.03, 3mm; : J^o~6.00, 1.5mm. All three data sets correspond to a 30mm optical path length cell. The line fitted to data set in (c) corresponds to a an effective non-ideal isodesmic association (see legend to Fig. 8), with $M_1 = 1.5 \times 10^6$, $B = 0.013 \times 10^{-6}$ mol.ml^{-1}fringe^{-1} and k= 2.1 fringe^{-1} where the fringe concentration units this time refer to a 30mm path length cell. Adapted from Creeth & Cooper, 1984.

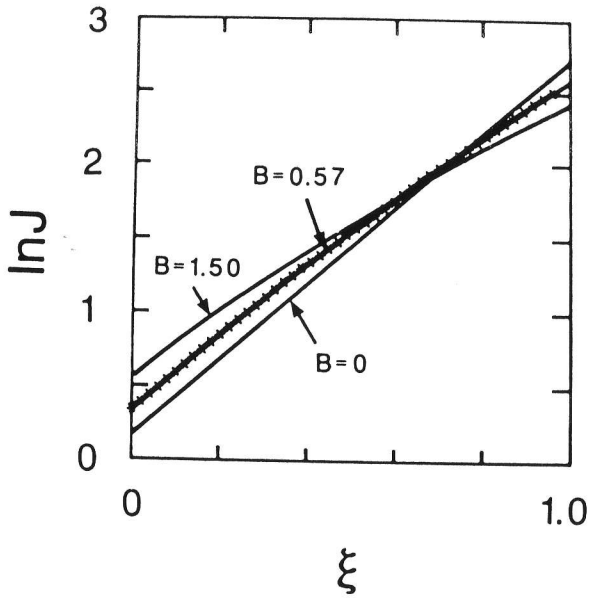


Fig. 10. Molecular weight distribution modelling of bronchial mucin CF PHI by Method II. Non-ideal three-component fit to the observed log concentration versus distance squared data for a low speed sedimentation equilibrium experiment on bronchial mucin CF PHI. The line fitted corresponds to the following parameters for each of the three components: component 1, M_1 = 1.2 x 10^6g/mol, J^o_1 = 0.9 fringe; component 2, M_2=1.8 x 10^6, g/mol, J^o_2= 3.6 fringe ; component 3, M_3 = 2.4 x 10^6g/mol, J^o_3 =0.9 fringe. B has been expressed as its value x 10^4 (ml.mol.g^{-2}). The 3 component model is based on observations from platinum-shadowed electron microscopy. From Harding (1985).

which at least partially represent mucins {Harding, 1985, 1989} (Fig. 10). The problems associated with the modelling of the concentration distribution for the non-ideal case have been further examined by Lechner (1992).

Method III. Equivalent self-association fit A much easier way, although theoretically less elegant than the previous method, is to use to our advantage the property of indistinguishability *from a single experiment* between a non-ideal polydisperse system and a non-ideal

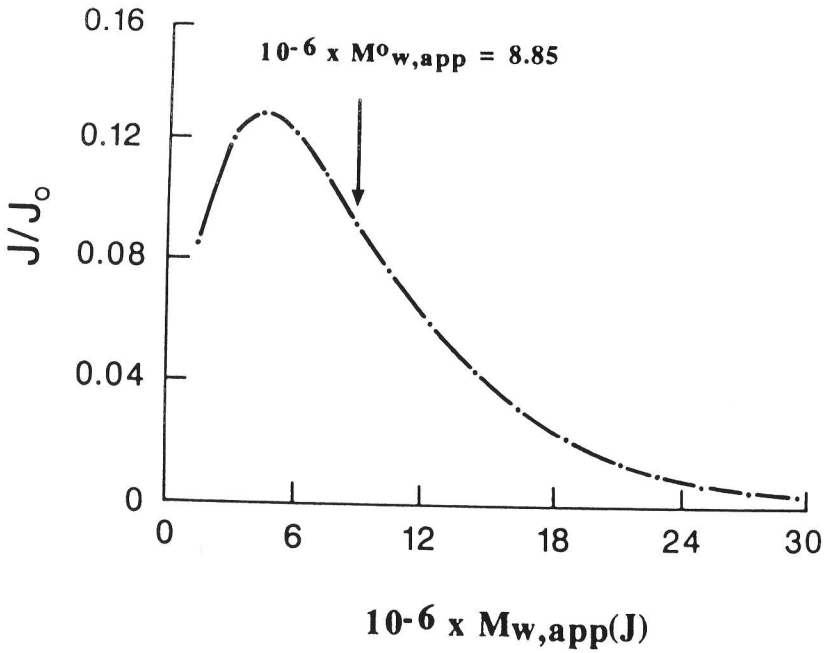


Fig. 11. Molecular weight distribution of pig gastric mucin, evaluated according to Method III. The distribution corresponds to the fit shown in Fig. 9c. The value marked by an arrow corresponds to the weight average (apparent) molecular weight for the whole solute distribution. Adapted from Creeth & Cooper, 1984 (see also Harding, 1989).

self-associating system. It is therefore possible to apply the much easier to handle equations of for example a non-ideal isodesmic association to calculate a constant which, when applied to a static system will define a distribution of molecular weight, no matter what the cause of the distribution is (Creeth & Cooper, 1984; Harding, 1989) and again, this has been successfully applied to mucins (Fig. 11).

Method IV. Off-line coupling to gel permeation chromatography. From a practical point of view, we find the best procedure is to use sedimentation equilibrium in conjunction with gel permeation chromatography (gpc) to provide an absolute calibration for the latter (Harding et al, 1988). The idea is to isolate fractions of narrow bandwidth (in terms of elution volume) from the gpc eluate, deter-

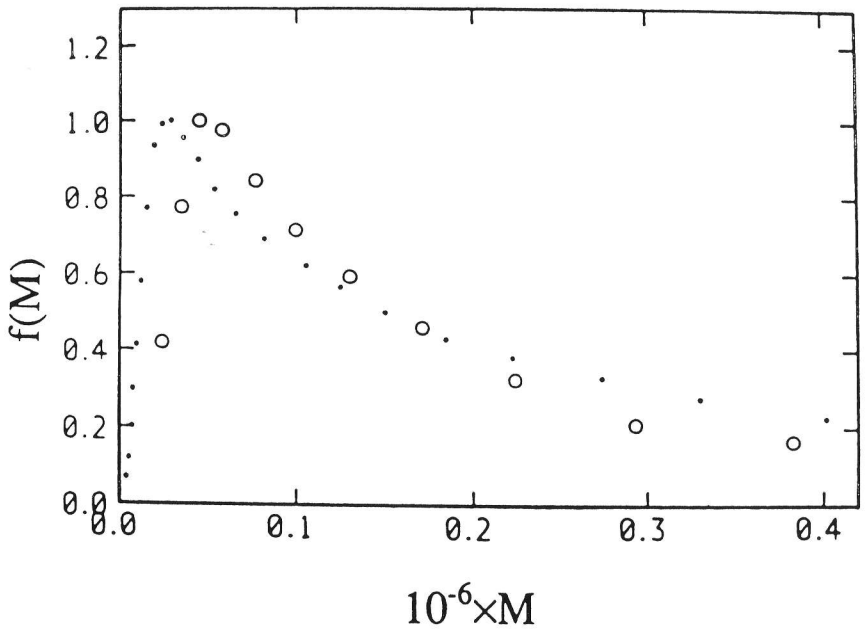


Fig. 12. Molecular weight distribution of citrus pectin, evaluated according to Method IV (open circles). The filled circles corresponds to the distribution on the same material evaluated by classical light scattering procedures coupled to gel permeation chromatography. Method IV is our method of choice. From Harding et al, 1991.

mine their molecular weights using sedimentation equilibrium using short solution columns (0.7mm - 1.5mm) and multi-channel cells to speed things up, thereby giving an absolute calibration for the gpc columns. Distributions of molecular weight found in this way have been in remarkable agreement with similar procedures involving light scattering as shown in Fig. 12. Method IV is in my opinion the method of choice. It is called "off-line" because the eluate is not fed directly into the ultracentrifuge cell whilst the ultracentrifuge is running {this distinguishes itself from certain light scattering photometers which are *"on-line"* - i.e. directly connected between gpc columns and a concentration - usually refractive index based - detector}.

THE FINAL PROBLEM: SEDIMENTATION EQUILIBRIUM VERSUS LIGHT SCATTERING

The decline of sedimentation equilibrium and other ultracentrifuge techniques in the 70's and 80's in the protein biochemistry field (largely because of the advent of electrophoretic procedures and gel permeation chromatography) and followed by the revival of interest has been well documented (see e.g., Schachman, 1992). In the polysaccharide and synthetic polymer fields the decline of sedimentation equilibrium as a routine absolute molecular weight tool has been largely because of the advent of laser light scattering techniques (both "classical" and "dynamic"). Probably the bulk of the apparatus and the length of time required to reach equilibrium have contributed to its downfall, but in reality light scattering - although the apparatus is more compact and measurements themselves are a lot quicker suffers far worse disadvantages (Harding, 1988), largely through sample clarification and for this reason it is fair to say that light scattering results have a greater degree of uncertainty than sedimentation equilibrium, when used in isolation. Although sedimentation methods would be my own method of choice, we find particularly for molecular weight distribution work confirmatory measurements from both techniques (and used in conjunction with gel permeation chromatography) extremely valuable (see, e.g. Harding et al, 1991).

GLOSSARY

b	Radial position at the cell base (cm)
B	Second thermodynamic virial coefficient ($ml.mol.g_{-2}$)
B_{eff}	Effective 2nd thermodynamic virial coefficient ($ml.mol.g^{-2}$)
C	Solution concentration (g/ml)
C^o	Initial loading solution concentration (g/ml)
C_a	Solution concentration at the meniscus (g/ml)
j	Solution concentration in fringe numbers relative to the meniscus

J^o	Initial loading concentration (fringe numbers)
J_a	Meniscus solution concentration in fringe numbers
M	Molecular weight (g/mol)
M_1	Molecular weight of a monomer (in self-association)
M*	Star average (operational) average molecular weight ($g.mol^{-1}$)
M^o_n	Whole-cell (i.e., over all radial positions in the solution column of the cell) number average molecular weight ($g.mol^{-1}$)
M^o_w	Whole-cell weight average molecular weight ($g.mol^{-1}$)
M^o_z	Whole-cell z-average molecular weight ($g.mol^{-1}$)
M_n	Point (i.e., at a local radial position in the cell) number average molecular weight ($g.mol^{-1}$)
M_w	Point weight average molecular weight ($g.mol^{-1}$)
M_z	Point z-average molecular weight ($g.mol^{-1}$)
R	Gas constant (8.314×10^7 $erg.mol^{-1}.K^{-1}$)
r	Radial position (cm)
$\bar{v}$	Partial specific volume (ml/g)
T	Temperature (K)
ρ	Solution density (g/ml)
ρ_o	Solvent density (g/ml)
ω	Angular velocity of rotor ($rad.sec^{-1}$)
ξ	Normalized radial displacement squared parameter (= 0 at meniscus, 1 at cell base)

REFERENCES

Berth, G., Dautzenberg, H., Lexow, D. and Rother, G. (1990) The determination of the molecular weight distribution of pectins by calibrated gel permeation chromatography Part I: Calibration by light scattering and membrane osmometry. *Carbohyd. Polym.* 12, 39-59

Creeth, J.M. and Cooper, B. (1984) Studies on the molecular weight distributions of two mucins. *Biochem. Soc. Trans.*, 12, 618-621

Creeth, J.M. and Harding, S.E. (1982a) Some observations on a new type of point average molecular weight. *J. Biochem. Biophys. Meth.* 7, 25-34

Creeth, J.M. and Harding, S.E. (1982b) A simple test for macromolecular heterogeneity in the analytical ultracentrifuge. *Biochem. J.*, 205, 639-641

Creeth, J.M. and Pain, R.H. (1967) The determination of molecular weights of biological macromolecules by ultracentrifuge methods. *Prog. Biophys. Mol. Biol.* 17, 217-287

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

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

Fujita, H. (1975) *"Foundations of Ultracentrifuge Analysis"*, Chap. 5., J. Wiley and Sons, New York

Giebler, 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"* (Harding,. S.E., Rowe, A.J. and Horton, J.C. eds.), Chap.2, Royal Society of Chemistry, Cambridge, UK

Gilbert, G.A. and Gilbert, L.M. (1980) Determination in the ultracentrifuge of protein heterogeneity by computer modelling, illustrated by pyruvate dehydrogenase multienzyme complex. *J. Mol. Biol.* 144, 405-408

Harding, S.E. (1984) An analysis of the heterogeneity of mucins. No evidence for a self association. *Biochem, J.*, 219, 1061-1064

Harding, S.E. (1985) The representation of equilibrium solute distributions for non-ideal polydisperse systems in the analytical ultracentrifuge. Application to mucus glycoproteins. *Biophys. J.* 47, 247-250

Harding, S.E. (1988) Polysaccharide molecular weight determination: which technique? *Gums and Stabilisers for the Food Industry* 4, 15-23

Harding, S.E. (1989) The macrostructure of mucus glycoproteins in solution. *Adv. Carb. Chem. & Biochem.*, 47, 345-381

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

Harding, S.E., Ball, A. and Mitchell, J.R. (1988) Combined low speed sedimentation equilibrium/ gel permeaion chromatography approach to

molecular weight distribution analysis. *Int. J. Biol. Macromol.*, 10, 259-264

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

Harding, S.E. and Creeth, J.M. (1982) Self-association, polydispersity and non-ideality in a cystic fibrotic glycoprotein. *IRCS (Int. Res. Commun. System) Med. Sci. Lib. Compend.*, 10, 474-475

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"* (Harding,. S.E., Rowe, A.J. and Horton, J.C. eds.), Chap.15, Royal Society of Chemistry, Cambridge, UK

Harding, S.E. and Johnson, P. (1985) Physicochemical studies on turnip yellow mosaic virus: Homogeneity, molecular weights, hydrodynamic radii and concentration dependence of parameters. *Biochem. J.*, 231, 549-555

Harding, S.E., Rowe, A.J. and Creeth, J.M. (1983) Further evidence for a flexible and highly expanded spheroidal model for mucus glycoproteins in solution. *Biochem.J.*, 209, 893-896

Herdan, G. (1949) Estimation of polymer distributions on the basis of differences between the average molecular weights of different order. *Nature*, 163, 139

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.L., Jeffrey, P.D. and Nichol, L.W. (1972) *J. Phys. Chem.* 76, 77

Jumel, K., Mitchell, J.R. and Harding, S.E. (1993) in preparation

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

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

Lecacheux, D., Mustiere, Y., Panaras, R. and Brigand, G. (1986) Molecular weight of scleroglucan and other extracellular microbial polysaccharides by size-exclusion chromatography and low-angle light scattering. *Carbohyd. Polym.* 6, 477-492

Lechner, M.D. (1992) Determination of molecular weight averages and molecular weight distributions from sediemtation equilibrium. In *"Analytical Ultracentrifugation in Biochemistry and Polymer Science"* (Harding,. S.E., Rowe, A.J. and Horton, J.C. eds.), Chap.16, Royal Society of Chemistry, Cambridge, UK

Mannion, R.O., Melia, C.D., Launay, B., Cuvelier, G., Hill, S.E., Harding, S.E. and Mitchell, J.R. (1992) Xanthan/ locust bean gum interactions at rom temperature. *Carbohyd. Polym.* 19, 91-97

Muzzarelli, R.A.A., Lough, C. and Emanuelli, M. (1987) The molecular weight of chitosans studied by laser light scattering. *Carbohyd. Res.* 164, 433

Roark, D. and Yphantis, D.A. (1969) *Ann. N.Y. Acad. Sci.* 164, 245-278

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

Sato, T., Norisuye, T and Fujita, H. (1984) Double-stranded helix of xanthan - dimensional and hydrodynamic properties in 0.1M aqueous sodium chloride. *Macromolecules*, 17, 2696-2700

Schachman, H.K. (1992) Is there a future for the Ultracentrifuge? In *"Analytical Ultracentrifugation in Biochemistry and Polymer Science"* (Harding,. S.E., Rowe, A.J. and Horton, J.C. eds.), Chap.1, Royal Society of Chemistry, Cambridge, UK

Teller, D.C. (1965) *PhD Dissertation*, University of California, Berkeley, California

Teller, D.C. (1973) Characterization of proteins by sedimentation equilibrium in the analytical ultracentrifuge. *Meth. Enzymol.*, 27, 346-441

Wedlock, D.J., Baruddin, B.A. and Phillips, G.O. (1986) Comparison of molecular weight determination of sodium alginate by sedimentation-diffusion and light scattering. *Int. J. Biol. Macromol.* 8, 57

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. *Carbohyd. Polym.* 1983, 3, 143

Yphantis, D.A. (1964) Equilibrium ultracentrifugation of dilute solutions. *Biochemistry*, 3, 297-317