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Some recent developments in the analytical ultracentrifugation of food proteins

(A review)

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Summary

In the past, the ultracentrifuge has provided much information on the sizes, shapes and interaction of food proteins. The availability of new on-line instrumentation has now extended the possibilities for analysis even further. In this short review we examine the state of the art of the technique and its past, present and potential future contributions to the field of food protein research. After a consideration of the developments in instrumentation and their implications, we focus on the contribution to the following areas: 1. gross conformation analysis (ellipsoid, bead and general conformation representations). 2. Molar mass and subunit composition analysis. 3. Self-association 4. Protein-polysaccharide and protein-ligand interactions 5. Gels and matrix diffusion phenomena.

Zusammenfassung

Einige neuere Entwicklungen der analytischen Ultrazentrifugation von Nahrungsproteinen (Übersichtsbericht)

Die Ultrazentrifuge hat in der Vergangenheit viele Informationen über Größe, Form und Wechselwirkungen von Nahrungsproteinen geliefert. Neue verfügbare Modelle mit on-line-Instrumentierung haben die analytischen Möglichkeiten deutlich erweitert. In diesem kurzen Überblick werden der Stand der Technik sowie seine bisherigen, gegenwärtigen und möglichen zukünftigen Beiträge für das Gebiet der Nahrungsprotein-Forschung überprüft. Nach Darlegung der Entwicklung der Instrumentierung wird der Schwerpunkt des Beitrages auf folgende Gebiete gelegt: 1. Gesamt-Konformationsanalyse (ellipsoide, kugelförmige und allgemeine Konformationsdarstellungen). 2. Molare Masse und Analyse der Zusammensetzung von Untereinheiten. 3. Selbst-Assoziation. 4. Protein-Polysaccharid- und Protein-Ligand-Wechselwirkungen. 5. Gel- und Matrix-Diffusionsphänomene.

Introduction

The analytical ultracentrifuge has been available for almost three quarters of a century as a tool for the investigation of the solution behaviour of macromolecules. Although it would certainly be untrue to claim that one of the major focusses of attention has been on food macromolecules, it is fair to say that our understanding of how food macromolecules behave in solution – in terms of their sizes, shapes and interactions – and even in gel form, has been considerably enhanced because of work emanating from this now "classical" – some would say dated – technique. In terms of research on food proteins, T. SVEDBERG – widely recognised as the founder of the ultracentrifuge – himself co-authored papers in this area, using both sedimentation velocity and sedimentation equilibrium ultracentrifugation. This included papers on egg albumin [1] and gelatin solutions [2]. This work preceded the large expansion of interest in the technique which occurred in the period

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1940 – 1970, when most of the remaining methodology concerning the various ultracentrifuge approaches (sedimentation velocity, sedimentation equilibrium, isopycnic density gradient methods, diffusion coefficient analysis and gel analysis) was established. It was during this period for example that the self-assembly properties of milk proteins became largely understood [3], progress was made with our understanding of the solution structure of myosin [4, 5] and P. JOHNSON and coworkers [6, 7] developed our understanding of the structure of gelatin gels.

The purpose of this article is to examine the state of the art of analytical ultracentrifugation and its past, present and potential future contribution to the field of food protein research. After a consideration of developments in instrumentation and its implications we will focus on the contribution to (I) gross conformation analysis in terms of ellipsoid, bead and general conformation representations; (II) molar mass ("molecular weight") and subunit composition analysis; (III) the analysis of self-association of food proteins in solution; (IV) protein-polysaccharide and protein-ligand interactions; (V) the analysis of protein gels as a complementary approach to conventional rheological methods, and finally (VI) protein diffusion through matrices.

Apart from a consideration of protein-polysaccharide interactions we will not consider the contribution that analytical ultracentrifugation is making towards our knowledge of the nature of other types of food biopolymers such as food polysaccharides. These present different problems and this subject has been dealt with elsewhere [8, 9].

Developments in instrumentation: The XL-A ultracentrifuge

There are five types of analytical ultracentrifuge in use. Four of these are "old generation" types, varying in vintage from 10 to 35 years old. These are: the BECKMAN (Palo Alto, USA) Model E (equipped with "photographic" Schlieren and Rayleigh interference optics, and either photographic or "scanning" absorption optics), the MOM (Magyar Optical Works, Budapest) Model 4170 (equipped with photographic Schlieren, Interference and absorption), the MSE (Crawley, UK) Analytical Mark II (photographic Schlieren and Interference) and the MSE Centriscan (scanning Schlieren and absorption). The select band of workers who have kept these instruments in active use over the years have between them constructed several modifications, such as laser light sources [10], LED [11] light sources and on-line (i.e. where the output is transferred directly into a PC) data capture for the Model E absorption [12] and interference [13] systems, off-line data capture for the Model E, MSE Mk. II and MOM interference [14, 15] and Schlieren [15] systems, and a fluorescence optical system for the Model E [16].

The Optima XL-A

The most significant recent development has been the development and launch of a completely new analytical ultracentrifuge, the "Optima XL-A" from Beckman instruments [17, 18] with full on-line computer data capture and analysis facilities. The initial model – of which we were fortunate to have one of the prototype or test instruments – was equipped with scanning absorption optics, a 4-hole rotor taking double sector cells (1 counterpoise cell, 3 for solution cells) of maximum optical path length 12 mm. For sedimentation equilibrium which requires only short solution columns it is possible to use

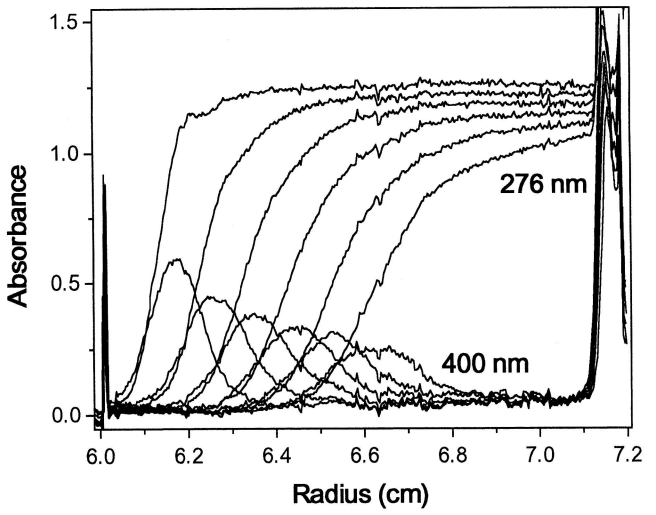

Fig. 1
Sedimentation velocity analysis on the XL-A ultracentrifuge. Sedimentation velocity scans on bovine serum albumin at a loading concentration of 2 mg/ml, rotor speed = 50000 rev/min, temperature = 20 °C. Standard 12 mm double sector cell, with ≈0.4 ml in solvent channel, 0.35 ml in solution channel. The direction of movement is from left to right. Top traces: conventional UV absorption profiles recorded at a wavelength of 276 nm. Lower traces: 'accidental' knife-edge Schlieren profiles recorded at a wavelength of 400 nm. Time between each scan at a given wavelength = 20 min. Time between an absorption and Schlieren scan = 2 min. From [20]

multi-channel cells each consisting of 3 solvent/solution pairs thereby allowing a capacity of 9 solutions to be run in a single experiment. The scanning absorption optical system, with signal to noise comparable to that of the MSE Centriscan and a considerable improvement on the scanning system on the Model E, permits (I) a wavelength scan at any radial position (II) radial scans at 3 different selected wavelengths for a given cell during a run and (III) multiple data capture and averaging. Examples of the quality of the data to be obtained are presented in Fig. 1 for sedimentation velocity and Fig. 2 for sedimentation equilibrium. So long as the solvent is reasonably transparent and the appropriate cell windows are employed, the "far" part of the ultraviolet spectrum can be used, down to ≈200 nm. This means for food proteins not only can we use conventional absorption detection at ≈278 nm, but also make use of the strong UV absorption of the peptide bond (210–230 nm).

Another feature which distinguishes it from all previous analytical ultracentrifuges is that the monochromator arm for the optical system resides in the rotor chamber (Fig. 3). The rationale behind this is apparently increased optical transmission properties. This comes at a price: it has to be removed at the end of each run and replaced at the start of the next run. A more serious restriction, deriving from the specific design of the monochromator is that because the gap between the top of the rotor and the monochromator is less than 1 cm it is impossible to use a rotor which would accommodate longer path length cells, e.g. of the type (30 mm) available for the Model E, thus limiting the lower concentration limit. For higher concentrations it is possible to purchase shorter path length cells (6 mm). (We have in-house machined cell centrepieces of 3 mm path length: any further

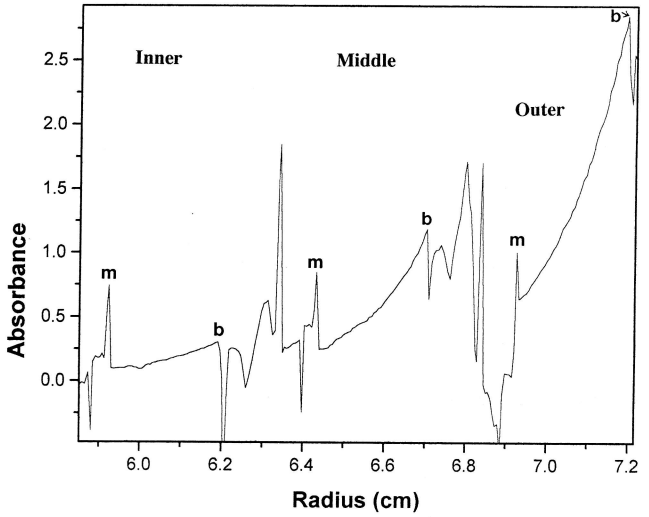

Fig. 2
Sedimentation equilibrium analysis on the XL-A ultracentrifuge. Sedimentation equilibrium profiles for beta-lactoglobulin B. Absorption optics, wavelength = 280 nm, Rotor speed = 15000 rev/min, temperature = 20.0 °C. Because of the shorter solution columns for sedimentation equilibrium work, a (12 mm path length) multi-channel cell could be used (3 solvent/solution pairs) with ≈0.12 ml in solvent channels, ≈0.10 ml in solution channels. Inner profile: loading concentration, c = 0.1 mg/ml; middle: 0.2 mg/ml; outer = 0.3 mg/ml. Because of restriction of the Lambert-Beer law, with the outer channel only absorbances <1.5 could be used

shortening runs the risk of pattern distortions through the proportionally greater surface tension effects from the windows).

A new version of the XL-A is being launched in 1996 with on-line Rayleigh interference optics and for sedimentation velocity work advantage can be made of highly sensitive data capture procedures permitting the analysis of macromolecular concentrations down to ≈100 µg/ml. Although an eight-hole rotor is planned the restriction on maximum cell path length remains (12 mm), so the lower limit for Rayleigh interference detection of sedimentation equilibrium records will be 0.5–0.8 mg/ml.

One of the greatest benefits of the new model, with simultaneous absorption/interference capabilities (for both velocity and equilibrium ultracentrifugation) for food protein research

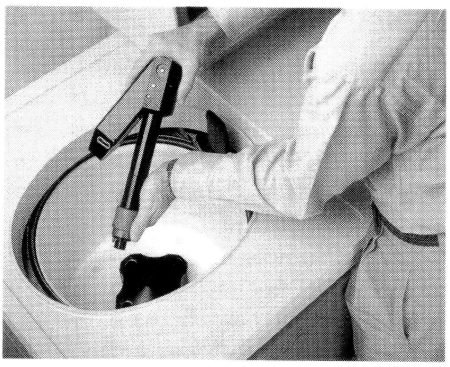

Fig. 3
Installation of the monochromator prior to a run on the XL-ultracentrifuge

will be for the investigation of food protein-polysaccharide interactions, with interference detecting solute distributions of both components together but the absorption optics giving selective detection of the protein within these distributions.

Gross conformation analysis using sedimentation velocity

With the new ultracentrifuge, the sedimentation coefficient of a food protein can be relatively easily obtained from the optical absorption records as in Fig. 1 or from interference optical records [19]. In principle the "second moment" of the sedimenting boundaries should be used; in practice the "first moment" (i.e. the point of inflection) is perfectly adequate. It is possible, in certain circumstances even to obtain Schlieren images on the XL-A ultracentrifuge [20] (Fig. 1). After correction to standard conditions of temperature, solvent density and viscosity (water at 20 °C) to give $s_{20,w}$, and from a linear fit of $s_{20,w}$ versus concentration (c, g/ml) data to $s_{20,w} = s^0_{20,w}(1 + k_s c)$ the infinite dilution sedimentation coefficient $s^0_{20,w}$ (s or SVEDBERGS, S) and the concentration regression parameter k_s [ml/g] can both bee obtained: both parameters are important for gross conformation analysis, and Table 1 gives values for a range of food proteins.

For shape analysis of food proteins there are three basic approaches. One approach – which applies to *relatively* rigid structures (which could, for many food protein systems, be reasonably claimed) – is to apply "whole body" ellipsoid (prolate, oblate or even triaxial) models to represent the overall structure of these molecules *in solution* (see, e.g. [34]): that is to say, the axial dimensions of a macromolecule and/or how its subunits are

Table 1
$s^0_{20,w}$ and k_s values for some food proteins

Food protein	M	$s^0_{20,w}$ [S]	k_s [ml/g]	Reference
Ovalbumin	45000	3.42[a]	6.2	[22, 23, 24]
Bovine serum albumin	67000	4.31	5.4	[25, 26]
β-Lactoglobulin (B) (dimer)	36000	2.83	4.6[b]	[27, 28]
Myosin	490000	5.6	94	[29]
Collagen	374000	3.02	265	[30]
Collagen sonicates:	336000	2.95	250	[30]
	297000	2.87	227	[30]
	250000	2.79	202	[30]
	217000	2.68	182	[30]
	192000	2.64	166	[30]
	170000	2.59	154	[30]
	149000	2.54	142	[30]
Lipase (*Chromobacter viscosum*)	35000	3.17	18.7	[31]
Lipase (*Pseudomonas* spp.)	38000	2.99	7.0	[31]
Lipase (*Aspergillus* spp.)		3.01	9.5	[31]

$s^0_{20,w}$ values for proteins normally correct to $\sim \pm 1\%$ or better. k_s values normally correct to $\sim \pm 2\%$. Some k_s values have been corrected for "radial dilution" and to "solution density" [32]. Most determinations done in (aqueous) solvents of ionic strength 0.1 M buffered to pH between 6 and 7. a: after correction from 25 °C to 20 °C. b: because this system is a reversible association, k_s value obtained using a procedure described in [33]

arrayed. This type of modelling can usually be done without making serious assumptions concerning the extent of molecular hydration or solvation of the protein. A more refined approach is to model the conformation as an array of beads [35]. In this way more complicated representations can be made but assumptions usually have to be made involving hydration. It is possible also to represent flexibility of the "hinge" type with this methodology [36] and this approach has been applied to myosin [36, 37]. The final approach is to perform more general modelling on a homologous polymer series, such as for the series of collagen sonicates cited in Table 1. The idea of this latter approach is to first identify the conformation type (random coil, rigid rod or compact sphere or something in between these extremes) and once established to make further deductions about the conformation. For example, if the conformation is that between a random coil and rigid rod, one can deduce information about 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 approach is followed, sedimentation data is best used in conjunction with other hydrodynamic data in order to minimize the problems of non-uniqueness of a particular model.

Ellipsoid approach to food protein shape

This can be done via calculation of the so-called "frictional ratio", or ratio of the friction coefficient, f, of the macromolecule to the frictional coefficient of a spherical particle, f_0, with the same mass and *anhydrous* volume. f/f_0 is related to the sedimentation coefficient by

$$f/f_0 = [M(1 - \bar{v}\varrho)/N_A \cdot (6\pi\eta_0 s^0_{20,w})] \cdot [4\pi N_A/3\bar{v}\, M]^{1/3} . \quad (1)$$

M is the molar mass [g/mol], $\bar{v}$ the partial specific volume [ml/g] (typically $\sim$0.73 ml/g for proteins, and calculable to a reasonable approximation from the amino acid sequence [38]), ϱ is the density [g/ml] of the solution (which after the correction to standard conditions and extrapolation referred to above is the density of water at 20.0 °C [39]), η_0 (poise) is the solvent viscosity (in this case, also after the correction referred to above, that of water at 20.0 °C) and N_A is AVOGADRO's number [mol^{-1}]. f/f_0 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 function", P (also known as "the frictional ratio due to shape"), in recognition of F. PERRIN [40] who developed the theory for the frictional coefficients of ellipsoids:

$$P = f/f_0 \cdot [(w/\bar{v}\varrho) + 1]^{-1/3} . \quad (2)$$

The factor $[(w/\bar{v}\varrho_0) + 1]$ is sometimes referred to as the "swelling of a macromolecule due to hydration" $= v_s/\bar{v}$ with v_s the swollen specific volume [ml/g] i.e. the volume occupied in solution by the swollen protein per unit anhydrous mass. For a food protein $v_s/\bar{v}$ is typically $\sim$1.4.

If the macromolecule is fairly rigid, P can be related directly to the axial dimensions of the macromolecule using ellipsoid of revolution models (i.e. ellipsoids with 2 equal semiaxes). A prolate ellipsoid of revolution has semi-axes a, b, b and an oblate ellipsoid of revolution a, a, b with $a > b$ in both cases. Table 2 gives the dimensions of some food 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, viz. the prolate case).

Table 2
Axial dimensions of food proteins from x-ray crystallography

Protein	Dimensions [Å]	Reference
Lipase *(Humicola lanuginosa)*	43 × 43 × 40	[41]
Ovalbumin	70 × 45 × 50	[42]
β-Lactoglobulin	81 × 47 × 40	[43]
Myoglobin	43 × 35 × 23	[44]
Hemoglobin	64 × 55 × 50	[45]

Rather complicated formulae exist relating P to the axial ratio (a/b), but extensive tabulations are also available (see, e.g. [8]). Arguably of more practical use, simple inversion formulae enabling the calculation of a/b from a given value of P are now available [46]. These are of the form of a polynomial expansion:

$$a/b = a_0 + a_1x + a_2x^2 + a_3x^3 + a_4x^4 + a_5x^5 + a_6x^6 \quad (3)$$

where in this case $x = P$ and the coefficients $a_0 - a_6$ for the three axial ratio ranges (1 – 2, 2 – 10 and 10 – 100) for both prolate and oblate ellipsoids are given [46].

A problem with this treatment is that f/f_0 (and hence P and the sedimentation coefficient) is a relatively insensitive function of shape. A more serious problem is than an assumption has to be made concerning the hydration w (in some texts "δ") or equivalently $v_s/\bar{v}$. A value of $w = 0.35$ has in the past been popularly taken for proteins although a survey of 21 proteins by SQUIRE et al. [47] suggests w values in the range (0.53 ± 0.26) (see also [48, 49]). An alternative is to use the hydration independent shape function referred to as the "WALES/VAN HOLDE ratio" $k_s/[\eta]$ [50], which is often referred to by the symbol "R" [32, 51]. This combination of the concentration dependence parameter k_s with the intrinsic viscosity $[\eta]$ gives, after certain assumptions and approximations a shape function which does not depend on knowledge of w or v_s, and is a more sensitive function of axial ratio a/b. The full functional dependence of R on a/b, together with extensive tabulations can be found in [32] and [8]. Again, like P, of more practical use are the simple polynomial inversion formulae of eq. (3) with $x = R$ this time and the corresponding coefficients $a_0 - a_6$ given in [46]. Its application to various food proteins is illustrated in Table 3.

Another shape function not requiring knowledge of the hydration level is the Π function [56]. This comes from measurements of the thermodynamic second virial coefficient B, $[\eta]$, and the molar mass, M:

$$\Pi = 2BM/[\eta] - f(Z, I)/[\eta]\, M \quad (4)$$

where $f(Z, I)$ is a function of the net charge on the macromolecule and the charge-suppressing effect of the ionic strength, I, of the solvent. $f(Z, I) = 0$ at the isoelectric point for proteins and tends to zero at higher ionic strengths. If it is still significant, it can be measured by for example titration (see e.g., JEFFREY et al. [57] 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. Again, as with P and R, complicated formulae and extensive tabulations are available [8, 56] as are the more practically useful coefficients $a_0 - a_6$ given in [46] for

Table 3
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)*	Reference
Ovalbumin	5.45	3.49	1.56	1.5	[22, 23, 24]
Bovine serum albumin	5.4	3.9	1.38	2.3	[26, 53]
β-Lactoglobin (B) (dimer)	4.6	2.86	1.61	1.0	[28, 54]
Collagen (374 kDa)	265	1250	0.212	>100	[30, 55]
Sonicates: 336 kDa	250	1075	0.232	100	[30]
297 kDa	227	865	0.262	70	[30]
250 kDa	202	625	0.323	43	[30]
217 kDa	182	495	0.368	33	[30]
192 kDa	166	400	0.415	25	[30]
170 kDa	154	325	0.474	18	[30]
149 kDa	142	245	0.580	14	[30]

* Of the equivalent prolate ellipsoid. k_s values are normally corrected for "radial dilution" and to "solution density" (see [32, 52]).

the inversion formulae of eq. (3). Table 4 illustrates the application of Π to three well-known food proteins. It is in particular interesting to note that the overall shape of the ovalbumin molecule from both the Π and R functions in 1981 [23] is almost exactly as found some 10 years later by x-ray crystallography [42].

Table 4
Axial ratios of food proteins from the Π function

Protein	Π	Axial ratio (a/b)*	Ref.
Hemoglobin	3.20	1.0	[56]
Ovalbumin	3.18	1.0–2.0	[24]
Myosin	0.47	80	[59]

* Of the equivalent prolate ellipsoid
$\Pi = \{2BM/[\eta]\} - \{f(Z, I)/[\eta]\, M\}$

Another "combined function" involving $s^0_{20,w}$ and [η] (referred to as the SCHERAGA and MANDELKERN [58] β-function) was the first published hydration independent shape parameter but it is extremely insensitive to a/b especially at low axial ratios, and is really only of use as a quasi-constant parameter for evaluating the molar mass from $s^0_{20,w}$ and [η] measurements. Although of limited use, the coefficients for evaluating a/b from the polynomial expansion of eq. (3) are nonetheless also given in [46].

Tri-axial ellipsoids

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. Further, a decision has to be made with some shape functions *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 2 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. As an example, by using a combination of Π with a radius of gyration function (from light scattering) the overall rod conformation and axial ratio of myosin has been successfully predicted as (a/b, b/c) = (80, 1) *without assuming a priori a prolate ellipsoid* [59]. Although this is rather a crude, simplistic picture of the myosin molecule and fails to take into account the hinge-type of flexibility, the overall conformation is still faithfully reproduced.

Hydrodynamic bead modelling

This provides a complimentary approach to classical "whole-body" or ellipsoidal modelling. Instead of considering directly the hydrodynamic behaviour of the whole particle, approximated as an ellipsoid, by approximating instead the structure as an array of spherical beads of not necessarily equal size and effectively summing the contributions the hydrodynamic properties of the particle as a whole can be predicted or modelled. This approach takes advantage of the fact that the hydrodynamics of spheres is much simpler than those of ellipsoids – even ellipsoids of revolution – and the hydrodynamics of interactions between spheres has also been well established (see, e.g., [35]). For food protein research, this approach would appear to be of particular value for modelling the assembly of multi-subunit proteins, such as seed globulins, if each subunit is approximated by a sphere, and in modelling macromolecules which have limited segmental flexibility, such as myosin. The basic idea is that the frictional ratio f/f_0 (and hence P and $s^0_{20,w}$) can be calculated (using formulae considerably more complicated than eq. (3) – see [35, 36] for a given array of spherical beads which do not have to be equal in size). This is done for an array of possible models for a given macromolecule, and the one which gives the predicted f/f_0 (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) (I) other hydrodynamic or scattering data (such as $[\eta]$, rotational diffusion coefficients or the radius of gyration, with the latter from neutron or x-ray scattering measurements, or, in the case of large macromolecular assemblies, from light scattering) and (II) a close starting estimate (from electron microscopy, x-ray crystallography etc.) to the true conformation are usually required.

Examples of its applications to a range of macromolecular systems can be found in [35]. Surprisingly, as 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 e.g. modelling the arrays of subunits, although similar approaches using light or low-angle x-ray scattering have been applied (see, e.g. PLIETZ et al. [61]). It has however been applied to myosin. Initial work of GARCIA DE LA TORRE and BLOOMFIELD [37], where the rod-shape portions (the LMM and S2 regions) of the myosin molecule were modelled as a linear tube of 20 Å beads and the S1 heads as an array of 4 unequal larger spheres of radii 14–34 Å confirmed flexibility at the 2 hinge points of the molecule (between the S1 and S2 regions and between the S2 and LMM regions). In further work, GARCIA DE LA TORRE et al. [62, 63, 36] cha-

racterized the partial flexibility between the S2 and LMM domains in terms of a flexibility parameter, Q (related to the bending potential) and the bend angle α. The latest developments are summarized in [36].

General modelling and general flexibility analysis

For polydisperse and not-so-rigid macromolecules we have to use shape analysis by sedimentation velocity in a general sort of way, first of all to indicate what conformation *type* the macromolecule is (random coil, rigid rod or compact sphere or whatever between these three extremes): these extremes are often represented by the so-called "HAUG triangle" (see [65]). 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 $s^0_{20,w}$ and the molar mass, M:

$$s^0_{20,w} = \text{const. } M^b \tag{5}$$

(similar coefficients exist for the intrinsic viscosity, the diffusion coefficient, and radius of gyration with M [64, 65, 60]). The MHKS b coefficient is usually obtained by preparing a "hormologous" series (i.e. the same polymer but different molecular weights) of the polymer (by e.g. chromatographic separation or heat degradation) and then taking the slope of a double logarithmic plot of log $s^0_{20,w}$ versus log M. The characteristic values of b for spheres, random coils and rods are respectively 0.667, 0.4–0.5 and 0.15 [65, 60]. The WALES VAN HOLDE parameter $k_s/[\eta]$ ($\equiv R$) is also useful in this context, having values of ~ 1.6 for spheres *and* random coils, and <1 for rods. Flexibility can also then be analysed in terms of worm-like coil type of approaches [66–70]. Although the main class of food macromolecule to benefit from these general approach are the polysaccharides, largely because of their inherent polydispersity [9], our understanding of linear protein molecules would also appear to benefit, such as titin, collagen and myosin filaments.

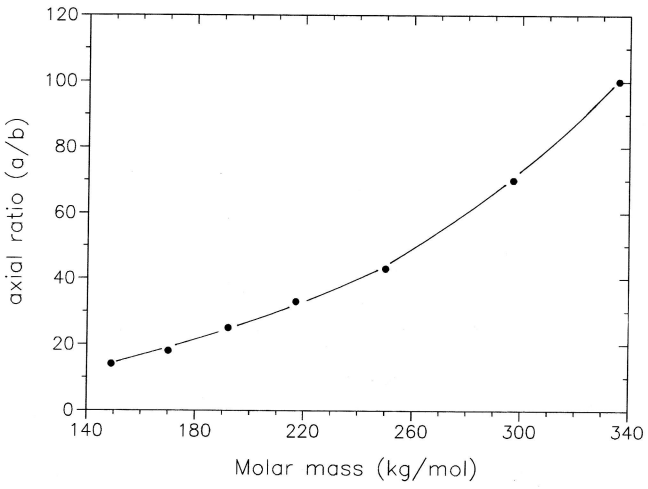

Fig. 4
Axial ratio of collagen sonicates, estimated from the WALES-VAN HOLDE ratio, $k_s/[\eta]$, as a function of molar mass

MARK-HOUWINK (-KUHN-SAKURADA) *analysis*

So-named because of the contributions from four different independent workers [71 – 75]. To illustrate the application of equation (5) consider the data for collagen and its sonicates of Table 3. In their original paper, NISHIHARA et al. [30] evaluated b from the slope of a plot of log $s^0_{20,w}$ versus log M and obtained a value of 0.20, consistent only with a fairly rigid rod model. This was confirmed from a similar plot of log $[\eta]$ versus log M, where $[\eta]$ is the intrinsic viscosity (ml/g): the slope obtained (i.e. the MHKS "a" coefficient) of 1.80 is again consistent with a rigid rod. Since we can therefore reasonably assume a rigid rod, advantage can then be taken of the availability of the R function to demonstrate the change in the axial ratio of the collagen rod as a function of molar mass (Table 3 and Fig. 4).

Flexibility: Worm-like coils

Worm-like coil modelling [67 – 70] permits the representation of flexibility between that of a rigid rod and a random coil. A variety of parameters have been used to represent this type of modelling, such as the ratio L/a of the contour length L to its persistence length, a. In the limits $L/a \to 0$ and $L/a \to \infty$ the conformation is rod-like and random-coil respectively. Other popularly used criteria of flexibility include for example the characteristic ratio, C_∞, the mass per unit length M_L and the "KUHN statistical length" with a variety of notations such as l_k, σ^{-1} (where $L\sigma = 0$ for a rod) or λ^{-1}. For example, FUJIME et al. [76] used a combination of dynamic light scattering diffusion measurements with sedimentation velocity to show, that, when the ionic strength of a myosin filaments is lowered (from 74 mM to 44 mM) there is an increase of the product $L\sigma$ from ≈ 0 to 0.1, corresponding to an increase in flexibility of the filaments. This observation was thought to have a connection with earlier observations from electron microscopy which showed the fraying of intact myosin filaments into three subfilaments [77].

Sedimentation velocity as a probe for heterogeneity

From the form of the sedimenting boundary it is possible to assess the heterogeneity of a sample – Fig. 5 shows the Schlieren images for a heterogeneous lipase preparation [31]. This type of analysis has been used to great effect for observing the heterogeneity of seed globulin preparations (see, e.g., [99, 107]). Sedimentation velocity can also be used to assay for interaction in a mixed solute system. In a recent study for example, the principle of

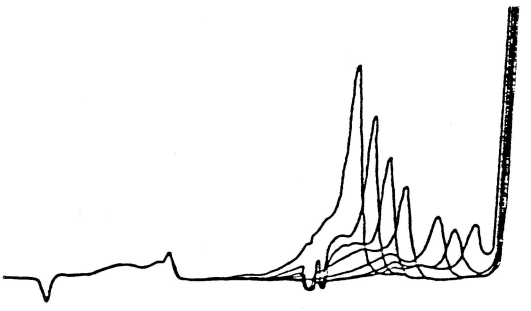

Fig. 5
Sedimenting boundaries for *Chromobacter viscosum* lipase using scanning Schlieren optics on the MSE Centriscan ultracentrifuge. Monochromator wavelength = 546 nm, scan interval = 10 min, rotor speed = 49000 rev/min; temperature = 20.0 °C. Two clear sedimenting boundaries are seen, at $s_{20} = (3.1 \pm 0.1)$ S (faster, major peak) and $s_{20} \approx 1.0$ S (slower, minor peak). From [31]

co-sedimentation was used to demonstrate an interaction between bovine serum albumin and an alginate [78]. Advantage can also be taken if the species in a mixture have chromophores absorbing in different regions of the UV or visible spectrum, whereby the absorption optical system can be used to optimal effect [79].

Sedimentation equilibrium: Molar mass, subunit composition and interaction constants

In the lower speeds used for sedimentation equilibrium, since the final steady state patterns are at equilibrium and involve no net movement of the molecules, frictional effects $\rightarrow 0$ and hence the solute distribute will be an absolute function of the molecular weight or "molar mass" M (g/mol) of a food protein and related parameters (such as association constants if the protein is self-associating). Its' absolute nature distinguishes it of course from relative methods such as gel filtration and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) which require calibration standards. Gel filtration, unless coupled to an absolute molar mass detector (usually light scattering [80]) requires assumptions concerning the conformation of the calibration standards whereas SDS-PAGE gives only the polypeptide molar mass, unless complicated procedures involving cross-linking agents are employed. Although of course sequence and mass spectroscopy provide the most accurate determinations of M, these only apply to proteins which have no non-covalently linked subunits – if not, only *subunit* molar masses are obtained. Mass spectrometry is also unable to help with the intact molar masses of the very large food proteins such as myosin (490000 g/mol) or the other muscle proteins such as the titins (Table 5) ($M > 1 \times 10^6$ g/mol,

Table 5
Molar mass (weight averages) of food proteins from sedimentation equilibrium analysis. These values are normally precise to $\pm 5-15\%$

Protein	M_w	References
β-Lactoglobulin	38000	[103]
	36000	[28]
Ovalbumin	44000	[96]
Bovine serum albumin	68000	[104]
Lipase (*Chromobacter viscosum*)	35000	[31]
Lipase (*Pseudomonas* spp.)	38000	[31]
Titin (sheep muscle)	1400000	[105]
Titin (pig muscle)	1500000	[106]
Titin (trout muscle)	1900000	[106]
Titin (tilapia muscle)	3000000	[106]
Carmin (from Safflower seed)	260000	[107] and refs. therein
α-Globulin (from Sesame seed)	270000	[107] and refs. therein
Arachin (from peanut)	330000*	[107] and refs. therein
Brassin (from rapeseed)	300000	[108]
	300000*	[99]
	290000**	[99]
Helianthin (from sunflower seed)	300000*	[107] and refs. therein

* From combining the sedimentation coefficient with the diffusion coefficient
** From combining the sedimentation coefficient with Stokes radius from gel filtration

depending on the source). 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 sample clarification etc.), and with the ability to run samples multiply (up to 9 at a time in the BECKMAN Optima XL-A), coupled with full on-line computer data capture and analysis [18] it may become the method of choice once again, especially for the analysis of interacting systems [17].

The most accurate way of recording these final steady state concentration distributions is using Rayleigh interference optics [81, 82] and the new ultracentrifuge from Beckman instruments will have this facility. For proteins however we can take advantage of absorption of the amino acids or the peptide bond to use the much more convenient (although less precise) absorption optical system and Fig. 2 illustrates the use of a multi-channel cell permitting the analysis of 3 solutions at a scanning wavelength of 280 nm. The signal for the inner channel of Fig. 2 is clearly only small for the loading concentration used (0.1 mg/ml): this can be considerably enhanced if a wavelength picking up the peptide bond absorption (210 – 230 nm) is used [8]. As noted above, absorption, interference or Schlieren data for sedimentation equilibrium analysis can be captured using a variety of on-line [12, 13, 18] or off-line [14, 15] automatic or semiautomatic procedures.

Molar mass analysis

Table 5 lists molar masses of some food proteins determined by the sedimentation equilibrium method. To obtain this sort of information from for example the XL-A the interfaced computer converts the digitised information from either absorption or interference optical records into an accurate record of concentration, c (expressed in terms of absorbance units, A or fringe displacement units, J) versus radial distance, r from the axis of rotation. Despite the greater precision of the optical records (helped by the opportunities for FOURIER types of averaging over all the fringe profiles [14, 15]) a drawback of the interference method is that the optical record is one of concentration of macromolecular solute *relative to that at the meniscus* versus radial displacement, r. Thus, either a procedure for obtaining the meniscus concentration has to be found, or a speed is chosen so that the concentration at the meniscus is effectively zero (this runs the risk of losing material from optical registration at the cell base, especially if the system is self-associating or contains a mixture of components – see [94]). The latter procedure known as the "meniscus depletion" method [93] is indeed more convenient – if achievable – but procedures are available for evaluating the meniscus concentration [86, 21] where this is not possible. With the absorption optical system the meniscus depletion method is not necessary, although adequate baselines need to be taken (to allow for the presence of non-macromolecular species contributing to the net absorbance): these can normally be obtained by overspeeding or depleting the meniscus from macromolecular solute at the end of a run.

For fairly ideal monodisperse systems (e.g. dilute solutions of some small proteins) a plot of $\ln c(r)$ versus r^2 will be linear, and from the slope the molar mass can be obtained. For heterogeneous (i.e. mixed or interacting) systems these plots will be curved upwards, or for proteins at higher concentration (> 1 mg/ml) thermodynamic non-ideality effects can become significant resulting in downward curvature: these effects can superimpose and in some cases almost cancel out, giving the false impression of a pseudo-ideal monodisperse system [83, 84]; if heterogeneity is suspected, these misleading effects can be avoided by recording equilibrium distributions at more than one initial loading concentration [83, 84, 85]. In case

of heterogeneity, the average slope of ln $c(r)$ versus r^2 plots, linear or otherwise, gives what we call the "whole distribution weight molar mass", or "whole cell weight average molar mass", $M_{w,\,app}$. For the extraction of $M_{w,app}$ an alternative procedure to simple slope evaluation using the properties of a function known as M^* [86] usually gives a more reliable estimate, particularly where the effects of heterogeneity or non-ideality are significant. Mainframe FORTRAN [88] or PC BASIC [89] programmes of a routine known as MSTAR – which uses the M^* function – are available for the extraction of $M_{w,app}$. Another highly useful molar mass routine available on PC is "XL-Aase" [87].

With most simple food protein systems run in solvents of sufficient ionic strength (normally ≈ 0.1) to suppress polyelectrolyte effects deriving from the charged groups on the protein, thermodynamic non-ideality effects are negligible for loading concentrations < 1 mg/ml, and it is usually no problem (either at ≈ 278 or $210-230$ nm so long as the buffers are transparent) satisfying this criterion, so that the approximation

$$M_w \approx M_{w,app} \tag{6}$$

can be reasonably made. This is a definite advantage of a sensitive optical detection system. Caution needs to be expressed for a system containing more than one subunit (dissociation can take place at lower concentration). Some further caution also needs to be expressed if the interference optics on the new BECKMAN ultracentrifuge are used where, because of the cell path length concentration restriction, the approximation of eq. (6) may not be appropriate.

In cases where non-ideality effects are significant (arising from excluded volume or unsuppressed charge effects) the classical method of measuring $M_{w,app}$ at several concentrations and fitting to the relation

$$1/M_{w,app} = (1/M_w) \cdot (1 + 2BM_w c) \tag{7}$$

can be employed [90] where B (A_2 in some texts) [ml · mol · g^{-2}] is the so-called "thermodynamic" or "osmotic pressure" second virial coefficient.

Local slopes along plots of ln $c(r)$ versus r^2 can also be taken (using sliding strip types of analysis [84]) to give local or "point" average molecular weights [$M_{w,app}(r)$] at a given radial positions in the ultracentrifuge, cell, and routines such as MSTAR [88, 89] and XL-Ase [87] also incorporate this type of analysis. Although the data can be rather noisy (especially when captured from absorption optical records) – which can be compensated for up to a limit by increasing the width of the sliding strip moving along the ln c versus r^2 curve – these point average representations can be particularly useful especially when given as a function of the local concentration, $c(r)$ in the cell. For example eq. 7 can be applied directly to data from a single sedimentation equilibrium experiment, in terms of a plot of $1/M_{w,\,app}(r)$ versus $c(r)$. Further, from estimates of the point average $M_{w,app}(r)$'s at the meniscus in the cell ($r = a$) and at the cell base ($r = b$) the z-average $M_{z,app}$ can also be escimated:

$$M_{z,app} = [M_{w,app}(b) \cdot c(b) - M_{w,app}(a) \cdot c(a)] / [c(b) - c(a)] \,. \tag{8}$$

As with M_w the approximation $M_z \approx M_{z,app}$ can usually be made for loading concentrations < 1 mg/ml, or an equivalent relation to eq. (7) (using the square root of $M_{z,app}$ [92]) can be used. In some cases the number average molar mass can also be obtained from Rayleigh optical records – absorption optical records are generally not precise enough, except in cases where the meniscus depletion method can be employed [93, 94]. For older

ultracentrifuges (not the XL-A) equipped with phase-plate Schlieren optics, $M_{z,app}$'s can also be obtained directly from the LAMM equation (see, e.g. [21]). Although more useful for food polysaccharides rather than food protein systems [9] the ratio of the z-average to the weight average molecular weight can be used as a gauge of the heterogeneity of a preparation [95].

Sedimentation-diffusion, sedimentation-gel filtration, sedimentation-viscosity and sedimentation-k_s methods

In the past molar masses have also been determined by combining the sedimentation coefficient with the diffusion coefficient via the SVEDBERG equation [96]. This like sedimentation equilibrium is also absolute, i.e. not requiring assumptions about conformation. Diffusion coefficients can be obtained from boundary spreading measurements in the ultracentrifuge [26] or more popularly these days by dynamic light scattering measurements (see, e.g. [97]). This will tend to yield the weight average molar mass, even if the z-average diffusion coefficient (from dynamic light scattering) is employed [98]. This method has been applied to for example seed globulins by SCHWENKE et al. [99, 102, 110]. Recent examples of applications of this method have been to mixed systems involving bovine serum albumin with alginate [78] and xylose [100]. An equivalent procedure to the sedimentation-diffusion method is the sedimentation-STOKES radius (from gel filtration) method [101], and this has also been applied to molar mass measurements on seed globulins [102]. Table 5 shows the good agreement between sedimentation equilibrium, sedimentation-diffusion and sedimentation-gel filtration for the 11 S rapeseed protein ("Brassin"). The molar mass can also be estimated by combining the sedimentation coefficient with the intrinsic viscosity. This takes advantage of the insensitivity of the SCHERAGA-MANDELKERN β-function, and either a globular conformation is assumed ($\beta \approx 2.112$) or if the molecule is rod-like like collagen an adjustment is made based on the axial ratio: the molar mass values for the collagen sonicates of Tables 1 and 3 were evaluated in this way [30]. It is possible to estimate molar masses also by combining the sedimentation coefficient with the concentration dependence regression coefficient k_s, and this has been shown to give reliable estimates for moderately solvated systems [32, 52]. Finally it is worth mentioning that the simplest method would be to estimate the molar mass from the sedimentation coefficient directly, and this can indeed be done but only if a conformation is assumed (cf. eqn. (5)).

Subunit composition analysis

A comparison of sedimentation equilibrium and sedimentation velocity analyses on proteins performed in non-dissociative compared with potentially dissociative solvents (8 M urea, 6 M GuHCl, SDS etc.) can give information on the subunit composition and nature of the interaction between subunits (hydrophobic, electrostatic etc.), and this procedure has been used extensively on seed proteins by for example SCHWENKE, PRAKASH and coworkers (see, e.g. [99, 102, 107, 109]). A significant recent development has been the advance of mass spectrometry based methods of analysis, which when combined with sedimentation equilibrium should be of considerable value for the analysis of food protein heterodimers ($\alpha_1\beta_1$) and other proteins of the $\alpha_n\beta_m$ type.

Self-associating systems

Sedimentation equilibrium is arguably the method of choice for the characterisation of self-associating systems, and was no doubt the driving force behind the so-called renaissance of ultracentrifugation in the late 1980's [17]. There has been a wealth of literature published on the methodology in the past, and key references are for example reviews by TELLER [84] and KIM et al. [111], a book EISENBERG [112] and an article by ROARK and YPHANTIS [85]. There are three approaches:

1. Comparison of M_w with the monomer molar mass, M_1 from which a stoichiometry and/or association constant can be deduced from the normal equations of mass action.

2. Model fitting plots of point average molar mass $M_{w,app}(r)$ versus local concentration $c(r)$, using M_1 as a constraint. This has been the most widely applied method in the past [84, 85, 111, 112] and is very useful for assessing the stoichiometry of an association, so long as non-ideality effects are not severe: particularly at higher loading concentrations an extra term needs to be taken into consideration in the evaluation of association constants. One of the classic examples of the application of point average procedures has been a study of the effect of temperature on the association/dissociation equilibria of beta lactoglobulin *B* at low pH [91]. This latter work also took advantage of the demonstration by ROARK and YPHANTIS [85] that if an associating system is genuinely reversible then plots of $M_{w,app}(r)$ versus $c(r)$ should superimpose for different initial cell loading concentrations c: this requirement was satisfied for the β-lactoglobulin system. Further studies [113] have examined the octamerisation of β-lactoglobulin under certain conditions. The sedimentation properties of milk proteins as a whole has been extensively covered in a book by MCKENZIE [3]. ROARK and YPHANTIS [85] also developed, "ideal" point averages $M_{y1}(r)$ and $M_{y2}(r)$ respectively, formed by combining $M_{w,app}(r)$ with respectively point number, $M_{n,app}(r)$ and point z-average $M_{z,app}(r)$ molar masses in a "simultaneous equation" fashion thereby eliminating first order non-ideality effects (i.e. the second virial coefficient, *B*). The extraction of $M_{n,app}(r)$ and $M_{z,app}(r)$ normally requires data of the highest possible precision (that is to say, normally beyond the capabilities of absorption optics), and even with interference optics, to extract the $M_{n,app}(r)$ realistically, the meniscus depletion method needs to be followed [93, 94]. Nonetheless, these "compound" point averages have been applied to study the monomer-dimer equilibria of myosin [5, 112]. Further "compound" combinations of $M_{w,app}(r)$, $M_{n,app}(r)$ and $M_{z,app}(r)$ as diagnostic tests for self-association have also been given [84, 85, 111, 112].

3. Direct modelling of the concentration distribution ($c(r)$ versus r) at sedimentation equilibrium [111]. The commercial software available with the new ultracentrifuge is largely based around this methodology. This in effect is equivalent to "2" but in principle avoids an extra differentiation of the data required to obtain the $M_{w,app}(r)$. A popular approach of this types, favoured by specialists from the Southern Seas is the omega function approach [114 – 116] where the concentration distribution is modelled with respect to the concentration at a given fixed reference position r_F in the equilibrium solute distribution. By extrapolating the omega function to zero concentration $c(r) = 0$ the thermodynamic activity of the monomer, $a_1(r)$ (or lowest molar mass species in a mixture) can be obtained as a function of r. This can be model fitted to assess the stoichiometry and association constants. As with $M_{w,app}(r)$ versus $c(r)$ data sets, superposition of the $a_1(r)$ versus $c(r)$ data-sets can be used as a criterion for the reversibility of a self-association. The method can also be used as an alternative procedure to eq. (7) to obtain the second virial coefficient *B* (or equivalently the activity coefficient, γ) of a system, useful amongst other things for assessing the

conformation of a food protein via the hydration independent Π function [56]. The B value obtained from omega analysis [57] has been used for example to show that egg albumin (ovalbumin) is essentially egg-shaped in solution [24].

Sedimentation equilibrium of mixtures and heterologous associations

Much of the theory behind the sedimentation of non-interacting mixtures was established almost at the birth of the ultracentrifuge, and derives from the work of SVEDBERG and RINDE [117, 118] and later developed by WALES [119]. Extension of this work to incorporate thermodynamic non-ideality has been difficult (see, e.g., [120, 94, 121, 122]). Although sedimentation velocity can give a relatively rapid qualitative and even semiquantitative picture of an interaction between different molecular species, sedimentation equilibrium, without the complications of conformation or JOHNSTON-OGSTON types of effects (see, e.g. [90]) can be used to assess the strength of an interaction. Again the omega function approach is of value here, and as an example, has been employed to examine the interaction of lysozyme with chitosan (used a food product in e.g. Japan) at low levels of acetylation. Very little free protein was found in solution ($a_{protein} \approx 0$) where the components were initially mixed on $a \approx 1:1$ basis by weight, thus confirming an almost stoichiometric reaction [123].

Food protein gels and matrix diffusion

In the pioneering work of JOHNSON et al. [124, 125], followed by the work of BORCHARD et al. [126–129] the utility of both sedimentation velocity and sedimentation equilibrium for examining the structure of gelatin and other gels was established, providing complimentary information to conventional rheological approaches. The Schlieren optical system appears to give the clearest records [126–129] and, because the network concentration/concentration gradient 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. Although not yet applied, it should be possible to use also the absorption optical system and, after selection of an appropriate wavelength (i.e. in which the gel matrix is invisible), to follow the diffusion of small molecules – including small proteins – through the gel, as a probe into gel structure. A similar approach has been applied to the study of the diffusion of lipases and other proteins through incompatible two-phase systems [130–132]. A low rotor speed is chosen simply to minimise convection effects. In this way the absorption optical system was used to examine the diffusion of various lipases (being increasingly considered in oil and fat processing [133]), and other proteins towards and through the interface separating incompatible aqueous polyethylene glycol/dextran two phase systems (transparent at 280 nm).

Concluding remarks

In conclusion, it is hoped this article has given some idea as to the breadth of application of modern ultracentrifuge procedures to problems in food protein research. Although it is by no means comprehensive, the examples quoted should give an idea of the types of

problem that can be solved using this type of methodology more often than not used in conjunction with other techniques. There is certainly every chance that the appearance of the new generation ultracentrifuges will provide a major impetus into our knowledge of the nature of food protein systems and how they interact with other macromolecules to govern some of the basic functional properties of foods.

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