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Analytical Ultracentrifugation and the Genetic Engineering of Macromolecules

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Introduction

There has developed an interesting 'cause and demand' hierarchy in molecular biology. The great advances in nucleic acid technology produced first of all a vast variety of recombinant and modified 'mutant' macromolecules, either directly (proteins) or indirectly (such as glycoproteins). It was then realized that these needed to be understood in terms of their chemical nature: protein chemists suddenly found they, too, were involved in this molecular biology revolution. Then it was realized that the physical properties needed understanding too. The stage was set for the physical chemist.

Physical chemistry, or 'biophysics', offers a wide variety of techniques for characterizing both the genetically engineered proteins (and derivatives thereof) and also those molecules used as the tools for their production, namely nucleic acids and nucleic acid binding proteins. These techniques can be classed as 'hydrodynamic' (e.g. viscometry, sedimentation velocity and sedimentation equilibrium in the analytical ultracentrifuge, gel permeation chromatography and gel electrophoresis), which give primarily overall size and shape information, 'scattering' (static and dynamic light scattering and X-ray scattering), 'spectroscopic' (absorption, fluorescence, NMR and ESR) and, finally, 'imaging' techniques (electron microscopy – including image reconstruction – optical or X-ray diffraction by fibres and X-ray crystallography), which can give low- and high-resolution structure of macromolecules.

Abbreviations: AMP, adenosine 5′-phospate; ATCase, aspartate transcarbamyolase; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; CAM, cell adhesion molecule; cDNA, circular DNA; CMC, *N*-cyclohexyl-*n*′-β-(4-methylmorpholinium) ethyl carbodiimide-*p*-toluene sulphonate; CSF, colony stimulating factor; CTP, cytidine triphosphate; EcoSSB, *E. coli* single-strand protein; ESR, electron spin resonance; GC, guanine cytosine; IFN-γ, natural human interferon; mRNA, messenger RNA; NANA, *N*-acetylneuraminic acid; NMR, nuclear magnetic resonance; PDGF, platelet-derived growth factor; RTP, replication terminator protein; SCF, stem cell factor; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TF, tissue factor; T_m, melting temperature (DNA, RNA); TNF, tumour necrosis factor; tRNA, transfer RNA; UV, ultraviolet.

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Table 1. Techniques for characterizing the physical properties of macromolecules

Technique	Type of macromolecule examined	Minimum amount of material required[a]	Information obtained
Hydrodynamic			
Viscometry	All	10 mg	Intrinsic viscosity → shape and molecular weight
Sedimentation velocity	All	100 μg–1 mg	1. Homogeneity 2. Sedimentation coefficient → shape and molecular weight[a] 3. Interaction studies
Sedimentation equilibrium	All	100 μg–1 mg	1. Molecular weight 2. Interaction studies
Analytical density gradient sedimentation	All		1. Purity, homogeneity 2. Composition (including G-C fraction for nucleic acids)
Diffusion analysis (in the analytical ultracentrifuge)	Reasonably monodisperse samples		1. Diffusion coefficient 2. Interface transport phenomena
Calibrated gel permeation chromatography	Proteins ($M<10^6$)	10 μg–1 mg	1. Homogeneity 2. Apparent molecular weight
SDS-PAGE	Proteins and nucleic acids	10μg	1. Homogeneity/subunit composition 2. Apparent molecular weight of polypeptide/polynucleotide
Scattering (from solutions)			
Static light scattering	All (M>50 000)	10 mg–1 g	1. Molecular weight 2. Rg → shape
X-ray scattering	All	100 mg–1 g	Rg → shape
Dynamic light scattering	All (M>50 000)	10 mg–1 g	Translational diffusion coefficient → shape and molecular weight[a]
Spectroscopic			
Absorption spectroscopy	1. Protein	100 μg	Concentration, ligand-induced conformational changes
	2. Nucleic acid		% G-C content (from T_m); % helical content (if composition known)
Circular dichroism	Proteins and nucleic acids		Secondary structure
Fluorescence	Macromolecules with natural or synthetic chromophore	μg–mg quantities	Concentration, ligand-induced conformational changes and (from depolarization studies) rotational diffusion
Nuclear magnetic resonance	1. Small proteins and nucleic acids 2. tRNA; globular proteins; membranes 3. Polysaccharides	100 mg+(depends on size of macromolecule)	1. Parts of 3-D structure in atomic detail 2. Ligand-induced conformation changes 3. Water relations
Electron spin resonance		100 mg+(depends on size of macromolecule)	Ligand-induced conformational changes
Mass spectrometry	Molecules <150 000 mol. wt.		Precise molecular weight of covalently intact species

Table 1. cont.

Technique	Type of macromolecule examined	Minimum amount of material required[a]	Information obtained
Imaging			
Image reconstruction from electron microscopy	Large macromolecules (e.g. polysaccharides, mucins), macromolecular assemblies, membrane proteins in 2-D array	μg–mg	Clarification of EM images, low-resolution structure, polydispersity
Optical or X-ray diffraction by fibres	Proteins, polysaccharides, DNA	Grams	Distance between repeat units
X-ray crystallography	Crystalline globular proteins and tRNAs	Grams	Electron density map, complete tertiary structure (provided sequence known)

[a] Ballpark figures only. Adapted from Furth and Moore (1986).

Table 1 summarizes the information available and the amount of material required. The interested reader can find a very useful summary of all these techniques in Furth and Moore (1986). Progressively more detailed considerations can be found in van Holde (1985), Cantor and Schimmel (1980) and the now dated, but still regarded by many as the authoritative, book of Tanford (1961).

From *Table 1* it is quite clear that the four analytical ultracentrifuge techniques – sedimentation velocity, sedimentation equilibrium, analytical isopycnic density gradient analysis and diffusion analysis – represent only a small part of the armoury of techniques available. Despite this, there are many features which make them highly useful for characterizing macromolecules, particularly engineered ones and assemblies thereof. Compared with other techniques, they are generally:

relatively rapid;

non-invasive;

do not require vast amounts of material (as is necessary with, for example, NMR and X-ray crystallography);

give size, structural (including subunit), heterogeneity and interaction information – and in some cases, in surprising detail.

These reasons make it worthy of serious consideration by the molecular biologist. It is no surprise that the demand for techniques to characterize 'gene products' has coincided with a revival of interest in analytical ultracentrifugation. It is worth stressing, however, that as with any 'low-resolution' technique like this, it is at its most powerful when used in conjunction with other complementary techniques, particularly solution X-ray scattering (conformation analysis), dynamic light scattering (size analysis), gel permeation chromatography (size distribution analysis) and gel electrophoresis (subunit composition and homogeneity analysis).

What sort of information about the macromolecules concerned with, and the products of, genetic engineering can we get from analytical ultracentrifugation? It depends on the type of ultracentrifuge technique we apply, which are all possible with the same sort of instrumentation. *Sedimentation velocity* (see, e.g. Harding, 1993a) can provide us with information on the sample homogeneity, shape information and also interaction information (in the case of ligand binding, assaying for what we call co-sedimentation phenomena). At lower rotor speeds, *sedimentation equilibrium* (see, e.g. Harding, 1993b) can provide absolute (i.e. not requiring calibration using standards) molecular weight and subunit composition information, together with association constants for a self-associating system. *Analytical isopynic density gradient equilibrium* (see, e.g. Creeth and Horton, 1977) can provide us with information on the purity and composition of a sample: the classic experiments of Meselson and Stahl (1958), in which DNA replication was shown to be semi-conservative, were performed using this technique. The analytical ultracentrifuge – or at least its optical system – can also be used as a powerful tool for *diffusion analysis* (see, e.g. Harding and Tombs, 1989). Although dynamic light scattering is now the method of choice for measuring translational diffusion coefficients, the optical system on the analytical ultracentrifuge is proving very useful for investigating the diffusion of molecules through matrices and towards and through interfaces between two phase systems (providing a good way of modelling membrane-based processes). A volume in which the state of the art of these various sedimentation techniques can be obtained has recently appeared (Harding, Rowe and Horton, 1992).

The technique is not new – the principles and general shape of the equipment have remained relatively unchanged since the inception of the technique by Svedberg and co-workers in the 1920s (much of this early work is reviewed in Rånby, 1987). Without doubt the great boom period was between 1950–70, when almost every biochemistry department was in possession of one of these instruments, most usually the famous Model E from Beckman Instruments (Palo Alto, CA, USA). These instruments were used mostly for routine sedimentation coefficient and molecular weight measurements. By the end of the 1970s, biochemists and molecular biologists were not content with such information (approximate molecular weights could be obtained anyway by using the much simpler and less expensive techniques of gel permeation chromatography and gel electrophoresis), and crystallography and nuclear magnetic resonance (NMR) had become the main focus of attention. By the mid-1980s, it was clear to many that NMR – at least in high-resolution mode – and crystallography could only be applied to a relatively limited number of biological macromolecules, and that techniques like gel filtration and gel electrophoresis were nowhere near as reliable for getting precise molecular weights, something that was essential for evaluating the subunit composition of assemblies of macromolecules. The need for a relatively rapid method for investigating the sizes, shapes [in terms of simple ellipsoids, with either two *or three* unequal axes (Harding, 1989), or sophisticated bead models (Garcia de la Torre, 1989)], subunit composition and interaction properties became acute with the appearance of this glut of newly

engineered molecules. The final *coup de grâce* was the launch in 1991 of a new computer-interfaced analytical ultracentrifuge. A good precis of this history has been given by Schachman (1992). Much of the published work using the analytical ultracentrifuge over the last few years – which this chapter attempts to review – has been on molecules concerned with genetic engineering, and using this and other more 'seasoned' analytical ultracentrifuges. It is appropriate to comment here that the author was stunned to discover so many references: a clear manifestation of the re-emergence of sedimentation methods in modern molecular biology.

Physical principles of analytical ultracentrifugation

An analytical ultracentrifuge is simply an ultracentrifuge with an appropriate optical system for observing and recording solute distributions both during and at the end of a sedimentation process (see, e.g. van Holde, 1985). A typical analytical ultracentrifuge cell contains one or two sector shape channels which can take up to 0.8 ml of solution or reference solvent. The amounts required depend on the type of ultracentrifuge experiment and the information required.

SEDIMENTATION VELOCITY: HOMOGENEITY, CONFORMATION AND INTERACTION ANALYSIS

In a *sedimentation velocity* experiment (see, e.g. Harding, 1993a), the rotor speed is sufficiently high to sediment the macromolecular species. If the macromolecule is a protein or nucleic acid, one can detect the position of the sedimentating boundary using absorption optics at the appropriate wavelength. Concentrations as low as 0.1 mg ml^{-1} can be routinely used, depending on the path length of the cell. Even lower concentrations are possible if a fluorescent chromophore is used (see, e.g. Schmidt and Riesner, 1990, 1992). For these, and macromolecules such as polysaccharides without sufficient chromophore, we can also use one of two types of optical system based on the refractive index: Rayleigh interference optics (this can also be extremely sensitive; see, e.g. Stafford, 1992) and Schlieren (viz. 'refractive index gradient') systems. The latter requires concentrations in excess of 1 mg ml^{-1}, but gives the classical 'Schlieren peak', which most biochemists associate with the analytical ultracentrifuge and, indeed, which has adorned the literature for the last 50 years. The sedimenting boundary is recorded on photographic film and then digitized or recorded via photomultipliers onto chart paper (or as analague output directly into a computer). The presence or not of more than one sedimenting boundary has been one of the classical ways of demonstrating homogeneity or heterogeneity (see, e.g. Morgan *et al.*, 1989, for the Pf1 gene 5 protein system).

The rate of movement of the sedimenting boundary per unit centrifugal field gives the sedimentation coefficient s, which is one of our shape parameters. By correcting this using simple formulae and standard conditions (water as solvent at 20°C) and extrapolating this (or the reciprocal thereof) to

zero concentration, one can get from the intercept the corrected sedimentation coefficient, $s^{o}_{20,w}$ (unit = seconds or Svedbergs, S, where 1 S = 10^{-13} s), and from the slope we can get the sedimentation concentration regression coefficient, k_s (unit = ml g^{-1}), and then from *both parameters* our shape information.

For conformation analysis there are three lines of attack. If the macromolecule is fairly rigid, one can combine the sedimentation coefficient with other techniques such as intrinsic viscosity, rotational diffusion (from fluorescence depolarization or electric birefringence measurements) or the radius of gyration (from 'static' light scattering or low angle X-ray scattering) to model the conformation in terms of simple ellipsoids of revolution, general triaxial ellipsoids (see, e.g. Harding, 1989) or sophisticated but beautiful bead models (see, e.g. Garcia de la Torre, 1989), and these approaches have been paticularly successful for the study of proteins, including antibodies and complement (see, e.g. Perkins, 1989). For more extended and more flexible macromolecules like nucleic acids and polysaccharides, one can model the conformation in terms of more general shapes using the 'Wales/van Holde' ratio of k_s to the intrinsic viscosity $[\eta]$, or the 'Mark-Houwink-Kuhn-Sakurada' b coefficient from the relation between $s^{o}_{20,w}$ and the molecular weight, M (similar coefficients exist for the intrinsic viscosity, the diffusion coefficient, and the radius of gyration with M; see, e.g. Harding *et al.*, 1991), to permit the modelling of the conformation in terms of general shapes, between the three extremes of compact sphere, rigid rod and random coil. A useful general construction for representing this type of modelling is the 'Haug triangle' (Smidsrød and Andresen, 1979; see also Harding *et al.*, 1991). We can use similar relations of $s^{o}_{20,w}$ etc. *vs* M to model the flexibility of the molecule in terms of the ratio of the contour length (L) to the persistence length (a) (Freire and Garcia de la Torre, 1992).

Sedimentation velocity is also a powerful probe into interaction phenomena. For self-associating systems, the dependence of the sedimentation coefficient $s^{o}_{20,w}$ on concentration can be used to estimate the stoichiometry and strength of the association (a good example is for tubulin; see Weisenberg, Borisy and Taylor, 1968), although sedimentation equilibrium is a more absolute probe into these phenomena. It also provides a quick and easy-to-use assay for interactions in a mixed solute system (including ligand binding) using the principle of co-sedimentation; a recent example of this, for the interaction of methylmalonyl mutase with its B12 cofactor, is given by Marsh and Harding (1993).

The sedimentation coefficient can also be used to give an absolute measure of molecular weight (by 'absolute' we mean not requiring calibration standards as are necessary with SDS-PAGE – which gives directly only subunit molecular weight of proteins – or gel permeation chromatography), but only when combined with the translational diffusion coefficient, which is now usually measured using dynamic light scattering (see, Pusey, 1989; Bloomfield, 1981). A more direct way is to use a different analytical ultracentrifuge technique known as *sedimentation equilibrium*.

SEDIMENTATION EQUILIBRIUM: MOLECULAR WEIGHT AND SUBUNIT STRUCTURE ANALYSIS

Whereas in a sedimentation velocity experiment at relatively high rotor speeds (for a polysaccharide, say 40 000–50 000 rev min^{-1}) the sedimentation rate and hence sedimentation coefficient depends on the size and shape of the molecule, at much lower speeds (say 20 000 rev min^{-1} or less) in a sedimentation *equilibrium* experiment (see, e.g. Harding, 1993b) the forces of sedimentation and diffusion on the macromolecule become comparable, and instead of getting a sedimenting boundary one obtains a steady-state equilibrium distribution of macromolecule with a low concentration at the meniscus building up to a high concentration at the cell base. This final steady-stage pattern is a function *only* of molecular weight and related parameters (virial coefficients and association constants where appropriate) and not of molecular shape (apart from entering into non-ideality considerations, again when appropriate), since at equilibrium there is no further movement of the macromolecule. Hence frictional effects through shape variation do not come into play and we have an absolute way of getting (weight-average) molecular weights.

Solute concentration distributions at sedimentation equilibrium are recorded most accurately using Rayleigh interference optics. These can be read directly on-line into a computer (Laue, 1992) or 'off-line', i.e. recorded on photographic film and then digitized into a computer (Rowe *et al.*, 1992), using, for example, a laser densitometer of the type found in many biochemistry departments used for scanning electrophoresis gels. Alternatively, the absorption optics can be used; although less precise, these can be much more convenient to use for proteins and nucleic acids. There are various ways of processing the concentration versus distance data.

1. Molecular weight or 'molar mass' (weight average) of all the macromolecular contents in the ultracentrifuge cell: M°_{w}. From routines (such as 'MSTAR': see Harding, Horton and Morgan, 1992) *almost* equivalent to getting the average slope (it is not *quite* as easy as that) of plots of log concentration against distance from the centre of the rotor squared, one can get the apparent weight average molecular weight, $M_{w,app}$, which after an extrapolation (of $M_{w,app}$ or $1/M_{w,app}$) to zero concentration (to remove non-ideality problems) gives us the molecular weight (this will be a weight average for a heterogeneous system). Number and z-averages can also be obtained if the data are good enough. For most proteins and nucleic acids at low concentration (0.5 mg ml^{-1} and less), non-ideality corrections can be quite small and a concentration extrapolation is often not necessary. The molecular weight so found will be of the intact molecule (not, like SDS-PAGE or mass spectrometry, of the subunits) (see, e.g. Marsh *et al.*, 1989). For multi-subunit systems where the subunits are of comparable size, by use of appropriate dissociative solvents (such as 6 M GuHCl) it is possible also to obtain the subunit molecular weight, hence the subunit composition. When the subunits are not equal in size, some combination of sedimentation equilibrium, SDS-PAGE (for proteins) and possibly sedimentation velocity is

necessary to give similar information. It is worth mentioning that although the favoured unit of molecular weight or molar mass is g mol^{-1}, since most molecular biologists think in terms of Da or kDa, we will tend to use this unit here.

2. Point average molecular weights (usually weight averages). These can be obtained from local slopes of the log concentration versus distance squared data, and are quite useful for obtaining the stoichiometry and association constants for self-associating systems. A good example has been given by Tang and Adams (1973), who used this procedure to monitor the effect of temperature on the dissociation equilibria of beta-lactoglobulin dimers. Again, point number and point *z*-averages can also be obtained if the data are good enough (see below).

3. Omega function analysis. Association constants can also be obtained by modelling the concentration versus distance squared data directly (see, e.g. Ralston and Morris, 1992).

4. Molecular weight distribution analysis. This is useful for polydisperse macromolecules, like nucleic acids and polysaccharides. There are four principal ways of obtaining distributions (see, e.g. Harding, in press), the most useful being 'Method IV', i.e. a combined approach with gel permeation chromatography.

5. Number and *z*-average molecular weights. These are useful for representing heterogeneous systems, whether they be self-associative, complex forming or polydisperse. If conventional absorption or Rayleigh interference optics are used, the weight average molecular weight is the principal average or 'moment', but it is in principle possible to obtain number average molecular weights [but only for those systems where the so-called 'meniscus depletion' method (Yphantis, 1964) can be employed, a method applicable only to a rather limited number of macromolecular systems) and also *z*-average molecular weights, but only after a noise-sensitive double differentiation of the basic concentration versus distance record. However, if the Schlieren optical system is used, *z*-averages can be obtained directly, and these optics can be as sensitive as the Rayleigh optical system (Rowe *et al.*, 1992).

ANALYTICAL ISOPYCNIC DENSITY GRADIENT ULTRACENTRIFUGATION

This is really a modified sedimentation equilibrium method (see, e.g. Creeth and Horton, 1977), the modification being a heavy salt – usually CsCl (also CsBr, Cs_2SO_4 and LiBr) is used at the appropriate molar concentration as solvent. At sedimentation equilibrium, the salt will redistribute, giving an equilibrium density gradient between the meniscus and cell base. This gradient will be a function of the salt, the rotor speed and the solution column length. A suspended macromolecule will at equilibrium 'band' at the isodensity point, which will either be at the meniscus, the base, or, if the density gradient has been appropriately chosen, somewhere between these two extremes. For example, the buoyant densities of proteins and polysaccharides are ~1.3 g ml^{-1} and ~1.6 mg ml^{-1}, respectively. The buoyant density of

nucleic acids is ~ 1.7 g ml^{-1}, depending on the GC content.

This method has been invaluable for assaying the purity of macromolecular preparatons, the extent of glycosylation of proteins and the base content (see, e.g. Schildkraut, Marmur and Doty, 1962) and extent of ligand binding to nucleic acids, and indeed was the classical method used to show that the replication of DNA is semi-conservative (Meselson, Stahl and Vinograd, 1957; Meselson and Stahl, 1958).

DIFFUSION ANALYSIS

Athough still used occasionally, the days of boundary spreading in the ultracentrifuge being used *on a routine basis* for the measurement of translational diffusion coefficients (see, e.g. Tanford, 1961) have long since gone, being replaced by far faster and *usually* more accurate dynamic light-scattering methods (see, e.g. Pusey, 1989). However, the optical systems on the analytical ultracentrifuge, coupled with the stabilizing effect (against convection phenomena) that a small centrifuge field gives (run at ~2000 rev min^{-1}) make it ideal for monitoring the diffusion of small proteins or other molecules through matrices (polysaccharide or other; see, e.g. Comper and Preston, 1992), or for monitoring the diffusion of proteins towards and through interfaces separating two or more incompatible (aqueous or non-aqueous) phase systems (Tombs and Harding, 1988; Harding and Tombs, 1989). The significance of this for monitoring membrane-based and commercial processes has been recognized (involving, for example, lipases), although no work has yet been done on the performance of *engineered* proteins of this type.

THE MACROMOLECULES REVIEWED HERE

Although the main focus of this review is on the engineered macromolecules themselves – largely protein and glycoprotein – it would be instructive to first briefly review the contribution ultracentrifuge methods have made to our understanding of those macromolecules associated with the engineering process, namely nucleic acids and nucleic acid binding proteins. Unfortunately, those arguably of the greatest interest – restriction endonucleases and ligases – responsible for the scissision and re-glueing together of genetic information – are themselves only produced in tiny quantities; they are themselves ideal candidates for gene cloning and overproduction for physical study.

Nucleic acids

The application of the ultracentrifuge to nucleic acid research dates back almost 50 years. For example, Kahler (1948) investigated the concentration dependence of the sedimentation coefficient of DNA. The classic application was the demonstration by Meselson and Stahl (1958; see also Meselson, Stahl and Vinograd, 1957) using analytical density gradient ultracentrifugation, and

its ability to separate DNA labelled with ^{15}N from unlabelled ^{14}N DNA, that DNA replication was semi-conservative.

SIMPLE HOMOGENEITY/PURITY AND COMPOSITION CHECKS

Buoyant density measurements using CsCl isopyenic density gradient methods (see, e.g. Weinblum, Geisert and Ostwald, 1990) are still regarded as prime purity assays for a nucleic acid preparation (see, e.g. Durante *et al.*, 1987; Ramachandran and Narayan, 1990). Bouyant density measurements are routinely used to establish the base composition (Schildkraut, Marmur and Doty, 1962; Kirchhoff and Flossdorf, 1987; McIntyre *et al.*, 1987; Ligon *et al.*, 1989) and even estimates for molecular weight from the band shape (see, e.g. Thanaraj and Pandit, 1987). Sedimentation velocity is also routinely used as a criterion for purity (see, e.g. Weller and Hill, 1992), and reproducibility of a sedimentation coefficient is regarded as an indicator of successful reconstitution of rRNA subunits (Bogdanov *et al.*, 1988).

STRUCTURAL ANALYSES

In DNA, sedimentation equilibrium and velocity analyses have been used in conjunction with other techniques such as circular dichroism and gel electrophroresis to investigate the formation of hairpin and cruciform structures (Howard *et al.*, 1991; Ross, Howard and Lewis, 1991; Scaria, Shire and Shafer, 1992), linking number anomalies (Ringquist, Shinn and Hanlon, 1989), B-Z transition phenomena (Chen, Ringquist and Hanlon, 1987), the structure of a short promoter region, in conjunction with neutron and dynamic light scattering (Lederer *et al.*, 1986), and salt-dependent changes in the structure and dynamics of circular DNA, in conjunction with dynamic light scattering (Schaper *et al.*, 1991), and the association properties of DNA nonamers (Braswell *et al.*, 1992). For mRNA, a combined approach (including Mark-Houwink-Kuhn-Sakurada analyses) has been used (Freerksen *et al.*, 1990). Broitman, Im and Fresco (1987) have looked at the triple helical poly (AAU). Steger *et al.* (1986) have also used a combined approach (sedimentation velocity and equilibrium with electron microscopy, high-performance liquid chromatography and spectrophotometry) to investigate the structural properties of the RNA transcripts from cloned oligomeric potato spindle tuber viroid.

LIGAND-BINDING STUDIES

Several recent studies have involved either sedimentation velocity or isopycnic density gradient methods. For example, Bickhardt *et al.* (1991) used sedimentation velocity to look at the interaction of clupeine with DNA. Thomas, Nessler and Katterman (1989) examined the degree of 5-bromodeoxyuridine binding to DNA using isopycnic density gradient analysis in CsCl, and Wang, Sullivan and Libowitz (1989) used the same type of analysis to monitor the extent of binding of CMC (*N*-cyclohexyl-*n*-β-(4-

methylmorpholinium) ethyl carbodiimide-*p*-toluene sulphonate) to supercoiled DNA as a probe for unpaired and mismatched sites in DNA. Richard (1987) had earlier applied scaled particle theory to gels formed by DNA in the ultracentrifuge to obtain estimates for the effective length and radius of a DNA particle, before and after treatment with ethidium bromide (causing extensive lengthening, interpreted in terms of denaturation) or acridine orange (causing modest changes, interpreted in terms of binding). Trohalaki, Frisch and Lerman (1991) have also used scaled particle theory to investigate the effects of LiCl, RbCl, CsCl and $MgCl_2$ on the close packing of persistence length DNA fragments. Westkaemper and Richard (1987) used sedimentation equilibrium analysis to investigate the effects of diadenosine tetraphosphate and ATP on the higher-order structure of DNA.

DNA–RNA INTERACTIONS

White, Wood and Hill (1988) used sedimentation velocity in conjunction with dynamic light scattering and sucrose density gradient centrifugation (on radiolabelled material) to investigate the extent and effects of the interaction between a cDNA 'probe' and the α-sarcin region of 23S ribosomal RNA from *Escherichia coli* while still integral with the 50S ribosomal subunit from which it is derived. This interaction was explored under conditions where the ribosome was in its native state and under 'collapsed state' conditions. Binding of the probe was confirmed while the subunit was in a collapsed state, although the probe itself was shown not to be responsible for the collapse itself, refuting earlier beliefs.

Nucleic acid binding proteins

DNA PACKING PROTEINS

These have been the most widely studied with an extensive literature. Most involve studies on *histones* and their binding to DNA in nucleosomes and chromatin. The application of ultracentrifuge methods ranges from simple purity checks using CsCl isopynic density gradient equilibrium (Amero *et al.*, 1988), right through to specific details of the interaction.

The basic unit of chromatin is the nucleosome: a histone core protein complex about which the DNA is wrapped (see, e.g. van Holde, 1989). Sedimentation analysis combined with gel filtration studies were instrumental in establishing that the histone core complex is an octameric assembly of eight histone protein molecules (two each of histones H2A, H2B, H3 and H4) (Godfrey, Eickbush and Moudrianakis, 1980). Recent studies have focussed on the nature of the interaction between the core proteins themselves and the DNA. Moehs *et al.* (1992) used meniscus depletion sedimentation equilibrium with gel filtration to show that the plant variants of H2A and H2B are responsible for enhanced stability of the octamer compared with its verterate counterpart. The octamer itself is thought to self-associate to give larger structures. Prevelige and Fasman (1987) used similar approaches to investi-

gate the effects of acetylation on this and the conformation, whereas Baxevannis, Godfrey and Moudrianakis (1991) looked at the effect of ionic strength on the association of the $(H3–H4)_2$ tetramer. Other studies have focused on the core proteins themselves (Ausio, 1988; Jutglar *et al.*, 1991) including the effect of H2a specific proteoloysis as a probe into the nature of the assembly process (Eickbush *et al.*, 1988).

In so far as the interactions with DNA are concerned, sedimentation velocity has been used to look at the cooperative inducing effect of Mg^{2+} (Triebel *et al.*, 1988), limitations of the polyglutamic acid reconstitution method for nucleosomes (Pennings, Muyldermans and Wyns, 1986), oligomerization of nucleosomes (Hansen *et al.*, 1989; Hansen, van Holde and Lohr, 1991), the significance of mobile histone tails in nucleosomes (Smith and Hill, 1989), two-sturt double helex formation (Osipora *et al.*, 1990) interactions at low ionic strength (Hirai *et al.*, 1988), the reversibility of the low salt transition of the core particles (Libertini and Small, 1987), the deleterious effect of higher ionic strengths (Yager, McMurray and van Holde, 1989), the influence of chromatin folding on inhibition of transcription initiation and elongation by RNA polymerase III (Hansen and Wolffe, 1992), the effect on the interaction of UV damage to the DNA (Gale and Smerdon, 1988) and B-Z DNA transition phenomena in reconstituted nucleosomes (Ausio, Zhou and van Holde, 1987). Sedimentation velocity distributions have been used to model the distribution of nucleosome positioning (Robert and van Holde, 1992). Sedimentation velocity and sedimentation equilibrium have been used to investigate the role of histone 'tails' in the stabilization of the nucleosome (Ausio, Dong and van Holde, 1989), in H1-depleted oligonucleosome folding (Garcia-Ramirez, Dong and Ausio, 1992) and the role of the $(H3-H4)_2$ tetramer in positioning the nucleosome (Dong and van Holde, 1991).

Besides histones, other packing-related proteins have been studied. Using sedimentation velocity analysis with low angle X-ray scattering, Herranz *et al.* (1990) have shown that conformational changes caused by proteolysis of the *ϕ29 connector* prevents DNA binding activity.

TOPOISOMERASES

Besides packing-proteins, other non-specific binding proteins have been investigated. Topoisomerases, for example, are enzymes that catalyse the breakage and rejoining of the DNA backbond. Riou *et al.* (1986) used isopycnic density gradient equilibrium to show that topoisomerase preparations from *Plasmodium berghei* were free from leukocytes, and combined the sedimentation coefficient measured in glycerol with the Stokes radius from gel filtration to obtain a molecular weight of 300 kDa, consistent with a dimer, whereas topoisomerase I gave 104 kDa, interpreted as monomer.

OTHER NON-SPECIFIC DNA BINDING PROTEINS

Brenner, Złotnick and Stafford (1990) have used sedimentation equilibrium to study the self-association properties of RecA (37.8 kDa) from *E. coli*. Monomers were in reversible equilibria with trimers, hexamers and dodecamers to an extent depending on the temperature, the ionic strength, pH and presence or not of ATP. Sarfert *et al.* (1989) have used sedimentation velocity analysis to investigate the DNA binding properties of the transcription affecting *Streptomyces hygroscopicus* DNA binding protein.

GENE5 PROTEIN

The gene5 protein of the filamentous bacteriophage Pf1 is a dimeric protein (30 kDa) which binds to single stranded DNA during phage replication to form a helical nucleoprotein complex. Sedimentation velocity and equilibrium studies (Morgan *et al.*, 1989) have been used to characterize solutions of the protein. Sedimentation diagrams have shown the existence of mostly a 2.6S species – corresponding to the monomer, with a significant proportion of a high molecular weight aggregate (35S). Point weight average molecular weight analysis confirmed that the monomer form was the predominant species, with point average molecular weights of 32 500±3000 dominating, except near the cell base.

REPLICATION TERMINATOR PROTEIN (RTP)

Specific DNA replication terminators operate by slowing down or arresting replication forks. Using sedimentation, Lewis *et al.* (1990) have shown that *Bacillus subtilis* RTP (14.5 kDa) exists as a dimer (29 kDa) at neutral pH and concentrations above 0.2 mg ml^{-1}.

POLYMERASES

RNA polymerase from *E. coli* (450 kDa) is a dimeric protein under normal solution conditions. Using co-sedimentation experiments, Blazy, Takahashi and Baudras (1980) have shown that RNA polymerase interacts with the cyclic AMP-cyclic AMP receptor protein complex in solution. Further sedimentation velocity experiments by Pinkney and Hoggett (1988) have shown that the cyclic AMP receptor protein itself binds predominantly to the monomeric rather than the dimeric form of the enzyme. Butzow, Oehrl and Eichorn (1991) have used sedimentation diagrams from sedimentation velocity to assay the proportion of free and template-bound *E. coli* RNA polymerase. Suzuki (1990) has shown from a comparison of sedimentation coefficients of synthetic peptides that a heptad repeat in the largest subunit of RNA polymerase II binds to DNA, a result supported by fluorescence quenching measurements. Hansen and Wolffe (1992) investigated the influence of chromatin folding on the inhibition of transcription initiation and elongation by RNA polymerase III. Sedimentation equilibrium studies

(Lewis *et al.*, 1992) have shown that DNA β-polymerase (39 kDa, consisting of 8 kDa and 31 kDa domains), a symmetric monomer, did not self-associate on binding of oligodeoxynucleotide. By investigating the binding as a function of temperature, the enthalpies and entropies of the reaction were evaluated.

POLYMERASE 'ACCESSORY PROTEINS'

The polymerase 'accessory proteins' of phage T4, such as the gene 44, 62 and 45 proteins which show ATPase activity, are able to carry out leading strand DNA synthesis *in vivo*. Jarvis, Paul and von Hippel (1989) used sedimentation equilibrium and velocity, together with dynamic light scattering and gel filtration, to show that the gene 44 and 62 proteins associate to form a tight complex containing four gene 44 protein subunits and one gene 62 subunit, with a total molecular mass of 163.7 kDa, with an axial ratio of 5 : 1 (assuming a prolate ellipsoid model). Sedimentation equilibrium and chemical cross-linking studies suggest the gene 45 protein self-associates to form a trimer, which is also asymmetric.

OTHER GENE CONTROL PROTEINS

Overman, Bujalowski and Lohman (1988) used sedimentation velocity to estimate the average lengths of synthetic homopolynucleotides (based on polyA and polyU calibrations) to investigate cation and anion effects and polynucleotide specificity on the equilibrium binding of *E. coli* single-strand protein (EcoSSB) to single stranded nucleic acids in the $(SSB)_{65}$ binding mode. Using model fitting sedimentation coefficient versus concentration curves, Callaci *et al.* (1990) have shown that the *Xenopus* transcription factor III complexes with 5S RNA in a 1 : 1 ratio, which self-associates to a dimer.

A combination of sedimentation velocity with gel filtration has shown that the Nu1 subunit (21.2 kDa) of bacteriophage γ terminase exists as a M>500 kDa aggregate (Paris *et al.*, 1988). The physical properties and potential for self-association as a function of ionic strength of the *E. coli* transcription factors σ and NusA have been examined by Gill, Yager and von Hippel (1991), using sedimentation velocity and equilibrium coupled with chemical cross-linking, dynamic light scattering and low-angle X-ray scattering.

Jaenicke *et al.* (1990) have used a combined sedimentation velocity/equilibrium approach to show that the raf repressor protein from *E. coli* under intracellular conditions exists as a 144 kDa tetramer ('dimer of dimers'), although it undergoes concentration-dependent dissociation at lower concentrations, under meniscus depletion conditions. Sedimentation equilibrium has been used by Richet and Raibaud (1987) to show that the MalT protein, the transcription activator of the *E. coli* maltose regulon, exists as a monomer (100 kDa), while using the same technique Starovasnik *et al.* (1992) have shown the myogenic determination factor MyoD, a sequence-specific DNA binding protein, is best described as a dimer-tetramer, with a dissociation constant of 17.3 μM.

Geiselmann *et al.* (1992) have used the same technique to give a molecular weight of 264 kDa for the *E. coli* transcription termination factor rho I, confirming a hexamer, in agreement with the 'Svedberg equation molecular weight' (sedimentation coefficient combined with the translational diffusion coefficient, the latter from dynamic light-scattering measurements), and to give estimates for the gross conformation consistent with electron microscopy and X-ray scattering that the hexamer is planar. Further studies have investigated the binding of ATP (Geiselmann and von Hippel, 1992) and the binding of DNA to termination factor rho II (Geiselmann, Yager and von Hippel, 1992).

TRANSLATION FACTORS

A similar approach adopted by Sam *et al.* (1990), combining sedimentation velocity and equilibrium measurements with dynamic light scattering, showed that under non-self-associating conditions (as confirmed by sedimentation analysis), the bacterial elongation factor EF-Tu : GDP complex is a near-spherical particle in solution. Bommer *et al.* (1988) have also used sedimentation equilibrium (molecular weight), together with sedimentation velocity and diffusion analysis (molecular weight and frictional ratio), to investigate the size and shape of the eukaryotic initiation factor eIF-2. Sedimentation velocity together with fluorescence quenching and chemical modification studis have been used to investigate the interaction of tryptophanyl-tRNA synthetase with $tRNA^{Trp}$ (Fournier *et al.*, 1987).

INTEGRASE

Retroviral integrase is responsible for the integration of viral DNA into host DNA, with regard to the endonucleolytic processing of viral DNA ends and the cleavage and joining of host DNA to the processed viral DNA termini. In an attempt to understand the nature of the enzyme and the kinetics of the process, short-column sedimentation equilibrium studies were employed by Jones *et al.* (1992) which revealed a substrate-dependent reversible equilibrium among the monomeric (31.7 kDa) dimeric and tetrameric forms of retroviral integrase from Rous sarcoma virus. This information, combined with steady-state kinetic studies, suggests that the minimal functional unit of the enzyme required for both the processing and joining of each viral DNA end is the dimer.

REVERSE TRANSCRIPTASE

Reverse transcriptase from avian myeloblasts is an $\alpha\beta$ type of protein. Lin *et al.* (1991) used sedimentation velocity and equilibrium to investigate the association state under native and glycerol-containing buffer systems. In the absence of glycerol, it was deduced that the enzyme behaved as a heterodimer (152 kDa). In the presence of glycerol, after taking into account solvent effects, the enzyme dimerized to give an $(\alpha\beta)_2$ structure. Recombinant HIV-1

reverse transcriptase, also an αβ heterodimer, has also been the subject of extensive analysis by ultracentrifugation, and will be considered below (p. 341).

RETROTRANSPOSON VIRUS-LIKE PARTICLES

Without wanting this review to drift into the realm of viruses – DNA–protein complexes in the broadest sense – it is worth mentioning that the RNA-based retrotransposon particles, which replicate by a reverse transcriptase step followed by an integration reaction into the host genome, have also been characterized by the 'Svedberg equation route', viz sedimentation coefficients combined with dynamic light-scattering translational diffusion coefficient measurements to size these particles. A value of 142 kDa has been obtained (Burns *et al.*, 1992).

RIBOSOMAL PROTEINS

Sedimentation velocity and equilibrium analysis, together with dynamic light-scattering diffusion coefficient measurements, have shown that the ribosomal protein S4 from *E. coli* was monomeric (23kDa), but asymmetric with an axial ratio of 5 : 1 based on a prolate ellipsoid model (Dodd and Hill, 1987). Georgalis *et al.* (1989) have used sedimentation equilibrium to show that another *E. coli* ribosomal protein complex, L7/L12, is tetrameric (50 kDa), consistent with measurements from other less accurate techniques.

Engineered proteins and glycoproteins

The last few years has seen a plethora of published work on engineered proteins, ranging from small peptides like interleukins and hirudin right through to large multi-enzyme complexes. The sort of questions being addressed include: Does a recombinant protein have the same degree of monodispersity or a tendency to associate as its native or 'wild-type' analogue? (see, e.g., Pingoud *et al.*, 1988). Does site-directed mutagenesis significantly affect the activity of an enzyme by altering its conformation or its relations with a cofactor? (see, e.g., Slodowski *et al.*, 1991). Can we probe the significance of particular amino acids using mutagenesis? Does altering a protein subunit affect the mode of self-assembly into a multi-enzyme complex or alter the shape and size of a viral coat?

MONOCLONAL ANTIBODIES

Because of their flexibility, there is as yet no high-resolution crystallographic structure for an intact, immunologically active antibody molecule, only structures worked out for fragments and mutant antibodies which lack a hinge region. Sedimentation analysis, combined with other solution techniques, such as low-angle solution X-ray scattering, have provided the only real handle on the conformation of the intact molecule in solution.

Byron (1992) used sedimentation equilibrium and velocity to confirm that chimeric monoclonal B72.3 does not self-associate in dilute solution and used hydrodynamic bead modelling of the sedimentation coefficient and radius of gyration to show that the Fab and Fc regions do not form a planar structure. Morgan, Byron and Harding (1992) used the same techniques to show the monodispersity of preparations of Fab and $(Fab)_2$ fragments of this antibody, and used bead models to show that the Fab domain has dimensions approximately 50 × 80 × 50Å, inclusive of hydration.

Wilhelm *et al.* (1987) have looked at size using sedimentation equilibrium analysis and conformation by low-angle X-ray scattering on complexes of monoclonal Fab fragments with the β_2-subunit of *E. coli* tryptophan synthase as the 'antigen', to show (1) that the C-terminal F_2 domains lie at the distal ends of the subunit, and (2) how steric hindrance stops the (dimeric) β_2 from binding more than one Fab 93-6 fragment per dimer. Similar binding studies, involving the whole antibody complexed with ricin (via a galactose-binding domain), have been performed by Colombatti *et al.* (1987). Jentoft and Bolinger (1987) have used sedimentation velocity to investigate the interaction of monoclonal IgG1 with human serum albumin. King *et al.* (1993) have shown by velocity and equilibrium sedimentation analysis that the two Fv domains of the chimaeric B72.3 monoclonal are associated at high concentrations at pH values close to neutral, but dissociate at concentrations lower than $\sim$0.5 mg ml^{-1}.

The behaviour of antibody fragments and constructs thereof ('mini-antibodies') is of topical interest at the moment. Pack and Plückthun (1992) have shown by sedimentation velocity analysis that a mini-antibody (consisting of two single chain Fv domains joined with a flexible hinge region from IgG3 and an amphiphatic helix fused to the C-terminus) dimerizes in solution. In another study using the 'combined approach', Morgan *et al.* (1988) used a combination of sedimentation velocity analysis with monoclonal antibody binding to probe the molecular determinants of haemopexin-mediated haem transport. Haem binding causes domain I of the protein to become more compact and induces an association with domain II.

RECOMBINANT COMPLEMENT

One of the biggest problems of recombinant technology is post-translational modification of the gene product: the problem is particularly acute for glycoproteins, of which complement is an example. Luo *et al.* (1992) measured the sedimentation coefficients of recombinant complement CI subcomponent lacking in beta-hydroxyasparagine, sialic acid and one of its two carbohydrate chains, and found that it still reassembles with Clq and Clr sub-components to form a functional CI complex.

RECOMBINANT INTERLEUKINS

Other recombinant proteins from the immune defence system have also been studied. Proudfoot *et al.* (1990) used sedimentation velocity to demonstrate

the homogeneity of human interleukin 5 preparations (after gene expression in *E. coli*), and using sedimentation equilibrium showed that the recombinant protein behaves as a homodimer (viz a covalently linked dimer with no evidence for further self-association behaviour), consistent with observations from gel permeation chromatography. Both recombinant interleukin 1α (Wingfield *et al.*, 1987) and 1β (Wingfield *et al.*, 1986) also show no tendency to self-associate in dilute solution, and adopt compact globular conformations.

RECOMBINANT MACROPHAGE FACTORS

Wingfield *et al.* (1988) used a combination of sedimentation velocity and equilibrium ultracentrifugation in conjunction with urea-gradient polyacrylamide gel electrophoresis and several spectroscopic methods to study the conformation and stability of recombinant human and mouse granulocyte-macrophage colony stimulating factors (after its gene had been expressed in *E. coli*). The proteins were demonstrated to be physically homogeneous monomers with compact globular shapes, findings consistent with gel permeation chromatography studies.

RECOMBINANT HUMAN TISSUE FACTOR (TF)

Human tissue factor, a membrane anchored cell-surface protein that initiates coagulation upon tissue damage, is an essential activator for the serine protease Factor VIIa. The gene for the soluble cytoplasmic domain (sTF) of TF has been cloned and expressed in sufficient quantity to enable sTF : VIIa complexes to be assayed and Laue *et al.* (1992b) have used sedimentation velocity analysis to show that sTF, VIIa and the complex are probably highly asymmetric (high frictional ratio) with sTF and VIIa associating end-to-end rather than side-to-side in the complex. This shape could be physiologically significant in terms of perturbation of blood flow (see also Waxman *et al.*, 1992). Another human blood factor, XIIIa, has had its gene expressed in *Sacharomyces cerevisae* in sufficient quantity for a sedimentation equilibrium study (Bishop *et al.*, 1990); see also Rinas *et al.* (1990).

RECOMBINANT HUMAN TUMOUR NECROSIS FACTOR (TNF)

There are two types, TNFα and TNFβ. TNFα is essentially another macrophage factor, since its cellular origin is macrophages, whereas TNFβ is a related cytokine produced by lymphocytes. Arakawa and Yphantis (1987) demonstrated by sedimentation equilibrium analysis using both the short column and long solution column methods that recombinant TNFα (after its gene expression in *E. coli*) exists as trimeric structures in solution with a (z-average) molecular weight of 52 kDa, almost exactly the trimer value and only slightly higher than an earlier relative molecular weight determination by gel filtration, suggesting the trimeric molecule has a compact structure. Schoenfeld *et al.* (1991) obtained a slightly lower result (49 kDa), suggesting

the presence of some dimer in their preparations. They also examined TNFβ, again expressed in *E. coli*, and obtained a result (56 kDa) that was also consistent with a trimeric structure.

Recombinant TNF receptor, and its complexes with TNFα and TNFβ have also been studied (see, e.g., Sreekrishna *et al.*, 1989). Using sedimentation equilibrium, Loetscher *et al.* (1991) found a molecular mass of 25 kDa for the receptor (less than half of the value estimated by gel filtration – probably a reflection of a non-compact structure) and 140 kDa for complexes of the receptor with either TNFα or TNFβ, strongly suggesting a stoichiometry of three receptor molecules binding to each TNFα or TNFβ trimer. Values for the sedimentation coefficients and also estimates for the translational diffusion coefficient from dynamic light scattering suggested an extended conformation for both the receptor and its complexes. In an independent study, Pennica *et al.* (1992) obtained similar results for the molecular weights of the receptor determined by sedimentation equilibrium (25 kDa) and gel filtration, although the molecular weight of the complex was somewhat less (114 kDa). The authors concluded that 2–3 receptor molecules bind to each TNFα (see also Shire, Pennica and Goeddel, 1992).

RECOMBINANT HEAT SHOCK PROTEIN HSP25

The heat shock protein hsp25 (23 kDa), which is associated with Ehrlich ascites carcinoma, has had its gene expressed in *E. coli* in sufficient quantities for detailed hydrodynamic analysis. Behlke *et al.* (1991) have used a combination of sedimentation velocity (sedimentation coefficient determination) and boundary spreading (translational diffusion coefficient) measurements in the ultracentrifuge with electron microscopy to observe the formation of high-order association products. A molecular mass of 750 ± 25 kDa was obtained from the Svedberg equation method, corresponding to a 32 subunit structure. From the frictional ratio, a compact hexagonal type of packing was proposed, consistent with electron microscopy. The resistance of the complex to disruption by detergent was also investigated.

RECOMBINANT HUMAN STEM CELL FACTOR (SCF)

Stem cell factor (SCF), also known as 'mast cell growth factor', acts on primitive hematopoietic progenitor cells of the marrow, and can operate synergistically in conjunction with other factors such as the interleukins Il-1, IL-3, IL-6 and IL-7, granulocyte and granulocyte-macrophage colony stimulating factors and erythropoietin (the latter considered below). In a recent study (Arakawa *et al.*, 1991), sufficient quantities of this recombinant glycoprotein (in both unglycosylated form, after gene expression in *E. coli*, and glycosylated form, as expressed in Chinese hamster ovary cells) have been produced to enable reasonably detailed physicochemical investigations. Both the glycosylated and unglycosylated forms were shown to be dimeric by combining the sedimentation equilibrium molecular weights (53 090 and

36 080 Da, respectively) with estimates for the monomer molecular weights from SDS-PAGE (18 500 and 28 000–35 000 Da, respectively) after allowance for anomalous binding of SDS to the glycosylated form: glycosylation does not appear to hold the key to the self-association process. The finding that SCF exists as a dimer is interesting in that other similar factors are also dimeric, such as the macrophage colony stimulating factor (CSF) and the platelet-derived growth factor (PDGF).

RECOMBINANT HUMAN ERYTHROPOIETIN

This factor is related to the stem cell factor and the gene for it has also been expressed – as a glycoprotein – in Chinese hamster ovary cells in sufficient quantities for physicochemical study (Davis *et al.*, 1987). Conventional long column sedimentation equilibrium was used to obtain a molecular weight (z-average) of 30 400 Da with no evidence for any polydispersity or self-association behaviour. This compares with an amino acid sequence molecular weight of 18 399 Da, suggesting the weight fraction of carbohydrate, like that for stem cell factor, is very high (0.395 ± 0.008). Comparison with the estimated hydrodynamic radius of the protein from gel filtration suggests either high expansion through solvation or considerable asymmetry.

RECOMBINANT CELL ADHESION MOLECULES

Cell adhesion molecules (CAMs) play a crucial role in the development and upkeep of adult tissues of multicellular organisms. A synthetic peptide has been produced, reproducing the Ca^{2+} binding motif of the cell adhesion molecule uvomorulin (Ozawa, Engel and Kemier, 1990). After point mutagenesis, where the first Asp was replaced by a Lys, a loss of Ca^{2+} binding and cell adhesion properties was observed. Sedimentation equilibrium analysis has confirmed that the monomeric state of this peptide is still retained after mutagenesis.

RECOMBINANT INTERFERONS

Natural human interferon γ (IFN-γ) is known to be unstable at low pH, although the nature of this instability, in terms of associative behaviour or other mechanisms, is largely unknown. In an attempt to address this issue, Yphantis and Arawaka (1987), and Arakawa and Hsu (1987) used sedimentation equilibrium in conjunction with circular dichroism to look at the effect of lowering the pH on the state of association and conformation of IFN-γ after its gene had been expressed in sufficient quantities in *E. coli*. After due care about the selection of the rotor speed because of the large sizes of the macromolecular species present in some solvent conditions – notably in the presence of 0.1 M NaCl – sedimentation equilibrium (using Rayleigh optics) yielded a largely unknown state of self-association of human interferon γ, expressed in *E. coli*. Acid-induced unfolding was confirmed by circular dichroism. At low pH (3.5) in the absence of salt, almost complete dissocia-

tion of the protein into monomers was observed; in the presence of salt, large aggregates were present at this pH. At a higher pH (6.9), the 'natural' dimeric form is reproduced; the presence of salt (0.1 M NaCl) does not appear to produce significant amounts of aggregate as observed at pH 3.5. The association state of human and murine interferon γ has also been investigated by Nagata *et al.* (1987).

The stoichiometry of the interaction between interferon γ and its receptor produced in both *E. coli* (unglycosylated) and baculovirus infected insect Sf9 and CHO cells (the latter giving glycosylated receptor molecules), also cloned in *E. coli*, has been studied by Fountoulakis *et al.* (1992). Single species only were observed in sedimentation diagrams from sedimentation velocity if the molecule and its (glycosylated) receptor were in a 1 : 2 molar ratio mixture. Both sedimentation equilibrium and the sedimentation coefficient (combined with the translational diffusion coefficient from dynamic light scattering) were used to obtain estimates of the molecular weight of the complex, and were in general agreement. The presence of glycosylation appeared to be important, with a binding stoichiometry (ligand : receptor) of 1 : 1 for unglycosylated and 1 : 2 for glycosylated receptors. This is another good demonstration of the importance of post-translational processing on the properties of recombinant proteins, and the perils of ignoring this fact.

RECOMBINANT HIRUDIN

Hirudin, a small peptide hormone (7 kDa), is so named because of the organism that produces it naturally (*Hirudo medicinalis*, the leech), and is responsible for the anti-blood coagulation reputation of this organism. Attempts have been made to clone its gene so that quantities can be made available without destroying the world's supply of leeches.

Recombinant hirudin produced in yeast was studied using sedimentation equilibrium analysis by Rowe *et al.* (1989) using Rayleigh optics with a novel data capture system. A value for the (weight average) molecular weight of 7080 ± 200 Da was obtained, almost in exact agreement with the amino-acid sequence molecular weight of 6964 Da, confirming that the recombinant peptide, like the wild-type peptide (Triebel and Walsmann, 1966), does not self-associate under dilute solution conditions. This observation was confirmed over a range of different solvent conditions by Otto and Seckler (1991) from both sedimentation equilibrium and sedimentation coefficient measurements.

PYRUVATE DEHYDROGENASE POINT MUTANTS

One of the most popular 'metabolic pathway' proteins studied by sedimentation analysis is pyruvate dehydrogenase (see, e.g. Gilbert and Gilbert, 1980). Attention has recently focused on the effects of site-directed mutagenesis on the mode of assembly of this multi-enzyme complex. Using sedimentation velocity analysis combined with gel filtration, Schulze *et al.* (1991) investigated the effect of point mutations in the E2p (dihydrolipoyl transacetylase)

component on its binding of the peripheral components E1p and E3, and showed that for example large deletions of the protease-sensitive region of E2p resulted in a total loss of the E1p and E3 binding. In another study (Schulze *et al.*, 1992), *chimeric* E2p components were constructed by exchanging the three domains between E2p from *E. coli* and *Azotobacter vinelandii* at the gene level, and the six chimeric E2p's have been expressed and purified from *E. coli*. Sedimentation coefficient analysis, in conjunction with gel filtration, was again used, this time to study the binding or 'reconstitution' of these chimeric E2p's with E1p and E3. *Escherichia coli* E3 was shown to interact with all the chimeras, whereas *A. vinelandii* only interacted with those chimeras containing its own binding domain. The binding of the E2p's to either the *E. coli* or *A. vinelandii* E1p's also depended strongly on whether or not the catalytic or lipoyl domains were from the same organism or not.

RECOMBINANT NITRITE REDUCTASE

This dimeric enzyme from *Pseudomonas aeruginosa* has been difficult to clone, because of the presence of two different prosthetic groups (haem c and haem d1), which rule out cloning in *E. coli*. The gene for the protein has, however, been successfully cloned in the related *Pseudomonas putida*: the recombinant protein, containing only the chemically bound haem c is water-soluble (unlike the chemically prepared apoprotein), and available in sufficient quantities for characterization (Silverstrini *et al.*, 1992). Based on a comparison of sedimentation coefficients (6.55S native, 6.2S cloned), the authors conclude that this difference is small enough for the cloned enzyme also to be in a dimeric state.

RECOMBINANT PHOSPHOLIPASE

The significance of lipases in membrane-based processes is well recognized. The gene for human phospholipase has been cloned and expressed in sufficient quantities for sedimentation equilibrium analysis (Levin *et al.*, 1992).

RECOMBINANT 4-OXALOCROTONATE TAUTOMERASE

4-Oxalocrotonate tautomerase is a bacterial enzyme and is part of a set of enzymes that oxidatively catabolize toluene, *m*- and *p*-xylene, 3-ethyltoluene and 1,2,4-trimethylybenzene to intermediates in the Krebs cycle. The entire pathway is encoded by the TOL plasmid pWW0 and enables strains of soil bacteria carrying this plasmid to utilize simple aromatic hydrocarbons such as these as their sole sources of carbon and energy.

Chen *et al.* (1992) obtained a molecular mass of 32 000 Da from the sedimentation coefficient and diffusion coefficient for the recombinant enzyme of *Pseudomonas putida* expressed in *E. coli*. Because of the lack of tyrosine and tryptophan, far-uv absorption optics had to be employed to

record solute distributions. This value compares with a value of 37 000 Da obtained by gel filtration for both the cloned and the wild-type enzyme. After comparison with the sequence molecular mass of 6811 Da, a pentameric structure for both the wild-type and cloned enzyme was proposed.

RECOMBINANT AMINOTRANSFERASE

This enzyme enables one pathway of amino acid catabolism, leading to the synthesis of intermediate metabolites that can enter the Krebs cycle. Although the molecular weight of the mammalian enzyme is known (50 kDa) and that it exists as a 'homodimer' (two covalently linked subunits), not much else is known because of insufficient purified material. It has been over-expressed in sufficient quantities in both *Saccharomyces* and *E. coli*. Dietrich, Lorber and Kern (1991) have used a combination of sedimentation velocity (sedimentation coefficient) and boundary spreading (translational diffusion coefficient) in the ultracentrifuge with other techniques (notably gel filtration, SDS and native PAGE) to assay the physical characteristics of the recombinant protein. The results for the molecular weight (110 ± 10 kDa) and frictional ratio (1.8 ± 0.2) of the protein are consistent only with a dimeric protein that is either asymmetric and/or significantly hydrated.

ASPARTATE TRANSCARBAMOYLASE POINT MUTANTS

Aspartate transcarbamoylase (ATCase) is one of the most extensively studied multi-subunit enzymes in terms of ligand binding, conformational change and its allosteric activity (see, e.g. Schachman *et al.*, 1984), and has been the subject of several studies on the effects of point mutagenesis. This 'new tool' has been invaluable, since previously quantitative information on the energetics of the various interchain interactions had been difficult to obtain because of the strength and multiplicity of the subunit contacts stabilizing the quaternary structure of the wild-type enzyme (see, e.g. Eisenstein, Markby and Schachman, 1989). ATCase catalyses the first committed reaction in pyrimidine biosynthesis in *E. coli*, exhibiting classical sigmoidal kinetics and subject to feedback inhibition by CTP and activation by ATP. It consists of 12 subunits, two catalytic trimers and three regulatory dimers linked non-covalently. The complex exists in either of two states: a low-affinity 'R' ('relaxed' or swollen) state or a high-affinity 'T' ('taut') state, affected by CTP and ATP binding. Boundary spreading sedimentation velocity experiments on the wild-type enzyme compared with a missense 'T-state' mutant ATCase554 (in which serine at position 52 in the catalytic chains was replaced by phenylalanine) indicated no significant intermediate conformation states (Werner and Schachman, 1989; see also Werner *et al.*, 1987) in the reaction.

Eisenstein, Markby and Schachman (1989) reported the effects of a number of point mutations in a limited region of the zinc-binding domain of the regulatory subunits. They compared changes in activity with changes in conformation, as manifested by a fractional change in the sedimentation coefficient of the enzyme, in the absence and in the presence of the ATP and

CTP ligands. For example, replacement of an asparagine residue at the interface between a regulatory and a catalytic chain in the opposing catalytic trimer causes a complete loss of the homotropic and heterotropic effects characteristic of wild-type ATCase. This corresponded with observations from the fractional change in sedimentation coefficient upon binding a bisubstrate ligand 'PALA' (viz 'difference sedimentation coefficient' experiments), that this mutant enzyme exists in the R conformation in the absence of active site ligands due to preferential destabilization of the T conformation relative to the R state.

To assist in the detection of any possible global conformation change caused by the binding of the inhibitor CTP, Eisenstein, Markby and Schachman (1990) replaced Lys143 in the regulatory chain Ala, thereby perturbing interactions at the regulatory–catalytic interface and destabilizing the T-state. Difference sedimentation velocity experiments involving measurements of the changes caused by the binding of a bisubstrate analogue demonstrated that the sedimentation coefficient of the mutant enzyme was intermediate between that observed for the T and R states of the wild-type ATCase. Together with equilibrium binding data, these results provide convincing evidence that heterotropic effectors cause an alteration in the gross conformation of ATCase and thereby regulate the activity of the enzyme by perturbing the $T \rightleftharpoons R$ equilibrium, refuting suggestions that only local changes occur on binding of CTP or ATP.

In point mutation studies on the catalytic chain (Newell and Schachman, 1990), a Lys164-Glu, Glu239-Lys or both showed that the mutationally altered enzymes were devoid of both homotropic and heterotropic effects, supporting the view that the allosteric properties of the wild-type enzyme are linked to a ligand-induced alteration in quaternary structure.

Advantage has been taken of crystallographic studies which have pinpointed the nucleotide (i.e. CTP or ATP) binding region on the regulatory chains. Wente and Schachman (1991) have shown that different point mutations at the same positions (Lys-60 and Lys-94) cause 'bewildering' changes in allosteric properties, again using the fractional change in the secimentation coefficient upon binding of the bisubstrate ligand as the conformational probe. Similar studies have explored the effects of replacement of the active site residues Gln231 (Peterson, Burman and Schachman, 1992) and Gln288 in the near-C-terminus α-helical region (Peterson and Schachman, 1992) in the catalytic chains.

RECOMBINANT AND POINT MUTANT NUCLEIC ACID-BINDING PROTEINS

The tools of genetic engineering have themselves been the subject of recombinant technology and point mutagenesis. The HIV-1 transactivating protein HIV-1 Rev (13 kDa) has been produced in *E. coli*. This regulates gene expression by binding to specific regions of viral mRNA. Wingfield *et al.* (1991) used both sedimentation velocity and sedimentation equilibrium to investigate the solution properties of the recombinant protein. Upward curved log concentration versus distance squared plots were observed from

sedimentation equilibrium, consistent with a significant self-association [whole cell weight average ($M_w°$) 56 kDa, with point average molecular weights ranging from 47 000 (cell meniscus) to 74 000 (cell base) at the rotor speed used]. This evidence for a self-association was confirmed by sedimentation velocity with a classical increase in the sedimentation coefficient with concentration.

Chakerian *et al.* (1991) used sedimentation velocity measurements in conjunction with other techniques to look at evidence for a zipper motif in the Lac repressor protein, and in a related study (Chakerian and Mathews, 1991) investigated the effects of point mutations at positions 281 or 282 on the oligomerization domain of the protein. The wild-type protein is a tetramer, with an $s°_{20,w}$ of 7.5S. Depending on the substition, up to a three-fold decrease in $s°_{20,w}$ was observed, indicating this region is essential for oligomerization.

Blondel and Bedouelle (1990) investigated the hydrodynamic properties (sedimentation velocity and gel filtration) of the gene5 protein of phage M13 hybridized with the maltose binding protein of *E. coli* and expressed in the latter. This hybridization route is considered a possible way of producing and purifying proteins like gene5 protein. Both sedimentation velocity and gel filtration are consistent with a monomer–dimer equilibrium for this hybrid.

Wallis *et al.* (1992) have over-produced colicin E9 immunity protein, a bacterial anti-suicide DNase protein, in sufficient quantity for sedimentation velocity and equilibrium analysis. The molecular weight (9.4 kDa) came out close to the sequence value, confirming a monomer. Some concern was expressed about the very high concentration dependence of the (apparent) molecular weight with concentration for a molecule so small. The sedimentation coefficient of 1.14S combined with the translational diffusion coefficient from dynamic light scattering yields a very similar molecular mass.

HIV-1 reverse transcriptase, as considered above, is an αβ heterodimer (M_α=66 kDa, M_β=51kDa). The α and β subunits, known as 'p66' and 'p51', respectively (Becerra *et al.*, 1991), have been expressed individually in *E. coli*, and have been the subject of extensive study by sedimentation equilibrium analysis. Studies in a solvent of moderate ionic strength (0.25 M NaCl) revealed that p66 was in a monomer–dimer equilibrium (K_A 5.1 × 10^4 M^{-1}), whereas p51 was monomeric. Mixing the p66 and p51 resulted in reconstitution of the 1 : 1 heterodimer with K_A 4.9 × 10^5 M^{-1}. This binding was resistant to a high salt concentration (1 M NaCl), suggesting a strong hydrophobic interaction, whereas a 20 kDa C-terminal truncation of p51 eliminated all complex formation with p66 and a peptide corresponding to the C-terminal 15 kDa of p66 was not able to bind to p66, suggesting that the central region of RT may contain sequences required for the dimerization process.

Point mutagenesis has been used to explore the tetramerization and function of the single-stranded DNA binding protein, EcoSSB of *E. coli* (Curth *et al*, 1991). In the native state, EcoSSB exists as a tetramer. Amino acid 55 (His) in the polypeptide chain was thought to play a key role: mutants were produced with tyr, glu, lys, phe and ile in this position. A combination of sedimentation velocity (concentration dependence of the sedimentation

coefficient), NMR, gel filtration and fluorimetric detection methods revealed that whereas the phe and ile substitutions did not change the properties of the protein appreciably, the others caused dissociation and loss of affinity for poly(dT).

The class II restriction endonuclease EcoRI has also been the subject of point mutagenesis studies (Geiger *et al.*, 1989). Sedimentation velocity, coupled with gel filtration, isoelectric focusing and circular dichroism studies have shown an altered monomer/dimer equilibrium for the Gln44Lys145 and Gln144Lys145Lys200 mutants.

RECOMBINANT POST-TRANSLATIONAL PROTEASES

Post-translational processing has been the thorn in the flesh for recombinant technology, until this was at least partially circumvented by the use of eukaryotic hosts, such as Chinese hamster ovary cells. One such post-translational processing protein is the MAS gene-encoded processing protease of yeast mitochondria, which cleaves amino-terminal presequences of proteins imported from the cytoplasm across the mitochondrial inner membrane. This has two subunits, the MAS1 gene encoded subunit and the MAS2 gene encoded subunit. These have themselves been the subject of over-production experiments and the MAS2 gene encoded subunit has been produced in sufficient quantities for sedimentation analysis (Geli *et al.*, 1990). Sedimentation velocity and sedimentation equilibrium experiments have shown that the isolated MAS2 subunit is a monomer, with a molecular weight (52 kDa) close to that predicted by the gene sequence, but with the presence (20–30%) of some larger species of molecular weights 90 kDa and 254 kDa.

Glycosylation is one of the most crucial forms of post-translational processing; among many other things, carbohydrate moieties are the key elements of molecular recognition phenomena. One of the key sugars is *N*-acetylneuraminic acid (NANA). Zapata *et al.* (1989) have cloned and expressed the K1 CMO *N*-acetylneuraminic acid synthetase (49 kDa) gene from *E. coli* and shown by sedimentation equilibrium analysis and gel filtration that the enzyme is active as a monomer, although it may form aggregates.

RECOMBINANT ANTENNAPEDIA HOMEODOMAIN

Homeotic genes and several other genes controlling development share a characteristic DNA segment, the 'homeobox'. Müller *et al.* (1988) have expressed the Antennapedia gene of *Drosophila* in *E. coli* and analysed the properties of the protein using sedimentation equilibrium and velocity analyses. Under reducing conditions, the protein behaves as a monomer, with a sedimentation equilibrium weight average molecular weight (9.04 ± 0.34 kDa) close to the sequence value (8.545 kDa). By combining this with the sedimentation coefficient (1.0 ± 0.1S), a globular structure was inferred, from the axial ratio (1.4–1.8) of the equivalent prolate ellipsoid,

assuming 'typical' hydration. In the absence of reducing conditions, the protein showed a tendency to dimerize.

RECOMBINANT LAMIN

Chicken lamin B2, a structural filament protein related to myosin, has been expressed in *E. coli*. The structure and assembly properties of the recombinant protein were examined by a combination of electron microscopy and sedimentation velocity and equilibrium ultracentrifugation (Heitlinger *et al.*, 1991). This protein was shown to form myosin-like 3.1S dimers, which associate longitudinally to form polar head-to-tail polymers.

RECOMBINANT N-TERMINAL GLOBULE OF COLLAGEN VI

The size and shape of a recombinant collagen fragment, N9–N2, has been studied using sedimentation equilibrium and velocity analyses by Specks *et al.* (1992). A molar mass from sedimentation equilibrium of 180 ± 5 kDa was inferred, consistent with an octameric structure. From the frictional ratio, a high degree of asymmetry was inferred (axial ratio 9, assuming a prolate ellipsoid model and 'typical' hydration), consistent with electron microscopy.

RECOMBINANT ADENOVIRUS PROTEIN IIIa

The gene for this has been cloned and expressed in *E. coli*, in both wild-type and ts112 mutant forms (Cuillel *et al.*, 1989). Both proteins have been shown by sedimentation velocity analysis (in sucrose density gradients) to be monomeric. Both the hydrodynamic and electron microscopic data were consistent with a cylindrical conformation of 20 nm length and 2.8 nm diameter.

VIRAL COAT PROTEIN MUTANTS

Shire *et al.* (1990) have looked at the self-assembly properties of recombinant DNA derived tobacco mosaic virus protein, expressed in *E. coli*. The recombinant protein lacks an acetyl group on the amino terminus of the polypeptide chain, so by comparison with earlier results of self-assembly of wild-type protein, important conclusions about the role of amino terminal acetylation in the self-assembly process could be inferred. Sedimentation velocity and equilibrium analysis was used in conjunction with electron microscopy and circular dichroism to demonstrate a reversible endothermic self-association, although the detail of the assembly process was different from the wild-type. For example, in contrast to the wild-type assembly process, the recombinant assemblies contain some unpolymerized disk-like structures, which suggest either two structures for the 28S aggregates (not seen in the wild-type), two parallel polymerization pathways or both. The effect of the extra charge through acetylation was thus shown to have important consequences for quaternary structure, even though this end of the

protein chain is on the outside of the protein in the assembly and in the virus.

Molina-Garcia *et al.* (1992) used sedimentation velocity and equilibrium analyses to investigate the effect of coat protein point mutations on the self-assembly of bacteriophage fd. Significant increases in the molecular weight of the virus (from 15 000 to 20 000 kDa) were observed by the substitution of the lysine residue in position 48 with either glutamine or alanine. Combining with the measured sedimentation coefficients, these results imply an increase in length of the virus from 900 to 1400 nm, consistent with observations from electron microscopy.

Engineered polysaccharides

Genetic engineering – recombinant technology or mutagenesis – produces directly engineered proteins, and also engineered glycoproteins so long as an appropriate host organism is used. The technology is also having an impact on the synthesis and properties of polysaccharides, although the only example I could find where ultracentrifuge analyses are involved was one involving ourselves at Nottingham.

Pectins (polyuronides), with molar masses from a few thousand to several million, are an important constituent of the cell walls of fruits, and are used extensively by the food industry as thickening and gelling agents. In tomatoes, the softening of the tomato during the green–red–rotten ripening process is associated with a progressive degradation of the cell wall pectins: a comparative sedimentation equilibrium and gel filtration study on green and red tomato pectins has confirmed this (Seymour and Harding, 1986). One of the key enzymes responsible for the degradation of cell wall pectins is polygalacturonase. Smith *et al.* (1990) have produced novel mutant tomato lines in which expression of the polygalacturonase gene has been downregulated by antisense RNA: this antisense gene was inherited stably after two generations. Molar masses (apparent values at a single low concentration) of pectins from red tomatoes – which had either 20% (GR95 progeny) or 1% (GR105 progeny) residual polygalacturonase activity – were measured by sedimentation equilibrium and compared with values for green and wild-type red tomato pectins. Samples from green tomatoes of antisense and wild-type plants had a similar apparent M_w of 158 kDa. As ripening progressed, pectin breakdown occurred, resulting in an increase in the amount but decrease in the size of soluble polyuronide fragments. In normal fruit, M_w had dropped to 80 kDa by 14 days, whereas it was 95 kDa for GR95 and as high as 135 kDa for GR105. These results demonstrate that while *in vivo* solubilization of pectin was not affected by substantially reduced polygalacturonase levels, depolymerization of the pectin was largely prevented.

The engineering of polysaccharides is clearly an untapped gold mine – and not just for the ultracentrifuge.

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