Laser Light Scattering and Ultracentrifuge Studies on Sheep Liver Cytosolic Aldehyde Dehydrogenase

A thesis presented in partial fulfilment
of the requirements for the degree of

Master of Science in Chemistry

at

Massey University
New Zealand

Jacqueline Jean Harvey
1995

Abstract

The techniques of laser light scattering and ultracentrifugation were used to investigate the association - dissociation behaviour of sheep liver cytosolic aldehyde dehydrogenase. Diffusion and sedimentation coefficients were obtained by these techniques.

The enzyme was studied at pH 7.4, a pH at which the enzyme was in an active, associated form, and also at pH 5.2 where the enzyme was thought to be in an inactive, dissociated form (Buckley *et al.*, 1991).

Whilst the gel chromatography results reported in this thesis agreed with those observed by Buckley *et al.* (1991), laser light scattering and ultracentrifuge results displayed no sign of any dissociation taking place. These results led to the proposition of the existence of a predissociated, inactive state of the enzyme. It was thought that this state was able to be converted back to the associated, active form of the enzyme through use of known methods for preventing dissociation and promoting association and activation of the inactive enzyme, but that this state could also dissociate into a smaller species.

Laser light scattering studies were also performed on the enzyme in the presence of Mg^{2+} or propanal, since these were known to promote association of the enzyme in some instances, as well as inhibit it in other cases (Buckley *et al.*, 1991). It was found that the addition of Mg^{2+} had no significant effect on the diffusion coefficient of the enzyme, but that the presence of propanal at pH 7.4 promoted large-scale aggregation of the enzyme, whilst having little effect at pH 5.2.

Acknowledgements

I wish to thank my supervisors, Assoc. Prof. Paul Buckley and Assoc. Prof. Neil Pinder, for their unfailing interest, encouragement and advice.

I would also like to thank Dr. Jim Lewis for his invaluable help and interest with the ultracentrifuge studies, Dr. Kathy Kitson for use of the affinity column and general help and advice and Mr. Dick Poll for the gel chromatography. Thanks also to other staff and graduate students who gave advice and interest.

I thank, of course, all those who provided me with support and encouragement throughout this thesis, especially my mother and Ron.

Table of Contents

Chapter 1
Introduction

Chapter 2
Aldehyde Dehydrogenase - Preparation, Storage and Gel Chromatography

Chapter 3
Ultracentrifuge Studies on Aldehyde Dehydrogenase

Chapter 4
Laser Light Scattering Studies on Aldehyde Dehydrogenase

Chapter 5
Laser Light Scattering Studies on Aldehyde Dehydrogenase at Elevated Temperatures

Chapter 6
Summary

Appendix 1
Ultracentrifuge Data

Appendix 2
Laser Light Scattering

Appendix 3
Data from Laser Light Scattering Studies on Aldehyde Dehydrogenase at Elevated Temperatures

List of Figures

List of Tables

Chapter 1
Introduction

1.1 Introduction to Aldehyde Dehydrogenase

1.1.1 Introduction to Aldehyde Dehydrogenase

The main role of aldehyde dehydrogenase is to catalyse the oxidation of acetaldehyde in the liver to form acetate. Acetaldehyde is a product of the metabolism of ethanol by alcohol dehydrogenase, and an excess of acetaldehyde in the body can lead to unpleasant symptoms similar to those of a hangover. Whilst there are several enzymes involved in the oxidation of acetaldehyde in the body, aldehyde dehydrogenase has been shown to have a much higher affinity for aldehydes (Feldman and Weiner, 1972, Crow *et al.*, 1974) and is thus considered to be the main enzyme involved in the metabolism of acetaldehyde in mammals.

1.1.2 History of Isolation of Aldehyde Dehydrogenase

Aldehyde dehydrogenase was first isolated by Racker (1949) from bovine liver. Aldehyde dehydrogenases from yeast (Steinman and Jakoby, 1967) and pseudomonas aeruginosa (Tigerstrom and Razzell, 1968) were isolated and purified in 1967 and 1968, but these two non-mammalian aldehyde dehydrogenases exhibit significantly different properties to those found in mammals. The first mammalian aldehyde dehydrogenase to be purified to homogeneity was that from horse liver, by Feldman and Weiner (1972). Aldehyde dehydrogenase from sheep liver was isolated and purified by Crow *et al.* (1974).

1.1.3 Sources of Mammalian Aldehyde Dehydrogenases

Apart from those obtained from non-mammalian sources, aldehyde dehydrogenases have been found in a variety of sites in the bodies of mammals, reflecting their role in the oxidation of aldehydes arising from other metabolic processes as well as those due to alcohol consumption. This distribution includes the liver, kidneys, adrenal glands, intestine, heart, lungs and brain (Dietrich, 1966) as well as other locations. Aldehyde dehydrogenase has been obtained and purified from bovine and monkey brains (Erwin and Dietrich, 1966) as well as from the stomach (Eckey *et al.*, 1990) and the cornea (Lindahl *et al.*, 1978). The main source of aldehyde dehydrogenase is the liver since this is the main site of ethanol oxidation in the body, and it has been isolated from bovine (Racker, 1949), horse (Feldman and Weiner, 1972), human (Kraemer and Dietrich, 1968, Greenfield and Pietruszko, 1977), rat (Shum and Blair, 1972), and sheep livers (Crow *et al.*, 1974) for example. Various isoenzymes of aldehyde dehydrogenase have

been found in mammalian livers, the most significant being those from the cytoplasm and the mitochondria, although a microsomal form has also been detected (Crow *et al.*, 1974).

Aldehyde dehydrogenases are present predominantly in the mitochondrial and cytosolic compartments of the cell in humans, sheep, horses and cattle. In human liver, aldehyde dehydrogenases are distributed approximately equally between mitochondria and cytosol. The brain and kidneys also exhibit aldehyde dehydrogenase activity and the subcellular distribution and characteristics of the isozymes are generally similar to those of the liver. In other tissues, such as the cornea, lung, stomach and urinary bladder, the majority of the aldehyde dehydrogenase activity is found in the cytosol, to the extent that in the cornea, more than 90% of the total aldehyde dehydrogenase activity is cytosolic (Lindahl, 1992).

1.1.4 Classes of Aldehyde Dehydrogenases

There are three major classes of mammalian aldehyde dehydrogenases based on primary sequence analysis. Classes 1 and 3 contain cytosolic aldehyde dehydrogenases, both constitutively expressed and inducible forms, whilst class 2 consists of constitutive mitochondrial enzymes. The non-mammalian aldehyde dehydrogenases, such as those from spinach or yeast, share some positional identity with the three classes but vary in the degree of relativity to them (Lindahl, 1992, Lindahl and Hempel, 1990). Sheep liver cytosolic aldehyde dehydrogenase belongs to class 1.

1.1.5 Mechanism of Sheep Liver Cytosolic Aldehyde Dehydrogenase

The mechanism below, a compulsory order mechanism with NAD^+ as the leading substrate (Scheme 1), is generally agreed to be that which the sheep liver cytosolic aldehyde dehydrogenase oxidation of aldehydes follows (Hill *et al.*, 1991, Buckley *et al.*, 1991). *E.NADH represents a conformationally rearranged form of the enzyme which must isomerise before NADH can be released from its binding site (Hill *et al.*, 1991).

$$E + NAD^+ \rightleftharpoons E.NAD^+$$

$$E.NAD^+ + \text{aldehyde} \rightleftharpoons E.NAD^+.\text{aldehyde}$$

$$E.NAD^+.\text{aldehyde} \rightleftharpoons E.NADH.\text{acyl}$$

$$E.NADH.\text{acyl} \rightarrow {}^*E.NADH + \text{acid}$$

$$^*E.NADH \rightleftharpoons E.NADH \rightleftharpoons E + NADH$$

1.1.6 Esterase Behaviour of Aldehyde Dehydrogenase

Aldehyde dehydrogenase also catalyses the hydrolysis of esters, as well as the dehydrogenation of aldehydes, via a covalent intermediate. There is disagreement as to

whether the ester and aldehyde substrates interact with aldehyde dehydrogenase at the same active site or not (Blackwell *et al.*, 1983, Duncan, 1985, Loomes and Kitson, 1986, Motion *et al.*, 1988) and whether the aldehyde dehydrogenase mechanism involves acylation of a different group from that involved in ester hydrolysis. Blackwell *et al.* (1983) proposed separate binding domains for aldehydes and esters. Tu and Weiner (1988a,b) proposed that the active site for the dehydrogenase reaction was cysteine-49 and that ester hydrolysis occurred at cysteine-162, and that inhibition of esterase activity was caused by modification of cysteine-162 and not by modification of cysteine-49. Duncan (1985) and Loomes and Kitson (1986) proposed that both aldehyde dehydrogenation and ester hydrolysis occur at the same site and involve the intermediacy of a common acyl-enzyme. Dickinson and Haywood (1986), through experiments in the presence and absence of Mg^{2+} also support the view that esterase and dehydrogenase activities occur at the same site. Loomes *et al.* (1990) after various labelling experiments, proposed that serine-74 was the catalytic residue for aldehyde dehydrogenation, and not a cysteine. Kitson *et al.* (1991) identified cysteine-302 as the essential enzymic nucleophile involved in the esterase activity of sheep liver cytosolic aldehyde dehydrogenase. They argue that esterase and dehydrogenase reactions are mediated by the same catalytic nucleophile and proposed cysteine-302 to be that group, based on modification studies and that cysteine-302 is the only cysteine residue that is conserved in all aldehyde dehydrogenases that have been sequenced to date. Blatter *et al.* (1992) also support the single-site model and used substrates for human liver aldehyde dehydrogenase (both aldehyde and amide, since the enzyme hydrolyses amides as well as esters) which formed chromophoric covalent intermediates. After labelling experiments, they specifically labelled cysteine-302 and found that the covalent intermediate was not formed from either the aldehyde or amide substrates. From this, and the conservation of the cysteine-302 residue, they also proposed that cysteine-302 was the residue that formed a covalent intermediate with both aldehyde and ester substrates.

1.1.7 Mg^{2+} and Aldehyde Dehydrogenase

The presence of Mg^{2+} ions may have an activating or inhibiting effect on sheep liver cytosolic aldehyde dehydrogenase, depending on pH and propanal concentrations (Buckley *et al.*, 1991). Weiner and Takahashi (1981) found that Mg^{2+} ions activated the mitochondrial but inhibited the cytosolic form of rat and beef liver aldehyde dehydrogenases. Takahashi and Weiner (1980) found that Mg^{2+} ions enhanced the activity of mitochondrial horse liver aldehyde dehydrogenase at pH 7.5 by a factor of 2 and proposed that the activation of the enzyme by Mg^{2+} was associated with a change in the number of functioning subunits and not with an alteration in the catalytic property of any existing active site, that the tetrameric enzyme functioned with half-of-sites reactivity and then underwent alteration to a dimeric form that functioned with all-of-sites activity. They also found that the apparent molecular weight of the enzyme decreased with

increasing Mg^{2+} concentration until it reached a limiting value of half the original, tetrameric molecular weight, suggesting that the tetrameric enzyme dissociated into dimers upon Mg^{2+} addition. Takahashi *et al.* (1981) found that the tetrameric enzyme also dissociated into the more active dimeric form with increasing pH in the absence of Mg^{2+} ions.

Sheep liver cytosolic aldehyde dehydrogenase is strongly inhibited by low concentrations of Mg^{2+}, Ca^{2+}, and Mn^{2+} ions, but some activity, 8 - 15 %, remains even at high concentrations of these ions and the addition of excess EDTA reversed these inhibition effects (Dickinson and Hart, 1982). Evidence for the interaction of Mg^{2+} with NADH complexes of the enzyme was provided by fluorescence-titration and stopped-flow experiments and whilst the low, micromolar, concentrations of Mg^{2+} that gave half-maximal effect at pH 7.5 were not enough to affect the esterase activity of the enzyme, high Mg^{2+} concentrations appeared to activate it (Dickinson and Hart, 1982). Dickinson and Haywood (1986) found that deacylation of the acyl-enzyme was the rate limiting step and was accelerated selectively by the presence of NADH or NADH and Mg^{2+}. They argued that bound Mg^{2+} accelerates the acyl-enzyme hydrolysis and found that at pH 5.1, the inclusion of millimolar amounts of Mg^{2+} accelerated the dehydrogenase activity as opposed to the 85 % inhibition observed at pH 7.0, and proposed that this was due to the acyl-enzyme hydrolysis being an important rate-limiting step at pH 5.1.

Buckley *et al.* (1991) found that when the enzyme was in the associated form, induced by propanal at pH 5.22, Mg^{2+} addition inhibited the enzyme by up to 85 % through a slowing of the rate-determining release of NADH, but when the propanal concentration was too low to cause significant association of the enzyme, addition of Mg^{2+} activated the enzyme by up to 50 % by causing it to associate.

1.1.8 Predilution, Propanal and Aldehyde Dehydrogenase

Predilution of the enzyme in the absence of propanal and NAD gave lower oxidation rates, indicating that dilution inactivated the enzyme (Buckley *et al.*, 1991, Blackwell *et al.*, 1987). Blackwell *et al.* (1987) found that at pH 7.6, sheep liver aldehyde dehydrogenase that was prediluted and left to stand for some time gave lower rates from assays than stock enzyme did. The presence of NAD in the prediluted samples did not give any protection from the dilution-time effect, thought to be caused by the inactivation of the enzyme, and a halving of the active-site concentration (Blackwell *et al.*, 1987).

Gel column results (Buckley *et al.*, 1991) showed that, even at pH 7.4, a concentrated sample of enzyme (44 μM) consisted of a major peak,presumed to be the active species, and a minor amount of a dissociated species which was presumed to be inactive. At 10-fold dilution of this sample, they found a significantly greater amount of the inactive species and a correspondingly decreased amount of the active species. At pH 5.0, they

found that the amount of the inactive, dissociated species had increased markedly compared to that at pH 7.4 for both the 44 μM and the 4.4 μM enzyme samples, again at the expense of the active species. Thus they proposed that dilution of the enzyme promoted dissociation into an inactive species, and this behaviour was more marked at pH 5.0 than at pH 7.4. Buckley *et al.*, (1991) also found that the ionic strength of the buffers affected the enzyme's behaviour. They found that lower ionic strength had a greater inactivating effect on the enzyme at pH 7.4 than at pH 5.22 and that the presence of propanal in the prediluted sample prevented the dissociation caused by predilution. Thus there are several, factors that affect the functional concentration of the enzyme in assays, including predilution, predilution time, pH, ionic strength, substrate presence or absence, and enzyme concentration (Buckley *et al.*, 1991).

The presence of the substrate propanal may promote association and thus activation of the enzyme when the enzyme is present in low concentration which would otherwise allow dissociation and inactivation of the enzyme sample. Hart and Dickinson (1982) found that for sheep liver cytosolic aldehyde dehydrogenase at pH 7.0, high (500 μM) concentrations of NAD^+ and low concentrations of propanal gave linear reciprocal plots, but for high propanal concentrations (greater than 50 μM), activation (about 3-fold) of the enzyme took place. At low NAD^+ concentrations, a high concentration of propanal produced substrate inhibition. They also observed that preincubation of the enzyme with NAD^+ activated the enzyme in the pre-steady state but that premixing of the enzyme with aldehyde had no effect.

Hart and Dickinson (1982) suggested that the activation by high aldehyde concentration at high NAD^+ concentrations was due to participation of an alternate route of product release which involved the formation of an abortive complex containing the substrate aldehyde, as opposed to an ordered mechanism with NAD^+ being the first substrate to bind as suggested by the linear plots at low aldehyde concentrations.

Blackwell *et al.* (1987) proposed that the active site concentration of sheep liver cytosolic aldehyde dehydrogenase was halved when the enzyme, at pH 7.6, was prediluted to a low concentration (1 μM) before the addition of NAD^+ and substrate. They also found that if the enzyme was diluted with NAD^+ instead of buffer, the presence of NAD^+ did not confer any protection against the dilution time effect.

Low enzyme and low propanal (171 μM) concentrations gave a linear plot of V_{max} versus enzyme concentration, but with a slope that corresponded to the k_{cat} value of approximately half that obtained from the linear plot at higher enzyme concentrations. A change was observed in the slope at propanal concentration of 17 mM, where substrate activation occurred. Blackwell *et al.* (1987) proposed that the rate of the E.NADH isomerisation step controlled the steady-state rate of oxidation at pH 7.6.

Buckley *et al.* (1991) found that inactivation by predilution of sheep liver cytosolic aldehyde dehydrogenase occurred at pH 5.0, as a plot of propanal oxidation rate versus enzyme concentration was linear at high enzyme concentration but was non-linear at low enzyme concentrations of less than 0.3 μM. They observed that the Lineweaver-Burke

plot for the oxidation of propanal by the enzyme at pH 5.22 was curved at low propanal concentration but linear at high propanal concentration, as opposed to plots at pH 7.0 and pH 7.6. Buckley *et al.* (1991) proposed that this non-linearity at low propanal concentrations was because the levels of propanal were insufficient to prevent the dissociation of the inactive form of the enzyme. Their determination of k_{cat} supported the proposal that the release of NADH from the enzyme is rate limiting and that substrate activation of NADH release from the enzyme does not occur at low pH.

1.2 Aldehyde Dehydrogenases and the Ultracentrifuge

1.2.1 Introduction

The analytical ultracentrifuge is a technique used for the determination of the sedimentation and diffusion coefficients, the molecular weight and other properties of a species. It has been used to determine various properties of species, including aldehyde dehydrogenases, although determination of molecular weight by gel filtration is also a method used. The technique also provides a check for the purity or homogeneity of the sample being centrifuged. The main use of the analytical ultracentrifuge with respect to aldehyde dehydrogenase has been confirmation of homogeneity and determination of the molecular weight via sedimentation equilibrium experiments, but samples of the enzyme have not always been spun at high speeds during these studies.

1.2.2 Ultracentrifuge Studies on Aldehyde Dehydrogenase from Yeast

Yeast aldehyde dehydrogenase was purified to a state of homogeneity, by Steinman and Jakoby (1967), as determined by a single symmetrical peak obtained from the ultracentrifuge. At a rotor speed of 60000 rpm they obtained a sedimentation constant for the enzyme that was independent of the protein concentrations used, those of 1.7 - 5.0 mg/mL. The average value of the sedimentation constant was 9.65 S. They obtained the diffusion coefficient for yeast aldehyde dehydrogenase at 5000 rpm, and, corrected to water at 25 °C, they determined the value to be 4.407×10^{-11} m^2s^{-1}. From these values and an assumed partial specific volume of 0.73 mL/g, Steinman and Jakoby (1967) calculated the molecular weight of yeast aldehyde dehydrogenase to be 200000.

1.2.3 Ultracentrifuge Studies on Aldehyde Dehydrogenase from Pseudomonas Aeruginosa

Aldehyde dehydrogenase from pseudomonas aeruginosa has also been studied in the ultracentrifuge and the sedimentation and diffusion coefficients of the enzyme and its molecular weight were obtained, as well as the observation of the sedimentation behaviour of the dissociated and reassociated enzyme (Von Tigerstrom and Razzell, 1968).

A sedimentation coefficient of 9.4 S was obtained for the aldehyde dehydrogenase at 59780 rpm and only a single peak was observed (Von Tigerstrom and Razzell, 1968). The diffusion coefficient was found to be 4.4 x 10^{-11} m^2s^{-1} after correction to 20 °C and water. From this and an assumed value for the partial specific volume they calculated a molecular weight of 191000. Using other sedimentation coefficient values of 9.0 S and 9.2 S and the same diffusion coefficient, they calculated an average molecular weight of 187000 ± 4000 for aldehyde dehydrogenase from pseudomonas aeruginosa (Von Tigerstrom and Razzell, 1968).

They also observed that although aldehyde dehydrogenase in 0.1 M potassium phosphate buffer at pH 7.0 showed only one component in the sedimentation pattern obtained from the ultracentrifuge, dialysis against a solution of low salt concentration, pH 7.2, deactivated the enzyme with an accompanying change in the sedimentation pattern observed in the ultracentrifuge. Whilst this change in the sedimentation coefficient might have been due to dissociation or unfolding of the enzyme, starch gel electrophoresis confirmed that dissociation had taken place. A partially dissociated sample was observed to have two major components with sedimentation coefficients of 7.1 S and 5.0 S and a minor component with a value of 2.8 S. They reassociated and reactivated the enzyme through addition of potassium phosphate and dithiothreitol and in the ultracentrifuge they then observed one major component with a sedimentation coefficient of 9.0 S and a minor component with a coefficient of 5.5 S. Both samples were incubated at 30 °C for 1 hour prior to centrifugation (Von Tigerstrom and Razzell, 1968).

Aldehyde dehydrogenase, 5.0 mg/mL, at pH 3.0 gave a single homogeneous peak when spun at 59780 rpm in the ultracentrifuge with a sedimentation coefficient of 2.0 S. From this and further experiments, they calculated the diffusion coefficient to be 2.72 x 10^{-11} m^2s^{-1} and the molecular weight to be 95000, approximately half that of the enzyme at pH 7.0. They found that the low sedimentation coefficient was accompanied by a very high viscosity and a relatively low diffusion coefficient, as compared to the values obtained for the enzyme at pH 7.0, and proposed that this indicated that extensive unfolding of the molecule from its assumed globular shape had taken place. They proposed that the molecular weight value for this material might represent the unfolded subunits which were observed in the low ionic strength buffer (Von Tigerstrom and Razzell, 1968).

Dissociation and reassociation as well as unfolding of the enzyme were able to be observed through use of the ultracentrifuge.

1.2.4 Ultracentrifuge Studies on Aldehyde Dehydrogenase from Horse Liver

Sedimentation equilibrium studies have been performed on horse liver aldehyde dehydrogenase using an ultracentrifuge by Feldman and Weiner (1972). They used samples with protein concentrations in the range 0.14 to 0.24 mg/mL, and sedimentation equilibrium data were obtained at 6800 rpm over several days. In this case, the data was obtained via voltage determinations which were proportional to the optical density of the protein, and a computer program calculated the relative optical density and distance from

the centre of rotation of each data point so that values of ln of the optical density as a function of the square of the distance were obtained and plotted. The linearity of this data was an indication of the homogeneity of the enzyme and an average molecular weight of 264000 was calculated from the sedimentation equilibrium data.

1.2.5 Ultracentrifuge Studies on Aldehyde Dehydrogenase from Sheep Liver Mitochondria

Hart and Dickinson (1977) performed sedimentation equilibrium studies on sheep liver mitochondrial aldehyde dehydrogenase at 9000 rpm and an initial enzyme concentration of 0.5 mg/mL. They calculated protein concentrations from absorbance measurements at 280 nm and their plot of log concentration versus the square of the radius exhibited linearity, providing confirmation of the homogeneity of the sample. The slope of the plot and an estimated value of the partial specific volume were used to calculate the molecular weight of the enzyme to be 198000. They also confirmed the homogeneity via gel filtration which gave a value of 190000 for the molecular weight.

Studies on aldehyde dehydrogenase with the ultracentrifuge have been mainly confined to sedimentation equilibrium studies and confirmation of homogeneity. The use of the ultracentrifuge in determining diffusion coefficients does not seem to have been explored to its full potential with regard to aldehyde dehydrogenase.

1.3 Laser Light Scattering and Enzymes

1.3.1 Introduction

Laser light scattering is a technique that has been used in the determination of the diffusion coefficients and molecular weights of polymers and enzymes. The hydrodynamic radius of the particle under observation is obtained from its diffusion coefficient via the Stokes-Einstein equation. The uses of dynamic laser light scattering with regard to proteins is varied and includes investigations into the native and denatured states of enzymes, the effect of metal ions on protein structure and the aggregation behaviour. Some examples of these various applications are quoted below.

1.3.2 Laser Light Scattering Studies on Native and Denatured States of Enzymes

Gast *et al.* (1992) used the technique of laser light scattering, in conjunction with X-ray scattering to investigate the conformational states of some proteins. In particular they studied the compactness of the native and denatured states of lysozyme, streptokinase, human alpha-lactalbumin and apo-cytochrome c. They investigated the change in the hydrodynamic radii of these proteins under various denaturing conditions, such as acidic pH, guanidine hydrochloride and thermal denaturation, and whether the changes that were induced under these conditions were reversible. They also observed the molten

globule state of human alpha-lactalbumin, a specific intermediate state on the non-random pathway of protein folding, in an attempt to lead to an increased understanding of the pathways of protein folding.
Others (Nicoli and Benedek, 1976, McDonnell and Jamieson, 1976, Nemoto *et al.*, 1993) have also used light scattering to characterise, and examine the behaviour of, native and denatured proteins and their aggregates, and the differences between the two states.

1.3.3 Laser Light Scattering Studies on Aggregates of Proteins

Horne (1992) examined concentrated casein micelle suspensions via dynamic light scattering. Casein micelles are aggregates of the casein family of phospho-proteins with calcium phosphate, and are found in skim milk, which is densely white, despite the removal of fat , the droplets of which scatter light and give the milk its white colour. The whiteness of skim milk, indicates that other scattering particles are present and are of smaller size than the average scattering particles in whole milk. Dynamic light scattering was used in determining the size of these micelles, and in investigating the gelation of casein micelles, induced by acidification, proteolysis with chymosin or a combination of the two. The technique was thought to have possible uses with milk systems where there was uncertainty over the intrinsic stability of casein micelles to dilution which had cast doubt over conventional quasi-elastic light scattering measurements.
Rarity *et al.* (1992) have studied the aggregation of a variety of antibodies in the presence of their respective antigens, in order to gather more evidence for a common fractal dimension for aggregates formed in antibody-antigen aggregation.

1.3.4 Laser Light Scattering Studies on Concentration Effects

Harper *et al.* (1985) investigated the concentration dependence of proteoglycan diffusion and found that whilst the mutual diffusion coefficient of the bovine nasal cartilage proteoglycan subunit, obtained from the analytical ultracentrifuge, increased rapidly with increasing concentration and decreasing ionic strength, the apparent diffusion coefficient, obtained by dynamic light scattering, was found to decrease with increasing concentration. They suggested that the reason for this was that there were two populations of proteoglycan in proteoglycan subunits preparations. The major fraction present (>95%) consisted of the proteoglycan subunit, whilst the minor fraction consisted of an aggregate form of the proteoglycan subunit. The major fraction was monitored in the ultracentrifuge and gave rise to an increasing mutual diffusion coefficient with concentration under physiological conditions, whereas the minor fraction, as an aggregate of the proteoglycan subunit would markedly influence the scattering intensity in the dynamic light scattering method which is biased towards large particles.

1.3.5 Laser Light Scattering Studies on Proteins of Various Shapes

Fujime *et al.* (1992) investigated the hydrodynamic behaviour of synthetic myosin

filaments of rabbit skeletal muscle, since, for suspensions of long filaments, laser light scattering provided information about translational, rotational, and bending motions of the filament. They found the translational diffusion coefficient of the myosin filaments at various ionic strengths and expect laser light scattering to provide information complementary to that obtainable from other sources.
Jamieson *et al.* (1992) also studied proteoglycan subunit and aggregate through dynamic light scattering and found that they behaved hydrodynamically like impermeable ellipsoids of uniform segmental density. They also studied human tracheobronchial mucins and found that results indicated that their configuration is a linear, semi-flexible chain.

1.3.6 Laser Light Scattering Studies on the Effect of Metal Ions on a Protein

Varma *et al.* (1990) studied the effects of Calcium ions on the solution properties of porcine submaxillary mucin by dynamic light scattering. Their results suggested that the conformation of the mucin was more compact in a solution containing calcium chloride than in solutions of sodium chloride or guanidine hydrochloride. They found these results to be in agreement with the compact packaging of mucin in the secretory granules of mucin-secreting cells which were known to have calcium ions present in high concentrations.

1.3.7 Laser Light Scattering Studies on Aggregation Properties

Kadima *et al.* (1993) studied the aggregation properties of zinc-free insulin using both dynamic and static laser light scattering. They investigated the aggregation as a function of ionic strength, pH and insulin concentration and determined the hydrodynamic radii and weight-averaged molar mass of the various aggregates. They found that insulin varied from a monomer at pH 10 and low salt and insulin concentration to the hexamer at pH 7.5 and high salt and insulin concentration. They suggest that the agreement between theory and experiments for the weight average molar mass raises the possibility of prediction of the aggregational properties of mutant forms of insulin.