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.

Chapter 2
Aldehyde Dehydrogenase - Preparation, Storage and Gel Chromatography

2.1 Introduction

2.1.1 First Isolation and Purification of Mammalian Aldehyde Dehydrogenase

Aldehyde dehydrogenase (EC 1.2.1.3) was first isolated from bovine liver by Racker (1949), who showed that the dismutation of aldehydes in liver tissue was catalysed by two separable enzymes, the already known alcohol dehydrogenase and the then newly isolated aldehyde dehydrogenase. Racker (1949) prepared an acetone-dried powder of the frozen beef liver which was extracted with water. The aqueous solution underwent centrifugation and ethanol additions, followed by nucleic acid additions, adjustments to pH 5.2 and centrifugation before the final precipitate was dissolved and the nucleic acid was removed from it. Further purification by ammonium sulphate fractionation was not pursued as the fraction was found to be free of alcohol dehydrogenase. The first mammalian aldehyde dehydrogenase purified to homogeneity was horse liver aldehyde dehydrogenase, by Feldman and Weiner (1972), who first homogenised the liver, then used centrifugation, ammonium sulphate precipitation, ion exchange chromatography and isoelectric focussing to obtain the homogeneous enzyme preparation.

2.1.2 Isoenzymes of Aldehyde Dehydrogenase

Two enzymes with aldehyde dehydrogenase activity were separated and partially purified from rat liver by Shum and Blair (1972). The resolution of the two enzymes was effected by DEAE-cellulose chromatography after preliminary fractionation with ammonium sulphate and treatment with CM-cellulose to remove alcohol dehydrogenase and NADH oxidase activity. Gel filtration of one of the enzymes removed the lactate dehydrogenase, but with the loss of some aldehyde dehydrogenase activity. These two enzymes differed in substrate specificity, pH optima, and arsenic inhibition. At least two isoenzymes were found in mammalian livers, one in the cytoplasm and one in the mitochondria. Since these exhibit different kinetic behaviour (MacGibbon *et al.*, 1978) and different catalytic properties (Kitson, 1975), separation of the isoenzymes is necessary for further investigations into aldehyde dehydrogenase. The presence of disulfiram was found to inhibit the enzyme from the cytoplasm to a much greater extent than it did the mitochondrial form of the enzyme, obtained from sheep liver (Kitson 1975). The presence of these two isoenzymes was observed by Feldman and Weiner (1972) during the DEAE-cellulose column chromatography step of their purification to homogeneity of horse liver aldehyde dehydrogenase. These isoenzymes of horse liver aldehyde

dehydrogenase were purified to homogeneity by salt fractionation followed by ion exchange chromatography, (which included the use of a DEAE-cellulose column to separate them), and gel filtration chromatography and their approximate molecular weights and subunit molecular weights were found to be similar (Eckfeldt *et al.*, 1976). The two isoenzymes of aldehyde dehydrogenase from human liver were separated and purified to apparent homogeneity via classical chromatography combined with affinity chromatography by Greenfield and Pietruszko (1977).

2.1.3 Isoenzymes of Aldehyde Dehydrogenase from Sheep Liver

The aldehyde dehydrogenases of sheep liver were found to originate in the cytoplasm and the mitochondria and were isolated by Crow *et al.* (1974) who used centrifugation and chromatography procedures similar to those above to separate and purify them. Dickinson and Berrieman (1979) found, by isoelectric focussing, that preparations of sheep liver aldehyde dehydrogenase from the cytoplasm were still contaminated by some of the mitochondrial enzyme. They obtained purified enzyme by fractionation with ammonium sulphate. This method was based on the cytosolic enzyme being less soluble and therefore preferentially precipitated at its isoelectric point and was monitored by isoelectric focussing. Another technique for the purification of sheep liver cytosolic aldehyde dehydrogenase was developed by Dickinson *et al.* (1981). This method involved pH-gradient ion-exchange chromatography and was viable for samples of the cytosolic enzyme that were heavily contaminated with the mitochondrial form as well as samples that were relatively pure to begin with. The enzymes had different isoelectric points and it was found that at pH values very close to the isoelectric points, the two species no longer had sufficient negative charge to bind to the DEAE-cellulose resin and eluted off the column in two distinct peaks with good separation and retainment of enzymic activity. Later methods used DEAE-Sepharose instead of DEAE-cellulose for the ion-exchange chromatography (Allanson and Dickinson, 1984). Preparation and purification of sheep liver aldehyde dehydrogenase was carried out according to the method of Motion (1986) with some modifications having been made to it .

2.1.4 Freezing of Aldehyde Dehydrogenase

Since aldehyde dehydrogenase was observed to undergo loss of enzymic activity over extended periods of time when stored in a refrigerator, investigations were made into another method of long-term storage. Freezing the enzyme was a method of storage that was used by some laboratory co-workers. This method was investigated for the appropriate buffer, and the optimum concentration and pH thereof, and for the retainment of enzymic activity upon freezing. Some investigations into freezing environments for yeast alcohol dehydrogenase had already been performed (Hill and Buckley, 1991), and these formed the basis for the investigations that were made. Freezing various buffer solutions can cause changes in the pH of these solutions. A notable example of this is phosphate buffer which shows a marked decrease in pH on freezing. Hill and Buckley

(1991) reported that yeast alcohol dehydrogenase loses activity in phosphate buffer upon freezing, but is stable in other buffers that show little change in pH when frozen. Thus buffers should be examined for any changes in pH upon freezing before freezing actual biological samples in them. Frozen pH indicator solutions provide information about the pH of the frozen solution by the colour of the frozen indicator solution (Hill and Buckley, 1991, Williams-Smith *et al.*, 1977). Since the buffers used in conjunction with aldehyde dehydrogenase are phosphate, bis-tris, and acetate - acetic acid, these are the logical ones to investigate for freezing suitability. Acetate - acetic acid buffer was not investigated as the enzyme lost activity and was thought to dissociate in the lower pH environment of this buffer (Buckley *et al.*, 1991), making it unsuitable for storage of the enzyme. Control samples of phosphate and bis-tris buffers, of various concentrations, containing universal pH indicator were frozen. These results were used to select buffers for freezing enzyme samples in order to investigate the loss of activity of the enzyme upon freezing.

2.1.5 Gel Chromatography of Aldehyde Dehydrogenase

Gel chromatography results for sheep liver cytosolic aldehyde dehydrogenase obtained by Hill *et al.* (1991) showed that two distinct peaks in absorbance at 280 nm were eluted at pH 7.4 and pH 5.0 and that at both pH's there exists two forms of aldehyde dehydrogenase, an enzymatically active form of molecular weight 200 000, and an inactive form of mass 50 000 to 100 000. They found that the ratios of these two forms varied depending on the solution pH and enzyme concentration. At pH 7.4 and 44 μM aldehyde dehydrogenase, they observed one large enzymatically active peak and one very minor inactive peak. At 10-fold dilution of the enzyme, the amount of the inactive peak increased.

At pH 5.0 and the same enzyme concentrations, they found that the amount of the inactive peak had increased compared to that at pH 7.4, at the expense of the active peak, until, at 10-fold dilution, the amount of the inactive peak was almost the same as that of the active peak. From these results it was expected that both species would be observed through ultracentrifuge and laser light scattering techniques. Although the larger species might dominate the light scattering data, the amount of smaller, dissociated species present at pH 5.2 should still be detectable. It was thought to repeat this gel column work for comparison with the results obtained for the enzyme from the ultracentrifuge and the laser light scattering.

2.2 Methodology

2.2.1 Preparation and Purification of Cytosolic Aldehyde Dehydrogenase from Sheep Liver

Cytosolic aldehyde dehydrogenase was obtained from sheep livers by following essentially the methods used by Motion (1986) and as further developed by Kitson (K.E. Kitson, personal communication). Livers were obtained from freshly slaughtered sheep

and frozen. All buffers and columns were stored and used at 4 °C. When the time came for use, the frozen livers were partially defrosted under running water to facilitate the dicing of the livers into small pieces. When not in use, the diced and yet to be diced livers were stored on ice. Samples were taken at intermediate stages of the enzyme preparation and assayed for enzyme activity, or lack of it in the cases of discarded supernatants, as well as the fractions from the columns and the final product that were assayed for enzyme activity. SDS gels were run to determine the purity of the final enzyme product obtained. Dithiothreitol (0.1% w/v) was added to all buffers. The DEAE ion exchange columns were cleaned by running a salt wash through them (about 0.5 M NaCl solution). They were prepared for use as per the manufacturer's instructions. The affinity column was washed immediately after use with the affinity buffer that contained 0.5 M NaCl, and then washed with a similar amount of the buffer without the high NaCl concentration.

Diced livers (750 g) were homogenised with 0.005 M phosphate buffer, pH 7.4, containing 0.25 M sucrose (1.5 litres), using either an ultraturrax or a food blender, in 3 batches. The homogenate was centrifuged at 9000 rpm for 10 minutes. The supernatant was filtered through glass wool and then centrifuged at 13000 rpm for 30 minutes. Again the supernatant was filtered through glass wool, and the volume of the filtrate was recorded. PEG 8000 was added to the continually stirred filtrate over 20 - 30 minutes to give a concentration of 12% w/v PEG 8000. This was then stirred for a further 15 minutes. The solution was centrifuged at 9000 rpm for 15 minutes and the volume of the supernatant was recorded. PEG 8000 was again added to the continually stirred supernatant over 20 - 30 minutes to bring the final concentration of PEG 8000 up to 20% w/v. The solution was centrifuged at 9000 rpm for 15 minutes. The supernatant was discarded and the precipitate was dissolved in 0.005 M phosphate buffer, pH 7.4 (about 250 mL).

The redissolved precipitate was loaded onto a DEAE 32 column and washed with 0.005 M phosphate buffer, pH 7.4 (0.5 - 1 litre) overnight. The enzyme was then eluted with 0.022 M phosphate buffer. The fractions were assayed for activity and the active fractions were combined and dialysed against 0.010 M bis-tris buffer, pH 6.5. The dialysed enzyme was then loaded onto the pH column and washed with about 300 mL 0.010 M bis-tris buffer, pH 6.5. The column was then eluted with about 150 mL of 0.010 M bis-tris buffer, pH 5.8, followed by 0.010 M sodium acetate - acetic acid buffer, pH 4.8. This elution was sometimes performed as a gradient mix of the two buffers, and sometimes as a step gradient. The fractions that displayed activity were dialysed against 0.025 M phosphate buffer pH 7.4.

When the sample was to be passed through an affinity column for further purification, the active fractions were dialysed instead into 0.025 M phosphate buffer, pH 7.4 containing 0.015 M NaCl, and 0.3 mM EDTA. This buffer was run through the 4-acetylphenoxy sepharose affinity column until the absorbance of the eluant at 280 nm against the buffer blank was < 0.05. The enzyme was loaded onto the column and washed with about 200 mL of buffer until the absorbance of the eluant against that of the buffer blank at 280 nm was < 0.05. The enzyme was then eluted with the affinity buffer above that also contained 0.010 M 4-hydroxyacetophenone, and the fractions were assayed for enzyme

activity. The active fractions were combined and dialysed overnight against 0.025 M phosphate buffer, pH 7.4.
An ammonium sulphate concentration step was sometimes used after the affinity column step. The active fractions were dialysed at least overnight in 0.05 M phosphate buffer, pH 7.4 (2 litres) containing 400 g ammonium sulphate per litre and an extra 4 g per 10 mL of sample to be dialysed, and 0.15 mM EDTA. The sample was then spun at 10000 rpm for 20 minutes, the supernatant was removed and the precipitate was dissolved in a small volume of 0.05 M phosphate buffer pH 7.4 and then dialysed against several changes of the same buffer.

2.2.2 Enzyme Assays

Samples were assayed for aldehyde dehydrogenase activity. They were assayed by monitoring spectophotometrically the increase in absorption at 340 nm due to the formation of NADH. The k_{cat} was assumed to be 0.56 s^{-1} (Blackwell *et al.*, 1987).
The assay mixture consisted of:

0.85 mL 0.05 M phosphate buffer, pH 7.4
50 μL 0.020 M NAD^+ solution, final concentration 0.001 M
50 μL enzyme sample
50 μL 0.400 M propanal solution, final concentration 0.020 M

The reaction was initiated by the addition of the propanal, which was always added last for that reason. The concentration of the enzyme that was obtained was an active site concentration expressed as a μM concentration.

2.2.3 Freezing Experiments on Aldehyde Dehydrogenase

0.050 M potassium dihydrogen phosphate and 0.050 M bis-tris buffers were prepared and the appropriate dilutions and pH adjustments were made. 50 μL universal pH indicator solution were added to 1.5 mL buffer in plastic 2 mL vials. Triplicate samples were prepared and one each was stored at room temperature and in the refrigerator (4 °C), and in the freezer (-25 °C). The samples from the refrigerator were warmed by holding in the palm of the hand and the frozen samples were defrosted by warming to room temperature in warm water (30 - 40 °C) and in the palm of the hand. The pH of the samples after storage were noted as were the changes that they underwent on warming. The pH was determined by the comparison with the colour chart on the universal pH indicator bottle, having first been checked with the original samples.
The most suitable buffers were chosen and enzyme samples were dialysed into the buffer, (from 0.025 M phosphate buffer, pH 7.4) and frozen overnight. They were quickly defrosted by the above method. The samples were assayed before and after freezing. The above method was used for defrosting to enable the enzyme to pass quickly

through the state of partial thawing when part of the enzyme was solid and part of it was not - when the solution was cloudy.

2.2.4 Gel Chromatography of Aldehyde Dehydrogenase

Samples of the enzyme at pH 7.4 and pH 5.2 and of the same concentrations as those used in the ultracentrifuge (Chapter 3) were passed through a Superose 12, gel filtration column. The systems used were 0.025 M potassium dihydrogen phosphate buffer, pH 7.4 or 0.010 M sodium acetate - acetic acid, pH 5.2, and acetonitrile/water 2 : 1.
Samples of aldehyde dehydrogenase at pH 5.2 (Chapter 3) that had been subjected to the ultracentrifuge were retained and the most concentrated and dilute of those three samples were then passed through a gel column a week later. Samples of aldehyde dehydrogenase at pH 7.4 that had not been subjected to the ultracentrifuge were passed through the gel column. These samples at pH 7.4 were from the same enzyme stock as the samples that had been subjected to the ultracentrifuge and were diluted to the same extent that the other samples were, although only the non - diluted and most dilute samples were passed through the gel column as were the pH 5.2 samples. It was found that 10 μL of the diluted samples and 5 μL of the concentrated samples were the appropriate amounts of sample to inject into the column. The samples were filtered before injection.
The enzyme at pH 7.4 had activity of 34.0 μM the day before the ultracentrifuge experiments were initiated. This enzyme was diluted 1 : 3 with the appropriate buffer to give the most dilute sample. The same stock solution was passed through the gel column 12 days later, as was a freshly diluted 1 : 3 sample of it. The dilution was left until the day before it was passed through the gel column so that any loss of activity due to dilution was not significant. Some of the same stock solution was dialysed to pH 5.2 and then diluted in the same manner as above for use in the ultracentrifuge and gel column. The stock solution at pH 7.4 had not undergone any significant loss of activity between the time that it was first used for ultracentrifuge samples and when the gel column enzyme samples were taken from it, (a week after the gel column samples were run, the activity of the stock solution had decreased by less than 7% of what it was at the time of the ultracentrifuge experiments, to 31.7 μM). The activity of the pH 5.2 sample (non-diluted) that had been subjected to the ultracentrifuge was around 20 μM at the time of the gel column experiment. There seemed to be no major loss of activity of either of the concentrated samples between the time of the ultracentrifuge and gel column experiments, regardless of whether the samples had been subjected to the ultracentrifuge or not.

2.3 Results

2.3.1 Freezing of Aldehyde Dehydrogenase

Table 2.1 - Frozen Phosphate Buffers

Conc. (M)	Initial pH	pH (room temp.)	pH (4 °C)	pH (-25 °C)
0.050	7.4	7.0 - 7.5	7.0 - 7.5	6.5 - 7.0
0.025	7.4	7.0 - 7.5	7.0 - 7.5	7.0 - 7.5
0.010	7.4	7.0 - 7.5	7.0 - 7.5	7.0 - 7.5
0.050	6.5	6.5 - 7.0	6.5 - 7.0	5.0 - 5.5
0.025	6.5	6.5 - 7.0	6.5 - 7.0	5.0 - 5.5
0.010	6.5	6.5 - 7.0	6.5 - 7.0	5.5 - 6.0

Table 2.2 - Frozen Bis-tris Buffers

Conc. (M)	Initial pH	pH (room temp.)	pH (4 °C)	pH (-25 °C)
0.050	7.3	7.0 - 7.5	7.0 - 7.5	7.5 +
0.025	7.3	7.0 - 7.5	7.0 - 7.5	7.5 +
0.010	7.3	7.0 - 7.5	7.0 - 7.5	7.5 +
0.050	6.5	6.5 - 7.0	6.5 - 7.0	6.5 - 7.0
0.025	6.5	6.5 - 7.0	6.5 - 7.0	6.5 - 7.0
0.010	6.5	6.5 - 7.0	6.5 - 7.0	6.5 - 7.0

Table 2.3 - Frozen Aldehyde Dehydrogenase

Buffer	Conc. (M)	pH	Activity (pre-frozen) (%) *	Activity (defrosted) (%) *	Activity (defrosted) (%) **
phosphate	0.025	7.4	100.0	105.8	105.8
phosphate	0.010	7.4	57.0	63.9	112.1
bis-tris	0.025	7.4	6.7	6.1	91.0
bis-tris	0.010	7.3	27.6	22.7	82.2
bis-tris	0.025	6.5	50.6	54.2	107.1
bis-tris	0.010	6.8	52.7	72.1	136.8

* Activity expressed as % of the activity of the stock solution (0.025 M phosphate buffer, pH 7.4) before dialysis into freezing buffer.

** Activity expressed as % of the activity of the sample, in the freezing buffer, before it was frozen.

The activity was quoted as a percentage of the unfrozen stock solution from which all samples were taken and dialysed in the required buffer. The stock enzyme solution was 33.0 μM in 0.025 M phosphate buffer, pH 7.4. The pH's of the buffers were measured at room temperature before being frozen.
A sample of enzyme (initially 33.0 μM in 0.025 M phosphate) was kept in the refrigerator and assayed the next day when the frozen samples were assayed. The activity of this control sample was 34.0 μM. Another control sample, (34.0 μM), was frozen for longer term storage than a few days. It was thawed and assayed three weeks later, (28.4 μM), and then refrozen and defrosted and reassayed the following day, (32.4 μM).
Some samples of enzyme were frozen and later thawed, over periods of time from days to weeks, but significant loss of activity occurred and colloidal matter was present. This occurred despite the enzyme having been purified by the three column prep. which gave enzyme that was essentially pure by SDS gels.

2.3.2 Gel Chromatography of Aldehyde Dehydrogenase

At pH 7.4, the gel chromatography showed the presence of a large species as well as a minor peak due to a smaller species, along with some contamination from other species (Figure 2.1).
At pH 5.2, the smaller species was still present, as were the contaminants, but the larger species was no longer present (Figure 2.2).

2.4 Discussion

2.4.1 Freezing of Aldehyde Dehydrogenase

The choices of pH's of the buffers that were frozen were influenced by their use in the enzyme preparation and by those investigated by Hill and Buckley (1991). From the results it was found, as expected, that none of the buffers underwent any pH changes when stored in the refrigerator or at room temperature. Phosphate buffer at pH 6.5 underwent a significant decrease in its pH when frozen, rendering it unsuitable for frozen storage of the enzyme. However, phosphate buffer, 0.025 M and 0.010 M, pH 7.4 did not change pH when frozen, although the more concentrated 0.050 M phosphate buffer underwent a small change in pH. None of these changes in pH were as great as were expected from the previous studies of Hill and Buckley, (1991), where they found that phosphate buffer at pH 6.5 or pH 7.5 decreased in pH to below pH 4.0 upon freezing, but was in agreement with the results of Williams-Smith *et al.*, (1977), who reported that whilst some buffers exhibited concentration effects upon freezing, those caused by phosphate buffers were small. The bis-tris buffer samples either remained at about the same pH or else their pH increased a little. This was in agreement with the results of Hill and Buckley (1991) who found that the pH of bis-tris buffer increased from pH 6.5 and pH 7.5 to pH 7.0 - 8.0 upon freezing.
The concentrations of the phosphate buffers affected the pH changes upon freezing, in

Figure 2.1 - Gel Chromatogram of Aldehyde Dehydrogenase at pH 7.4

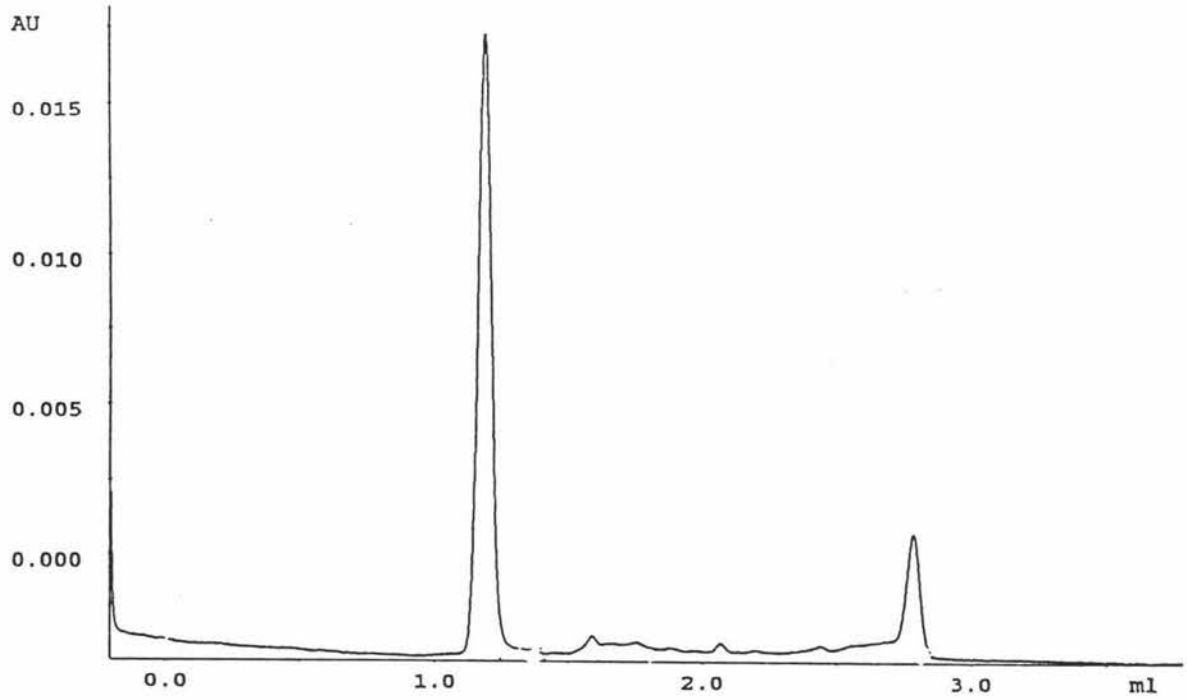
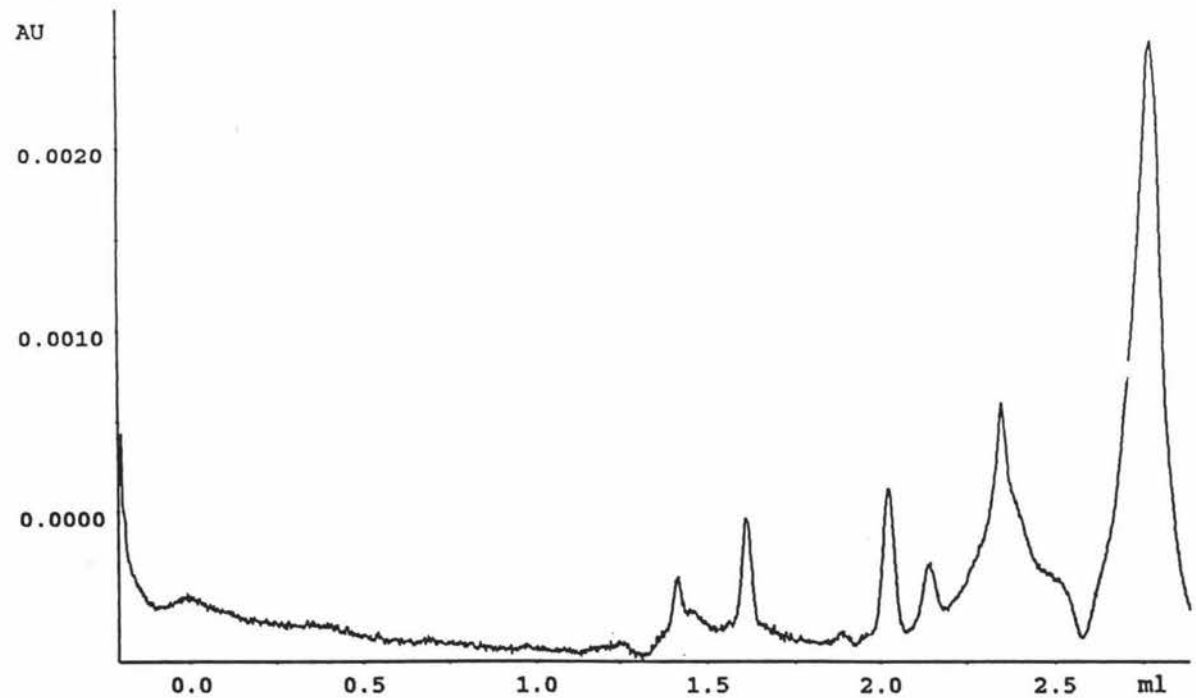


Figure 2.2 - Gel Chromatogram of Aldehyde Dehydrogenase at pH 5.2



that the more dilute buffers seemed to undergo lesser changes in pH. Since the buffers studied previously by Hill and Buckley (1991) were of 0.050 M concentration, this result meant that there was still a reason to investigate the more dilute phosphates for freezing suitability, especially since the enzyme was stored in 0.025 M phosphate buffer, pH 7.4 in the refrigerator.

After samples of aldehyde dehydrogenase were frozen in phosphate buffer pH 7.4 and bis-tris buffer pH 7.4 and 6.5, (0.025 M and 0.010 M), the most appropriate buffer for freezing was found to be 0.025 M phosphate buffer, pH 7.4. The enzyme retained its activity in this buffer after being frozen. Whilst the enzyme also retained its activity upon being frozen in the other buffers used, it had lost a large portion of its initial activity upon being dialysed into those buffers. 0.025 M phosphate buffer, pH 7.4 also had the advantage of being the buffer in which the enzyme was routinely stored.

However, enzyme that was frozen in this buffer did sometimes lose a significant amount of activity after being frozen and colloidal matter was sometimes present upon thawing also. This may have been due to the ability of concentrated proteins to buffer against pH changes in the storage buffer, and that the samples that exhibited instability upon freezing were not of a sufficiently high enzyme concentration to buffer against solvent changes upon freezing. Williams-Smith *et al.* (1977) reported that samples that were frozen slowly (in a refrigerator cabinet) to -20 °C sometimes exhibited signs of separation of the solute, and they proposed that, for some cases at least, it was the freezing process itself, rather than the low temperatures, that gave rise to the apparent pH changes. They also reported that the presence of bovine serum albumin, in the buffers, largely suppressed that pH changes that occurred upon freezing, and that the suppression was greater at higher protein concentration. The non-enzyme, 0.025 M phosphate buffer sample, pH 7.4, did not exhibit any noticeable pH change upon freezing, so the reason for the occasional instability of the enzyme on freezing remains unclear.

2.4.2 Gel Chromatography of Aldehyde Dehydrogenase

The gel column results indicated that for the samples at pH 7.4 and for both concentrations used (34 μM and a 4-fold dilution thereof), there was a major large species present and another smaller species, with a little contamination from other species. At pH 5.2 there was none of the large species, but rather the smaller species was present, with again a little contamination from other species, the amount of which had increased a little compared to the amount present in the pH 7.4 samples. This seemed to suggest that aldehyde dehydrogenase either existed in partial dissociation at pH 7.4 or complete dissociation at pH 5.2, of which the latter did not agree with the gel filtration column results reported by Buckley *et al.* (1991), and the ultracentrifuge (Chapter 3) and laser light scattering(Chapter 4) results.

The gel column result, obtained by Buckley *et al.* (1991), of the concentrated enzyme sample, (44 μM), at pH 5.0 showed that the active peak was still very dominant over the inactive peak. This domination agrees with the light scattering and ultracentrifuge results that found little difference between the diffusion coefficients of the enzyme samples at pH

7.4 and pH 5.0. The diffusion coefficients obtained from the light scattering results agree with the gel column results of the concentrated (44 μM) enzyme solutions (Buckley *et al.*, 1991), but even if the inactive form of the enzyme was so minor as to be undetected by light scattering and ultracentrifuge techniques, the increased amount of it present at lower concentrations (Buckley *et al.*, 1991) should have been observed by these techniques. Concentrations as low as 8 μM were used which approached the 4.4 μM used by Buckley *et al.* (1991).

Whilst the time spent by the sample in the ultracentrifuge may have promoted compacting and aggregation of the sample which would affect the diffusion coefficient obtained by the light scattering method (and not all light scattering samples were subjected to the ultracentrifuge), this should not have any significant effect on the gel column results. Indeed, whilst the samples at pH 5.2 that had first been subjected to the ultracentrifuge showed extremely long decays and consequently much smaller diffusion coefficients than were expected when observed via the light scattering technique, the gel column results showed no sign of any species comparable in size to the associated form of the enzyme at pH 7.4, but instead the smaller species was present.

The technique of gel column chromatography observes the individual particles, whilst that of laser light scattering is biased towards observing the behaviour of large particles or aggregates of particles.

A possible explanation for the absence of the major peak that was present in the pH 7.4 is that the enzyme does indeed dissociate at pH 5.2, but the dissociated species also undergoes aggregation and it is these aggregates that are observed by light scattering or the ultracentrifuge. These aggregates may then break apart on the gel column and thus the smaller dissociated species may be observed. Another explanation is that the time spent on the gel column may affect the association - dissociation equilibrium of the enzyme.

The latter argument seems to be the more likely since the diffusion coefficients obtained from light scattering were very similar for samples of the enzyme at both pH's. The enzyme had shown a decided tendency towards aggregation. The diffusion coefficients were obtained from non-aggregated samples (Chapter 4, Tables 4.1 and 4.5a), and those from aggregated samples were discarded (Appendix 1, Tables A1.1a - b and A1.2). Thus if the samples at pH 5.2 that were passed through the gel column were aggregated and consequently broke apart on the column, it would be likely that the samples at pH 7.4 were aggregated also, (since it is unlikely that the dissociated sample at pH 5.2 would aggregate only to the extent that it had the same diffusion coefficient as the pH 7.4 species), and thus would be expected to fall apart on the column also.

If, however, the samples at pH 7.4 were not aggregated, they would not be expected to fall apart on the column, and thus would agree with the results from all three techniques (ultracentrifuge, light scattering and gel column) of a major large species taken to be the tetrameric form of the enzyme. The enzyme samples at pH 5.2 could consist of mainly non-dissociated enzyme, (the same as at pH 7.4), with a small amount of dissociated species that was not enough to be detected by the light scattering technique, (in a similar manner to the behaviour of the pH 7.4 samples), being overshadowed by the larger non-dissociated species. This dissociated species was either not present in the ultracentrifuge

sample or the technique pushed the equilibrium in the direction of association (since no smaller species was detected despite being spun at very high speeds). If some of the very small amount of dissociated species present was somehow trapped on the column, over the half an hour taken for the running of the column, the equilibrium could have shifted towards complete dissociation of the enzyme. This might also explain the presence of a small amount of dissociated species present at pH 7.4. If there was only a trace of this species present in the pH 7.4 samples before they were passed through the gel column (as borne out by light scattering and ultracentrifuge results) and the gel column did affect the association-dissociation equilibrium to promote dissociation, this could explain the presence of the minor peak due to the dissociated species.

The gel column results for pH 7.4 agree with those of Buckley *et al.* (1991) with a major peak due to the associated form of the enzyme and a minor peak due to the dissociated form, although the amount of the minor peak did not increase at lower enzyme concentrations. The results for pH 5.2 showed complete dissociation at both concentrations, unlike the partial dissociation observed by Buckley *et al.* (1991), which did not agree with results obtained from other techniques, leading to the postulation that the gel column itself promoted the dissociation.

Chapter 3
Ultracentrifuge Studies on Aldehyde Dehydrogenase

3.1 Introduction

3.1.1 Introduction

The analytical ultracentrifuge is a useful technique for the determination of sedimentation and apparent diffusion coefficients and other physical properties of biological macromolecules.
The technique consists of spinning a sample in the ultracentrifuge at extremely high speeds (in this instance 48000 rpm and higher) and monitoring the sample's progress as it sediments. The ultracentrifuge's monitoring system uses Schlieren optics. Photographs are taken of these images at appropriate intervals. By use of mirrors and lenses the Schlieren pattern can be deflected onto a photographic plate to allow it to be recorded at any stage of the run.
When varying amounts of protein are added to a solvent the refractive index of the solution will alter as a function of the protein concentration. During the ultracentrifuge run the protein moves centrifugally through the cell. The cell is wedge shaped so the concentration of the protein decreases as it moves radially through the cell. During an experiment, at any point in time, the concentration varies radially through the sedimenting boundary in the cell with a concomitant radial concentration gradient (Figure 3.1). The change in the refractive index of the solution reflects this changing concentration gradient. The sector shaped cell minimises the effect of the cell walls on the sedimentation process. The speed of sedimentation is dependent on the shape of the sedimenting species as well as its mass, but for a globular protein such as aldehyde dehydrogenase the contribution due to shape should be constant.

3.1.2 The Schlieren Pattern

The image of the Schlieren pattern on the photographic plate is that of a peak (or peaks for a heterogeneous solution) that moves across the plate (which corresponds to the cell) as the run progresses (Figure 3.2). The Schlieren peak, or boundary region, moves across the image, away from the meniscus, as the species sediments. It leaves behind it pure solvent and the peak broadens, owing to diffusion, and decreases in size, owing to radial dilution, as the sedimentation proceeds.
The image is actually a plot of dn/dr versus r, where n is the refractive index and r is the radial distance from the centre of rotation. However since the changes in the refractive index are considered directly proportional to the changes in the protein concentration the

Figure 3.1 - Plots of Concentration versus Radial Distance and of Rate of Change of Protein Concentration with Radial Distance

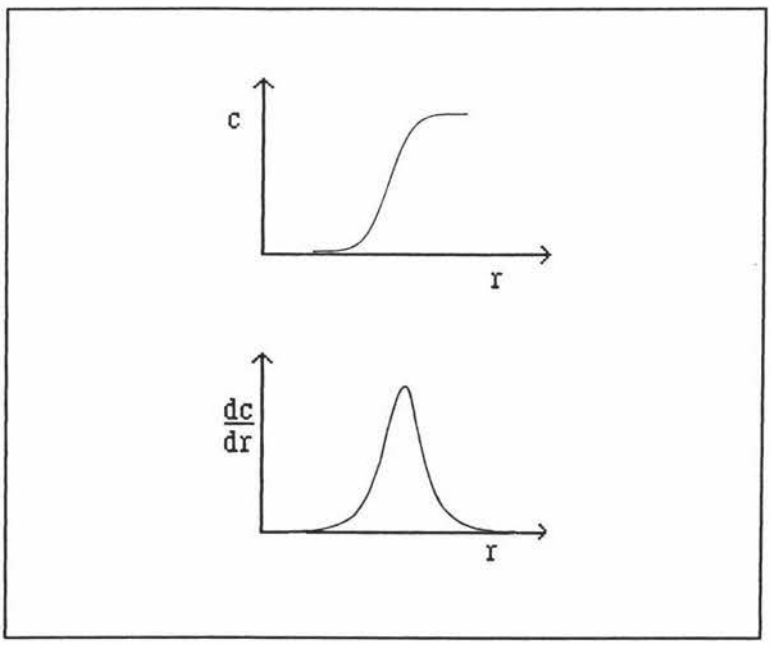
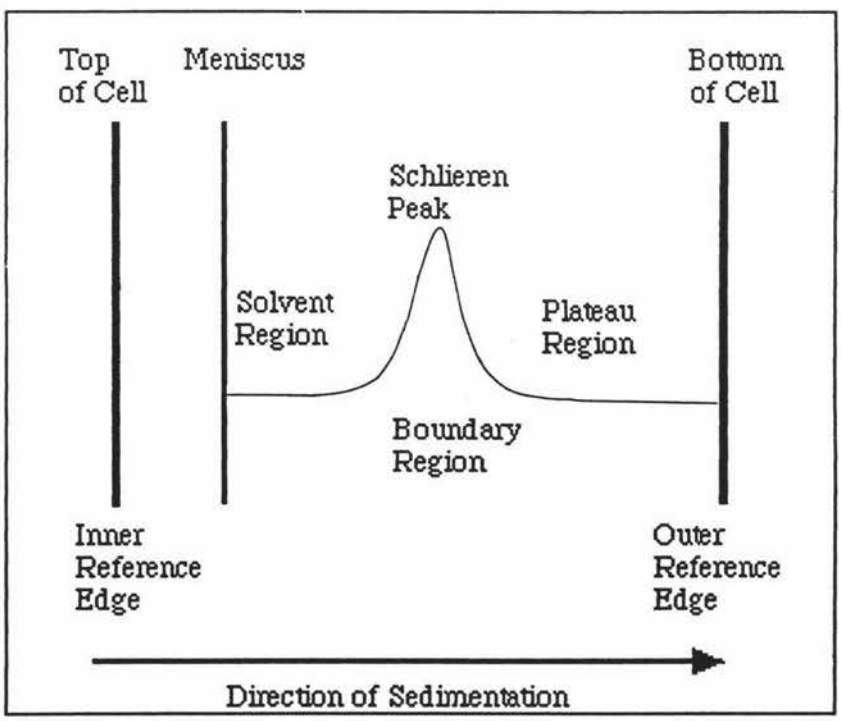


Figure 3.2 - Diagram of a Schlieren Pattern of a Homogeneous Solution



Schlieren pattern becomes a plot of the rate of change of the protein concentration with radius (ie. a plot of dc/dr versus r) (Figure 3.1).
Thus the area under the peak is proportional to the concentration of the protein. Since the protein concentration decreases as it moves across the cell, the Schlieren peak decreases in height and becomes more spread out as it moves across the image as sedimentation progresses. However, if self-interactions occur the system will show marked non-ideality. The sedimentation and diffusion coefficients are determined from the movement and spreading of the boundary with time.

3.1.3 Peak Behaviour

For a homogeneous solution one peak only should be present, whilst for heterogeneous solutions the number of peaks generally reflects the number of components present. The peaks move across the cell at different rates. The larger the species the faster it sediments, and thus the faster the peak moves. Therefore contamination by dust or dirt is not the problem it can be in laser light scattering, (except for possible marking of the windows of the cell), as these particles would sediment very quickly before they could obscure any peaks due to the sample components.
Gaussian shaped peaks usually indicate that there is a single species present causing that peak and that it is not undergoing any interactions with other species present, although self-interactions may be occurring. However, non-ideality and self-association reactions may compensate each other to give Gaussian curves. Trailing edges indicate interactions between different species and very broad peaks suggest that there may be two or more species contributing to that peak.

3.1.4 Aims

The purpose in subjecting sheep liver cytosolic aldehyde dehydrogenase to the ultracentrifuge was to investigate the association - dissociation behaviour of the enzyme at pH's which have been reported to affect the association - dissociation of the enzyme (Buckley *et al.*, 1991). The aims were also to obtain the sedimentation and diffusion coefficients of the enzyme using this technique and to use them to calculate the molecular weight of the enzyme.The diffusion coefficients will be compared with those obtained by laser light scattering and the molecular weights will be compared with those obtained from gel column chromatography results (Buckley *et al.*, 1991).
The use of the ultracentrifuge complements laser light scattering results since both allow the diffusion coefficient of the sample to be measured. Pinder *et al.* (1995) found good agreement between the diffusion coefficients obtained from the ultracentrifugation and laser light scattering of a ternary polymer solution. Comparison of the diffusion coefficients obtained by these methods for aldehyde dehydrogenase should be useful as each method has its possible susceptibilities to the nature of the enzyme samples - possible pressure-induced association or aggregation for the ultracentrifuge and bias towards large particles for the light scattering technique.

The ultracentrifuge has been used to study the association - dissociation behaviour of aldehyde dehydrogenase from pseudomonas aeruginosa (Von Tigerstrom and Razzell, 1968), so observation of changes in the association - dissociation behaviour for sheep liver cytosolic aldehyde dehydrogenase should be possible.

3.2 Methodology

3.2.1 Use of the Ultracentrifuge

The ultracentrifuge used was a Beckman model E analytical ultracentrifuge with an An-H rotor with Schlieren optics set at 546.1 nm and an analyser angle set at 60° and an aluminium 2.5° single sector cell. The rotor was kept at 25 °C throughout the experiments. Before its initial use the components of the ultracentrifuge cell were assembled as per the instruction manual (Chervenka, 1969), rinsed with buffer solution and left immersed in it overnight and then rinsed again with it the following day.
The cell was filled with enzyme solution (0.4 - 0.45 mL) and weighed. Weights were added to the counterbalance to adjust its mass to within 0.5 g of that of the filled cell. The mass of the counterbalance was always kept less than that of the filled cell to ensure that if any solution leaked out of the cell the difference between the mass of the cell and that of the counterbalance would narrow instead of widen.
The enzyme samples were centrifuged in order of increasing concentration and decreasing pH. The speeds used were 48000 rpm and 56000 rpm. 56000 rpm was the maximum speed available due to the derating, over time, of the ultracentrifuge rotor from its original maximum speed of 60000 rpm. Use of the ultracentrifuge at 56000 rpm was discontinued once it was established that there were no species present that were detectable only at that speed.

3.2.2 Monitoring of each Ultracentrifuge Run

The progress of each ultracentrifuge run was monitored visually and photographs were taken at appropriate intervals. The photographic plates contained five frames and one plate per ultracentrifuge run was used (Figures 3.3a - b). These photographs had an exposure time of 40 seconds and the time of each photograph was recorded as that at halfway through the exposure time and relative to time zero of the run.
Time zero of the run was taken as being when 2/3 final speed was reached. This was because the current to the motor was kept constant at 12 amps, giving constant torque and thus constant acceleration. So from the time at 2/3 final speed to the time when final speed was reached the same amount of the protein would have moved the same distance as would have if the ultracentrifuge had been operating at final speed from the start. A plot of speed, or angular velocity, (ω) versus time (t) up until constant speed is reached yields a triangle, the median of which is $t_{2/3speed}$.

Figure 3.3a - Photograph of an Ultracentrifuge Run of Aldehyde Dehydrogenase, pH 7.4

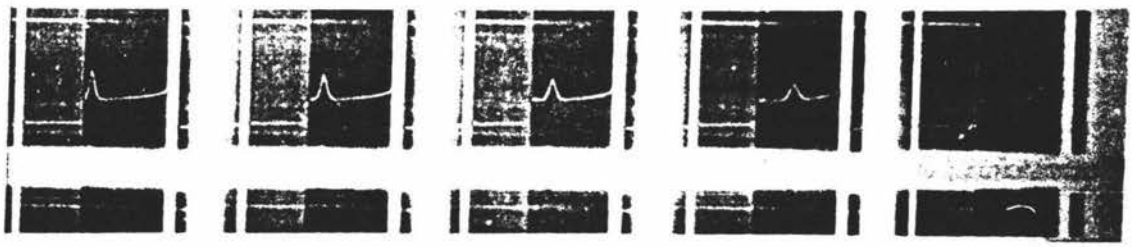
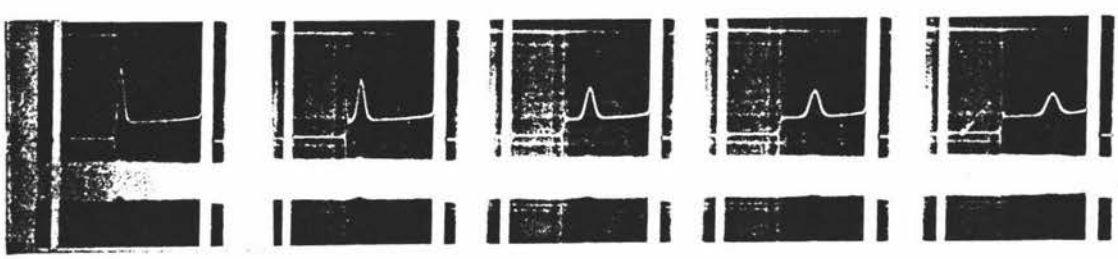


Figure 3.3b - Photograph of an Ultracentrifuge Run of Aldehyde Dehydrogenase, pH 5.2



3.2.3 Samples

Stock aldehyde dehydrogenase, in 0.025 M phosphate buffer, pH 7.4, with an initial concentration based on activity of 34.0 μM was diluted with buffer to give a series of 1:1 and 1:3 dilutions. No further dilutions were made as 1mg/mL (5 μM) was the arbitrary limit set by the operator based on the size of the enzyme. The three samples were centrifuged in order of increasing concentration. The cell was rinsed well with the appropriate buffer solution between uses.
The stock enzyme was then dialysed in 0.010 M sodium acetate/acetic acid buffer, pH 5.2 and the same dilution series was prepared and centrifuged, again in order of increasing enzyme concentration. The most dilute sample at pH 5.2 was kept for approximately one week and then centrifuged again to observe if any time-related changes had taken place.
Where possible, samples were retained after ultracentrifugation for assays and laser light scattering experiments.

3.2.4 Analysis of Results

The photographic plates were developed by the operator using fresh Kodak fixing and developing solutions as per accompanying instruction leaflet.
The length of the cell was measured using an optical comparator. The developed plates were placed in a photographic enlarger and the distance from the inner to outer reference edges was measured for each frame. From these and the known length of the cell, the magnification factor was calculated for each frame. The distances from the inner reference edge to the meniscus and to the boundary position (the maximum ordinate of the peak) were also measured. Tracings were made of each peak. Using a ruler, the height of each peak was measured at 1 mm intervals, starting at 0.5 mm in from the peak edge, as well as the width at 1/2 maximum peak height.

3.3 Results

3.3.1 Analysis of Photographic Plates

All photographic plates were analysed using a photographic enlarger and were measured with a ruler to 0.05 cm. The radial distances of the images measured, such as to the meniscus or the boundary position (the maximum of the Schlieren peak), were made relative to the image of the inner reference edge of the counterbalance. The distance of this inner reference edge from the centre of the rotor was taken as 5.71 cm.(Chervenka, 1969) This was because rotors stretch as rotor speed increases. For aluminium rotors what was 5.71 cm at low speed remains 5.71 cm at 40000 rpm and becomes 5.72 cm at 60000 rpm. This figure is only an approximation since other factors such as the age of the rotor affect the stretch of the rotor. The measurements of the stretch of titanium rotors were not available but an error of 0.01 cm for this distance would be insignificant for the

usual measurements made in the ultracentrifuge.
5.71 cm was multiplied by the magnification factor for each frame and added to the measured quantity (made relative to the image of the inner reference edge of the counterbalance). This final measurement was then divided by the magnification factor to obtain the actual radial distance from the centre of the rotor. Thus the actual distance of the boundary position (x) from the centre of the rotor was obtained.
The magnification factor was obtained by placing the counterbalance in an optical comparator and measuring the distance between the two reference edges. This was found to be 1.586 cm. For each frame the distance between the Schlieren image (in a photographic enlarger) of the two reference edges was measured and divided by the actual distance of 1.586 cm to calculate the magnification factor. This factor was calculated for each frame since there were sometimes slight variations in the distances measured due to variations in the clarity of the images. This factor was also recalculated when the images were subjected to further measurements at a later date.

3.3.2 Sedimentation Coefficient of Aldehyde Dehydrogenase

Time (min.) versus $\log_{10}x$ was plotted and the slope obtained via linear regression. The zero time was taken as the time when 2/3 speed was reached, as explained previously. The value of the slope, $d\log_{10}x/dt'$, was used to obtain the sedimentation coefficient, s, via the equation below.

$$s = [1/(\omega^2 r)](dr/dt) = [2.303/(60\omega^2)](d\log_{10}x/dt') \qquad (3.1)$$

where ω is the angular velocity in radians per second,
t is the time in seconds, and
t' is the time in minutes.
Data for the calculation of the sedimentation coefficient of aldehyde dehydrogenase at pH 7.4 and 5.2 is located in Appendix 1, Tables A1.1 and A1.2.

$$s = [2.303/(60\omega^2)](d\log_{10}x/dt') = [2.303/(60\omega^2)](\text{slope}) \qquad (3.2)$$

The values of ω^2 were obtained from the reference manual (Chervenka, 1969).

At 48000 rpm $\omega^2 = 2.527 \times 10^7$ and so $2.303/(60\omega^2) = 1.579 \times 10^{-9}$, and

at 56000 rpm $\omega^2 = 3.439 \times 10^7$ and so $2.303/(60\omega^2) = 1.116 \times 10^{-9}$.

The slopes and sedimentation coefficients from the above graphs for the enzyme at pH 7.4 and pH 5.2 are given in the tables below. The sedimentation coefficient (s) is expressed in Svedbergs (S) which are $s/10^{-13}$.

Table 3.1 - Sedimentation Coefficients of Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	Conc. (g/dL)	Speed (rpm)	$d\log_{10}x/dt'$ (10^{-4})
8.5	0.18	48000	7.29 ± 0.05
17.0	0.36	56000	8.8 ± 0.2
34.0	0.72	48000	6.4 ± 0.1

The stock sample of aldehyde dehydrogenase had activity of 34.0 μM.
The other samples were diluted from the stock sample, 1 : 1 and 1 : 3 with buffer and their activity was calculated from the dilution of the stock enzyme.

Table 3.2 - Sedimentation Coefficients of Aldehyde Dehydrogenase at pH 5.2

Conc. (μM)	Speed (rpm)	$d\log_{10}x/dt'$ (10^{-4})	s (S)
*8.5	56000	9.3 ± 0.1	10.4 ± 0.1
*17.0	48000	6.2 ± 0.2	9.4 ± 0.3
*34.0	48000	6.8 ± 0.2	10.3 ± 0.3
**8.5	56000	9.2 ± 0.5	10.3 ± 0.6

*34.0 was the stock sample of aldehyde dehydrogenase that had activity of 34.0 μM at pH 7.4 and was dialysed to pH 5.2 and then centrifuged.
*17.0 and *8.5 were samples of the stock enzyme, at pH 5.2, that were diluted 1 : 1 and 1 : 3 with pH 5.2 buffer and centrifuged.
The activities quoted were those inferred from dilutions of the enzyme at pH 7.4.
**8.5 was the sample that was recentrifuged a week after it was first centrifuged.

Sedimentation coefficient versus concentration was plotted for the enzyme at each pH (Figures 3.4 and 3.5). The enzyme exhibited no consistent variation of the sedimentation coefficient with concentration at pH 5.2. However the plot did show a trend of decreasing sedimentation coefficient with increasing concentration at pH 7.4. This plot was linearly regressed to obtain the slope of the plot, since the sedimentation coefficient was expected to vary with concentration according to the equation:

$$s_{(c)} = s_0 (1 - k_s c) \qquad (3.3)$$

where c is the concentration, in g/dL

$s_{(c)}$ is the sedimentation coefficient at concentration c

s_0 is the sedimentation coefficient at zero concentration and

k_s is the fractional change in the sedimentation coefficient per unit increase in concentration.

$$k_s = - \text{slope}/s_0 \qquad (3.4)$$

and k_s is expressed in dL/g.

Figure 3.4 - Sedimentation Coefficient versus Concentration for Aldehyde Dehydrogenase at pH 7.4

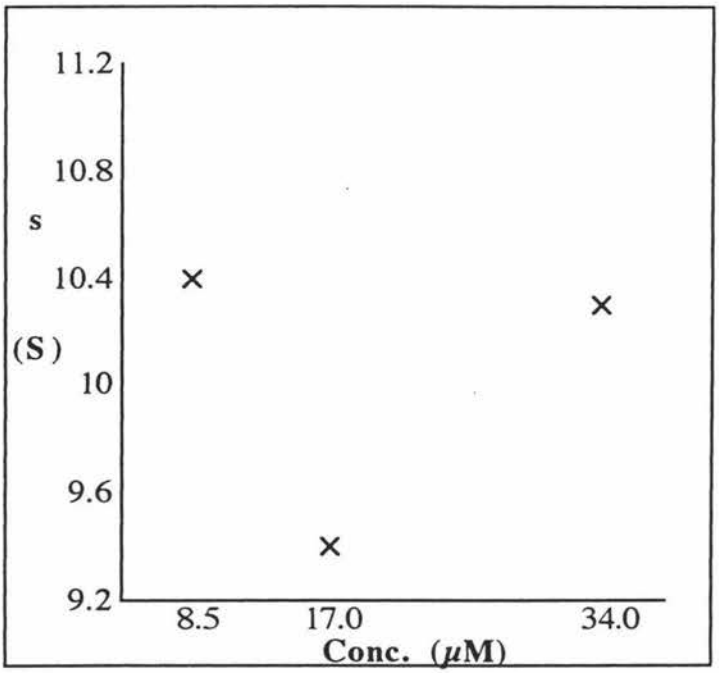
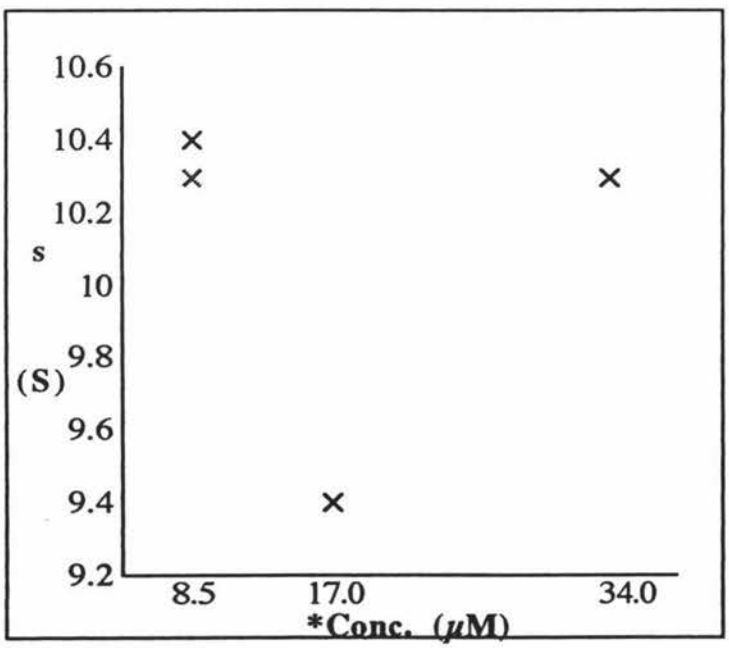


Figure 3.5 - Sedimentation Coefficient versus Concentration for Aldehyde Dehydrogenase at pH 5.2



*Conc. - concentrations at pH 7.4, see Table 3.2

Whilst the line was not of good fit for the plot at pH 7.4, the value of k_s was found to be 0.02 dL/g. This was smaller than the values of k_s for proteins which are usually around the value of 0.08 dL/g or higher.

3.3.3 Calculation of the Diffusion Coefficient of Aldehyde Dehydrogenase

After the sedimentation coefficient was obtained, further measurements were performed on the plates to determine the diffusion coefficient. The distances between the reference edges of the images were remeasured in order to recalculate the magnification factor where necessary. The image of each peak was traced and a baseline for each peak was drawn. All further measurements were then carried out on these tracings.

The area under each peak was determined using two separate methods. The first method involved the rectangular approximation approach, whilst the second was based on the assumption that the peaks were Gaussian in shape. In the rectangular approximation method the area under each peak was divided into sections of width 1 mm. Starting at 0.5 mm in from the edge of the peak the height of each trapezoidal portion was measured at 1 mm intervals, (ie. the height was measured at the centre of each 1 mm wide trapezoidal portion of the peak). The sum of the areas of the trapezoidal portions gave a close approximation of the area under the peak. That is, the area under the peak was taken as 1 mm multiplied by the sum of the measured heights.

Using the assumption that the peaks were Gaussian in shape, the areas under the peaks were also calculated from the product of the maximum peak height and the width of the peak at half of the maximum peak height. In both cases corrections for the magnification factor were left until a later stage in the calculations.

After the area under each peak was calculated by both methods, it was divided by the maximum peak height and then squared. This value was then divided by the square of the magnification factor to correct for the magnification of the initial tracings of the peaks. The apparent diffusion coefficient (D) was then able to be obtained via the equation

$$(A/H)^2 = 4\pi tD \qquad (3.5)$$

where A/H was the area of the peak divided by the maximum peak height and corrected for magnification effects, and t was the time in seconds.

Thus a plot of $(A/H)^2$ versus t would have a slope equal to $4\pi D$.

The enzyme at pH 7.4 did not exhibit a linear plot of sedimentation coefficient versus concentration. Calculation of a linear slope of the plot, in order to obtain a value for k_s since the enzyme did exhibit a trend of decreasing sedimentation coefficient with increasing concentration, yielded a value of k_s that was less than that expected for a protein. Since the plot was not linear and k_s was small, k_s was assumed to be zero, thus ignoring any variation in sedimentation coefficient with concentration. As the enzyme exhibited no consistent variation in sedimentation coefficient with concentration at pH 5.2, k_s was taken as zero for this case also.

Thus, assuming $k_s = 0$, by Lamm's method (Lamm, 1929) equation 3.6

$$(A/H)^2 = D[2\pi/(s\omega^2)][\exp(2s\omega^2 t) - 1] \quad (3.6)$$

becomes reduced to equation 3.5

$$(A/H)^2 = 4\pi tD \quad (3.5)$$

By using the assumption that the protein does not exhibit any variation in sedimentation coefficient with concentration, ($k_s = 0$), Lamm's method will show variations in the diffusion coefficient with time of centrifugation (Baldwin, 1957).

From the data (Appendix 1, Tables A1.3 and A1.4), $(A/H)^2$ versus t was plotted for each enzyme sample run in the ultracentrifuge, using both methods of calculating the area under the peaks.

The plots were linearly regressed in order to obtain the slope of each plot, and from these, the diffusion coefficient, D, was calculated for each enzyme sample. The values of the slopes and the diffusion coefficients determined from them and details of the regression are located in Appendix 1, Tables A1.5 and A1.6.

Tables 3.3 and 3.4 contain the diffusion coefficients of aldehyde dehydrogenase, determined from two separate methods of calculating the area under each peak, at pH 7.4 and pH 5.2 respectively.

Table 3.3 - Diffusion Coefficients of Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	D (10^{-11} m^2s^{-1})	D_G (10^{-11} m^2s^{-1})
8.5	6.0 ± 0.6	5.5 ± 0.6
17.0	4.5 ± 0.4	5.1 ± 0.3
34.0	3.5 ± 0.2	3.9 ± 0.2

Table 3.4 - Diffusion Coefficients of Aldehyde Dehydrogenase at pH 5.2

Conc. (μM)	D (10^{-11} m^2s^{-1})	D_G (10^{-11} m^2s^{-1})
*8.5	5.4 ± 0.4	5.8 ± 0.2
*17.0	6.1 ± 0.2	6.1 ± 0.2
*34.0	5.5 ± 0.2	6.6 ± 0.6
**8.5	5.0 ± 0.1	7.7 ± 0.4

* and ** see Table 3.2.

In order to determine how closely the calculations from the two different methods resembled each other, the differences between A and A_G, and$(A/H)^2$ and $(A_G/H)^2$ were calculated as percentages of A and $(A/H)^2$ respectively for the samples at both pH 7.4 and 5.2 (Appendix 1, Tables A1.7 and A1.8).

3.3.4 Determination of the Molecular Weight of Aldehyde Dehydrogenase

The molecular weight of the enzyme was calculated via the sedimentation and diffusion method, which is based on the Svedberg equation, and also via the Stokes - Einstein equation and using the assumption that the enzyme was spherical in shape. The sedimentation and diffusion method required that the sedimentation and diffusion coefficients used were calculated under the same conditions, in this case they were obtained from the same runs. The molecular weight was calculated from the Svedberg equation.

$$M = RTs / [D(1 - \upsilon\rho)] \qquad (3.7)$$

where M is the molecular weight

R is the gas constant, 8.314×10^3 $gm^2s^{-2}K^{-1}mol^{-1}$

T is the temperature, 298 K

s is the sedimentation coefficient, in s^{-1}

D is the diffusion coefficient, in m^2s^{-1}

υ is the partial specific volume of the enzyme, assumed to be 0.73 mL (Tanford, 1961), ρ is the density of the solution, assumed to be that of water, 1.00 gmL^{-1}

The values used of s and D were those at the lowest concentration since there was not enough consistent variation of either of these with concentration to allow extrapolation to zero concentration. The molecular weights were calculated for both pHs and using the D values obtained from both the Gaussian and non- Gaussian calculation of the areas under the peaks.

The same values of D were also used in calculating the molecular weight using the Stokes - Einstein equation and the assumption that the molecule was a sphere. The molecular weight was also calculated using the diffusion and sedimentation coefficients for all concentrations (Appendix 1, Table A1.9). The hydrodynamic radius was obtained from D via the Stokes - Einstein equation.

$$R_H = kT / (6\pi\eta D) \qquad (3.8)$$

where R_H is the hydrodynamic radius

k is the Boltzmann constant, 1.381×10^{-23} JK^{-1}

T is the temperature, 298 K

η is the viscosity of the solution, assumed to be that of water, 8.9×10^{-4}

D is the diffusion coefficient.

Using the assumption that the molecule was a sphere, and with the density of water (as in the previous method), the volume and mass of the molecule was calculated.

$$m = \rho V = \rho 4/3\pi R_H^3 \qquad (3.9)$$

where m is the mass of a single molecule

ρ is the density of the molecule, assumed to be that of water, 1×10^6 gmL

V is the volume of the spherical molecule.

From the mass of one molecule the mass of one mole of that molecule was calculated using Avogadro's number.

$$M = 6.02 \times 10^{23} m \quad (3.10)$$

where M is the molecular weight.

Table 3.5 - Molecular Weights Calculated from the Svedberg and Stokes-Einstein Equations

pH	D (10^{-11} $m^2 s^{-1}$)	M_S	M_{S-E}
7.4	6.0	168000	172000
	5.5_G	184000	224000
5.2	5.4	178000	236000
	5.8_G	166000	191000
	5.0*	193000	298000

Where M_S is the molecular weight obtained from the Svedberg equation (Equation 3.7) and M_{S-E} is the molecular weight obtained via the Stokes-Einstein equation (Equations 3.8 - 3.10) and

$_G$ denotes D obtained using assumption that peaks were Gaussian

* denotes sample that was respun a week later.

There is an uncertainty of about 10% in the values of M_S due to the uncertainty in the diffusion coefficient values, but the uncertainty in M_{S-E} is greater, owing to the equations used (Equations 3.8 - 3.10).

3.4 Discussion

3.4.1 Peak Shape and Behaviour

Each peak of the Schlieren pattern represents the sedimenting boundary of a species and, from the behaviour of the peak, information about the species is obtained. In general, the faster a peak is seen to move radially through the cell, the larger the species it represents. Increasing the speed that the sample is being spun at promotes faster movement through the cell, thus allowing smaller, slower-moving species to be observed sooner. Peak broadening suggests either self-interactions or interactions between species are occurring. A single, symmetrical peak that persists for most of the passage through the cell usually indicates that the sample is homogeneous, it contains one species only that is not undergoing any interactions. However, non-ideality and self-association reactions may compensate each other to give a Gaussian curve anyway. Certain types of reacting systems which are mixtures of macromolecular species also give a single peak.

If there is a single peak that spreads rapidly, then the solute is polydisperse, whilst the presence of two or more peaks indicates a paucidisperse solute. Systems of reacting

solutes which have pressure-dependent equilibrium constants can give anomalous Schlieren patterns. Peaks which move through the cell in a few minutes indicate a rapidly sedimenting species which is often the aggregation of part of the sample. If a rapidly sedimenting boundary occurs in a protein sample, it is very likely due to the presence of denatured protein aggregating.

3.4.2 Peak Behaviour of Aldehyde Dehydrogenase

Both the samples at pH 7.4 and at pH 5.2 gave a single peak. No other peaks were observed, even when spun at the maximum available speed of the ultracentrifuge. Whilst, in all cases, the peak moved fairly quickly through the cell, indicating a fairly large species present, as expected for aldehyde dehydrogenase, it did not move so fast as to suggest that the sample had denatured and was aggregating.

At first inspection the peaks all looked Gaussian in shape for the duration of each run. The peak in the first photograph for each run did not appear to be quite Gaussian, but this was because the photograph was taken early in the run, before the peak had moved completely clear of the meniscus. Concentration did not seem to affect the Gaussian shape of the peaks. The only concentration effect seemed to be the reduction in the height and size of the peaks and a subsequent slight broadening of them at lower concentrations, as was expected since the area under the peak is a function of concentration. The peak of the most dilute, pH 5.2 sample, that was left for a week before being respun, was noticeably smaller in size than its earlier counterpart.

A stringent way of determining if the peaks were truly Gaussian was if the area under each peak was the same as the area calculated by other means. Whilst the two areas occasionally came out to be the same, they generally differed. The differences between the areas and the $(A/H)^2$ values did not seem to exhibit any consistent variation with concentration, pH, or the progression of the run, although the respun sample at pH 5.2 did show considerably greater differences between the values calculated by the two methods than any other sample, including its first run. However, on the whole, the areas calculated using the Gaussian assumption were larger, to varying extents, than those calculated by the rectangular approximation method.

The samples at pH 5.2 gave slightly smaller peaks than their counterparts at pH 7.4. However, all runs were of very similar length, namely about one and a quarter hours. The last run, that of the sample at pH 5.2 which had already been spun once before, a week previously, was the longest in length, at about one and a half hours duration.

3.4.3 Sample Behaviour

There was the possibility that, due to the enormous pressures exerted on the sample in the ultracentrifuge, aggregation and/or denaturation of the protein may have occurred to some extent. This would have been expected to have altered the peak shape and behaviour, but the peaks remained approximately Gaussian in shape and gave values of the diffusion coefficient consistent with the known size (212 000) of the enzyme. As stated previously,

whilst the sample sedimented fairly quickly, it did not seem to sediment so rapidly as to suggest that aggregation and/or denaturation had taken place.

There was not as great a difference between the values calculated for the diffusion coefficient at pH 7.4 and pH 5.2 as was expected from the gel chromatography results of Buckley *et al.* (1991) who found that significant dissociation of the enzyme occurred at pH 5.2 and, from activity assays (Appendix 1, Tables A1.10 and A1.11), the sample at pH 5.2 seemed to retain its activity after ultracentrifugation to a greater extent than expected. So there is a possibility that the pressures of the ultracentrifuge discouraged the dissociation expected at the lower pH. However, once the pressure was no longer exerted, if the dissociation at pH 5.2 was indeed pressure dependent, dissociation and a further decrease in activity would be expected to occur. The activity did not decrease to the extent expected, over a period of time after the samples were subjected to the ultracentrifuge. Gel column chromatography results showed the presence of a major species of considerably smaller size than that at pH 7.4. However, the possibility of a pressure-promoted association of the enzyme at pH 5.2, to its tetrameric form, as a consequence of ultracentrifugation, so that the enzyme behaved similarly to the sample of it at pH 7.4 is not likely.

3.4.4 Sedimentation Coefficient of Aldehyde Dehydrogenase

The sedimentation coefficient is the rate of sedimentation of the average of all the molecules in a sample. It is measured by recording the position of the boundary in the ultracentrifuge cell which appears as the maximum of a Schlieren peak. The movement of this peak is obviously dependent on the speed that the sample is spun at but this is corrected for in the sedimentation coefficient calculations. The sedimentation coefficient was determined for a series of dilutions and then extrapolated to obtain the sedimentation coefficient at infinite dilution. This was done because the sedimentation coefficient is a function of the concentration in general. The corrected sedimentation coefficient, $S_{20,w}$ is a standard value for the sedimentation rate of the sample in a solvent with the density and viscosity of water at 20 °C. Since the sedimentation coefficient is a function of concentration, $S_{20,w}$ should be obtained by similar extrapolation to zero concentration. However, $S_{20,w}$ was not calculated due to the number of errors inherent in the calculations. In order to obtain $S_{20,w}$ the partial specific volume of the solute is required. This is not known for most proteins so the ultracentrifuge manual (Chervenka, 1969) suggests that a known value for a protein is used since partial specific volumes are similar for most proteins. The density of the solute is also required. For the purpose of this experiment the density of aldehyde dehydrogenase has been assumed to be that of water, so this introduces two assumptions into the calculation of $S_{20,w}$. Also, the further the temperature moves away from 20 °C, the greater the uncertainty of the extrapolation. Whilst it is useful to convert the experimental values to those under standard conditions, for some samples, such as some proteins, the uncertainties in the calculations seem too great for the final result to be meaningful to any great extent.

The sedimentation coefficient is expected to increase as the concentration decreases but not necessarily in a linear manner. This increase in the sedimentation coefficient is at least partly due to the frictional effect between the molecules which increases as they get closer together.

From the results obtained at both pHs, a linear extrapolation was not possible and the only trend was the expected one of increasing sedimentation coefficient with decreasing concentration. Whilst it was not possible to obtain an extrapolated sedimentation coefficient value at zero concentration, the values at the lowest concentration were sufficient indication that the enzyme at both pHs has a sedimentation coefficient value of approximately 11 S.

The fact that the sedimentation coefficient for the most dilute sample at pH 5.2, that was respun later, did not change from its previous value is an indication that the enzyme did not undergo any significant structural changes on standing.

The values of the sedimentation coefficient were very similar for all the samples, especially between the same dilutions at both pHs. This similarity indicates that it is the same system present at each pH and that there has been no radical change to the enzyme upon dialysis to the lower pH. The differences in the sedimentation coefficients at the two pHs would be due to salt and buffer effects that may have modified the enzyme's structure slightly. The points were all roughly within range of each other and if the experiment were to be repeated the values would vary somewhat from those obtained since the sample under analysis was part of a biological system with a multi-component solvent containing salts, etc, instead of a pure single solvent and the calculations are based on the assumption that the sample was in a single solvent.

The main aim of the ultracentrifuge experiments was to observe if association - dissociation occurred at the two separate pHs. The results of the sedimentation coefficient values showed that the same system was present at both pHs and that dissociation did not seem to have occurred.

3.4.5 Diffusion Coefficient of Aldehyde Dehydrogenase

As well as a sedimentation coefficient, a diffusion coefficient was able to be obtained from the ultracentrifuge experiments. The diffusion coefficient was determined from the spreading of the sharp boundary, formed in the cell by the solute, with time. The occurrence of sedimentation at the same time was not an obstacle to determining the diffusion coefficient and indeed it was very useful to obtain both the sedimentation and diffusion coefficients for the same sample under identical conditions, (ie from the same run). Since the diffusion coefficient is a function of concentration, like the sedimentation coefficient, a series of dilutions of the sample allowed observance of whether the sample exhibited appreciable concentration dependence, and if this was the case, extrapolation to zero concentration became possible in theory.

Whilst correction of the diffusion coefficient to standard conditions of 20 °C in a solvent with the viscosity of water is sometimes an option, the problems associated with the correction are similar to those associated with the correction of the sedimentation

coefficient to standard conditions, the temperature correction is an especial uncertainty. Since these experiments were performed to be used in comparison with the laser light scattering results, all measurement were made at 25 °C and no corrections were made.
The diffusion coefficient obtained from the ultracentrifuge experiments was the apparent diffusion coefficient, whilst that from the light scattering was the mutual diffusion coefficient. At the low concentrations used, the values should be very similar since divergence between the two types of diffusion coefficient does not occur until much higher concentrations are reached.
Calculation of the diffusion coefficient was obtained by using the initial slope of the plots of $(A/H)^2$ versus t. The advantage of using the assumption that the peaks are Gaussian is that less measurements are required for the calculation of the area under each peak, as well as a comparison of this area with that obtained by another method to allow an idea of how Gaussian in shape the peaks actually are.
The values of the diffusion coefficient calculated by the two separate methods for each run were very similar and within error region of each other. This was taken as an indication that the peaks (with the exception of the final respun sample) were all fairly Gaussian in shape. This was also indicated by the differences in the areas calculated by the two methods (Appendix 1, Tables A1.7 and A1.8). As seen from Table A1.8 there was a large difference between the two calculated areas for the final run of the respun sample at low pH. This lack of Gaussian character was reflected in the values obtained for the diffusion coefficient. The value obtained from the area calculated by the rectangular approximation method overlaps that of its earlier counterpart, but that calculated using the Gaussian assumption has an anomalously high value. The sedimentation coefficient for this sample was also the same as its earlier counterpart, as is its other non-Gaussian value of the diffusion coefficient. All this combines to indicate that the peak from that run was not Gaussian and that the values obtained from the Gaussian calculation should be discarded.
All the values of the diffusion coefficient obtained were very similar and further extrapolation to zero concentration could not be reliably performed to obtain a significant value since the error region increases as extrapolation towards zero concentration occurs. However, values from the lowest concentration in each case indicated a value for the diffusion coefficient of approximately $5.5 - 6.0 \times 10^{-11}$ m^2s^{-1} which was comparable to that obtained from the light scattering.

3.4.6 Molecular Weight of Aldehyde Dehydrogenase

Calculation of the molecular weight using both the sedimentation and diffusion (Svedberg equation) method and the method based on the Stokes-Einstein equation and the spherical molecule assumption gave mainly values in agreement at both pHs. A value of 0.73 mL was assumed for the partial specific volume in the Svedberg equation. This was the partial specific volume of catalase which has a similar molecular weight and sedimentation coefficient to that of aldehyde dehydrogenase (Tanford, 1961). The assumption of the partial specific volume based on a similar sample is an often-used

assumption when the actual value is not known.
The values obtained for the molecular weight by both methods, using values of the diffusion coefficient obtained in the two different ways, on the whole, gave similar values for the molecular weight. At pH 7.4, using the non-Gaussian diffusion coefficient, the molecular weights from both the Svedberg and the other method were within 2% of each other and within 21% of the expected molecular weight of 212000. All the values at both pHs obtained using the Svedberg equation were very close together, and whilst the value for the final, respun sample was somewhat different from these values, it was only closer to the expected 212000 molecular weight of the tetramer. The difference in the molecular weight from 212000 could be due to the values of the sedimentation and diffusion coefficients used not being those at zero concentration, due to the non-consistent variation of those values with concentration ruling out the possibility of extrapolation to zero concentration.
Differences between the molecular weights obtained using the two methods of calculating the molecular weight could also be due in part to the Svedberg method giving the molecular weight for the anhydrous molecule, whilst the Stokes-Einstein equation gives the hydrodynamic radius of the molecule, from which the molecular weight is calculated.

3.4.7 Aldehyde Dehydrogenase and the Ultracentrifuge

Thus from results, both pHs seem to yield the same system, that of the tetrameric form of the enzyme with a known molecular weight of 212000. The possibility of pressure effects causing the continued association of the enzyme at pH 5.2 is unlikely. Pressure effects in the ultracentrifuge are rare and usually occur when very large molecules (much larger than aldehyde dehydrogenase) are used. As stated earlier, the peaks showed no signs of pressure related broadening or other effects.
The similarity of the values of the sedimentation and diffusion coefficients for the enzyme at both pH's, and over time, indicates that the same system was present in each case. Differences in these values are due to the sample being a multicomponent system with salt and buffer effects being responsible for possible slight modifications to the enzyme structure, but no major conformational changes were observed.
The main aim of the ultracentrifuge experiments was to investigate the association - dissociation expected to occur at pH 7.4 and pH 5.2 respectively. Since these studies showed no evidence of this behaviour, no further ultracentrifuge studies were carried out. As there was no major conformational change, just a constant system, this technique was used for comparison of the diffusion coefficients (and calculated molecular weight) with those obtained from the light scattering experiments.

Chapter 4
Laser Light Scattering Studies on Aldehyde Dehydrogenase

4.1 Introduction

4.1.1 Introduction to Laser Light Scattering

Laser light scattering has been used in the determination of the molecular weights of various polymers. In this instance the technique was used to investigate the association - dissociation behaviour of cytosolic aldehyde dehydrogenase from sheep liver. Concentration, pH and the presence of cofactors, singly or together can affect the association - dissociation behaviour of the enzyme.

Dynamic light scattering is a non-perturbing process performed on only very small quantities of sample, hence many studies may be carried out on the same sample. The technique requires the sample to be suspended in a clear liquid, such as water. Dust is a major source of inaccuracies and false data, but filtration and a generally dust-free environment prevent it being a significant problem.

The sample is placed in a constant temperature water bath in the direct path of the laser beam. The laser light beam is scattered when it hits a particle. The intensity of the scattered light is a function of the scattering particle's radius, and whilst ideally, the scattering particles are all of the same size, in biological systems in particular the sample may be polydisperse. The larger the particle, the more the light is scattered. Large impurities such as dust cause much more scattering, sometimes overwhelming the data from the species under examination, since the intensity of the scattered light is proportional to $(\text{radius})^6$. However, a few dust particles will produce an increased intensity which fluctuates slowly, so there will be another decay due to these particles which will be so long that it will form a basically constant background and may not completely obscure the decay of interest (Cummins, 1974). The Rayleigh ratio is the intensity scattered into a unit solid angle per unit of solution divided by $2\sin^2\theta$, (Figures 4.1a - b). The Rayleigh ratio for lysozyme (molecular weight of 14000) at 1% concentration is about 20 times that of pure water (Dubin, 1970), so aldehyde dehydrogenase (with a molecular weight of 212000) should be readily observable.

4.1.2 Twinkling and Decay of Scattered Light

The scattered light from the moving particles "twinkles" because the conditions for constructive interference vary as the scattering particles diffuse in the solution. The photomultiplier monitors this twinkling and the intensity autocorrelation function of the

Figure 4.1a - Laser Light Scattering Apparatus

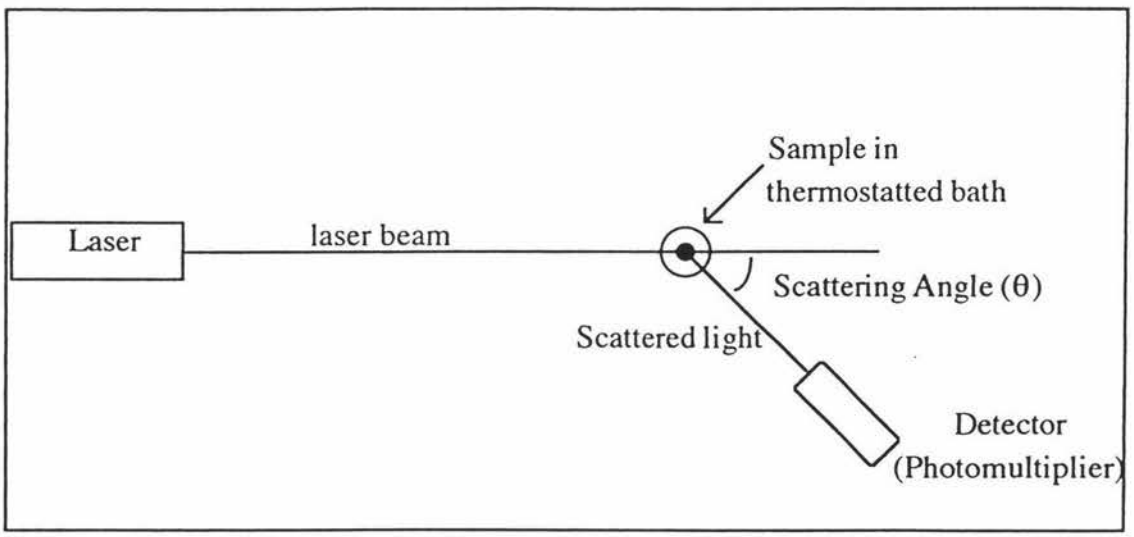


Figure 4.1b - Photograph of Laser Light Scattering Apparatus

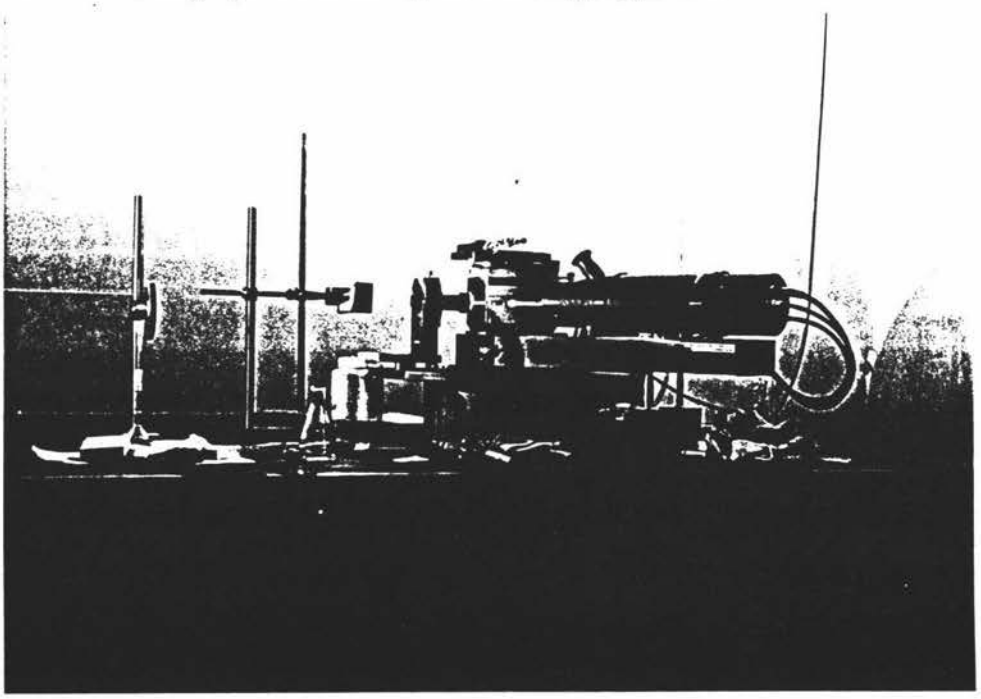
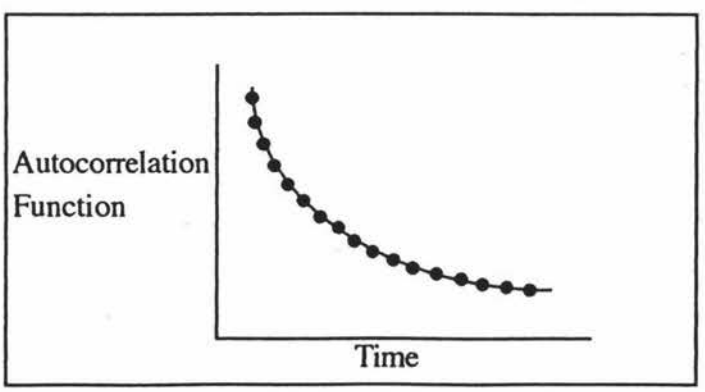


Figure 4.2 - Plot of Autocorrelation Function versus Time



scattered light decays exponentially with time. The decay is affected by particle size; the smaller the particle, the faster the decay. This autocorrelation function is "sampled" by 48 equally spaced points. The sample time is the spacing between adjacent points, (Figure 4.2).

4.1.3 Detector Angle and Sample Time

The detection angle chosen for making measurements depends somewhat on the the size of the species that scatters the light. Small particles may not generate enough signal from the scattered light at angles of 90° or more to make monitoring at those angles a viable option. The intensity autocorrelation function decays over shorter periods of time as the size of the scattering angle increases and the sample time must be altered accordingly. The run time of the instrument is the length of time over which it records the light intensity of the twinkling.

A plot of the log of the correlation function versus time should give a straight line if only one species is present. A curved plot indicates a polydisperse sample which may be due to an initially heterogeneous sample or to aggregation occurring over time. Thus the log plot provides a quick visual check on the data as they are obtained. The data are collected at a variety of angles and if the data are pure and homogeneous, similar results should be obtained at each angle. The sample time is chosen to give the best plot. If the sample time is too long then all the relevant decay information is in the first part of the plot, whereas if it is too short, not all of the information will be collected. Sample time and detector angle are both key factors in obtaining the best plot of the log correlation function versus time.

The relationship between the scattering angle, (θ), and the decay time, (t_d), is such that

$$t_d \sim [\sin^2(\theta/2)]^{-1} \qquad (4.1)$$

Thus, as θ approaches zero, t_d approaches infinity, and so the smaller the scattering angle, the longer the sample time required.

4.1.4 Data Collection

Data are collected as a set of runs (each consisting of a number of accumulations at a set sample time) which are then averaged. The number of accumulations per run is altered, depending on how strongly the sample scatters light and other aspects of the sample, such as purity and homogeneity. The runs can also be individually inspected before averaging, and those that are distorted can be excluded. During the runs there is a rejection scheme set up for the discarding of accumulations that show an unusually high scattering intensity as compared to the mean counts per sample time of the previous 10 accumulations. This has the advantage that the mean value can cope with the presence of a small amount of dirt and can adapt to small drifts in the average intensity.

4.1.5 Determination of the Diffusion Coefficient

The instrument measures the intensity correlation function, g^2, which is related to the intensity of the scattered light by the equation:

$$g^2(\tau) = [\ I(t)I(t+\tau)\ ] / [\ I(t)I(t)\ ] \qquad (4.2)$$

where $I(\tau)$ is the intensity at time τ.

This is then related to the theoretical electric field correlation function, g^1, by the Siegert relation.

$$g^2(\tau) = 1 + |g^1(\tau)|^2 \qquad (4.3)$$

g^1 is then used to determine the diffusion coefficient (D).

$$|g^1(K,\tau)| = \exp(-DK^2\tau) \quad \text{and} \qquad (4.4)$$

$$\log|g^1| = -DK^2\tau \qquad (4.5)$$

where K is the scattering vector.

Simple analysis, which assumes $|g^1|$ is a log function, then gives the plot of the log autocorrelation intensity function against τ. The diffusion coefficient is obtained from this plot. Collection of data at various angles and sample times allows the determination of the diffusion coefficient for those instances, and the diffusion coefficient may be under-extrapolated back towards zero angle or sample time in some cases.

4.1.6 Determination of the Hydrodynamic Radius and Molecular Weight

The hydrodynamic radius (R_H) of the macromolecule can be obtained from measurement of the diffusion coefficient via the Stokes-Einstein equation, for spherical molecules:

$$D = k_BT / 6\pi\eta_0R_H \qquad (4.6)$$

where k_B is the Boltzmann constant, T is the temperature and η_0 is the solvent viscosity.

The hydrodynamic radius is the radius of the "dry" particle plus its internal solvation and some externally associated solvent (Cummins, 1974). The difference between the "dry" radius and the hydrodynamic radius may be large, for example in the case of DNA from the virus fd where diffusion measurements gave a value for the hydrodynamic radius that was 4.35 times larger than the radius of a tightly packed sphere of a dry DNA molecule (Newman *et al.*, 1974). However, in that case the molecule was in the form of a loosely coiled strand, whilst aldehyde dehydrogenase is a globular protein and would be expected to exhibit less difference between its hydrodynamic and "dry " radii. Aldehyde dehydrogenase must be assumed to be spherical in shape in order to allow the Stokes-Einstein equation to be used. Using this assumption, a value for its molecular weight can be calculated since the volume of the enzyme (V) will be given by

$$V = (4/3)\pi R_H^3. \qquad (4.7)$$

The mass of the enzyme molecule (m) is obtained via

$$V = m/\rho \qquad (4.8)$$

where ρ is the density of the enzyme, assumed to be that of water. Thus the molecular weight (M) of the enzyme is given by

$$M = N_A m \qquad (4.9)$$

where N_A is Avogadro's number. The molecular weight obtained is the z-average molecular weight.

This method for calculating the molecular weight does call for certain assumptions to be made about the macromolecule being studied. The assumption that aldehyde dehydrogenase is spherical is allowable since it is a globular protein, but non-globular proteins and some polymers have R_H values greater than those predicted from their molecular weights and thus inaccuracy is introduced. The density of aldehyde dehydrogenase is assumed to be that of water. This assumption seems reasonable since the enzyme occurs in the body in an aqueous medium. The density would be expected to be within 10% of the density of water, so the error in the mass would be about 10%.

Since the molecular weight of aldehyde dehydrogenase is known to be 212000, the expected value of the hydrodynamic radius and hence the diffusion coefficient can be calculated using the equations and assumptions above. This would provide a means of determining whether the observed decay was due to the enzyme sample or some other particles or aggregates of the enzyme.

A comparison of the diffusion coefficient values could provide a quick check of whether association or dissociation has taken place.

4.2 Methodology

4.2.1 Instrumentation

The laser used was a Spectra Physics 165 argon laser and the wavelength used was 488 nm. The laser was used at 100 mW but this was sometimes increased, up to 300 mW, when the signal from the scattering was weak. The laser was not used at still higher power due to the risk of convection currents in the sample caused by the dissipation of laser energy in a small part of the sample volume.

4.2.2 Samples

The samples subjected to laser light scattering consisted of aldehyde dehydrogenase, prepared as stated previously (Chapter 2), at pH 7.4 and 5.2 in phosphate and acetate buffers respectively. A solution of Mg^{2+} was added to some of the samples to give a final concentration of 5 mM Mg^{2+}. The Mg^{2+} was dialysed into the sample or added at some point prior to the light scattering. Occasionally it was added after some scattering experiments had already been performed. Propanal was also added to some samples to give a final concentration of 20 mM propanal. Acetic acid was used to drop the pH of one

sample from pH 7.4 to pH 5.2.

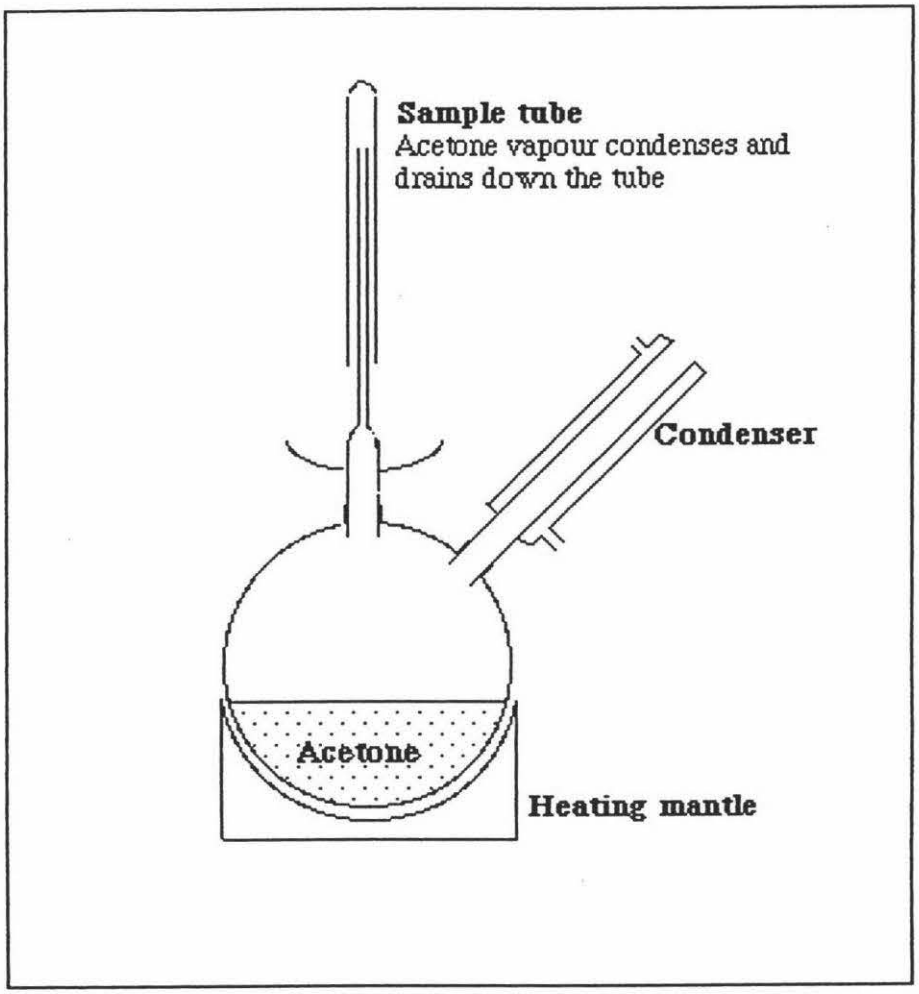
4.2.3 Cleaning of Glassware

In order to remove any large contaminants, such as dust, the samples were initially filtered through an Acrodisc 0.2 μm filter with a 0.8 μm prefilter into specially cleaned NMR tubes. The sample was filtered to a depth of just above the curved bottom of the tube. This minimum possible amount of sample was used to ensure that convection currents within the sample were kept to a minimum.
The NMR tubes and glass syringe were washed and then inverted over a spout above a flask of refluxing analytical reagent grade acetone, (Figure 4.3). The acetone vapour passed up the spout into the glassware and condensed down the inside of the glassware. This was done in order to obtain very clean, dry and dust-free glassware. Each item of glassware was rinsed in this manner for a minimum of half a minute in a dust reduced atmosphere. A fumehood was wiped down and then turned on, with the hood fully down, for at least 10 minutes before being turned off to create a dust reduced atmosphere. The glassware, still inverted, was transferred to a dessicator that had been pumped out with a water suction pump and then had had air filtered into it through a Millipore 0.2 μm filter. The dessicator was then pumped down again and left overnight, at least, until the glassware was required. Whilst the efficiency of using the fumehood to create a dust-reduced atmosphere was questionable, the above method of cleaning, drying and storing the glassware did provide dust-free glassware. This technique is regularly used in the preparation of samples by research workers in the Massey University laser light scattering group, who have no difficulty in producing dust-free samples.

4.2.4 Laser Light Scattering of Sample and Parameter Selection

The filtered sample was placed in the water bath set at 25 °C in the path of the laser so that the laser beam passed through the centre of the sample. If there were a large volume of sample present the tube was adjusted until the beam passed just below the surface of the sample. This minimised problems due to the settling of larger particles as they dropped down through the sample. The sample was left to equilibrate for a minimum of 30 minutes before scattering experiments were commenced. The twinkling pattern of light resulting from the scattering could be observed on the wall in front of the laser.
The signal was obtained by 48 equally-spaced channels, each one represented on the oscilloscope trace. The angle and sample time for each sample were chosen based on the behaviour of the oscilloscope trace and the amount of light scattered.
If the trace moved upwards on the screen in a rapid and uneven manner, this indicated that the sample was not clean and that large particles, such as dust, were passing through the beam. Where possible in these cases, the detector was set at larger angles, such as 90°, since the majority of the light from the dust particles was scattered forwards. However, if the sample was only weakly scattering a smaller angle was chosen as the time taken to collect data at the larger angle would be too long to be practical.

Figure 4.3 - Apparatus for Cleaning Glassware



The sample time chosen was also influenced by the behaviour of the trace. If the sample time was too long, all the relevant information about the decay would be contained in the first few channels of the trace. If the sample time were too short, not all the information about the decay would be obtained. The decay occurs over shorter periods of time as the detector angle is increased (Equation 4.1), so a smaller detector angle, requires a longer sample time. In general, the larger the particles, the longer the decay, and the longer the sample time required. For a homogeneous, clean sample the data obtained should be very similar, regardless of detector angles used. The ideal plot was a single straight line. If the plot was curved, different sample times for the different decays were used to determine, where possible, the diffusion coefficients of the different species present.
Each accumulation consisted of data collected over a length of time set by the operator. For very clean samples data were collected over a longer period. Thus more data were obtained per individual accumulation, so shortening the overall time taken for the experiment. The actual times for each accumulation ranged from as little as 0.4 s to 10 s. When there was a lot of signal present, due to strong scattering from a concentrated sample, smaller accumulation times were chosen to avoid overwhelming the photomultiplier with signal.

4.2.5 Data Collection

The data accumulations were collected in sets of runs which were then averaged. A rejection scheme discarded accumulations which varied considerably from the mean counts per sample time of the previous runs. If the sample was not very clean and too many accumulations were selected for a run, many rejections might occur, the data would take too long to collect and would be somewhat suspect, due to the repeated rejections. Each run was able to be inspected and if too many rejections had occurred, (this was an arbitrary amount selected by the operator and influenced by the cleanliness of the sample, the number of accumulations per run and the total number of runs) the run was discarded. All the remaining runs were averaged and a plot of these was obtained. The first point of the plot was often discarded as it was often anomalously high due to photomultiplier after-pulsing. Sometimes, for very weakly scattering samples, the first few points were unusually high and so were discarded when the sample time used was very short.

4.2.6 Obtainment of the Diffusion Coefficient

The computer program written by Daivis (1989) fitted first to fourth order polynomials to the plot. The data of most interest were the values of the diffusion coefficient and the hydrodynamic radius. When these diffusion coefficients from the various polynomials were in agreement, it was an indication that the data were reliable and the sample was fairly homogeneous. In these instances, the plot was usually a straight line, or only gently curved.

4.2.7 Sample Storage

The time taken for a set of runs varied from less than one hour up to several hours. After the scattering experiment was complete, the sample was replaced in the refrigerator for subsequent use or discarded. In general the sample was not kept for further scattering experiments for longer than a week at most. Samples were not removed from the NMR tubes for further use because of the difficulty in doing this without frothing occurring with resultant loss of activity.

4.3 Results

4.3.1 Introduction

Laser light scattering was performed on various batches of aldehyde dehydrogenase over a range of concentrations and at a variety of scattering angles. The most appropriate sample time for the sample at the particular angle was chosen and was then varied somewhat to observe what effect this had, if any, on the diffusion coefficient. For homogeneous samples, data that were collected over a range of angles gave similar diffusion coefficients. The technique of light scattering is biased towards large particles. Thus, if there were any particles of larger size than the enzyme present, such as aggregates of the enzyme or dust, they would dominate the data obtained. The diffusion coefficients yielded by many samples over both long and short sample times (Appendix 2, Tables A2.1a - b) were discarded as the values were significantly influenced by the presence of very large particles, thought to be aggregates of the enzyme.

4.3.2 Aldehyde Dehydrogenase at pH 7.4

Table 4.1 shows the diffusion coefficient, (D), for aldehyde dehydrogenase at pH 7.4 collected at a scattering angle of 60° over a range of concentrations, (Conc.), and sample times (S.T.).

Table 4.1 - Laser Light Scattering Results from Aldehyde Dehydrogenase at pH 7.4.

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
15.5	50	2.4 ± 0.2	4.8 ± 0.3
	20	4.1 ± 0.2	
	15	7.3 ± 0.5	
	10	5.3 ± 0.2	
20.8	7	5.1 ± 0.3	5.4 ± 0.3
	4	5.7 ± 0.2	
52	3	4.8 ± 0.1	5.0 ± 0.2
	2	5.0 ± 0.3	
	1	5.1 ± 0.3	

These data, (Table 4.1), were observed to be sensibly concentration independent and were preferred as the diffusion coefficients are similar to those predicted by the size and mass of the enzyme molecule using equations 4.6 - 4.9 previously. Whilst there were several reasons why the measured diffusion coefficient might have been lowered from the actual value for the enzyme, the only reason for an increase in the diffusion coefficient would be a preponderance of smaller particles in the solution. However, the SDS gels run on these enzyme samples showed that the samples were not contaminated by a significant small particle population. Thus these values of the diffusion coefficient were taken as the true values for the enzyme. These diffusion coefficients were not extrapolated to zero concentration, as is often the case for diffusion coefficients obtained via this technique, since they exhibited no consistent dependence on concentration. The data did not exhibit any consistent variation with sample time within the appropriate range chosen, so extrapolation to zero sample time was also inappropriate.

4.3.3 Aldehyde Dehydrogenase and Mg^{2+} at pH 7.4

Mg^{2+} was added to the enzyme and laser light scattering was carried out on the sample after it had equilibrated in the laser beam for about 30 min., (Table 4.2a). Mg^{2+} was also added to a sample some days prior to commencement of the light scattering to observe if the length of time that the Mg^{2+} was present had any effect on the sample, (Table 4.2b).

Table 4.2a - Addition of Mg^{2+} to Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	Angle (°)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
13.9	60	15	1.4 ± 0.1	3.0 ± 0.6
		7	4.6 ± 1	
21	60	20	1.1 ± 0.1	2.1 ± 0.1
		10	2.1 ± 0.1	
		4	3.1 ± 0.1	
52	60	3	4.8 ± 0.1	5.0 ± 0.3
	90	2	4.6 ± 0.4	
	120	1	5.5 ± 0.4	

Table 4.2b - Addition of Mg^{2+} to Aldehyde Dehydrogenase at pH 7.4 some days prior to Laser Light Scattering. Data was collected at a detector angle of 60°.

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
15.5	20	3.7 ± 0.1	4.6 ± 0.1
	10	5.4 ± 0.1	

The majority of the samples exhibited curved plots, if not two distinct slopes. This indicated that a range of scattering particle sizes were present. The diffusion coefficient increased as the sample time decreased. This effect was most easily observed for the most concentrated sample. This was due to the increased signal to noise ratio of the scattered light allowing shorter sample times to be used. Comparison with the sample which suffered no additions, (Table 4.1), indicated that the samples had aggregated on the addition of Mg^{2+}.

Whilst there was a definite decrease in the diffusion coefficient of most of the samples, this appeared to be a move towards aggregation of the enzyme rather than a uniform increase in size of all the enzyme particles present. The sample of 52 μM is an exception to this statement. The addition of Mg^{2+} had no discernible effect on the enzyme at this concentration, but the other samples exhibited a trend towards aggregation of the enzyme, especially if it was already predisposed towards aggregation, as many of the samples were (Appendix 2, Table A2.1a). The length of the time that had elapsed after the addition of Mg^{2+} and before light scattering was commenced did not seem to have any effect on the sample. The enzyme still displayed signs of aggregation, but the aggregation did not seem any more pronounced than for those samples that had been treated with Mg^{2+} immediately prior to the commencement of laser light scattering.

4.3.4 Aldehyde Dehydrogenase and Propanal at pH 7.4

Propanal was also added to the enzyme to observe if it caused any change to the sample, since it is a known substrate for aldehyde dehydrogenase, (Tables 4.3a - b). All data were collected at a scattering angle of 60° and the concentrations quoted were the original concentrations before propanal was added or laser light scattering was commenced.

Table 4.3a - Addition of Propanal to Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
15.5	70	0.036 ± 0.003	0.031 ± 0.001
	100	0.036 ± 0.002	
	500	0.0258 ± 0.0003	
	800	0.0245 ± 0.0004	
21	200	0.047 ± 0.001	0.044 ± 0.001
	400	0.040 ± 0.001	
52	50	0.034 ± 0.01	0.034 ± 0.01

Table 4.3b - Addition of Propanal to Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
15.5	15	0.08 ± 0.03	0.09 ± 0.01
	20	0.077 ± 0.009	
	50	0.103 ± 0.002	
21	10	0.27 ± 0.02	0.15 ± 0.01
	50	0.069 ± 0.007	
	50	0.096 ± 0.008	
52	3	2.7 ± 0.1	1.8 ± 0.1
	6	2.0 ± 0.2	
	10	1.59 ± 0.01	
	15	1.09 ± 0.01	

The data in Table 4.3a were those obtained when the sample times chosen were appropriate to the main, long decay present. Sample times that varied somewhat to either side of the optimum sample time for each sample were also included. The data in Table 4.3b were those from the same samples when the sample times chosen were much too short for the decay present, or to determine if there was any shorter decay present. They were also chosen to provide a qualitative comparison between the "pre-propanal" data and those after the addition of propanal. As observed from the change in sample times above, the value of the diffusion coefficient decreased by a factor of approximately 100. This correspondingly indicates a hundred-fold increase in the hydrodynamic radius of the particles. Even at the short sample times there was a decrease in the values of the diffusion coefficient. I concluded that the addition of propanal promoted large scale aggregation of the enzyme.

4.3.5 Summary of the Results from Aldehyde Dehydrogenase at pH 7.4

Table 4.4 - Summary of Results from Aldehyde Dehydrogenase at pH 7.4

Additive	Conc. (μM)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
-	15.5	4.8 ± 0.3	5.1 ± 0.3
-	20.8	5.4 ± 0.3	
-	52.0	5.0 ± 0.2	
Mg2+	13.9	3.0 ± 0.6	*
"	21.0	2.1 ± 0.1	
"	52.0	5.0 ± 0.3	
** Mg2+	15.5	4.6 ± 0.1	4.6 ± 0.1
§ propanal	15.5	0.031 ± 0.001	0.036 ± 0.004
"	21.0	0.044 ± 0.001	
"	52.0	0.034 ± 0.01	
§§ "	15.5	0.09 ± 0.01	0.68 ± 0.04
"	21.0	0.15 ± 0.01	
"	52.0	1.8 ± 0.1	

* No average as sample exhibits apparent concentration dependence

** Mg^{2+} added some days prior to light scattering

§ Longer sample times used

§§ Shorter sample times used

4.3.6 Aldehyde Dehydrogenase at pH 5.2

Laser light scattering was performed on aldehyde dehydrogenase after it was dialysed to pH 5.2. The enzyme has been reported to dissociate significantly at this pH (Buckley *et al.*, 1991) and light scattering was performed to attempt to observe the dissociated enzyme, (Tables 4.5a - b). Other results were obtained at longer sample times but were subsequently discarded (Appendix 2, Table A2.2).

Table 4.5a - Laser Light Scattering Results from Aldehyde Dehydrogenase at pH 5.2

Conc. (μM)	Angle (°)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
6.8	30	20	2.6 ± 0.1	2.7 ± 0.3
		10	2.7 ± 0.2	
		7	2.8 ± 0.5	
20.6	60	10	2.91 ± 0.04	
		3	4.2 ± 0.2	4.7 ± 0.3
	90	1.5	4.5 ± 0.6	
20.6	60	3	5.3 ± 0.2	

The data from Table 4.5a showed no signs of dissociation of the enzyme as compared to the associated form at pH 7.4 (Table 4.1). The diffusion coefficient did not increase as was expected for a dissociated sample. At very short sample times, of less than 7 μs, there was no significant change in the diffusion coefficient from that of it at pH 7.4, whilst at sample times of 7 μs and greater, the diffusion coefficient decreased. This indicated that there was a mixture of particle sizes present and whilst dissociation was not detected, some aggregation had taken place.

4.3.7 Aldehyde Dehydrogenase at pH 5.2 after Ultracentrifugation

Table 4.5b - Laser Light Scattering Results from Aldehyde Dehydrogenase at pH 5.2 after the enzyme had been being subjected to Ultracentrifugation

Conc. (μM)	Angle (°)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
15	60	100	0.085 ± 0.003	0.065 ± 0.002
		300	0.079 ± 0.001	
	90	150	0.031 ± 0.001	

The sample that had been subjected to ultracentrifugation, (Table 4.5b), did however exhibit a definite decay at the longer sample times, as well as a possible slope due to a shorter decay. There was an increased amount of aggregation in the sample as compared to those samples not centrifuged.

4.3.8 Aldehyde Dehydrogenase and Mg^{2+} at pH 5.2

Mg^{2+} was also added to the enzyme at pH 5.2 and light scattering was performed on the samples, both shortly after equilibration with the water bath was reached, and also at least one day after the addition of the Mg^{2+}, (Tables 4.6a - b). All data were collected at a scattering angle of 60°.

Table 4.6a - Addition of Mg^{2+} to Aldehyde Dehydrogenase at pH 5.2

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
6.8	10	2.8 ± 0.3	2.0 ± 0.2
	20	1.2 ± 0.1	
20.6	3	3.8 ± 0.2	3.0 ± 0.2
	20	2.2 ± 0.1	

Table 4.6b - Addition of Mg^{2+} to Aldehyde Dehydrogenase at pH 5.2 some days prior to Laser Light Scattering

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
1.7	10	1.33 ± 0.04	0.84 ± 0.02
	4	0.95 ± 0.02	
	200	0.58 ± 0.004	
	50	0.51 ± 0.004	

The sample treated with Mg^{2+} at least one day prior to the start of the light scattering seemed to have a second, longer slope than the other samples, suggesting that aggregation occurred continuously over this sample time. There did not seem to be any significant effect on the diffusion coefficients from the addition of Mg^{2+} to the enzyme at pH 5.2 as compared to the initial samples with no additives present, (Table 4.5a), but the aggregation could have masked an effect.

4.3.9 Aldehyde Dehydrogenase and Propanal at pH 5.2

Propanal was also added to aldehyde dehydrogenase at pH 5.2. Data were collected at a range of scattering angles and from three different enzyme batches, (Table 4.7). The concentrations quoted were those of the samples at pH 5.2 before the addition of propanal and the commencement of laser light scattering.

Table 4.7 - Addition of Propanal to Aldehyde Dehydrogenase at pH 5.2

Conc. (μM)	Angle (°)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
20.6	60	3	4.6 ± 0.2	5.0 ± 0.2
		5	4.9 ± 0.1	
	90	3	5.5 ± 0.4	
6.8	30	10	1.8 ± 0.2	1.5 ± 0.2
		20	1.7 ± 0.2	
		50	1.1 ± 0.1	
11	60	500	0.011 ± 0.001	0.010 ± 0.001
		1500	0.008 ± 0.001	

The sample times chosen in each case were those appropriate to the various decays of each sample. Each plot showed more than one decay, indicating the presence of particles of differing sizes. At the shorter sample times above, all plots showed two slopes and the sample times were chosen for first the shortest decay and then the next one. By 50 μs, the sample time had become too long for the second slope. There was a certain amount of

sample that had aggregated to the extent that there was a third, extremely long, decay that was indicative of aggregation on a similar scale to that at pH 7.4. The addition of propanal to the sample did not promote aggregation as it did at pH 7.4. It encouraged aggregates of smaller sizes rather than very large aggregates, although evidence for aggregation was obtained from the data collected at long sample times.

4.3.10 Summary of the Results from Aldehyde Dehydrogenase at pH 5.2

Table 4.8 - Summary of Results from Aldehyde Dehydrogenase at pH 5.2

Additive	Conc. (μM)	D (10^{-11} m^2s^{-1})
-	6.8	2.7 ± 0.3
-	20.6	4.2 ± 0.3
After U/C	15	0.065 ± 0.002
Mg2+	6.8	2.0 ± 0.2
"	20.6	3.0 ± 0.2
* Mg2+	1.7	0.84 ± 0.02
propanal	20.6	5.0 ± 0.2
"	6.8	1.5 ± 0.2
"	11	0.10 ± 0.001

U/C = Ultracentrifugation

* Mg^{2+} added some days prior to light scattering

4.3.11 Addition of Acetic Acid to Aldehyde Dehydrogenase

Acetic acid was added to a sample of the enzyme at pH 7.4 in order to lower the pH to 5.2. Two successive amounts of acetic acid were added and data were collected after each addition, (Table 4.9). This was compared to sample that had been dialysed to pH 5.2, shown in Table 4.9 marked with an asterisk (*). Both samples, at each concentration, exhibited two slopes. The sample time of 100 μs seemed to be appropriate to the longer decay.

Table 4.9 - Addition of Acetic Acid to Aldehyde Dehydrogenase to lower its pH to 5.2 The concentrations quoted were those of the sample at pH 7.4 and the corresponding values after dilution since the sample was not removed from the tube during the light scattering experiment for assays to be performed on it (Appendix 2). Data was collected at a detector angle of 30°.

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})
57*	30	1.8 ± 0.1
38	30	3.0 ± 0.1
	20	3.2 ± 0.1

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})
57*	100	0.97 ± 0.06
38	1000	0.048 ± 0.002
	100	0.32 ± 0.01
	100	0.45 ± 0.01
29	100	0.26 ± 0.2

*This sample was from same batch of enzyme and was dialysed to pH 5.2. The diffusion coefficient of this sample indicated that it had undergone some aggregation. The addition of acetic acid to the sample at pH 7.4 resulted in a sample with a higher diffusion coefficient than that of the sample dialysed to pH 5.2 and of the samples at pH 7.4 (Appendix 2, Table A2.1a). This increase in diffusion coefficient, while not sufficient to be that expected of a dissociated sample of the enzyme (Equations 4.6 - 4.9), was enough to suggest that some form of dissociation had occurred, perhaps the breaking up of aggregates.

4.4 Discussion

4.4.1 Data Interpretation

The diffusion coefficient for the samples from the laser light scattering was obtained via a computer program which applied the method of cumulants to yield the z-average diffusion coefficient. The program was devised by Daivis (1989). The computer displayed a plot of log $|g^2|$ against t. For the ideal case of a non-disperse non-interacting species, a straight line would be generated, otherwise a curve would be obtained. The degree of non-linearity was an indicator of the non-ideality of the sample. First to fourth order polynomials were fitted to each curve and the diffusion coefficient was obtained from the initial slope of the log graph for each of these fits. Usually the second order fit was appropriate as the data rarely gave linear plots, but were usually a little curved. This yielded the z-average diffusion coefficient. If the data were noisy, higher polynomial fits were suspect as they placed too much emphasis on the first point of the plot, and this sometimes led to negative diffusion coefficients being obtained for the cubic and quartic fits. The errors quoted were a measure of the uncertainty of the data and thus an indication of the data quality. Noisy data gave large errors and widely disparate values of the diffusion coefficient. Clean data resulted in similar diffusion coefficients for all fits

and small errors accompanying them. Data that were very noisy and contained large errors were unreliable and so were discarded.

4.4.2 Large Particle Contamination

The sample times were chosen to optimise the plots for each run. Errors were possible if more than one slope were present, especially if one slope was very much greater than the other. If this were the case, the larger slope could have been overlooked because the data for it were contained in only the first few points of the plot. The sample time was determined by comparison to previous results as well as by trial and error after visual inspection of the data decay at an unlimited multiplicity of the sample time on the screen. Thus, instead of collecting data, for example, in batches of 10^5 x 10 μs, (ie 1 second accumulations), the data were collected at an unlimited number of 10 μs. This allowed the slope due to the sample's decay to build up on the screen and it was possible to decide if the sample time did indeed give the optimum plot of the decay.

The sample time required for the same decay increases as the scattering angle decreases (Equation 4.1). At smaller angles, large particles have a greater effect on the data, so data collected at an angle of 30° are more likely to be influenced by larger species or impurities than are data collected at 90°. However, there is less light scattered at 90° and so data collection takes longer. For dilute samples, there may not be enough sample present to scatter enough light at larger angles for data collection at these angles to be practicable and smaller angles may have to be used, along with their inherent sources of error for inhomogeneous samples.

The technique of laser light scattering is weighted towards large particles. If there are large particles present in a sample, they will be observed, often at the expense of the smaller particles present. Thus the technique exhibits size selectivity whereby it "selects" large particles. This could influence the sample time chosen, so that data are collected on the larger species present, which might be a contaminant of some sort, thus overshadowing the smaller species. The larger contaminants might be aggregates of varying sizes of the particles, impurities such as dust particles, or, in the case of biological systems, they might be other proteins. Only a small quantity of large particles is enough to be misleading about the actual decay to be observed. A rejection scheme was set up to minimise the effect of any dust particles or other large contaminants randomly passing through the laser beam. If the sample were not very clean, or else very inhomogeneous, many rejections would occur and the data would be suspect.

4.4.3 Photomultiplier Artifact

The photomultiplier, at short sample times produced an artifact in the first point of the plot, (or more, depending on how short the sample time was) that was excessively high. This after-pulsing affected the estimation of the diffusion coefficient and these points were discarded before a polynomial was fitted to the slope. For each set of data a decision was made as to how many of the points were to be discarded. There was also the

possibility that at longer time scales the higher point(s) might have been a sign of the presence of another much faster decay. Whilst a first point (or points) that looked unusually high was discarded, with a subsequent decrease in the diffusion coefficient, instances occurred where the point(s) was left in. This was because after-pulsing is really only a small effect and is discernible only if there is a paucity of data. The very high first point should only have occurred at short sample times (~1μs) and at low light levels. If there were plenty of light (and thus data), the first point should not be high. When the first point was high at not so short sample times, the diffusion coefficients obtained from the higher polynomial fits were examined in order to determine whether or not the point should be omitted from the calculations. If the third and fourth order diffusion coefficient values, after the first point was removed, were high and very similar, (within the errors), to those values when the first point was included, the first point was kept in the calculations. This was because these higher order fits indicated that, in the case of both polynomials, the fit went up high on the plot, near to the first point, and that therefore the signal in the first point was needed, even if the quadratic value of the diffusion coefficient decreased when it was omitted.

4.4.4 Noisy Data

A very noisy plot with a wandering baseline indicated problems caused by large particles. Some runs that were collected had rejections scattered throughout the data - some runs had a large percentage of their required number of accumulation rejected, whilst others had few, if any, rejections. This indicated a "iumpy" solution, either due to a few large aggregates or non-uniform concentration. When these passed through the laser beam, they scattered much more light than the rest of the sample and consequently caused many rejections of that data. Some polynomial fits were attempted using data that had been censored, in that only runs that had less than a certain number of rejections were used in the calculations, (those with a high number of rejections were discarded). However, it was generally found that either there was not enough signal present when the data was edited in this way, so that all runs had to used to obtain any meaningful data, or else that the diffusion coefficient when all runs were used was not significantly different from that when only certain of the runs were used.

4.4.5 Samples of Little Signal

Data collection took a long time for samples that gave little signal and the data were noisy and unreliable. In such cases the laser power was increased from the usual power of 100 mW to 300 - 400 mW. Whilst there was an initial fear of the increased laser power 'driving' the solution and causing currents in the solution due to the solution being heated by the increased laser power, as well as the momentum of the photons knocking the molecules out of the beam, no circular currents appeared in the twinkling patterns on the wall, and the data did not reflect any changes due to increase in power. The use of laser power of 400 mW on protein solutions had occurred before with no reported derogatory

effects towards the data (Gast *et al.*, 1992).

4.4.6 Diffusion Coefficient of Aldehyde Dehydrogenase at pH 7.4

The diffusion coefficients obtained were not extrapolated to zero concentration, as is often the case with such data, as they exhibited no sensible concentration dependence, and indeed also showed no consistent variation with sample time within the appropriate range selected for the samples, thereby indicating that the second virial coefficient was very small. The lack of dependence of the diffusion coefficients on concentration is not an unusual occurrence, bovine serum albumen has been shown to have a weakly concentration-dependent diffusion coefficient in both its native and denatured states as have other denatured proteins (McDonnell and Jamieson, 1976).

Aldehyde dehydrogenase was expected to have a diffusion coefficient in the vicinity of 5.5×10^{-11} m^2s^{-1} at pH 7.4, from calculations based on a molecular weight of 212000 and reasonable assumptions made about its shape and density (Equations 4.6 - 4.9). From the data in Table 4.1 (and Table 4.7), the diffusion coefficient of aldehyde dehydrogenase at pH 7.4 was found to be 5.1×10^{-11} m^2s^{-1}. This was the average of the diffusion coefficients obtained from three pure samples of the enzyme. These values followed the expected trend of increasing diffusion coefficients with decreasing sample times. The diffusion coefficient for aldehyde dehydrogenase at pH 7.4 was independent of changes in the concentration of the enzyme, although at first sight this did not appear to be the case for later enzyme samples. However dilute samples with diffusion coefficients that exhibited an apparent concentration dependence were thought to be affected by a factor that was more noticeable at lower concentrations of the enzyme, thus giving rise to the appearance of a diffusion coefficient that was dependent on sample concentration. The more concentrated the sample, the greater the intensity of the scattered light and the signal generated by it and the less important the contaminating factor would be as the signal to noise ratio of the sample would be increased. Thus contamination of the sample by aggregates would have a more noticeable effect on the diffusion coefficient at low concentrations of the sample when there was less signal present. This contaminating factor seems unlikely to be due to the technique used in cleaning the apparatus used in the light scattering as it is a technique that has been in use for 20 years, but rather seems to be a medium effect of some sort. In this case, the effect would be more noticeable at lower concentrations of the sample under study. If the solvent used were a good solvent, the diffusion coefficient would be expected to increase with increasing sample concentration, whereas if it was a non-solvent the diffusion coefficient would decrease and if it was a poor solvent the diffusion coefficient would remain unaffected by increasing concentration. From the results in Table 4.1, it can be concluded that the phosphate buffer used was a poor solvent for the sample despite being the solvent regularly used for the enzyme at pH 7.4. The factor affecting the diffusion coefficient at low concentrations of the enzyme might be the phosphate present in the buffer. The presence of salts contributing to the intensity of the scattered light has been reported by Dubin *et al.* (1973) who found that for a 6 M guanidine hydrochloride solution, the salt

scattered 21% as much light as the 1% lysozyme solution. Gast *et al.* (1992) also found that for high concentrations of denaturants and protein concentrations of the order of 1 mg/mL, the scattering contribution of the salt became comparable to that of the protein solution. A contribution from the buffer might have an effect on the overall diffusion coefficient if there was little light being scattered by the sample itself. Thus it seems possible that at low enzyme concentrations this background effect starts to dominate, causing the difficulty in observing the enzyme at these concentrations, possibly contributing to the unusually high first point(s) that were sometimes a problem. The effect would be less significant for higher enzyme concentrations or larger species with greater refractive indices.

However, many samples had a diffusion coefficient that was less than 5.1×10^{-11} m^2s^{-1} by a factor of 4 or more, (Appendix 2, Tables A2.1a - b). The sample times chosen were appropriate to the samples, but even at very short sample times, (in particular for a sample that was later found to be contaminated with both smaller and higher molecular weight species), the value of the diffusion coefficient did not increase. This indicated that the majority of the particles were of similar size and the experimental value of the diffusion coefficient was taken as that of the enzyme. Later results (Table 4.1) gave values of the diffusion coefficient around 5×10^{-11} m^2s^{-1} as previously expected. Since the only reason for the increase of the diffusion coefficient was that of smaller particles being present, and these later samples were known, by SDS gels, to be uncontaminated by a significant population of small particles, the previous results were discarded. The enzyme samples were thought to have undergone aggregation to have produced these results. This, and later results, suggested that the enzyme had a tendency towards aggregation, which was lessened, (but not eradicated), when in very pure samples.

4.4.7 Treatment of Aldehyde Dehydrogenase with Mg^{2+} at pH 7.4

Mg^{2+}, which was known to have an effect on the dissociative behaviour of the enzyme, both in promoting association and preventing dissociation at certain enzyme concentrations, was added to aldehyde dehydrogenase. At pH 7.6, millimolar concentrations of Mg^{2+} inhibit aldehyde dehydrogenase via a slowing in the rate-determining release of NADH (Bennett *et al.*, 1983). Whilst it was not expected to have much effect on the enzyme at pH 7.4 it was added to a variety of enzyme concentrations. The value of the diffusion coefficient decreased in some of the samples after the addition of Mg^{2+}, but by not much more than a factor of 2 (Table 4.2a). Some of the diffusion coefficients obtained were comparable to those obtained earlier (Table 4.1). The plots were curved and there was a trend towards larger values of the diffusion coefficient at shorter sample times (Table 4.2a), as expected from curved plots. The higher value of the diffusion coefficient of the most concentrated sample as compared to the remainder of the samples was most likely due to the presence of more signal from the sample. This would then have allowed measurements to be taken at shorter sample times and thus higher

diffusion coefficients were obtained. There was no real difference in the diffusion coefficients, between the samples that had had Mg^{2+} added less than one hour prior to laser light scattering (Table 4.2a) and those that had had it added some days previously (Table 4.2b). Aldehyde dehydrogenase has demonstrated a trend towards self-aggregation and whilst the addition of Mg^{2+} had little effect on the enzyme (Tables 4.2a - b), it is likely that aggregation effects have masked any other effects that Mg^{2+} may have had on the enzyme. The cycling of samples from refrigerator to the laser beam and water bath and back again, with the possible addition of Mg^{2+} further down the line was a probable cause of furthering aggregation.

4.4.8 Addition of Propanal to Aldehyde Dehydrogenase at pH 7.4

Propanal, a substrate in the oxidation of aldehyde dehydrogenase, was added to the enzyme at a pH of 7.4. It was known that predilution of the enzyme in the absence of propanal gave lower oxidation rates in assays when compared to non-prediluted enzyme, and that the presence of propanal protected the enzyme from the inactivating effects of predilution (Blackwell *et al.*, 1987). Thus it seemed a reasonable proposition to investigate the possible effects of propanal addition to aldehyde dehydrogenase by a technique that focussed on the size related changes taking place in the species under observation. The addition of propanal had an immediate, noticeable effect on aldehyde dehydrogenase. Two decays were present. At short sample times (Table 4.3b), the diffusion coefficient had decreased by varying factors, signifying that varying degrees of aggregation had occurred, although an apparent concentration dependence was observed as the diffusion coefficient of the concentrated 52 μM sample was considerably less affected by the addition of propanal than the other samples. However, at the long sample times appropriate to the main decay (Table 4.3a), the values of the diffusion coefficient were similar and had decreased by a factor in the vicinity of 100. Such a large increase in the hydrodynamic radius of the enzyme (about 100-fold) was not due solely to the tendency of the enzyme towards self-aggregation, but rather was a definite change to large-scale aggregation, raft formation, brought about by the addition of the propanal to the sample. The concentration effect observed was unlikely to be due to insufficient propanal being present in the higher concentration sample, as propanal was added to ensure a final concentration of 20 mM propanal in all samples. This was the same concentration that was used in assaying the activity of the enzyme (Chapter 2) (Bennett, 1981) and was present in greater than 350 - fold excess, of the enzyme concentration in the light scattering samples. The spectacular effects of the addition of propanal to low concentrations of the enzyme could be due in part to the background effect observed previously at low concentrations of the enzyme, but mainly due to modification of the enzyme by the propanal. The site, or sites, of this propanal binding are not known, nor whether the propanal binds at the active site in the absence of NAD^+, the cofactor for the enzyme catalysed reaction.

4.4.9 Summary of the Behaviour of Aldehyde Dehydrogenase at pH 7.4

The tendency towards mild self-aggregation by aldehyde dehydrogenase (a calculated increase in the size of the radius by a factor of about 4.6, to give a diffusion coefficient of about 1.2 x 10^{-11} m^2s^{-1}) might have been caused by attractions between surface charges on the enzyme molecules, or hydrogen bonding between amino acids and functional groups exposed on the surface of the molecule. The addition of Mg^{2+} did not seem to alter this, whereas the addition of propanal caused a large-scale, 100-fold increase in the hydrodynamic radius of the sample. From the sudden and dramatic decrease in the diffusion coefficient, the addition of propanal must cause an alteration to the configuration of the enzyme promoting aggregation, perhaps by exposing more charged groups, or by bringing them into a more favourable conformation for intermolecular forces to have a greater effect. There is also the likelihood that the propanal formed Schiff bases with amine groups on the enzyme, thus modifying it and possibly promoting the aggregation. This altered conformation of aldehyde dehydrogenase did not decrease its activity, so aggregation would still leave the enzyme free to react, or else, in the process of the external enzyme molecules of the aggregate becoming involved in the catalytic reaction, the entire aggregate was broken up.

Thus aldehyde dehydrogenase at pH 7.4 has a diffusion coefficient of 5.1 x 10^{-11} m^2s^{-1}, which is not significantly affected by addition of Mg^{2+} at some time prior to light scattering measurements. The addition of propanal, however , has a immediate effect on the diffusion coefficient of aldehyde dehydrogenase that is unaccounted for by the enzyme's tendency towards aggregation (Table 4.4).

4.4.10 Diffusion Coefficient of Aldehyde Dehydrogenase at pH 5.2

Laser light scattering was performed on aldehyde dehydrogenase at pH 5.2, at which pH the enzyme was known to be able to exist in both the associated and dissociated forms, in order to probe the usefulness and sensitivity of this technique in observing the dissociative behaviour of aldehyde dehydrogenase. Results obtained by Buckley *et al.* (1991) from gel filtration chromatography on the enzyme at pH 7.4 and 5.0 have shown two peaks present, an enzymatically active peak that is the tetramer, and a dissociated form of a quarter to half the molecular weight of the active peak which was proposed to be enzymatically inactive. The enzymatically inactive peak increases as concentration of the enzyme decreases at pH 7.4. At pH 5.2 there is markedly more of the minor inactive peak to begin with, and the amount of it increases as enzyme concentration and the active peak decreases until the ratio of peaks is approximately 1 : 1 (Buckley *et al.*, 1991). The enzyme concentrations quoted are indicative only of the still active enzyme. Enzyme samples were dialysed to pH 5.2 but no dissociation was observed. Instead of the increase in the diffusion coefficient (calculated from Equations 4.6 - 4.9 to then be about 8.8 x 10^{-11} m^2s^{-1} for the monomer and somewhat less than that if the dissociated species was the dimer), the diffusion coefficient (Table 4.5a) had either decreased due to the

occurrence of aggregation, or else there was very little change from the pH 7.4 values. Somewhat longer sample times than those used for the enzyme at pH 7.4 were found to be appropriate for the decay observed. When shorter sample times (<7μs) were used, the diffusion coefficient was found to be 4.7 x 10^{-11} m^2s^{-1} which was similar to that obtained at pH 7.4, so no dissociation was observed. The somewhat longer sample times required for the samples at pH 5.2 and the accompanying decrease in the diffusion coefficients were most likely due to the medium effect that was observed to have affected dilute samples previously. There was a second decay present at very long sample times, but when data were collected there was little signal present, (Appendix 2, Table A2.2), indicating that there was little sample present to contribute to the long decay and that there were few large aggregates of the enzyme present. The enzyme had still undergone aggregation despite the lower pH and supposed dissociated form.

4.4.11 Treatment of Aldehyde Dehydrogenase with Mg^{2+} at pH 5.2

Mg^{2+} was added to aldehyde dehydrogenase at pH 5.2, as at this pH Mg^{2+} was known to affect the enzyme's association-dissociation behaviour. The gel chromatography results that showed that there were two peaks, active and inactive, present at pH 5.0 and that the inactive peak was favoured more at pH 5.0 than it was at pH 7.4, also showed that the presence of Mg^{2+} favoured the enzymatically active form of the enzyme (Buckley *et al.*, 1991). Propanal is also believed to induce the associated form of aldehyde dehydrogenase at pH 5.22. When the enzyme is in this form, the addition of Mg^{2+} inhibits the enzyme by slowing the rate-determining release of NADH. However at low pH and when the propanal concentration is not high enough to cause a significant amount of association of the enzyme, addition of Mg^{2+} promotes association of the enzyme, thus activating it (Buckley *et al.*, 1991). The samples that had been treated with Mg^{2+} no more than 30 minutes before light scattering was started (Table 4.6a) showed a small decrease in the diffusion coefficient as compared to the Mg^{2+} - free sample (Table 4.5a). The presence of Mg^{2+} in the sample at pH 5.2 seemed to promote either association or aggregation. Aggregation, rather than association, seemed to be the effect promoted by the addition of Mg^{2+} to the enzyme at pH 5.2, since the enzyme had shown no sign of dissociation at pH 5.2 (Table 4.5a) and had previously exhibited a tendency towards aggregation on treatment with Mg^{2+}. The background effect that seemed to appear at low enzyme concentrations would also be a factor in the decrease of the diffusion coefficient. The sample treated with Mg^{2+} some days prior to laser light scattering exhibited two decays. The initial slope yielded a diffusion coefficient less than half that of the untreated sample at similar sample times whilst the longer decay gave a smaller diffusion coefficient by a factor of two again (Table 4.6b). When the Mg^{2+} was added to the sample some days prior to commencing light scattering, it appeared that the sample had kept on

aggregating. The smaller particles (seen at <7 μs for the enzyme alone at pH 5.2) had disappeared. Any effect that Mg^{2+} might have had on the association-dissociation behaviour of aldehyde dehydrogenase was masked by the aggregation of the sample. Again, the dilute nature of the sample may have contributed to the decrease in the diffusion coefficient via the smaller signal to noise ratio of the scattered light.

4.4.12 Addition of Propanal to Aldehyde Dehydrogenase at pH 5.2

Propanal was also added to aldehyde dehydrogenase at pH 5.2. Unlike the same addition at pH 7.4, widespread large-scale aggregation did not occur. At short sample times (< 10 μs), (Table 4.7), there was no discernible change from the diffusion coefficients of the non-propanal sample (Table 4.5a), and at an average value of 5.0×10^{-11} m^2s^{-1}, they were very similar to the diffusion coefficient of the enzyme at pH 7.4 (Table 4.1). This agreed with the results obtained by Buckley *et al.* (1991), who found that at decreasing concentrations (down to 4.4 μM) at pH 5.2, the ratio of dissociated species to associated enzyme increased to maximum of about 1:1. However, they also found that addition of propanal provided 100% protection against the partial dissociation of the enzyme at pH 5.0 (Buckley *et al.*, 1991). In that case, the expectation would be for the enzyme to undergo aggregation to a similar extent as was observed for the samples at pH 7.4. Thus the results demonstrated that not all the enzyme dissociated at pH 5.2. Even at these short sample times there was another decay present. At a smaller scattering angle, the diffusion coefficient was smaller (Table 4.7) and somewhat less than (by about a factor of two) that at the same angle for the non-propanal sample (Table 4.5a). This slope was more influenced by the larger particles present as it was at a smaller scattering angle. Another, extremely long, decay was present (Table 4.7) and was indicative of aggregation on a similar scale to that at pH 7.4, but there was very little of this present. Overall, the addition of propanal did not promote aggregation at pH 5.2 as it did at pH 7.4. There was a range of decays, but these were mainly indicative of smaller-scale aggregates, as had occurred in non-propanal samples at both pH's. There was also some sample present that was non-dissociated enzyme, or else it was dissociated enzyme that had aggregated to a similar size as the associated form of the enzyme. The latter suggestion was unlikely as no very fast decay corresponding to the dissociated form of the enzyme was present in any of the samples (with or without any additives), and all aggregation observed was greater than 2 - 4 units of the enzyme combining. Whereas at pH 7.4, the propanal was postulated to have caused a conformational change, most likely by binding to the enzyme in some way that did not affect the active site, but promoted aggregation through increased ease of inter-enzyme attraction, at pH 5.2 this did not occur. This may have been caused by the protonation of amino groups on the enzyme at pH 5.2 that otherwise may have formed Schiff bases with propanal and undergone greater aggregation, as was postulated to have occurred at pH 7.4. Alternatively, the conformational change caused by the addition of propanal may have taken place near the active site of the enzyme or else somewhere that was altered or destroyed during dissociation, and was thus unable to

occur when the enzyme had dissociated. The small amount of very large aggregates present would have been due to the propanal-assisted aggregation of the non-dissociated enzyme. Again, attractive forces between dissociated enzyme units would have caused the aggregation observed.

4.4.13 Aldehyde Dehydrogenase at pH 5.2 after Ultracentrifugation

A sample of aldehyde dehydrogenase at pH 5.2 was subjected to ultracentrifugation (Chapter 3) and then to laser light scattering (Table 4.5b). The data were collected at long sample times and whilst there may have been a short decay present, it was obscured by the long decay, (ie if there was a short decay it would have been in the first few points of the long slope). The long decay seemed to be the main decay present and the values of the diffusion coefficient were indicative of very large aggregates. Not only were these aggregates some of the largest present at either pH, but the data were collected at scattering angles that were not as subject to influence by large particles as other, smaller angles were that gave small diffusion coefficients. These extremely small, (for aldehyde dehydrogenase), diffusion coefficients may have been due to the sample being first spun in the ultracentrifuge. If the sample had a tendency towards aggregation, the time spent in the ultracentrifuge would have promoted this. The ultracentrifuge tends to compact samples as they move through the cell, leaving pure solvent behind them. Even if the sample did not sediment completely, but remained in solution, (as the enzyme sample that was then subjected to light scattering did), a certain amount of compacting would have occurred and this would certainly have caused a substantial concentration increase anyway. This would have provided very favourable conditions for aggregation of an enzyme that already had tendencies in that direction.

4.4.14 Summary of the Behaviour of Aldehyde Dehydrogenase at pH 5.2

At pH 5.2, aldehyde dehydrogenase showed no signs of dissociation but rather its diffusion coefficient remained similar to that of the associated form at pH 7.4 or exhibited some aggregation. Treatment of the sample with Mg^{2+} gave a diffusion coefficient indicative of the occurrence of some aggregation that became a greater amount of aggregation when the treatment occurred some days prior to the light scattering. Addition of propanal did not cause the massive decrease in the diffusion coefficient that it did to the enzyme at pH 7.4. The diffusion coefficient for the sample that had first been subjected to ultracentrifugation showed that the enzyme had undergone aggregation, (Table 4.8).

4.4.15 In situ pH drop from 7.4 to 5.2 of Aldehyde Dehydrogenase

Acetic acid was added to a sample of aldehyde dehydrogenase at pH 7.4 in order to drop the pH to pH 5.2. A predetermined amount of acetic acid was added to the sample and after light scattering was performed on it, a further amount of acetic acid was added. Acetic acid was used to drop the pH instead of hydrochloric acid as addition of

hydrochloric acid caused precipitation of the enzyme, and acetic acid - acetate buffer was the buffer used when the enzyme was dialysed to pH 5.2. The acetic acid was filtered before addition to the sample and no contamination of the sample by dirt of any sort resulted from the addition of the acetic acid. The results (Table 4.9) were compared to a sample of the enzyme that had been dialysed to pH 5.2 and to results obtained at pH 7.4. Unfortunately the enzyme sample used was one which had undergone aggregation at pH 7.4, so the diffusion coefficient at pH 7.4 was not that of the non-aggregated enzyme (Appendix 2, Table A2.1a). Likewise for the enzyme sample dialysed to pH 5.2. When acetic acid was added to the sample, the diffusion coefficient increased from the values at pH 7.4. Whilst this value was not that expected of the dissociated species, or even as high as that of the non-aggregated tetramer, it showed that the drop in pH had caused some dissociation and had broken up the aggregates to some extent in order to do so. There was also a longer decay present in both the acetic acid added and dialysed samples.The longer decay had a diffusion coefficient that was about 1/3 to 1/2 that of the dialysed sample, indicating that some larger aggregates remained, despite the addition of the acetic acid. The data were noisy, possibly due to a 'lumpy' solution as was expected if the acetic acid was breaking up some of the aggregates. The laser power was increased to obtain more signal. The diffusion coefficient obtained at higher laser power was similar to that at lower power. The data collected after the second addition of acetic acid were noisy, and allowing for the large errors in the data, the diffusion coefficient was similar to those obtained after the first addition of acid. Addition of acetic acid which lowered the pH to pH 5.2 broke up the aggregates (and may have caused some dissociation) thus supporting the previous results that showed that at pH 5.2 aggregation was not as prevalent as at pH 7.4.

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

5.1 Introduction

5.1.1 Introduction

The behaviour of aldehyde dehydrogenase at elevated temperatures was observed in order to investigate the expected unfolding and to determine the magnitude of the change in size of its diffusion coefficient and thus its hydrodynamic radius. Denatured proteins can unfold to varying degrees and light scattering is a technique that is useful for monitoring the overall changes that occur in the dimensions and conformation of the protein, as opposed to the active site changes in the local structure of the protein that can be probed spectroscopically or by activity assays. Thermal denaturation was chosen as the method for promoting unfolding as it was an *in situ* method that also allowed the sample to be returned to pre-denaturation conditions and did not cause immediate precipitation of the enzyme as the addition of acid did. The advantage of returning the sample to pre-denaturation conditions was that it allowed the possibility of investigating the reversibility of the unfolding process. However there was also the likelihood that hydrophobic groups exposed during the unfolding process would lead to aggregation of the enzyme thus preventing the reverse process from occurring.

5.1.2 Denaturation of Other Proteins

In acidic conditions, Gast *et al.* (1992) found by light scattering that the three proteins, streptokinase, apo-cytochrome c, and α-lactalbumin, all increased their hydrodynamic radii by 12 - 86%. Gast *et al.* (1992) also found that the hydrodynamic radii of streptokinase and α-lactalbumin increased by 40 - 55% when denatured by guanidine hydrochloride and by 11 - 15% when thermally unfolded. Lysozyme has also been studied under similar conditions by light scattering (Dubin *et al.*, 1973, Nicoli and Benedek, 1976). Dubin *et al.* (1973) found that the hydrodynamic radius of lysozyme, at pH 4.2, increased by up to 45% upon addition of increasing concentrations of guanidine hydrochloride. Nicoli and Benedek (1976) reported that the thermal denaturation of lysozyme, at pH 1.45, produced an increase in the average radius by 18%. In all the above cases these globular proteins have increased the size of their hydrodynamic radii when subjected to various denaturing conditions by up to a maximum of 86%. This leads to the expectation that the unfolded form of aldehyde dehydrogenase caused by thermal denaturation will be measurable by light scattering unless the enzyme's previously

demonstrated tendency towards aggregation asserts itself as unfolding proceeds.

5.2 Methodology

A sample of aldehyde dehydrogenase of activity 30.4 μM, pH 7.4, was filtered in preparation for light scattering and placed in the laser beam in the water bath at 25 °C. At the same time a control sample was placed in a thermostatted water bath at the same temperature. Throughout the light scattering temperature experiment, the control sample was subjected to the same temperature changes and for the same amount of time, as the sample in the laser beam. Aliquots were removed from the control sample and assayed in the usual manner before each temperature increase. After the first batch of light scattering at 25 °C, the samples were kept in the refrigerator overnight and then briefly subjected to light scattering at 25 °C again before the temperature was increased to 40 °C in 5 ° increments. Once the required temperature was reached, time was allowed for the sample to equilibrate, about 20 minutes, before light scattering was performed. After light scattering was completed at 40 °C, the temperature was lowered to 25 °C and the samples were left at that temperature overnight. The next day, light scattering was performed on the sample at 25 °C and then again at 40 °C.

5.3 Results

The data were collected at a scattering angle of 60°. The diffusion coefficients obtained via light scattering are reported in Appendix 3, Table A3.1. The initial activity of the sample was 30.4 μM and the A_{280} was 0.3005. The activities of the control sample at the various temperatures experienced by the light scattering sample are reported in Appendix 3, Table A3.7.

The measurements of the diffusion coefficients were made over a total of 3 days. On the first day, some measurements of the diffusion coefficient at 25 °C were obtained. These values varied somewhat and scattering was repeated on the following day before the elevated temperature measurements were made. The diffusion coefficients at 25 °C were averaged and this average was nominated as the reference value. This value was in agreement with diffusion coefficients obtained previously. The averages of the diffusion coefficients (Appendix 3, Table A3.3), were plotted against the sequential temperatures used (Figure 5.1), as well as the averages of the diffusion coefficients relative to the diffusion coefficient at 25 °C, (Appendix 3, Table A3.4), (Figure 5.2). The diffusion coefficients, despite an initial increase, subsequently displayed significant decreases from the prior values of the diffusion coefficients as the temperature was altered. The initial runs at 25 - 35 °C displayed high initial points on the computer plot of the scattering trace. These points were discarded where possible, but in the majority of cases, they were kept as they seemed to contain genuine signal from the sample and were not due to after pulsing of the photomultiplier, (Chapter 4). The measures of hydrodynamic radius at each temperature, (Appendix 3, Table A3.5), were averaged, (Appendix 3, Table A3.6). Theses averages were plotted against temperature (Figure 5.3). The averages of

Figure 5.1 - Average Diffusion Coefficients versus Temperature

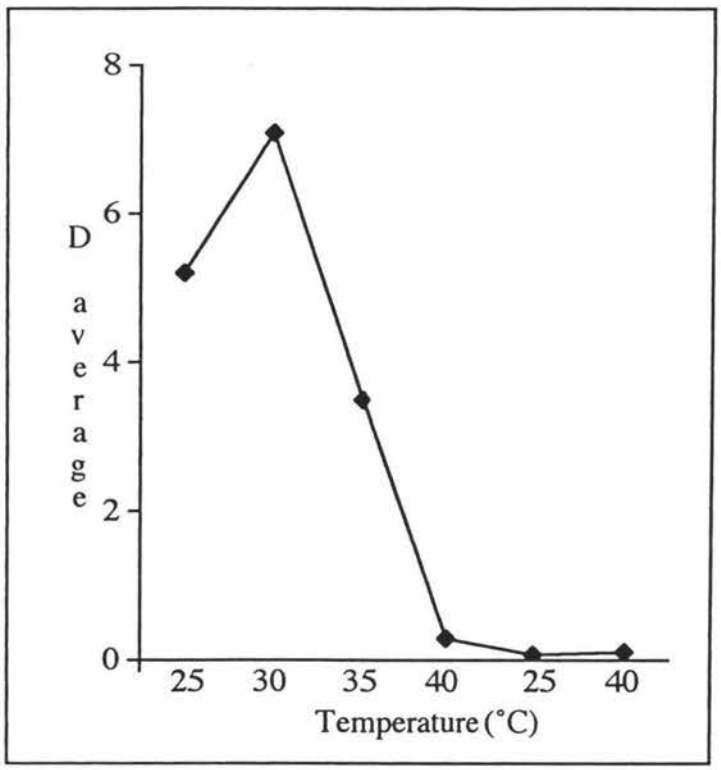


Figure 5.2 - Averages of the Diffusion Coefficients, Relative to that at 25 °C, versus Temperature

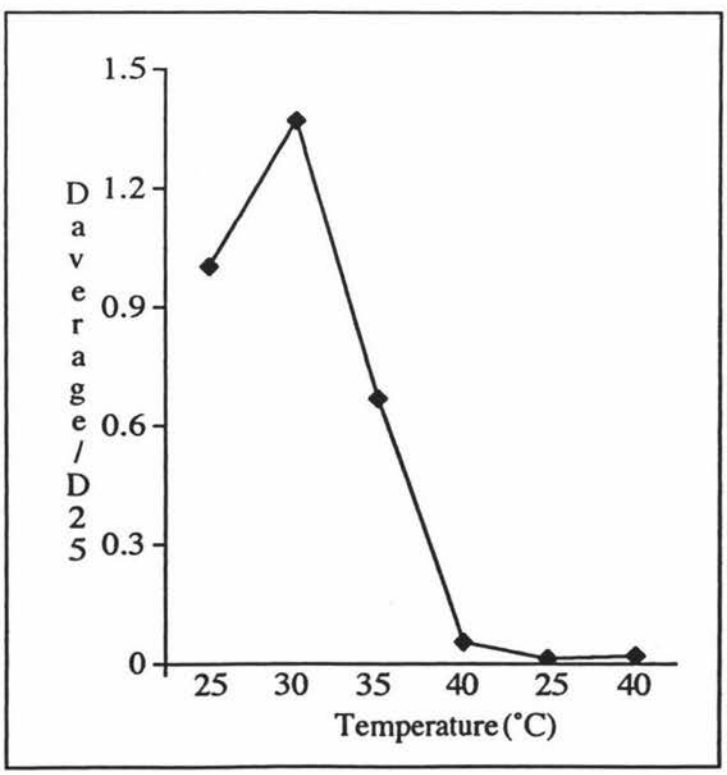


Figure 5.3 - Average Hydrodynamic Radii versus Temperature

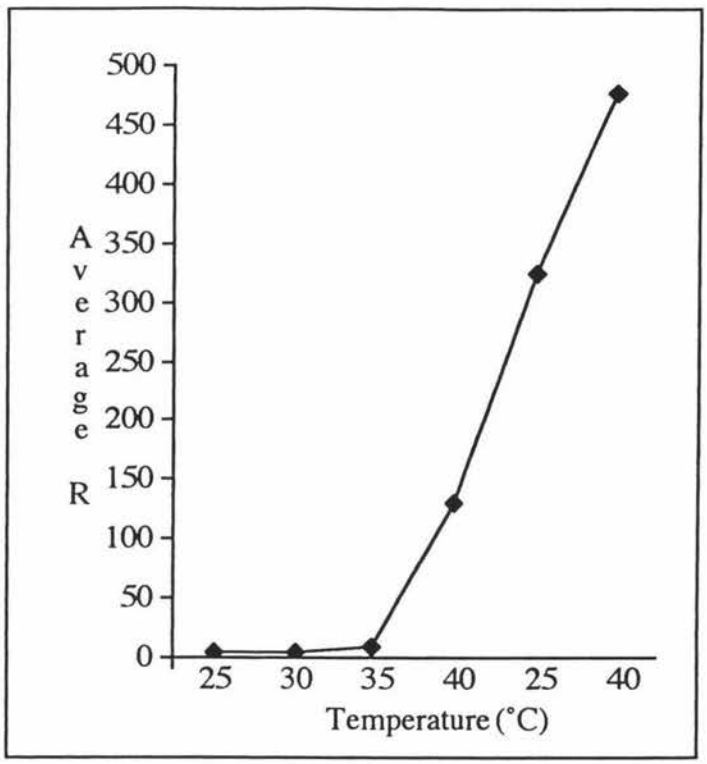
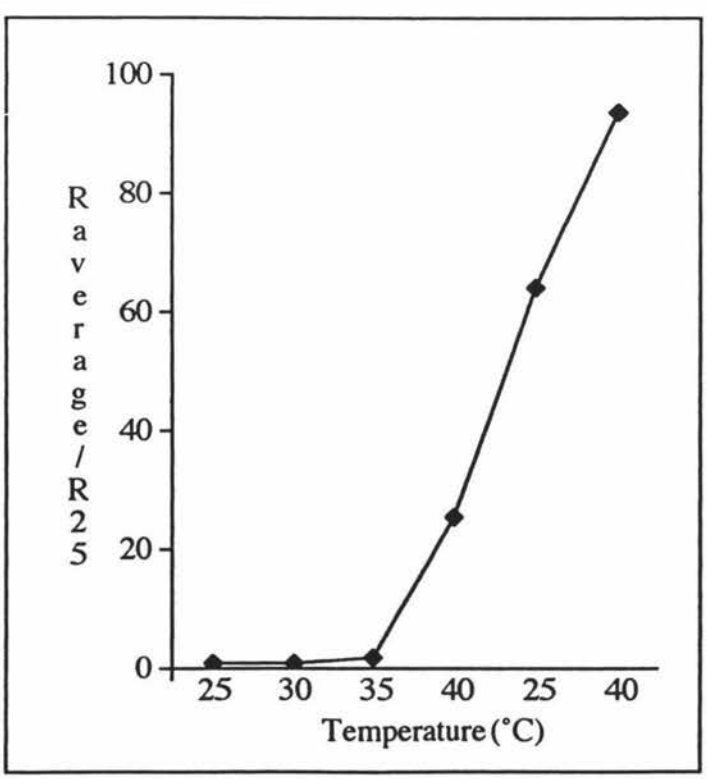


Figure 5.4 - Averages of the Hydrodynamic Radii, Relative to that at 25 °C, versus Temperature



the hydrodynamic radius relative to that at 25 °C, (Appendix 3, Table A3.7), were also plotted against temperature (Figure 5.4).

5.4 Discussion

5.4.1 Behaviour of the Diffusion Coefficient of Aldehyde Dehydrogenase at Elevated Temperatures

Aldehyde dehydrogenase was monitored at elevated temperatures in order to observe, through the diffusion coefficient and activity, the effect this had on the behaviour of the enzyme. The enzyme was expected to undergo thermal denaturation and after reaching 40 °C, the sample was allowed to cool back down to 25 °C to determine if the unfolding caused by the increased temperature were reversible, as Gast *et al.* (1992) found for streptokinase, and if raising the temperature a second time promoted yet more changes to the enzyme. The diffusion coefficient would be expected to revert to the initial 25 °C value, when allowed to cool, if the unfolding of the enzyme were reversible. This did not occur. The diffusion coefficient of the sample, after remaining at 25 °C overnight, had decreased in value somewhat further and remained near this value, even when heated back up to 40 °C again. Thus the hydrodynamic radius of the enzyme, after an initial decrease at 30 °C, had increased in size by a factor of approximately 26 compared to its value at 25 °C. This does not agree with light scattering results obtained by Gast *et al.* (1992) for the proteins H.α-lactalbumin and streptokinase, and by Nicoli and Benedek (1976) for lysozyme. Gast *et al.* (1992) found that in all three cases, the effect of heat on the hydrodynamic radius was only moderate. They reported an increase in size by 11 - 18% only, and that, for streptokinase at least, the effect was reversible. Gast *et al.* (1992) also found that, despite the small difference in the increase of the hydrodynamic radii of streptokinase and α-lactalbumin, streptokinase adopted a different conformation, that of a disordered chain, when denatured by heat, whilst α-lactalbumin remained in a native-like conformation. This is an indication that an increase in size does not necessarily indicate a change in the type of conformational state of the protein. Gast *et al.* (1992) concluded that the approximately 10% increase in the hydrodynamic radius of the temperature denatured form of α-lactalbumin was due to the protein adopting the molten globule state. However since aldehyde dehydrogenase underwent a 2600% increase in hydrodynamic radius upon heating to 40 °C and displayed an increase of some 200% at 35 °C, the possibility of the protein adopting a molten globule conformation for any significant length of time (ie long enough to be measured by light scattering as was the case for α-lactalbumin (Gast *et al.*, 1992)) seems remote. Since some of the enzyme eventually precipitated out of solution, it seems more likely that aldehyde dehydrogenase adopted an unfolded conformation whereby the exposure of hydrophobic regions promoted the aggregation of the enzyme. This would explain the sudden large increase in the hydrodynamic radius without an intermediate value.

It was also reported that the three proteins above underwent denaturation by guanidine hydrochloride and that again their hydrodynamic radii increased by similar amounts, albeit 40 - 55% increase as opposed to 11 - 18%, (Gast *et al.*, 1992, Dubin *et al.*, 1973). However, from the same data it was seen that lysozyme underwent a greater size increase when there were no intact disulphide bridges present. Light scattering on aldehyde dehydrogenase was carried out in a reducing environment so no disulphide bridges should have been present, but again the increase in the hydrodynamic radius of aldehyde dehydrogenase seems too large to be accounted for merely by unfolding alone, aggregation must have occurred.

Dubin *et al.* (1973) reported that, for the chemical denaturation of lysozyme, the diffusion coefficient decreased with increasing concentration of the denaturant until a plateau value was reached, which was taken as the diffusion coefficient of the denatured protein. From these results and other studies, they concluded that the protein existed in two states, either the native form or the denatured form. They proposed that the varying diffusion coefficients were due to lysozyme being present in both forms to varying degrees rather than unfolding in a single continuous process. While this may also be the case for aldehyde dehydrogenase, the results (Appendix 3, Table A3.1) indicate that the denatured form rapidly undergoes aggregation to such an extent that any contribution to the diffusion coefficient by the native form of the enzyme is overshadowed by the denatured and aggregated form present.

Dubin *et al.* (1973) also found that the reduction of disulphide bridges by dithiothreitol caused an increase in the hydrodynamic radius of the denatured lysozyme by an additional 28% . However, lysozyme was found to retain a compact configuration on denaturation (Dubin *et al.*, 1973, Gast *et al.*, 1992) and was not reported to undergo aggregation. The large increase in the hydrodynamic radius of aldehyde dehydrogenase that took place at elevated temperatures (Figures 5.3 and 5.4) indicated that aldehyde dehydrogenase did not retain a compact conformation, despite the presence of a reducing agent. The denaturation of ribonuclease, which has a similar molecular weight to lysozyme, has also been studied. Upon denaturation Rimai *et al.* (1970) reported that the diffusion coefficient of ribonuclease increased by more than an order of magnitude. The samples of ribonuclease that Rimai *et al.* (1970) used however, had undergone partial aggregation which was thought to have contributed to this large change in their diffusion coefficient. This is similar to the results for aldehyde dehydrogenase, (Appendix 3, Table A3.1), which also were found to have aggregated upon denaturation. Nicoli and Benedek (1976) reported that thermally denatured lysozyme in a relatively high salt environment (0.2 M KCl) partially aggregated upon its return to room temperature after being held at 50 °C or 60 °C for several minutes or longer.

5.4.2 Activity of Aldehyde Dehydrogenase at Elevated Temperatures

The diffusion coefficient increased when the temperature was raised to 30 °C before decreasing drastically at higher temperatures. This contraction of the hydrodynamic radius at 30 °C was accompanied by a small increase in the activity of the control sample,

before both the diffusion coefficient and the activity decreased as the temperature and duration of the experiment increased.

The activity of the control sample of aldehyde dehydrogenase increased a little from its 25 °C value after 4 hours at 30 °C but from then on the activity of the enzyme decreased until precipitation occurred towards the close of the experiment (Appendix 3, Table A3.7). The small increase in activity at 30 °C would most likely be due to k_{cat} varying with temperature. Hill *et al.* (1992) found that at physiological temperature, 37 °C, substrate activation of the enzyme occurred for propanal concentrations of 120 μM to 5 mM and that at higher propanal concentrations, inhibition occurred. Thus the enzyme was stable for the duration of the assay. Since the samples were left at elevated temperatures for hours rather than minutes, the initial slight increase in activity followed by the continual decrease and eventual precipitation was not unexpected.

5.4.3 Summary

Elevated temperatures were found to have an overall decreasing effect on the diffusion coefficient that was non-reversible. Despite an initial decrease of the hydrodynamic radius at 30 °C, the size of the enzyme increased with the temperature and duration of the study to an extent that was accountable for by aggregation of the denatured form of the enzyme. The activity of the enzyme decreased overall, until precipitation occurred, in agreement with the proposal of denaturation and massive aggregation of the enzyme taking place.

Chapter 6
Summary

6.1 Review of Data about Native Aldehyde Dehydrogenase

6.1.1 Introduction

Sheep liver cytosolic aldehyde dehydrogenase was purified by a three column preparation. Enzyme isolated in this way contained few contaminants, and all were of smaller size than aldehyde dehydrogenase (from SDS gels).In spite of a tendency towards aggregation, diffusion coefficients were obtained for non-aggregated enzyme via both laser light scattering and ultracentrifuge techniques. These diffusion coefficients, together with gel chromatography results, it was hoped would provide an insight into the dissociation - association behaviour of sheep liver cytosolic aldehyde dehydrogenase.

6.1.2 Gel Chromatography of Aldehyde Dehydrogenase

The enzyme was passed through a gel column in order to repeat the gel chromatography results of Buckley *et al.* (1991) who observed dissociation of the enzyme that increased with decreasing enzyme concentration and pH. The gel chromatography results reported in this thesis indicated that there was partial dissociation at pH 7.4, to a small extent, and complete dissociation at pH 5.2, in that none of the major, large species at pH 7.4 was present.

6.1.3 Ultracentrifuge Studies on Aldehyde Dehydrogenase

The diffusion coefficients from the ultracentrifuge (Tables 6.1 and 6.2) were determined using the assumption that the sedimentation coefficient did not vary with concentration, so some uncertainty due to this assumption must be associated with them.
No dissociation of cytosolic aldehyde dehydrogenase from sheep liver was observed in the ultracentrifuge at either pH 7.4 or pH 5.2 at concentrations that ranged from 1.8 - 7.2 mg/mL. Von Tigerstrom and Razzell (1968) observed dissociation and reassociation of aldehyde dehydrogenase from pseudomonas aeruginosa in the ultracentrifuge within this same concentration range. They obtained a sedimentation coefficient of 9.4 S at 59780 rpm and a molecular weight of 187000 for this aldehyde dehydrogenase. Their samples were of a similar concentration, molecular weight and sedimentation coefficient, and were spun at similar speeds to the samples of cytosolic sheep liver aldehyde dehydrogenase (Chapter 3). They obtained a diffusion coefficient of 4.4×10^{-11} m^2s^{-1} for the active enzyme.
They promoted deactivation (loss of enzymic activity) and its accompanying dissociation at pH 7.2 by dialysis against solutions of low salt concentration and reassociated the

enzyme by the addition of potassium phosphate. They also found that reactivation of the reassociated enzyme was more or less dependent on the presence of a reducing agent.
Von Tigerstrom and Razzell (1968) found that partially dissociated enzyme showed two major peaks with sedimentation coefficient values of 7.1 S and 5.0 S in the ultracentrifuge. The 7.1 S component was thought to be identical to the 9.4 S component of the native, active enzyme. Partial unfolding may occurred due to the low salt concentration of the dissociated sample which would result in a lower sedimentation coefficient. Starch gel electrophoresis showed one major zone for the active state of the enzyme, but after dissociation, a second zone of faster mobility appeared.
The reassociated enzyme showed a minor peak with a sedimentation coefficient of 5.5 S and a major peak with a value of 9.0 S. The 5.5 S component in the reactivated and the 5.0 S component in the dissociated sample were thought to be identical and to correspond to the fast zone in electrophoresis. The appearance of the second zone in electrophoresis with faster mobility was an indication that a smaller molecule was obtained. This supported the proposition that the change in the sedimentation coefficient was due, not to unfolding of the molecule, but to its dissociation. Their results suggested that partial dissociation of active aldehyde dehydrogenase occurred, probably into two subunits of similar or equal size. The dissociation was promoted by low salt concentrations and reversed by addition of salt to the protein solution, followed by incubation at elevated temperature (30 °C).
At pH 3.0, they found that the enzyme had a sedimentation coefficient of 2.0, but was not of smaller size than the active species at pH 7.0 from gel chromatography results. The enzyme also underwent a large increase in viscosity at pH 3.0 and had a lower diffusion coefficient than at pH 7.0. From the sedimentation and diffusion coefficients, von Tigerstrom and Razzell (1968) calculated the molecular weight to be approximately half that obtained for aldehyde dehydrogenase at pH 7.0. They proposed that extensive unfolding of the molecule occurred, resulting in a subunit that was different in shape from the assumed globular shape at pH 7.0.
Thus if sheep liver cytosolic aldehyde dehydrogenase had undergone dissociation whilst in the ultracentrifuge, it should have been observed. The sedimentation and diffusion coefficients obtained for the enzyme at pH 7.4 (Chapter 3) were similar to those obtained by von Tigerstrom and Razzell (1968) for active aldehyde dehydrogenase from pseudomonas aeruginosa, which they assumed to also be a globular protein and which had a molecular weight in the vicinity of that of cytosolic aldehyde dehydrogenase from sheep liver. The results obtained at pH 5.2 (Chapter 3) showed no significant variation from the pH 7.4 results that would be indicative of dissociation having occurred.The samples were not incubated at 30 °C prior to ultracentrifugation, but light scattering results at elevated temperature showed no large change in the diffusion coefficient and the increase in activity of the enzyme at that temperature was accounted for by the increase in k_{cat} with temperature.

6.1.4 Laser Light Scattering Studies on Aldehyde Dehydrogenase

Cytosolic sheep liver aldehyde dehydrogenase showed a tendency towards aggregation. This was a problem when carrying out laser light scattering on the enzyme as this technique is biased towards large particles and the presence of aggregates can overwhelm the data from the remaining particles. The diffusion coefficients obtained via laser light scattering (Tables 6.1 and 6.2) were in the vicinity of the value expected for aldehyde dehydrogenase (Chapter 4, Equations 4.6 - 4.9). The enzyme exhibited no noticeable signs of dissociation at pH 5.2, but remained in an associated form, when not aggregated. It is unlikely that the enzyme would have dissociated at pH 5.2 and then aggregated to the extent that it had a similar diffusion coefficient to that of its tetrameric form.

In studying association - dissociation behaviour, the dissociated form should be of one size, rather than a range of sizes, otherwise a curved plot will result that will be difficult to resolve into component decays. The greater the difference in size between the associated and dissociated forms the better the resolution of the plot into separate decays. The intensity of the light scattered is proportional to the square of the mass of the scattering object. Thus, for aldehyde dehydrogenase, a mixture composed of equal amounts of tetramer and monomer would scatter 16 times more light from the tetramer than from the monomer. If the enzyme had undergone dissociation to the extent that 20% of the mixture was of monomeric form, the tetramer would scatter 64 times more light than the monomer. Whilst this may suggest that there was very little chance of observing any dissociation, the case for so doing is not as bleak as first presented. The diffusion coefficient of the tetramer was known from previous light scattering studies and the diffusion coefficient of the dissociated species was expected to be greater than that of the tetramer. Thus the data were inspected, at shorter sample times, for a species that exhibited a faster decay and thus a greater diffusion coefficient, as expected for a dissociated form of the enzyme. This diffusion coefficient was not found, so whilst a small amount of dissociation might be overshadowed by the non-dissociated species, data that were specifically inspected for dissociation should reveal dissociation if a significant amount of it had occurred.

6.1.5 Diffusion Coefficients for Aldehyde Dehydrogenase - A Summary

Table 6.1 - Comparison of the Diffusion Coefficients of Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	U/C - D av. (10^{-11} m^2s^{-1})	Conc. (μM)	LLS - D av. (10^{-11} m^2s^{-1})
8.5	5.8 ± 0.6	15.5	4.8 ± 0.3
17.0	4.8 ± 0.4	20.8	5.4 ± 0.3
34.0	3.7 ± 0.2	52.0	5.0 ± 0.2

U/C - D av. - The average of the diffusion coefficients obtained from the ultracentrifuge

and calculated by both methods of determining the area under the peaks.
LLS - D av. - The average of the diffusion coefficients obtained by laser light scattering.
The concentrations reported were based on an activity assay. The concentrations of the ultracentrifuge samples were inferred from the dilutions of the stock solution, of activity 34.0 μM.

Table 6.2 - Comparison of the Diffusion Coefficients of Aldehyde Dehydrogenase at pH 5.2

Conc. (μM)	U/C - D av. (10^{-11} m^2s^{-1})	Conc. (μM)	LLS - D av. (10^{-11} m^2s^{-1})
5.8 **	5.6 ± 0.3	6.8	2.7 ± 0.3
11.5 *	6.1 ± 0.2	20.6	4.7 ± 0.3
23 **	6.1 ± 0.2		

* This activity value was obtained some time after the sample was subjected to the ultracentrifuge (Appendix 1, Table A1.10)
** These values were inferred from the above (*) value. All three values should be taken as the minimum activity value of the enzyme.

Stock enzyme held at pH 7.4 was used for all ultracentrifuge studies at higher pH , by suitable dilution. For pH 5.2 ultracentrifuge studies, the stock enzyme (pH 7.4) was dialysed to pH 5.2 and then diluted as per the ultracentrifuge samples at pH 7.4.
Laser light scattering samples were prepared by dialysis of stock enzyme to pH 5.2 and were assayed to determine their concentration, based on activity, prior to being subjected to laser light scattering.
Diffusion coefficients obtained from the ultracentrifuge and laser light scattering experiments indicated that at both pH 7.4 and 5.2, aldehyde dehydrogenase existed predominantly in the undissociated tetrameric form. No evidence was obtained for the presence of any dissociated enzyme by these techniques. Whilst the smaller, dissociated species was expected to be the minority species (Buckley *et al.*, 1991), it was not expected to be completely hidden by the larger species. Thus if the enzyme had dissociated to the extent expected, some trace of the smaller species should have been observed, and thus have had the appropriate effect on the diffusion coefficient. This was not the case, and it was concluded that the enzyme had not dissociated, under the conditions used in these experiments, at pH 5.2 to any greater extent than it had at pH 7.4. The diffusion coefficient at pH 7.4 was that which was expected of the non-dissociated, enzymatically active species of mass about 200 000, (from Chapter 4, Equations 4.6 - 4.9).
Whilst the enzyme demonstrated a tendency towards aggregation upon light scattering; it did not seem to aggregate upon ultracentrifugation, despite the possibility of the high pressures promoting this. A difference in the two techniques was the use of the laser at 488 nm in the laser light scattering technique, but the samples exhibited no signs of the convection currents that would have been expected if absorption was occurring at this

wavelength. Since not all light scattering samples underwent aggregation the use of the laser at 488 nm can be discarded as a possible source of promotion of aggregation of the enzyme. Calculation of the molecular weight of the enzyme via the sedimentation - diffusion method and that expressed in Chapter 4 (Equations 4.6 - 4.9) confirmed that the diffusion coefficients obtained were appropriate to the enzyme, despite the fact that the gel chromatography results indicated that there was partial dissociation at pH 7.4, to a small extent, and complete dissociation at pH 5.2, in that none of the major, large species at pH 7.4 was present.
An explanation for this is that some dissociation may occur at pH 5.2 and is undetected by the other two techniques - due to being overshadowed by the associated species in laser light scattering and to association being favoured by the high pressures in the ultracentrifuge. The act of passing the enzyme through the gel column may alter the association - dissociation equilibrium, promoting dissociation of the enzyme.

6.2 Effects of Additives and Temperature Increases on the Diffusion Coefficient of Aldehyde Dehydrogenase

Mg^{2+} had no discernible effect on the association - dissociation behaviour of the enzyme at pH 7.4 or pH 5.2, despite expectations to the contrary. Some aggregation was thought to have occurred, so that may have masked some small effects.
Propanal promoted large-scale aggregation of the enzyme at pH 7.4. This aggregation was thought to be caused by propanal inducing a change in the conformation of the enzyme. This change could be the exposure of more charged groups and/or the bringing of them into a conformation that was more favourable for the intermolecular forces to promote aggregation. The aggregation could also be caused by the propanal forming Schiff bases with amine groups on the enzyme resulting in a modified form of the enzyme that was more inclined towards large-scale aggregation. The retainment of activity suggests that the active site was left free to react and possibly the aggregates broke up as the enzyme reacted. At pH 5.2, the addition of propanal did not promote widespread large-scale aggregation. This may have been due to the protonation of the amino groups on the enzyme at pH 5.2 before the propanal was added, thus reducing the amount of Schiff bases formed that may otherwise promote large-scale aggregation.
Elevated temperatures had a non-reversible effect on aldehyde dehydrogenase. The diffusion coefficient decreased, and thus the hydrodynamic radius increased, to an extent that indicated that aggregation had occurred. The activity of the enzyme decreased and eventually precipitation of the denatured enzyme occurred.

6.3 Strengths and Limitations of the Techniques Used in the Studies on Aldehyde Dehydrogenase

Laser light scattering as a technique for studying enzyme behaviour is useful but certain criteria must be met. The main one of these is that the enzyme does not exhibit a tendency towards aggregation as the technique is biased towards large particles which will

overshadow any small particles present. This technique has the advantage of using small amounts of enzyme that are reusable, although repeated use over periods of days can lead to loss of activity and aggregation, probably through the repeated cycling of the sample in and out of the laser beam and refrigerator. Additions can be made to the sample at any stage in the experiments without removing it from the sample tube, so comparisons can be made with the same sample with and without the presence of an additive. The sensitivity of the technique towards large particles requires that the samples be clean (dust-free) and pure.

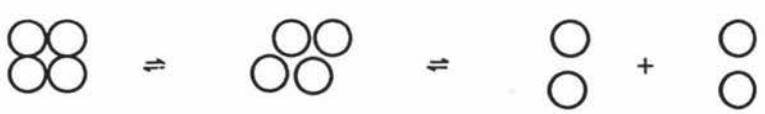
The use of the ultracentrifuge allows the determination of the diffusion coefficient via another method and is complementary to the technique of laser light scattering as small species that may be overshadowed in laser light scattering may be observed with this technique. The presence of dust or dirt in the sample is not such a problem as any very large particles such as these will quickly sediment during ultracentrifugation, leaving the sample free to be observed without their presence.

Gel chromatography also provides information on the various species present. It is a relatively quick technique, although it also requires very clean samples, and will detect any other species present.

The use of these techniques, in particular laser light scattering, may be useful in monitoring the behaviour of enzymes, providing the above criteria are met. They could also be used to provide information about mutant forms of enzymes that may not show activity but may be found to exhibit similar behaviour to the native form.

6.4 A Model for the Association - Dissociation Behaviour of Aldehyde Dehydrogenase

The following model has been developed in response to the results from the various techniques. It is proposed that the association - dissociation equilibrium behaviour of sheep liver aldehyde dehydrogenase incorporates a pre-dissociation state (Equation 6.1). The enzyme in the pre-dissociated enzyme state is supposed to be of a similar size to the non-dissociated tetramer, but has undergone a conformational change that has rendered it inactive.



(6.1)

A **B** **C**

Association **Pre-dissociation** **Dissociation**

States A and C, the associated and dissociated states, are those that were observed by gel chromatography (Chapter 2, Buckley *et al.*, 1991) - A being the active, tetrameric form of the enzyme, and C being the inactive, dissociated form.

At pH 7.4, the enzyme exists predominantly in the active, associated form, state A, as evidenced by gel chromatography results (Chapter 2, Buckley *et al.*, 1991). At pH 5.2, a

smaller inactive form of the enzyme, state C, is present to varying extents (Chapter 2, Buckley *et al.*, 1991).

Since B is of a similar size to A, the technique of laser light scattering would not distinguish between the two states. It is proposed that at pH 7.4, the enzyme is present predominantly as A. Any small amount of enzyme that was present in the dissociated state C would not be detected by laser light scattering as it would be hidden by the scattering of the larger particles of A. The ultracentrifuge results are consistent with the enzyme being present as A at pH 7.4.

However, at pH 5.2, it is proposed that the enzyme exists in all three states. The presence of A accounts for any activity shown by the sample at this pH and for the large species present in gel chromatography results (Chapter 2, Buckley *et al.*, 1991). However pH 5.2 favours the inactive pre-dissociated state B and the inactive dissociated state C. The state B is inactive, which accounts for the inactivity of the samples at this pH, and is of similar size to A, which explains why the diffusion coefficients obtained by laser light scattering and the ultracentrifuge show little difference to those obtained for the enzyme at pH 7.4.

The technique of laser light scattering is non-perturbing towards the sample, so it should not affect the association - dissociation equilibrium of the enzyme. The technique of ultracentrifugation, if it affected the association - dissociation behaviour of the enzyme at all, would be expected to favour association, that is states A and B, the larger particles. It is proposed that gel chromatography affects the equilibrium to the extent that state C is favoured. This is the small species present in the gel chromatography results (Chapter 2, Buckley *et al.*, 1991) and is why some of a small dissociated species was observed at pH 7.4. More of this species was present at pH 5.2 since there was a greater amount of the enzyme present as B and this was converted to C, thus affecting the equilibrium. Results at pH 5.2 (Chapter 2) indicate that all of A was converted to C on the gel column. Before being passed through the gel column, the sample had some activity, but less than the stock enzyme from which it was taken at pH 7.4. This suggests that some of the enzyme was present as A, (the form which contributed the activity of the sample), and the rest mainly as B, these two forms giving the diffusion and sedimentation coefficients that were similar to those of the enzyme at pH 7.4, and the remainder present as C. The act of passing the enzyme through the gel column then altered the equilibrium to the extent that all of A and B were converted to C. The fact that Buckley *et al.* (1991) observed some of the associated species at pH 5.2 after gel chromatography may be accounted for by the dilution effect. They observed that premixing of the enzyme at pH 5.0 with buffer decreased the rate of production of NADH and proposed that on predilution the enzyme dissociates into an inactive species and that the predilution time could also affect the assay concentration of the enzyme. Their samples were prediluted for 30 minutes before being loaded onto the gel column whilst the samples used in this study had been at their concentrations for a period of time in the order of days before they were passed through the gel column. This difference in the amount of time before the samples were subjected to gel chromatography may have contributed to the differing amounts of active and inactive species that were observed.

The addition of Mg^{2+} did not have any significant effect on the diffusion coefficient of aldehyde dehydrogenase at either pH as observed via laser light scattering. This was not surprising as the technique of laser light scattering observes the size of the particles in solution, not their activity or lack of it. At pH 7.4, Mg^{2+} is known to bind to the enzyme and inhibit NADH release from the enzyme, thus lowering the activity (Buckley *et al.*, 1991). At pH 5.2 Mg^{2+} binds to the enzyme, promoting association and activation (Buckley *et al.*, 1991). These results are consistent with equation 6.1 since Mg^{2+} primarily affects the activity of the enzyme rather than its size. At pH 7.4, Mg^{2+} would bind to the predominant A, inhibiting the release of NADH and hence the activity, but would not significantly affect the size of the enzyme. At pH 5.2, Mg^{2+} would bind to the enzyme in states A and B, promoting association to A and giving a net activation since the active form A is favoured. At both pH's the net result observed by laser light scattering was particles of similar size to the tetrameric form of the enzyme.

The addition of propanal to the enzyme promoted large scale aggregation of the enzyme at pH 7.4, as observed by laser light scattering, possibly through the formation of Schiff's bases with amino groups on the enzyme. At pH 5.2, the propanal binds to the enzyme, favouring formation of A, the active form. Buckley *et al.* (1991) found that premixing of the enzyme at pH 5.0 with propanal before assaying provided protection against dissociation of the tetramer. However, protonation of the amino groups on the enzyme may have occurred at this pH which would prevent the propanal reaction that would lead to the formation of the super-aggregates that were observed at pH 7.4.

In conclusion it is clear that the various techniques used in this thesis reveal different aspects of the association - dissociation behaviour of sheep liver cytosolic aldehyde dehydrogenase. The expectation that laser light scattering and ultracentrifugation would confirm the enzyme dissociation that was observed on the gel column was not met. Thus the hope that these techniques could be used to explore more subtly the association - dissociation behaviour was not fulfilled. The actual situation has proven much more complex and what has been proposed is a self-consistent model to describe the different insights obtained from each technique used.

Blackwell *et al.* (1987) found that the kinetic results obtained with low concentrations of aldehyde dehydrogenase that had been prediluted, (< 1 μM), differed from those obtained when the assay was carried out without predilution of the enzyme. Predilution led to a loss of about half the initial activity. A loss in activity (final activity about 20% of control assay) on predilution at pH 5.2 was observed by Buckley *et al.* (1991). The simplest explanation of both phenomena is that during the time of predilution, dissociation to an inactive form of the enzyme takes place. In both cases the enzyme concentration was much less than those used in either the laser light scattering or ultracentrifuge studies reported in this thesis and it is known that dilution favours dissociation.

It was not possible to use laser light scattering or ultracentrifugation to study the enzyme under assay conditions, with both substrate and cofactor present, as the reaction would be complete before significant measurements could be made. Association - dissociation

effects may be important at low pH under assay conditions but the laser light scattering and ultracentrifuge studies reported here do not provide new insights . The role of the predissociated complex may be that of an inactive, intermediate state that, with prompting, may either reassociate and reactivate - as in the case of Mg^{2+} addition in some instances or the presence of propanal (Buckley *et al.*, 1991), or that may dissociate into a smaller species as observed from gel chromatography results (Chapter 2, Buckley *et al.*, 1991). Further investigations, preferably involving non-perturbing techniques, may be of interest to examine the possible existence of this predissociated state and model of the association - dissociation behaviour of sheep liver cytosolic aldehyde dehydrogenase.

Appendix 1
Ultracentrifuge Data

A1.1 Sedimentation Coefficient Data

Table A1.1 - Data for the Calculation of the Sedimentation Coefficient of Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	Time, t' (min.)	x (cm)	$\log_{10} x$
8.5	0 (meniscus)	50.27	1.7013
	4.75	50.72	1.7052
	11.75	51.32	1.7103
	18.75	51.92	1.7153
	30.75	52.97	1.7240
	43.75	54.12	1.7334
17.0	0 (meniscus)	51.38	1.7108
	5.25	52.08	1.7167
	10.25	52.58	1.7208
	15.25	52.95	1.7239
	26.25	54.20	1.7340
	42.25	56.05	1.7486
34.0	0 (meniscus)	50.82	1.7060
	4.75	51.17	1.7090
	9.75	51.57	1.7124
	17.75	52.17	1.7174
	34.75	53.57	1.7289
	50.75	54.69	1.7379

Activity based on dilutions of stock enzyme solution known to be 34.0 μM.

Table A1.2 - Data for the Calculation of the Sedimentation Coefficient of Aldehyde Dehydrogenase at pH 5.2

Conc. (μM)	Time, t' (min.)	x (cm)	$\log_{10} x$
*8.5	0 (meniscus)	50.32	1.7017
	5.75	50.97	1.7073
	10.75	51.57	1.7124
	18.75	52.34	1.7188
	28.75	53.49	1.7283
	37.75	54.59	1.7371
*17.0	0 (meniscus)	50.45	1.7209
	5.50	50.90	1.7067
	11.50	51.27	1.7099
	21.50	51.84	1.7146
	36.50	53.09	1.7250
	50.50	54.29	1.7347
*34.0	0 (meniscus)	50.72	1.7052
	5.50	51.17	1.7090
	12.50	51.87	1.7149
	23.50	52.59	1.7209
	30.50	53.19	1.7258
	45.50	54.54	1.7367
**8.5	0 (meniscus)	52.05	1.7164
	5.50	52.75	1.7222
	9.50	53.25	1.7263
	13.50	53.80	1.7308
	17.50	54.02	1.7326
	23.50	54.72	1.7381

* Activity quoted as that of dilutions of stock enzyme at pH 7.4

** Sample was recentrifuged a week later

A1.2 Diffusion Coefficient Data

Table A1.3 - Data for the Calculation of the Diffusion Coefficient of Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	t (s)	H (m)	Σh (m)	A (10^{-5} m^2)	(A/H)^2 (10^{-7} m^2)	$W_{1/2}$ (m)	A_G (10^{-5} m^2)	(A_G/H)^2 (10^{-7} m^2)
8.5	285	0.013	0.062	6.2	3.55	0.005	6.5	3.90
	705	0.010	0.059	5.9	5.43	0.0065	6.5	6.59
	1125	0.007	0.050	5.0	7.96	0.0075	5.3	8.77
	1845	0.005	0.037	3.7	8.54	0.0085	4.3	11.27
	2625	0.0045	0.033	3.3	8.39	0.009	4.1	12.63
17.0	315	0.022	0.112	11.2	4.04	0.005	11.0	3.90
	615	0.018	0.100	10.0	4.81	0.0055	9.9	4.72
	915	0.015	0.093	9.3	5.99	0.0065	9.8	6.59
	1575	0.011	0.087	8.7	9.83	0.008	8.9	10.06
	2535	0.009	0.081	8.1	12.63	0.010	9.0	15.60
34.0	285	0.053	0.216	21.6	2.59	0.0045	23.9	3.16
	585	0.041	0.200	20.0	3.71	0.005	20.5	3.90
	1065	0.031	0.173	17.3	4.86	0.0065	20.2	6.59
	2085	0.021	0.154	15.4	8.39	0.008	16.8	9.98
	3045	0.0165	0.154	15.4	13.59	0.0095	15.7	14.07

t is the time for each frame,
H is the maximum peak height,
Σh is the sum of the measured heights for a peak,
A is the area under the peak,
(A/H)^2 has been corrected for magnification effects,
$W_{1/2}$ is the width of the peak at 1/2 the maximum peak height ,
A_G is the Gaussian area under the peak,
(A_G/H)^2 has been corrected for magnification effects.

Table A1.4 - Data for the Calculation of the Diffusion Coefficient of Aldehyde Dehydrogenase at pH 5.2

Conc. (μM)	t (s)	H (m)	Σh (m)	A (10^{-5} m^2)	$(A/H)^2$ (10^{-7} m^2)	$W_{1/2}$ (m)	A_G (10^{-5} m^2)	$(A_G/H)^2$ (10^{-7} m^2)
*8.5	345	0.014	0.066	6.6	3.47	0.005	7.0	3.90
	645	0.010	0.055	5.5	4.72	0.0055	5.5	4.72
	1125	0.008	0.049	4.9	5.90	0.0075	6.0	8.84
	1725	0.006	0.0485	4.9	10.27	0.009	5.4	12.73
	2265	0.005	0.052	5.2	17.00	0.010	5.0	15.72
*17.0	330	0.024	0.104	10.4	2.91	0.0045	10.8	3.13
	690	0.019	0.120	12.0	6.22	0.0065	12.4	6.59
	1290	0.015	0.115	11.5	9.24	0.008	12.0	10.06
	2190	0.011	0.114	11.4	16.88	0.0105	11.6	17.33
	3030	0.0105	0.1125	11.3	18.04	0.012	12.6	22.64
*34.0	330	0.046	0.226	22.6	3.76	0.0055	25.3	4.72
	750	0.032	0.201	20.1	6.15	0.007	22.4	7.64
	1410	0.024	0.190	19.0	9.77	0.008	19.2	9.98
	1830	0.022	0.205	20.5	13.65	0.010	22.0	15.72
	2730	0.017	0.180	18.0	17.62	0.0105	17.9	17.33
**8.5	330	0.005	0.018	1.8	2.01	0.005	2.5	3.87
	570	0.005	0.024	2.4	3.57	0.006	3.0	5.57
	810	0.005	0.029	2.9	5.21	0.007	3.5	7.58
	1050	0.0035	0.017	1.7	3.68	0.007	2.5	7.64
	1410	0.004	0.0265	2.7	6.84	0.008	3.2	9.98

Table A1.5 - Calculation of the Diffusion Coefficient of Aldehyde Dehydrogenase at pH 7.4

Conc (μM)	Slope (10^{-10}m^{2}s^{-1})	D (10^{-11}m^{2}s^{-1})	No. pts.	R	$Slope_G$ (10^{-10}m^{2}s^{-1})	D_G (10^{-11}m^{2}s^{-1})	No. pts.	R_G
8.5	7.5 ± 0.8	6.0 ± 0.6	3	0.873	6.9 ± 0.8	5.5 ± 0.6	4	0.816
17.0	5.6 ± 0.5	4.5 ± 0.4	5	0.888	6.4 ± 0.4	5.1 ± 0.3	5	0.918
34.0	4.4 ± 0.2	3.5 ± 0.2	5	0.976	4.9 ± 0.3	3.9 ± 0.2	5	0.957

Table A1.6 - Calculation of the Diffusion Coefficient of Aldehyde Dehydrogenase at pH 5.2

Conc (μM)	Slope (10^{-10}m^{2}s^{-1})	D (10^{-11}m^{2}s^{-1})	No. pts.	R	$Slope_G$ (10^{-10}m^{2}s^{-1})	D_G (10^{-11}m^{2}s^{-1})	No. pts.	R_G
*8.5	6.8 ± 0.5	5.4 ± 0.4	5	0.962	7.3 ± 0.3	5.8 ± 0.2	5	0.985
*17.0	7.7 ± 0.3	6.1 ± 0.2	4	0.993	7.7 ± 0.2	6.1 ± 0.2	5	0.995
*34.0	6.9 ± 0.3	5.5 ± 0.2	5	0.977	8.3 ± 0.7	6.6 ± 0.6	4	0.927
**8.5	6.3 ± 0.8	5.0 ± 0.1	3	0.999	9.7 ± 0.5	7.7 ± 0.4	3	0.961

where Slope is the slope of the plot of $(A/H)^2$ vs t,

D is the diffusion coefficient obtained from the slope,

No. pts. is the number of points from the plot used in the regression to obtain the slope, R is the value of multiple R from the regression data,

$Slope_G$ is the slope of the plot of $(AG/H)^2$ vs t,

D_G is the diffusion coefficient obtained from slopeG,

No. pts_G is the number of points from the plot used in the regression to obtain the slopeG,

R_G is the value of multiple R from the regression data used to obtain slopeG.

Table A1.7 - Comparison of Areas Determined by Trapezoidal and Gaussian Methods for Samples at pH 7.4

Conc. (μ**M**)	**Time** (s)	**$(A_G-A)/A$** (%)	**$[(A_G/H)^2-(A/H)^2]/(A/H)^2$** (%)
8.5	285	4.8	9.9
	705	10.2	21.4
	1125	6.0	10.2
	1845	16.2	32.0
	2625	24.2	50.5
17.0	315	-1.8	-3.5
	614	-1.0	-1.9
	915	5.4	10.0
	1575	1.1	2.3
	1535	11.1	23.5
34.0	285	10.6	22.0
	585	2.5	5.1
	1065	16.8	35.6
	2085	9.1	19.0
	3045	1.9	3.5

Table A1.8 - Comparison of Areas Determined by Trapezoidal and Gaussian Methods for Samples at pH 5.2

Conc. (μM)	Time (s)	$(A_G-A)/A$ (%)	$[(A_G/H)^2-(A/H)^2]/(A/H)^2$ (%)
*8.5	345	6.1	12.4
	645	0.0	0.0
	1125	22.4	49.8
	1725	10.2	24.0
	2265	-3.8	-7.5
*17.0	330	3.8	7.6
	690	3.3	5.9
	1290	4.3	8.9
	2190	1.8	2.7
	3030	11.5	25.5
*34.0	330	11.9	25.5
	750	11.4	24.2
	1410	1.1	2.1
	1830	7.3	15.2
	2730	0.6	1.6
**8.5	330	38.9	92.5
	510	25.0	56.0
	810	20.7	45.5
	1050	47.1	107.6
	1410	18.5	45.9

A1.3 Molecular Weight Calculations

Whilst the molecular weight was calculated, by both methods, using only the sedimentation and diffusion coefficients from the samples of the lowest concentration at each pH (Chapter 3, Table 3.5), the molecular weight was calculated using the sedimentation - diffusion coefficients method for each sample. The diffusion coefficients obtained by the trapezoidal and Gaussian methods (Chapter 3, Tables 3.3 and 3.4) were averaged for each sample (Table A1.9), except for when there was a significant difference in the two values, in which case the diffusion coefficient obtained by the trapezoidal method was used. The molecular weight was calculated from the averaged diffusion coefficients using the Svedberg method (Table A1.9), since this took into account both the diffusion and sedimentation coefficients, instead of the method based on the Stokes-Einstein equation which requires the diffusion coefficient only.

Table A1.9 - Molecular Weights Calculated from the Svedberg Equation using Averaged Diffusion Coefficients

pH	D av. (10^{-11} m^2s^{-1})	M_S
7.4	5.8 ± 0.6	176000
	4.8 ± 0.4	187000
	3.7 ± 0.2	241000
5.2	5.6 ± 0.3	170000
	6.1 ± 0.2	141000
	*5.5 ± 0.2	172000
	*5.0 ± 0.1	189000

D av. - the average diffusion coefficient

* - the diffusion coefficient calculated by the trapezoidal method only was used.

A1.4 Activity of Ultracentrifuge Samples

Samples of aldehyde dehydrogenase that underwent ultracentrifugation retained their activity to an extent that was comparable with samples that were not ultracentrifuged. In some instances, the samples that had been ultracentrifuged retained their activity to a slightly greater extent than those that had not been subjected to the ultracentrifuge, and to a much greater extent than those that were subjected to laser light scattering.

The activity of the stock enzyme solution, pH 7.4 was 34.0 μM on the day prior to the start of the ultracentrifuge experiments. Aliquots of these were diluted 1 : 4 and 1 : 1 with buffer and these and the stock solution were ultracentrifuged over a period of 5 days. 16 days later, these samples and the stock solution were assayed (Table A1.9).

A day after the start of the ultracentrifuge experiments, some of the stock enzyme solution was dialysed from pH 7.4 to pH 5.2, and was subjected to the ultracentrifuge in the same dilutions as the pH 7.4 samples over a period of 2 days after the ultracentrifugation of the pH 7.4 samples was completed. These samples and the non-ultracentrifuged, pH 5.2, stock solution were assayed on the same day as the pH 7.4 samples (Table A1.10). Some of these samples had been subjected to laser light scattering in the meantime.

Table A1.10 - Activity of pH 7.4 Samples

Sample	Activity (μM)
Stock	31.7
Stock + U/C	28.4
Stock + U/C + LLS	26.4
Stock + LLS	3.0

Stock - stock enzyme solution from which all samples were taken (was 34.0 μM on the

day prior to the start of the ultracentrifuge experiments).
+ U/C - sample has been subjected to the ultracentrifuge.
+ LLS - sample has been subjected to laser light scattering.

Table A1.11 - Activity of pH 5.2 Samples

Sample	Activity (μM)
Stock	11.1
(1 : 1) + U/C	11.5
Stock + U/C + LLS	7.0

Stock - stock enzyme solution that was pH 7.4 prior to dialysis
(1 : 1) + U/C - sample was stock solution that was diluted 1 : 1 and then subjected to the ultracentrifuge

Appendix 2
Laser Light Scattering

A2.1 Discarded Diffusion Coefficients

A2.1.1 Discarded Diffusion Coefficients of Aldehyde Dehydrogenase at pH 7.4

These values of the diffusion coefficient, over a range of concentrations and sample times, were discarded since they showed the effects of significant aggregation of aldehyde dehydrogenase at pH 7.4.

Table A2.1a - Laser Light Scattering Results, Subsequently Discarded, for Aldehyde Dehydrogenase at pH 7.4.

Conc. (μM)	Angle (°)	S.T. (μs)	D (10^{-11} m^2s^{-1})
13.7	30	70	1.1 ± 0.01
	45	70	1.2 ± 0.1
		30	1.6 ± 0.1
	90	6	1.5 ± 0.04
	30	70	0.84 ± 0.01
6.4	90	5	1.5 ± 0.1
	120	4	1.4 ± 0.1
	60	8	1.3 ± 0.03
	40	20	1.1 ± 0.02
	90	0.8	1.7 ± 0.4
		1	1.7 ± 0.4
		3	1.2 ± 0.04
		4	1.1 ± 0.1
		6	0.91 ± 0.04
		7	0.83 ± 0.03
50	90	10	1.2 ± 0.02
	30	50	0.99 ± 0.01
	60	10	0.99 ± 0.01
	120	7	1.0 ± 0.01
9	120	7	1.2 ± 0.03
	90	5	1.13 ± 0.01

From these data it was found that the appropriate sample time for these samples was that from 3 - 70 μs, depending on the angle chosen, since as the scattering angle approaches

zero, the sample time required approaches infinity. The diffusion coefficients from these data were all in the same region, indicating that similar sized particles were present in the majority in all cases. The average value of the diffusion coefficient obtained from these data was 1.2×10^{-11} m^2s^{-1}. These data, however, were subsequently discarded as later results indicated that a certain amount of aggregation had taken place. The technique favours the observation of larger particles since they have a much higher intensity of scattered light. Thus the values of the diffusion coefficients reported above were not true values for aldehyde dehydrogenase at pH 7.4, but rather those of the enzyme in a slightly aggregated state.

Table A2.1b - Laser Light Scattering Results at Long Sample Times for Aldehyde Dehydrogenase at pH 7.4

Conc. (μM)	Angle (°)	S.T. (μs)	D (10^{-11} m^2s^{-1})
7.5	60	500	0.02
		1000	0.05
		3000	0.02
57.6	30	100	0.4
		1000	0.05
		7000	0.03
-	90	400	0.038 ± 0.001
	35	800	0.045 ± 0.003
	45	2000	0.021 ± 0.0004
	60	1000	0.019 ± 0.001

These data collected at the very long sample times, (Table A2.1b), displayed at least two different decays and from the large errors present in most of the data, (especially for the higher degrees of polynomial fits), it was concluded that this extremely long decay was due to a small number of very large aggregates of the enzyme being present in the sample. These data were used only for comparison of the diffusion coefficient at very long sample times with that obtained in later experiments where a long sample time was an appropriate option.

A2.1.2 Discarded Diffusion Coefficients of Aldehyde Dehydrogenase at pH 5.2

Table A2.2 - Laser Light Scattering Results, Subsequently Discarded, for Aldehyde Dehydrogenase at pH 5.2. Data was collected at a detector angle of 60°.

Conc. (μM)	S.T. (μs)	D (10^{-11} m^2s^{-1})	Average D (10^{-11} m^2s^{-1})
11	400	0.17 ± 0.05	0.15 ± 0.04
	200	0.13 ± 0.02	

The sample at long sample times, (Table A2.2), had little signal, indicating that these were not the appropriate sample times to use, and that there was little sample present to contribute to the long decay, ie there were very few aggregates of the enzyme present.

A2.2 pH Jump and Laser Light Scattering

A2.2.1 Introduction

Various buffers were titrated with acid and bases to determine the quantity required to change the pH of the buffer solution to a predetermined value. This was so that a solution of enzyme that had been subjected to laser light scattering might then have its pH altered in situ by the addition of a predetermined amount of acid, base or buffer. The pH of the enzyme solution would be adjusted in one pH jump to that which was significantly different from the original and at which the behaviour of the enzyme was thought to differ so that it might be expected to give an altered laser light scattering plot.

A2.2.2 Methodology

The amount of additive used to alter the pH needed to be kept to a minimum to prevent dilution effects from becoming significant. Mixing was carried out by gently swirling the solutions and tapping the tube gently to aid the mixing. The amount of enzyme solution present was less than 0.5 mL so the amount of additive required was by necessity small and accurately determined by repeated titrations to ensure that the pH was altered to the desired extent. The tube that contained the enzyme solution was calibrated to 0.1 mL by addition of known volumes of water to the tube before use. Once the volume of enzyme solution was known to the nearest 0.05 mL, water was added to another empty tube by pipette until the two tubes contained an equal (determined by eye) volume of solution. The control tube was then emptied and the amount of water that had been determined was added to it to check that it was the right amount. If it was correct, it was then taken as the volume of the enzyme solution for use in calculating the amount of additive to be added.

A2.2.3 Results

It was found that 1 mL of 0.025 M phosphate buffer, pH 7.3, required 0.40 mL of 0.05 M HCl or 0.75 mL of 0.025 M HCl to decrease the pH to pH 5.6. When an extra 0.05 mL of HCl, (or 0.1 mL in the case of 0.025 M HCl), was added, the pH decreased sharply to pH 3.7 - 4.0. It was decided that the more dilute acid solution was not suitable for decreasing the pH to 5.6 as the dilution factor was too large.

A sample of aldehyde dehydrogenase (57.6 μM, 0.5 mL) in 0.025 M phosphate buffer, pH 7.4, was subjected to laser light scattering. 0.20 mL of 0.05 M HCl was added to the sample to drop the pH to pH 5.6. However, precipitation of the enzyme occurred due to high localised acid concentration, rendering the sample useless for further light scattering. From further titrations carried out with acetic acid in place of HCl, it was found that 0.20 mL or 0.40 mL of 0.10 M or 0.05 M acetic acid respectively were required to decrease the pH of 0.025 M phosphate buffer from pH 7.3 to 5.6.

Laser light scattering was performed on a fresh sample of aldehyde dehydrogenase (57.6 μM, 0.1 mL) in 0.025 M phosphate buffer, pH 7.3. 40 μL (0.040 mL) of 0.05 M acetic acid were added to the sample. 20 minutes later, light scattering was performed on the sample and then again on the following day. After this another 40 μL of acetic acid was added and more light scattering carried out (Chapter 4, Table 4.9).

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

A3.1 Diffusion Coefficients at Elevated Temperatures

Diffusion coefficients of aldehyde dehydrogenase, pH 7.4 were obtained at temperatures increasing in 5° increments to 40 °C, and then again at 25 °C and 40 °C on the following day. Data were collected at a scattering angle of 60°, (Table A3.1). Some runs were discarded on the basis that no real signal or data were present. As evidenced by the error values associated with the diffusion coefficients, the sample gave quite noisy data, at least until it underwent aggregation at elevated temperatures.

Table A3.1 - Diffusion Coefficients at Elevated Temperatures

Temp. (°C)	S.T. (μs)	D (10^{-11} m^2s^{-1})
25	50	7.0 ± 0.5
	25	6.2 ± 0.5
	20	5.6 ± 2
	12	3.5 ± 1
	8	6.1 ± 2
	5	3.1 ± 2
25*	20	5.0 ± 0.8
30*	10	7.2 ± 0.8
	5	7.0 ± 0.6
35*	20	3.03 ± 0.04
	4	4.0 ± 0.2
40*	70	0.416 ± 0.005
	50	0.265 ± 0.002
	10	0.20 ± 0.02
25**	100	0.062 ± 0.001
	50	0.067 ± 0.002
	20	0.077 ± 0.005
	10	0.11 ± 0.02
40**	150	0.103 ± 0.002
	100	0.057 ± 0.002
	50	0.041 ± 0.002
	20	0.17 ± 0.02
	10	0.10 ± 0.04

*This data was collected on the following day after being left in the refrigerator overnight.
**These runs were collected a day later again, after being left at 25 °C overnight.

The diffusion coefficients obtained at each temperature were averaged, (Table A3.2). The sample was observed to undergo changes during the time it spent at 35 °C which were reflected in the significantly changing diffusion coefficient from then onwards. The averages of the diffusion coefficients at each temperature were then divided by the average value for 25 °C, (Table A3.3).

Table A3.2 - Averages of the Diffusion Coefficients at Each Temperature

Temp. (°C)	Dav. (10^{-11} m^2s^{-1})
25	5.2
30	7.1
35	3.5
40	0.29
25	0.079
40	0.094

Table A3.3 - Averages of the Diffusion Coefficients Relative to that at 25 °C.

Temp. (°C)	Dav./D25
25	1.00
30	1.37
35	0.67
40	0.056
25	0.015
40	0.018

A3.2 Hydrodynamic Radii at Elevated Temperatures

The hydrodynamic radii, (Table A3.4), were averaged using the same criteria as for the diffusion coefficients, (Table A3.5), and the averages were divided by that of the sample at 25 °C, (Table A3.6).

Table A3.4 - Hydrodynamic Radii at Elevated Temperatures

Temp. (°C)	R (10^{-9} m)
25	7.0 ± 2
	4.0 ± 1
	4.4 ± 1
	3.9 ± 0.3
	8.0 ± 5
	3.5 ± 0.2
	5.0 ± 0.8
30	3.9 ± 0.4
	4.0 ± 0.3
35	7.7 ± 0.4
	10.4 ± 0.1
40	84 ± 1
	132.3 ± 0.9
	173 ± 20
25	369 ± 10
	220 ± 40
	397 ± 6
	317 ± 20
40	865 ± 50
	346 ± 100
	621 ± 20
	341 ± 5
	211 ± 30

Table A3.5 - Averages of the Hydrodynamic Radii at Each Temperature

Temp. (°C)	Rav. (10^{-9} m)
25	5.1
30	4.0
35	9.1
40	130
25	326
40	477

Table A3.6 - Averages of the Hydrodynamic Radii Relative to that at 25 °C

Temp. (°C)	Rav./R25
25	1.0
30	0.8
35	1.8
40	25.5
25	63.9
40	93.5

A3.3 Behaviour of the Control Sample

A control sample of aldehyde dehydrogenase was also subjected to the same temperatures for similar periods of time and its activity monitored, (Table A3.7).

Table A3.7 - Activity of the Control Sample of Aldehyde Dehydrogenase

Temp. (°C)	Activity (μM)	Time - Control (hours)	Time - Sample (hours)
25	25.2	1	2
30	29.6	4	4
35	23.1	2.5	3
40	15.6	1	1.5
25	16.9	15.5	14.5
25		2	2
40	*	5.5	5.5

* Precipitation had occurred.

Chemicals

Chemical	Company
Acetone	Univar
Ammonium sulphate, enzyme grade	Bethseda Research Laboratories
Bis-tris	USB
Dithiothreitol	Serva
EDTA	Pronalys
4-hydroxyacetophenone	BDH
Magnesium chloride hexahydrate	Riedel - de Haen
NAD+	Sigma
Polyethylene glycol 8000	Sigma
Potassium dihydrogen phosphate	Riedel - de Haen
Propanal	BDH
Sodium acetate	Sigma
Sodium dihydrogen phosphate	Sigma
Sodium hydroxide	Unilab

References

Allanson, S. and Dickinson, F.M. (1984) Biochem. J., 223, 163 - 169

Baldwin, R.L. (1957) Biochem. J., 65, 503 - 512

Bennett, A.F. (1981) M.Sc. Thesis, p. 5, Massey University

Bennett, A.F., Buckley, P.D. and Blackwell, L.F., (1983) Biochemistry, 22, 776 - 784

Blackwell, L.F., Bennett, A.F. and Buckley, P.D. (1983) Biochemistry, 22, 3784 - 3791

Blackwell, L.F., Motion, R.L., MacGibbon, A.K.H., Hardman, M.J. and Buckley, P.D. (1987) Biochem. J., 242, 803 - 808

Blatter, E.E., Abriola, D.P. and Pietruszko, R. (1992) Biochem. J., 282, 353 -360

Buckley, P.D., Motion, R.L., Blackwell, L.F. and Hill, J.P. (1991) in Enzymology and Molecular Biology of Carbonyl Metabolism 3, 31 - 41

Chervenka, C.H. (1969) A Manual of Methods for the Analytical Ultracentrifuge, Beckmann Instruments Inc.

Crow, K.E., Kitson, T.M, MacGibbon, A.K.H. and Batt, R. D. (1974) Biochim. Biophys. Acta, 350, 121 - 128

Cummins, H.Z. (1974) in Photon Correlation and Light Beating Spectroscopy (H.Z. Cummins and E.R. Pike, editors) Nato Advanced Study Institute Series, Vol. 3, Plenum Press, 285 - 330

Daivis, P.J. (1989) Ph.D. Thesis, Massey University

Dickinson, F.M. and Berrieman, S. (1979) Biochem. J., 179, 709 - 712

Dickinson, F.M. and Hart, G.J. (1982) Biochem. J., 205, 443 - 448

Dickinson, F.M., Hart, G.J. and Kitson, T.M. (1981) Biochem. J., 199, 573 - 579

Dickinson, F.M. and Haywood, G.W. (1986) Biochem. J., 233, 877 - 883

Dietrich, R.A. (1966) Biochem. Pharmacol., 15, 1911 - 1922

Dubin, S.B. (1970) Ph.D Thesis, M.I.T.

Dubin, S.B., Feher, G. and Benedek, G.B. (1973) Biochemistry, 12, 714 - 719

Duncan, R.J.S. (1985) Biochem. J., 230, 261 - 267

Eckey, R., Timmann, R., Hempel, J., Agarwal, D.P. and Goedde, H.W. (1990) in Enzymology and Molecular Biology of Carbonyl Metabolism 3, 43 - 52

Eckfeldt, J., Mope, L., Takio, K. and Yonetani, T. (1976) J. Biol. Chem., 251, 236 - 240

Erwin, V.G. and Dietrich, R.A. (1966) J. Biol. Chem., 241, 3533 - 3539

Feldman, R.I. and Weiner, H. (1972) J. Biol. Chem., 247, 260 - 266

Fujime, S. and Mochizuki-Oda, N. (1992) in Laser Light Scattering in Biochemistry, (S.E. Harding, D.B. Sattelle, V.A. Bloomfield, editors), Chapter 17, 245 - 259, Royal Society of Chemistry

Gast, K., Damaschun, G., Damaschun, H., Misselwitz, R., Zirwer, D. and Bychkova, V.A. (1992) in Laser Light Scattering in Biochemistry, (S.E. Harding, D.B. Sattelle, V.A. Bloomfield, editors), Chapter 14, 209 - 224, Royal Society of Chemistry

Greenfield, N.J. and Pietruszko, R. (1977) Biochim. Biophys. Acta, 483, 35 - 45

Harper, G.S., Comper, W.D., Preston, B.N. and Daivis, P. (1985) Biopolymers, 24, 2165 - 2173

Hart, G.J. and Dickinson, F.M. (1977) Biochem. J., 163, 261 - 267

Hart, G.J. and Dickinson, F.M. (1982) Biochem. J., 203, 617 - 627

Hill, J.P., Blackwell, L.F., Buckley, P.D. and Motion, R.L. (1991) Biochemistry, 30, 1390 - 1394

Hill, J.P. and Buckley, P.D. (1991) Analytical Biochemistry, 192, 358 - 361

Hill, J.P., Buckley, P.D., Blackwell, L.F., Sime, R.M. and Kingston, R.L. (1992) Biochem. Pharmacol., 44, 2425 - 2426

Horne, D.S. (1992) in Laser Light Scattering in Biochemistry, (S.E. Harding, D.B. Sattelle, V.A. Bloomfield, editors), Chapter 15, 225 - 239, Royal Society of Chemistry

Jamieson, A.M., Blackwell, J., Zangrando, D. and Demers, A. (1992) in Laser Light Scattering in Biochemistry, (S.E. Harding, D.B. Sattelle, V.A. Bloomfield, editors), Chapter 18, 260 - 274, Royal Society of Chemistry

Kadima, W., Ogendal, L., Bauer, R., Kaarsholm, N., Brodersen, K., Hansen, J.F. and Porting, P. (1993) Biopolymers, 33, 1643 - 1657

Kitson, T.M. (1975) Biochem. J., 151, 407 - 412

Kitson, T.M., Hill, J.P. and Midwinter, G.G. (1991) Biochem. J., 275, 207 - 210

Kraemer, R.J. and Dietrich, R.A. (1968) J. Biol. Chem., 243, 6402 - 6408

Lamm, O. (1929) Z. Phys. Chem., A143, 177

Lindahl, R. (1992) Critical Reviews in Biochemistry and Molecular Biology, 27(4, 5): 283 - 335

Lindahl, R. and Hempel, J. (1990) in Enzymology and Molecular Biology of Carbonyl Metabolism 3, 1 - 8

Lindahl, R., Roper, M., and Dietrich, R.A. (1978) Biochem. Pharmacol., 27, 2463

Loomes, K.M. and Kitson, T.M. (1986) Biochem. J., 238, 617 - 619

Loomes, K.M., Midwinter, G.G., Blackwell, L.F. and Buckley, P.D. (1990) Biochemistry, 29, 2070 - 2075

MacGibbon, A.K.H., Blackwell, L.F. and Buckley, P.D. (1978) Biochem. J., 171, 527 - 531

McDonnell, M.E. and Jamieson, A.M. (1976) Biopolymers, 15, 1283 - 1299

Motion, R.L. (1986) Ph.D. Thesis, Massey University, Palmerston North, New Zealand

Motion, R.L., Buckley, P.D., Bennett, A.F. and L. F. Blackwell, (1988) Biochem. J., 254, 903 - 906

Nemoto, N., Koike, A., Osaki, K., Koseki,T. and Doi, E. (1993) Biopolymers, 33,

551 - 559

Newman, J., Day, L., Berkowitz, S. and Swinney, H.L. quoted by Cummins, H. Z., (1974) in Photon Correlation and Light Beating Spectroscopy (H.Z. Cummins and E.R. Pike, editors) Nato Advanced Study Institute Series, Vol. 3, Plenum Press, 285 - 330

Nicoli, D.F. and Benedek, G.B. (1976) Biopolymers, 15, 2421 - 2437

Pinder, D.N., Ueleni, T. and Lewis, J.A. (1995) J. Molecular Structure, in press

Racker, E. (1949) J. Biol. Chem., 177, 883 - 892

Rarity, J.G., Owens, P.C.M., Atkinson, T., Seabrook, R.N. and Carr, R.J.G. (1992) in Laser Light Scattering in Biochemistry, (S.E. Harding, D.B. Sattelle, V.A. Bloomfield, editors), Chapter 16, 240 - 244, Royal Society of Chemistry

Rimai, L., Hickmott, J.T., Cole, T. and Carew, E.B. (1970) Biophys. J., 10, 20 - 37

Shum, G.T. and Blair, A.H. (1972) Can. J. Biochem., 50, 741 - 748

Steinman, C.R. and Jakoby, W.B. (1967) J. Biol. Chem., 242, 5019

Takahashi, K. and Weiner, H. (1980) J. Biol. Chem., 255, 8206 - 8209

Takahashi, K., Weiner, H. and Filmer, D.L. (1981) Biochemistry, 20, 6225 - 6230

Tanford, C. (1961) Physical Chemistry of Macromolecules p 358, 381, John Wiley and Sons

Tu, G-C and H. Weiner, H. (1988a) J. Biol. Chem., 263, 1212 - 1217

Tu, G-C and H. Weiner, H. (1988b) J. Biol. Chem., 263, 1218 - 1222

Varma, B.K., Demers, A., Jamieson, A.M., Blackwell, J. and Jentoft, N. (1990) Biopolymers, 29, 441 - 448

Von Tigerstrom, R.G. and Razzell, W.E. (1968) J. Biol. Chem., 243, 6495

Weiner, H. and Takahashi, K. (1981) in Monoamimine Metabolising Enzymes (E. Usdin, editor), Macmillan, New York

Williams-Smith, D.L., Bray, R.C., Barber, M.J., Tsopanakis, A.D. and Vincent, S.P. (1977) Biochem. J., 167, 593 - 600