MECHANISTIC STUDIES ON SHEEP LIVER ALDEHYDE DEHYDROGENASES

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

Doctor of Philosophy
in Chemistry

at

Massey University,
New Zealand.

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1976

ABSTRACT

The enzyme aldehyde dehydrogenase has been extensively purified from the cytoplasmic fraction of sheep liver and a study of its kinetic behaviour has been made.

Studies showed that the nucleotide fluorescence of NADH increased on binding to cytoplasmic aldehyde dehydrogenase and the 5.6 fold enhancement of fluorescence has been used to determine the binding site concentration of enzyme solutions. These binding studies showed that the NADH binding sites on the enzyme were all equivalent and possessed a dissociation constant for NADH of 1.2μM. No significant amounts of zinc were detected in the purified enzyme samples.

Steady-state kinetic studies at pH 7.6 showed that the enzyme was capable of utilizing a wide range of aldehydes as substrates and the enzyme also possessed the ability to hydrolyze p-nitrophenyl acetate. The mechanism of action of cytoplasmic aldehyde dehydrogenase using propionaldehyde as a substrate was found to be ordered, with NAD^+ binding prior to the aldehyde and the acid product being released before NADH. Michaelis constants for NAD^+ and propionaldehyde were 2.2μM and 1.4μM respectively while the dissociation constant for NAD^+ was 8μM. At high aldehyde concentration (both for propionaldehyde and acetaldehyde) substrate activation was observed. Steady-state kinetic results were also reported at pH 9.3.

Stopped-flow fluorimetric studies of NADH displacement from aldehyde dehydrogenase using a series of displacing agents (NAD^+, deamino-NAD^+, ADP-ribose and 1,10-phenanthroline) show that this process is biphasic with rate constants of $0.85s^{-1}$ and $0.22s^{-1}$. This has been interpreted as a two step displacement process. The $0.22s^{-1}$ rate constant is similar to the maximum enzyme reaction velocity in the steady-state at high aldehyde concentrations. The association of NADH with the enzyme was also found to be biphasic, one phase being dependent on the NADH concentration while the other was independent.

Stopped-flow experiments where aldehyde dehydrogenase was rapidly mixed with the coenzyme and propionaldehyde showed a burst of NADH formation followed by a slower steady-state turnover. The maximum burst rate constants were $11s^{-1}$ and $23s^{-1}$

for propionaldehyde and acetaldehyde respectively. A mechanism has been postulated for the observed burst and values for various individual rates constants derived.

The general features of the kinetics of sheep liver cytoplasmic aldehyde dehydrogenase have been compared with those of the mitochondrial enzyme from the same source and except for the value of the NAD^+ binding rate constant the two enzymes have been shown to be remarkably similar.

ACKNOWLEDGEMENTS

I wish to thank my supervisors Dr. Len F. Blackwell and Paul D. Buckley for their enthusiasm and invaluable advice throughout the course of this study.

I would also like to thank the other members of the alcohol research group, especially:

Kathy Crow for her assistance in isolating the cytoplasmic aldehyde dehydrogenase.

Dr. Trevor M. Kitson for supplying the mitochondrial aldehyde dehydrogenase.

Terry B. Braggins for his expert technical assistance.

Thanks are also extended to all the members of the Chemistry, Biochemistry and Biophysics Department for their help, and in particular to Nancy Reaburn.

Finally, I would like to thank Jenny Parry for typing the thesis.

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SECTION 1

INTRODUCTION

The consumption of alcohol has been a common feature of a variety of societies in the world for many thousand years. While low concentrations of alcohol may be derived from ingested food or from microbial fermentation of sugars in the intestine (Krebs and Perkins, 1970) the main source of the familiar effects of alcohol is from the consumption of large amounts of alcoholic beverages. Knowledge about the metabolism of ingested ethanol and the participating enzymes offers a better understanding of the biochemical processes involved in acute and chronic alcohol intoxication. It is possible that at some stage it may also aid in understanding the effects of alcoholism and the existence of any physiological basis of the alcohol dependence, in a similar manner to drug dependence.

The major site of ethanol metabolism is the liver. Here the ethanol is oxidized to acetaldehyde, then further oxidized to acetate and subsequently to the important intermediate acetyl coenzyme A. The acetate portion of the acetyl coenzyme A molecule can be oxidized completely to carbon dioxide and water or converted via the citric acid cycle to other biologically important compounds, such as fatty acids.

The major enzyme involved in the conversion of ethanol to acetaldehyde is alcohol dehydrogenase (EC 1.2.1.3), an NAD^+ dependent enzyme which can also catalyse the reverse reaction using NADH as the coenzyme. Since this enzyme was first purified (Bonichsen and Wassan, 1948) it has been the subject of intensive study. Another enzyme which may catalyse the reaction, but which is not as important, is the hydrogen peroxide dependent enzyme catalase (EC 1.11.1.6).

Several enzymes may catalyse the conversion of acetaldehyde to acetate. The flavoproteins xanthine oxidase (EC 1.2.3.1) and aldehyde oxidase (EC 1.2.3.2) may catalyse the reaction, producing hydrogen peroxide, however the most important enzyme is the NAD^+ dependent aldehyde dehydrogenase (EC 1.2.1.3) (Richert and Westerfield, 1957). Initially an enzyme system "aldehyde mutase" was thought to catalyse the

dismutation of acetaldehyde to ethanol and acetic acid (a Cannizzaro reaction). However it was subsequently shown by Racker (1949) that this mutase activity was in fact due to the presence of two enzymes, alcohol dehydrogenase and a previously unknown enzyme aldehyde dehydrogenase. Racker then purified this aldehyde dehydrogenase from bovine liver and reported the wide aldehyde specificity of the enzyme. The enzyme has since been isolated from various different sources, however it has been much less extensively investigated than alcohol dehydrogenase.

It has been suggested that since acetaldehyde is a much more reactive compound and is more lipid soluble than ethanol it, rather than ethanol itself, is responsible for the effects of alcohol consumption (Truitt and Walsh, 1971). Acetaldehyde may form Schiff-base addition products with many aromatic amines, including some with endogenous importance (Cohen and Collins, 1970). Mitochondrial oxygen consumption, respiratory control, oxidative phosphorylation and energized calcium uptake were all found to be inhibited by acetaldehyde; and the malate-aspartate, α-glycerophosphate and fatty acid shuttles, reconstituted with either ethanol-alcohol dehydrogenase or lactate-lactic dehydrogenase, were highly sensitive to the compound (Rubin and Cederbaum, 1974). Certain symptoms of the "hangover" after drinking such as headache, sweating, nausea and vomiting have been attributed to acetaldehyde (Himwich, 1956). The "aversion therapy" treatment of alcoholism using the drug disulfiram is attributed to the inhibition of aldehyde dehydrogenase and subsequent accumulation of acetaldehyde, producing unpleasant symptoms (Truitt and Duritz, 1967). However, although high blood acetaldehyde concentrations have been found after disulfiram treatment the usual level following alcohol consumption is very low (of the order of 20μM). It has been observed by Korsten et al. (1975) though, that the blood acetaldehyde concentration of alcoholics may be elevated (of the order of 40-50μM).

Aldehyde dehydrogenases have been shown to be capable of utilizing a very wide range of aldehydes as substrates and it has been demonstrated that the enzyme is involved in the oxidation of aldehydes arising from biologically active

amines (Erwin and Deitrich, 1966). Unlike alcohol dehydrogenase (which is mainly in the liver) it has been found that aldehyde dehydrogenase is widely distributed among the tissues with 70% in the liver, 10% in the kidney, 7% in the adrenals, 4% in the small intestine and 2% in the brain (Deitrich, 1966). It has been suggested by Walsh and Davis (1970) that acetaldehyde, from alcohol consumption, inhibits the use of aldehyde dehydrogenase to metabolize biogenic amines in the brain allowing the nonenzymatic formation of condensation products which resemble apomorphine and may be linked to the similarity in withdrawal symptoms of opium addicts and alcoholics.

Since acetaldehyde is an important compound in alcohol metabolism it was thought to be of interest to study the mechanism of action of aldehyde dehydrogenase as part of a wide ranging study of alcohol metabolism being carried out in this department. Sheep were used as the source of this enzyme since fresh livers were always in plentiful supply.

SECTION 2

PURIFICATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE FROM SHEEP LIVER

2.1 INTRODUCTION

Aldehyde dehydrogenase (EC 1.2.1.3) was first isolated by Racker (1949) from bovine liver, demonstrating the existence of a distinct aldehyde oxidizing enzyme which in combination with alcohol dehydrogenase had the properties of the "aldehyde mutase" enzyme. The preparation of the enzyme was carried out by ethanol precipitation, nucleic acid precipitation and finally addition of protamine sulphate, producing a 30 fold purification with a 20-40% yield. After purification the enzyme was quite unstable and lost activity reasonably quickly. However, long term stability was improved by freezing the enzyme in a dry ice box. The purification scheme of Racker was improved by Deitrich et al. (1962) who obtained a specific activity which was twice as high and was able to demonstrate the wide range of substrates capable of being oxidized by the enzyme.

Most studies of the behaviour of aldehyde dehydrogenase had been conducted on crude total cell homogenates, or fractions which had been purified to a small extent and it was not until after the sheep liver purification was commenced that Shum and Blair (1972) used column chromatography techniques in an attempt to purify the enzyme. Their study on the supernatant fraction of rat liver showed the presence of two enzymes, one of which was unretarded on DEAE cellulose and another which was bound, these enzymes exhibiting different substrate specificities. After ammonium sulphate precipitation, CM-cellulose, DEAE cellulose and Sephadex G-200 chromatography, the specific activity of the DEAE-bound enzyme was 390 nmoles $(min.mg)^{-1}$. Not long afterwards, Feldman and Weiner (1972a) reported the purification to homogeneity of horse liver aldehyde dehydrogenase, using similar chromatographic techniques.

Since reports in the literature indicated that there may be both a cytoplasmic and a mitochondrial aldehyde

dehydrogenase present in mammalian livers an enzyme separation and purification was carried out, in conjunction with Dr. T.M. Kitson and K. Crow, to attempt to obtain sheep liver enzyme samples which were pure enough to be used in kinetic and physical enzyme studies.

2.2 METHODS

2.2.1 Buffers

The phosphate buffers used in the enzyme preparation were prepared according to Dawson et al. (1969) from potassium dihydrogen phosphate and sodium hydroxide (the buffer concentration is expressed in terms of potassium dihydrogen phosphate concentration).

2.2.2 Protein Determination

The relative protein concentration was estimated by the absorbance at 280nm and the actual protein concentration was determined by the method of Lowry et al. (1951).

2.2.3 Ammonium Sulphate Precipitation

The ammonium sulphate precipitations were carried out by the slow addition of powdered ammonium sulphate to the enzyme solution while the temperature was maintained at 0-4°C. A table of the amount of ammonium sulphate necessary to produce the required percentage saturation is given by Dawson et al. (1969).

2.2.4 Temperature

All chromatography, centrifugation, ammonium sulphate precipitation and enzyme storage was carried out at 0-4°C.

2.2.5 Gel Electrophoresis

An Ortec 4200 electrophoresis system was used to carry out slab gel electrophoresis. All solutions were made up according to the manual supplied, usually a single layer 8% acrylamide gel run at pH 9.0. The gels were stained for protein using Amido Black and activity stained for aldehyde dehydrogenase using phenazine methosulphate and nitroblue tetrazolium solutions with NAD^+ and acetaldehyde, as described by Robbins (1966).

2.2.6 Preparation of the Affinity Column

The affinity column for aldehyde dehydrogenase was prepared following the method of Cuatrecasas (1970). The method involved the addition of an aliphatic 'arm' to a cyanogen bromide-activated beaded agarose gel followed by the addition of the enzyme-specific group to the column by the reaction of a carboxylic acid group with the amino group of the aliphatic 'arm'. Thus the enzyme specific group is separated from the agarose bead by the length of the 'arm' and so more able to bind to the enzyme without steric hinderance. Bio-gel A-15 agarose ($20cm^3$) was mixed with an equal volume of water and small crystals of cyanogen bromide (5g) were then added. The pH of the suspension was immediately raised to, and maintained at, pH 11 with addition of NaOH (4M) and the temperature was maintained at 20°C by adding ice. The reaction was complete in about 15 minutes as shown by cessation of proton release. Having formed the activated agarose a large amount of ice was added to the suspension, and the entire solution added to a buchner funnel, in which it was washed under suction with cold $Na_2CO_3/NaHCO_3$ buffer ($300cm^3$). The 3,3'diaminodipropylamine solution (5.25g in water ($20cm^3$) titrated to pH 10 with 6M hydrochloric acid) was added to the activated agarose, mixed well, and the entire solution transferred to a flask and very gently stirred for 3 hours. The mixture was then allowed to stand for 22 hours after which the gel was washed with seven litres of distilled water. Since the gel was stable at this stage the addition of the enzyme-specific group could be made at leisure. Carboxybenzaldehyde (200mg) in dimethylformamide ($8cm^3$) was mixed with the diaminopropyl agarose gel ($8cm^3$) and the pH of the suspension was brought to pH 4.7 with sodium hydroxide (1M). 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulphonate (0.2g) dissolved in dimethylformamide ($0.6cm^3$) was then added over a 5 minute period, the reaction being allowed to proceed overnight at room temperature. Finally the affinity column produced was washed with large quantities of distilled water.

2.2.7 Enzyme Assays

(1) Aldehyde dehydrogenase assay solution was as follows:

Pyrophosphate buffer pH 9.3	33.0mM
NAD^+	1.7mM
acetaldehyde	1.7mM
and sample	

The final volume was $3cm^3$ and the activity of the enzyme solution (expressed as μmoles of NADH produced per minute per cm^3 of enzyme solution) was determined by following the increase in absorbance at 340nm due to the formation of NADH. Interference in the assay from alcohol dehydrogenase was stopped by the addition of pyrazole (0.5mM), which is a potent inhibitor of that enzyme, to all samples suspected of containing alcohol dehydrogenase. Pyrazole did not affect the activity of aldehyde dehydrogenase.

(2) Alcohol dehydrogenase assay solution was as follows:

Pyrophosphate buffer pH 8.8	5mM
NADH	0.15mM
acetaldehyde	1.7mM
and sample	

The activity was determined by following the disappearance of NADH absorbance at 340nm.

(3) Lactate dehydrogenase assay solution was as follows:

Phosphate buffer pH 7.4	100mM
NADH	0.16mM
sodium pyruvate	0.76mM
and sample	

The disappearance of NADH absorbance at 340nm was followed.

(4) Malate dehydrogenase assay solution was as follows:

Sodium glycinate buffer pH 10	100mM
NAD^+	0.43mM
sodium-L-malate	33mM
and sample	

The increase in the NADH absorbance at 340nm was followed.

(5) Catalase

The relative catalase concentration was estimated by measuring the absorbance at 400nm. The actual catalase concentration was determined by measuring the decrease in absorbance at 240nm when hydrogen peroxide was added to the enzyme solution (Luck, 1965).

(6) Aldehyde oxidase

The activity was determined by following the decrease in the absorbance at 600nm when acetaldehyde and enzyme was mixed with the dye, 2,6-dichlorophenol-indolphenol (Hendtlass, 1973).

2.3 RESULTS

2.3.1 Development of the Enzyme Purification Procedure

At the time that the purification of the enzyme was being considered a new DEAE (diethylaminoethyl) anionic exchange resin, Protion, was being tested in the Department. The advantage of this resin was that it was capable of being used with a very fast flow rate and did not clog. Since it was not known whether there was more than one aldehyde dehydrogenase in the sheep liver it was thought that use of Protion resin as an initial purification step would enable enzyme elution patterns to be observed and would also be an efficient step in the purification procedure. Another reason for using the column as the first step was that initial studies showed a loss of enzyme activity if ammonium sulphate precipitation was used. Sheep livers, obtained fresh from the local freezing works, were homogenized in a Waring blender and diluted five times with 0.0012M phosphate buffer at pH 7.3. When this total homogenate was added to the Protion column it was found that the majority of the aldehyde dehydrogenase activity was unretarded on the column and came through with the void volume. On applying 0.1M sodium chloride to the column a further small amount of activity was removed. The column was rerun using the two eluted active fractions and it was found that the fraction which did not bind initially to the column was largely unretarded when rerun and similarly the other fraction was retarded as before. As the unbound fraction was by far the

largest activity peak an attempt was made to purify it further. The fractions were precipitated with a zero to sixty-five percent ammonium sulphate cut and after dialysis added to a second DEAE Protion column, from which the unretarded fraction was again bulked and precipitated with ammonium sulphate. After dialysis the enzyme solution was added to a double cationic CM (carboxymethyl) and anionic DEAE Protion column at pH 6.0. The activity was once again unretarded but only 0.1% of the activity was recovered. Addition of 2-mercaptoethanol and degassing of all the buffers increased this recovery to 0.7% in later preparations.

If, instead of 0.1M sodium chloride, a gradient of 0.0012M to 0.25M phosphate buffer was used to elute the protein off the column a large increase in the amount of activity retarded on the column was observed, suggesting that the strong sodium chloride solution was deactivating the enzyme. At the same time less activity was present in the void volume. A further improvement was observed if a stepwise increment to 0.025M phosphate buffer was used instead of the gradient. It therefore appeared that column overloading was playing a large part in the appearance of the unbound activity peak, even though protein overloading should not have been occurring at the concentrations used. Attempts were then made to purify the retarded fraction.

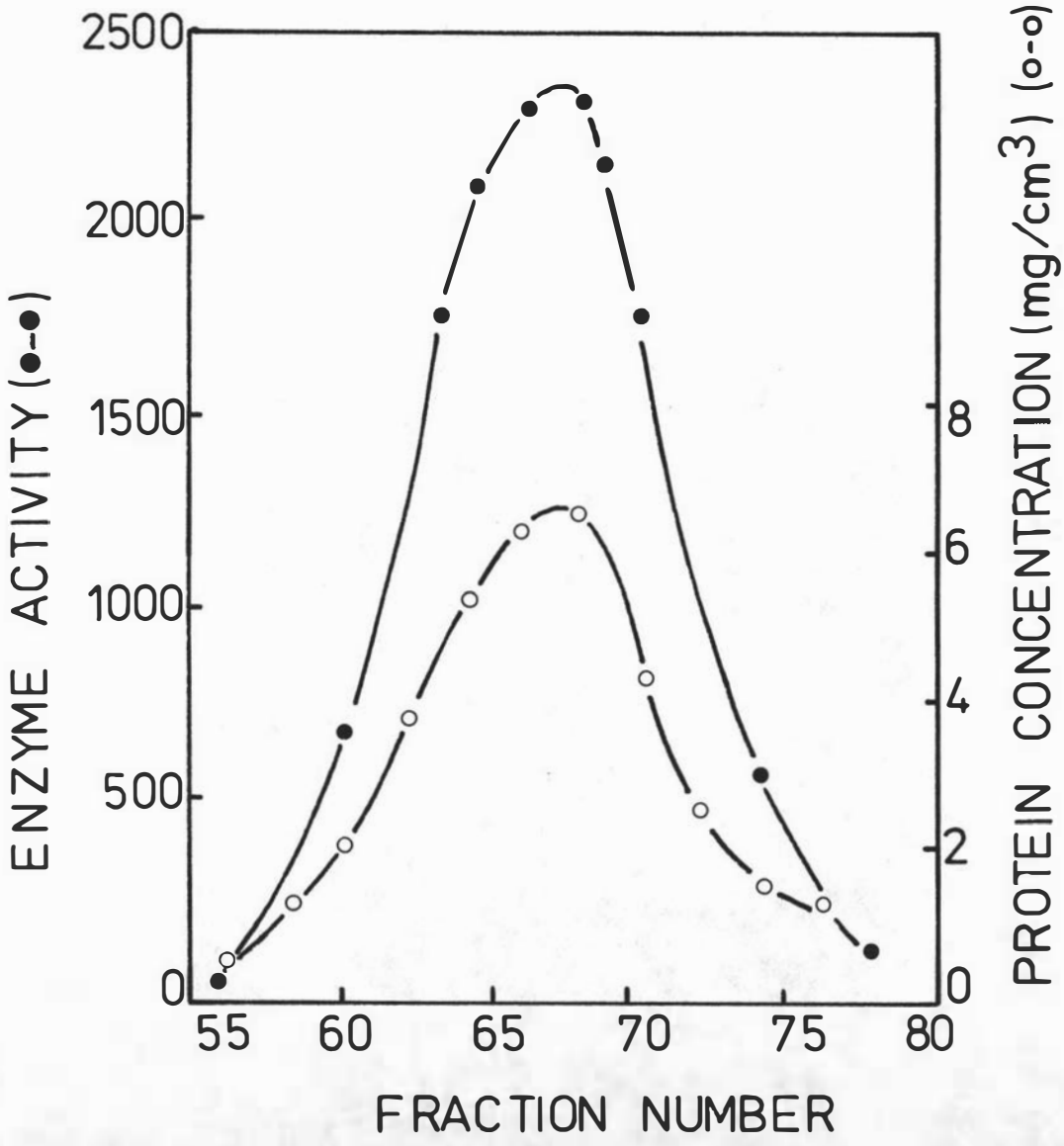
At this time T. Kitson, who was working in the same laboratory reported the existence of a mitochondrial sheep liver aldehyde dehydrogenase which was retarded on DEAE Protion columns. It therefore became necessary to isolate the separate cellular fractions, firstly to determine the enzyme activity in each of the cellular compartments and secondly to determine the elution patterns of the enzyme from each compartment. The cellular fractions were isolated by centrifugation and it was found that the cytoplasm contained 37% of the total activity, mitochondria 34% and the microsomes 10% while the remainder was present in whole cells which were unbroken by homogenization in a Potter-Elvenhjem homogenizer (Crow *et al.*, 1974). Elution patterns of these fractions showed that both the cytoplasmic and mitochondrial enzymes were retarded on the DEAE column while the microsomal enzyme was unretarded. It was apparent that cellular

fractionation should be carried out before any further purification so the mitochondrial and cytoplasmic enzymes could be separated. After fractionation the column behaviour of the cytoplasmic fraction was still erratic with various concentrations of activity being unretarded. However, if the unretarded peak activity was applied to a column with freshly prepared resin it was always retarded. Obviously the resin was regenerating very poorly and as the same column was being used, reduction in the binding capacity seemed to be taking place. Thus it was decided to use the more conventional technique of an ammonium sulphate precipitation as the first step so that less protein was added to the column in the second step. Preparations using this method, of an ammonium sulphate cut between 45% and 70% saturation followed by a column run, showed that 80% of the activity added to the column could be recovered in the retarded fraction. However, after a series of preparations it became obvious that the efficiency of the column was very inconsistent and could not be relied upon and large changes in column binding capacity were occurring. This column step was crucial for the removal of contaminating enzymes, such as alcohol dehydrogenase which should be unretarded and catalase which is retarded a little more than aldehyde dehydrogenase. Since the column run was now the second step in the purification, the fast flow characteristics of the Protion resin were no longer required and it was decided to replace the Protion resin by the well proven Whatman DEAE cellulose resin in the purification scheme. In Section 2.3.3 this final purification scheme is discussed in full.

2.3.2 Affinity Chromatography

To improve the purity of the enzyme after anionic exchange chromatography an attempt was made to design a column with an affinity which is specific for aldehyde dehydrogenase. To do this it is necessary to find a compound which will bind specifically to aldehyde dehydrogenase but is not a substrate and in addition it must be possible to attach this compound to a resin matrix in order to make a column. Since aldehyde dehydrogenase has few inhibitors (chloral hydrate is one but would be difficult to put on the

FIG. 2.1 GEL FILTRATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE



The enzyme activity is expressed as nmoles min^{-1} mg^{-1}
The column dimensions were 5.0 cm x 33 cm, flow rate $0.5 cm^3$/min and the volume of each fraction was about $4\ cm^3$. The total protein added to the column was 530 mg.

column) and will react with almost all aldehydes, the choice of compound was very difficult. It was decided to use p-carboxybenzaldehyde which was a poor substrate and once attached to the column should produce little reaction. After preparation of the column a $5cm^3$ enzyme sample in 0.05M phosphate buffer at pH 7.3 containing NAD^+ (16μM) (necessary because of the ordered addition of substrated with NAD^+ first) was added to the column and allowed to stand for half an hour. In washing the column to remove the unbound protein 20% of the enzyme activity was also eluted. The retarded enzyme activity was found to be very strongly bound to the affinity column and it required 0.5M phosphate buffer with 2.5mM acetaldehyde (to act as an alternative binding compound) to remove it from the column. Since the first column run was efficient and later runs were less effective it appeared that the aldehyde groups on the resin were slowly being converted to carboxylic groups. Thus the affinity column was not going to be practical for a large series of enzyme preparations and was not used further. However if the removal from the enzyme solution of apparently inseparable proteins was ever required the column would be very useful.

2.3.3 Purification Scheme for Cytoplasmic Aldehyde Dehydrogenase

Fresh sheep liver was obtained from the local freezing works and taken back to the laboratory in an ice bucket. The liver was cut into small pieces and homogenized in a Potter-Elvehjem homogenizer (with a teflon head) using 0.005M phosphate buffer at pH 7.3 containing 2-mercaptoethanol (14mM). The buffer also contained sucrose (0.25M) to prevent swelling of the mitochondria and hence possible contamination of the extract with mitochondrial enzymes. The homogenate was then centrifuged at 12,300g for one hour, the resulting precipitate was discarded and the supernatant centrifuged at 34,000g for two and a half hours to precipitate mitochondria and microsomes. Once again the precipitate was discarded and the supernatant (the cytoplasmic fraction) was taken to 45% saturation with ammonium sulphate, centrifuged and the supernatant retained. The supernatant was then

taken to 70% saturation with ammonium sulphate and centrifuged. The enzyme activity was contained in the precipitate which was redissolved in 0.005M phosphate buffer at pH 7.3 and dialysed against more buffer to reduce the high salt concentration. After dialysis the sample was centrifuged to remove any precipitate which may have formed and then loaded onto an anionic exchange column, Whatman DE 32 cellulose (5.5cm x 30cm) at a flow rate of 1 to 2cm^3 per minute. The column was then washed with 0.005M phosphate buffer (5cm^3/minute) until the protein concentration of the elutant was below 0.1mg/cm^3. At this stage 0.022M phosphate buffer at pH 7.3 was added to the column to elute the enzyme and fractions were collected. After assaying for enzyme activity and protein concentration, the most active fractions were bulked and concentrated (usually to 10-20cm^3) using an ultrafiltration Diaflow membrane (with XM100 filter) or by a 0% to 70% ammonium sulphate cut. The concentrated sample was then loaded onto a Biogel A 0.5M gel filtration column (5cm x 33cm) and the enzyme eluted with 0.022M phosphate buffer (Fig. 2.1). For a summary of the purification scheme see Table 2.1.

2.3.4 Purity of the Aldehyde Dehydrogenase Sample

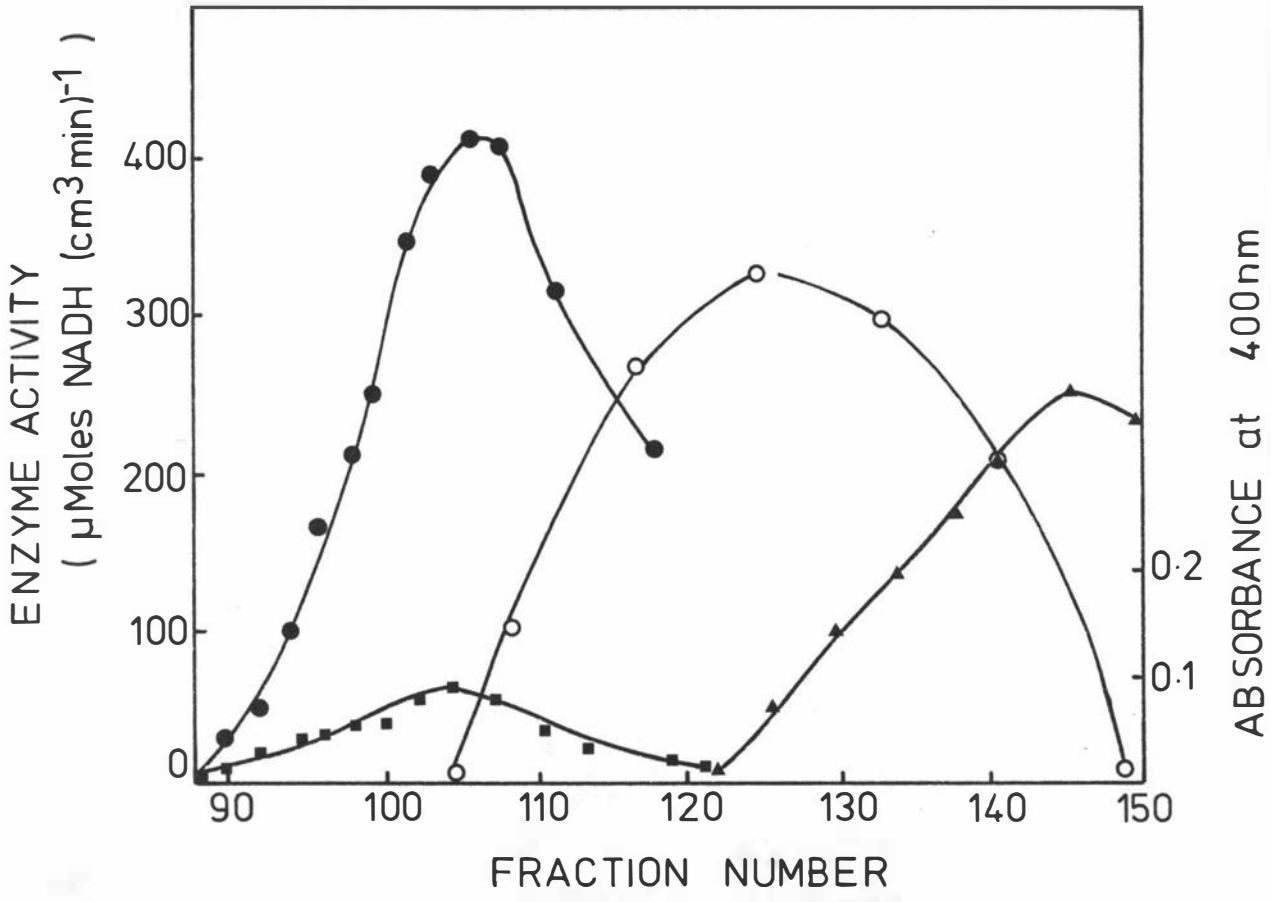
Polyacrylamide gels of the purified enzyme, stained for protein with Amido Black, showed only one band with a faint second band, even when large concentrations of enzyme were applied. (see photograph at back) The major band was shown to be aldehyde dehydrogenase by activity staining a similar gel and observing that the band formed in an identical position to the protein stain. The faint band was catalase contamination and was found, by measurement of peak areas on a scan of the gel, to be less than 5% of the total protein. The peak fractions of the Biogel column were assayed for other contaminating enzymes as shown in Fig. 2.2. No aldehyde oxidase or malate dehydrogenase could be detected however activities of alcohol dehydrogenase, lactate dehydrogenase and catalase could be detected. As seen in Fig. 2.2 the Biogel column is not efficient at removing these contaminating enzymes as their molecular weights are of the same order as that of aldehyde dehydrogenase and so only the initial fractions are not

TABLE 2.1 PURIFICATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE FROM SHEEP LIVER

Fraction	Volume (l)	Activity (μmoles NADH/min)	Protein (g)	Specific Activity (nmoles NADH. $min^{-1}.mg^{-1}$)	Recovery (%)
Cytoplasm	2.15	206	23.7	8.7	100
Dialysed 45-70% $(NH_4)_2SO_4$ precipitate	0.30	130	10.5	12.4	63
DEAE-cellulose eluted fraction	0.26	112	0.70	160	54
Dialysed 0-70% $(NH_4)_2SO_4$ precipitate	0.024	104	0.55	189	50
Biogel eluted fraction	0.057	95	0.25	380	46

The 11 peak tubes of the Biogel eluted fraction were bulked, however the specific activity of each tube was about the same. Over a series of 7 preparations the final specific activity varied from 130 to 390 and the % recovery varied from 10% to 46%.

FIG. 2.2 ELUTION PROFILE OF CONTAMINATING ENZYME ON BIOGEL GEL FILTRATION COLUMN



The activity of the dehydrogenases; aldehyde dehydrogenase (●–●), lactate dehydrogenase (○–○) and alcohol dehydrogenase (▲–▲) are expressed as moles of NADH produced or reacted every minute for each cm^3 of enzyme solution. The relative catalase activity is shown by the absorbance at 400 nm (■–■).

contaminated by other dehydrogenases. It must be remembered that though the activity of the contaminating enzymes is high the fact that they react much faster than aldehyde dehydrogenase means that their actual protein concentration is small. However, all these enzymes may be removed by DEAE cellulose chromatography and so it is apparent that the efficiency of that column is essential to a good enzyme purification. An indication of this efficiency is given by the movement of the green catalase containing band, which is retarded slightly more than aldehyde dehydrogenase, on the DEAE cellulose column. If this band remains on the column while the aldehyde dehydrogenase activity is being eluted then the level of contaminating enzymes will be very low. Thus by careful column chromatography, or by rerunning samples if necessary, the level of contaminating enzymes activity could be reduced to undetectable levels.

2.4 DISCUSSION

It appears that the original confusion over the retarded and unretarded peaks on DEAE Protion resin can be explained. The unretarded peak was due in part to the low binding capacity of the Protion column which allowed an activity overflow and also due to the presence of a microsomal enzyme. The cellular fraction of the sheep liver showed the existence of such an enzyme and it was found to be unretarded on Protion column. Another source of confusion was the fact that the original sodium chloride eluant deactivated the retarded activity and hence masked the presence of the enzyme on the column. These problems were solved by separating the cytoplasmic fraction of sheep liver before attempting enzyme purification.

The purification scheme (Table 2.1) shows that there is a forty-five fold increase in the specific activity of cytoplasmic aldehyde dehydrogenase from the initial cytoplasmic fraction (obtained after fractional centrifugation) to the enzyme solution eluted from the Biogel gel filtration column. The first mammalian aldehyde dehydrogenase purified to homogeneity, that from horse liver, was purified 250 fold from a total liver homogenate producing a specific activity of 720 nmoles.(min.mg)$^{-1}$ (Feldman and Weiner, 1972a). The final

purification step involved isoelectric focusing of the enzyme solution and improved the specific activity 6 fold. Later workers have shown that this enzyme was mitochondrial in origin and that a lesser activity peak, observed but ignored by Feldman and Weiner, was an aldehyde dehydrogenase of cytoplasmic origin (Eckfeldt et al., 1976) with a specific activity after purification of 370 nmoles.(min.mg)$^{-1}$. This observation of distinct mitochondrial and cytoplasmic enzymes is similar to that which has been found for sheep liver and reported by Crow et al. (1974). The degree to which the cytoplasmic sheep liver enzyme has been purified is similar to that for the most highly purified aldehyde dehydrogenase the horse liver enzyme, with the additional isoelectric focusing step. The specific activity of the cytoplasmic horse liver enzyme (called F_1) is almost identical to that found for the sheep liver enzyme and any difference with the horse liver mitochondrial enzyme (F_2) may simply reflect differences in catalytic-centre activity rather than any difference in purity. Recently a purification of human liver aldehyde dehydrogenase has been described (Sidhu and Blair, 1975a). Using a similar purification scheme to that used for the cytoplasmic sheep liver enzyme a specific activity of 1200 nmoles (min.mg)$^{-1}$ was found which is somewhat higher than that found for the horse liver and sheep liver enzymes, but which showed similar behaviour on columns during purification. This suggests that the aldehyde dehydrogenases from these sources are reasonably similar.

Further work has been carried out on the physical properties of cytoplasmic sheep liver aldehyde dehydrogenase by Kathy Crow in this laboratory (Crow, 1975). She found that the molecular weight of the enzyme was 212,000 $\pm$ 8,000 from gel filtration with marker enzymes and sodium dodecyl sulphate gel electrophoresis produced a single band of 53,000 $\pm$ 2,000 suggesting that the enzyme is a tetramer with identical subunits. Isoelectric focusing experiments in a glass column (110cm^3) in the range pH 4.5 to pH 5.5 showed a single band with an isoelectric point of 5.25. Once again these results are comparable with the recently reported horse liver enzymes with molecular weights of 230,000 and 240,000 for the F_1 and F_2 isozymes respectively and 52,000 and 53,000 for the subunit molecular weights (by sodium dodecyl sulphate-polyacrylamide

gel electrophoresis) for F_1 and F_2 isozymes again (Eckfeldt *et al.*, 1975). The isoelectric focusing of F_2 gave a band at pH 5.5, in the same region as the sheep liver enzyme, but in some preparations an additional band at pH 4.8 was reported.

Since the cytoplasmic enzyme from sheep liver appeared to be free from contaminating enzymes it was judged suitable for use in kinetic studies on the enzyme.

SECTION 3

EQUILIBRIUM STUDIES AND THIOL DETERMINATIONS

3.1 INTRODUCTION

3.1.1 Coenzyme Binding

Aldehyde dehydrogenase catalyses the reaction of aldehydes and the coenzyme NAD^+ to produce the corresponding acids and the reduced coenzyme NADH. Study of the coenzyme binding to the enzyme can aid the elucidation of the enzyme mechanism in addition to determination of the dissociation constants of the species involved. The enzyme-coenzyme complex formation is usually monitored by associated absorption or fluorescence changes. For example, horse liver alcohol dehydrogenase forms a complex with NADH which is characterised by a shift in the absorption maximum of the reduced coenzyme from 340 nm to 325 nm (Theorell and Bonnichsen, 1951) which allows the formation of the complex to be followed. On the other hand, for beef heart lactate dehydrogenase, NADH exhibits only a small shift in absorbance on binding to the enzyme, but there is a large enhancement and shift in the fluorescence maximum of NADH from 462 nm to 440 nm (Velick, 1958). These changes have been used to determine the dissociation constants of the complexes and the stoichiometry of the binding of coenzyme to enzyme. Other compounds which can form ternary complexes with the enzyme and coenzyme have been shown to induce further changes to the absorbance and fluorescence spectrum of enzyme-bound NADH. In beef heart lactate dehydrogenase oxamate, an inhibitor of the enzyme, was found to quench the fluorescence of the enzyme-coenzyme complex while the addition of L-lactate to the complex of rabbit muscle enzyme and NADH produced an additional shift and enhancement of NADH fluorescence (Fromm, 1970). This latter effect is due to the formation of the dead-end ternary complex enzyme-NADH-lactate, lactate being the product of the reaction of enzyme-NADH with pyruvate.

It was decided to attempt to detect any absorbance

or fluorescence changes that occurred on binding of NADH to sheep liver aldehyde dehydrogenase and determine whether such changes could be used to measure dissociation constants and the total concentration of binding sites. At that time the only aldehyde dehydrogenase to have been studied was from yeast (Bradbury and Jakoby, 1971b) in which the co-enzyme was found to bind to a binary complex composed of enzyme and aldehyde, and an enhancement of fluorescence was observed.

3.1.2 Theory of Ligand Binding

An enzyme often contains a number of potential binding sites for ligands. If the binding sites are all identical then they will act as independent entities such that the binding of a ligand at one site of the enzyme will not affect the likelihood of a second ligand binding to another site on the same enzyme. For ligand binding to a homogeneous population of independent binding sites, the dissociation constant, K_D, is defined by the equation

$$K_D = \frac{[E][NADH]}{[E\text{-}NADH]} \qquad (1)$$

where $[E]$ is the concentration of free binding sites
$[NADH]$ is the concentration of unbound ligand (in this example NADH)
$[E\text{-}NADH]$ is the concentration of bound ligand (or occupied binding sites)

Since the total concentration of each species (binding sites and ligand) is the sum of the bound and unbound species, then equation (1) becomes

$$K_D = \frac{([E]_o - [E\text{-}NADH])([NADH]_o - [E\text{-}NADH])}{[E\text{-}NADH]} \qquad (2)$$

where $[E]_o$ is the total concentration of binding sites
$[NADH]_o$ is the total ligand concentration.

If the proportion of the total number of binding sites that are occupied is defined as R, that is

$$R = \frac{[E\text{-}NADH]}{[E]_o} \qquad (3)$$

then on substituting $R[E]_o$ for $[E\text{-}NADH]$ equation (2) becomes

$$K_D = \frac{[E]_o(1 - R)([NADH]_o - R[E]_o)}{R[E]_o} \qquad (4)$$

Rearrangement of this equation gives

$$\frac{1}{(1 - R)} = \frac{[NADH]_o}{K_D.R} - \frac{[E]_o}{K_D} \qquad (5)$$

The utility of equation (5) is that often the fraction of binding sites occupied is known from some physical property while the total concentration of binding sites may be unknown. A plot of $\frac{1}{1 - R}$ against $\frac{[NADH]_o}{R}$ where different concentrations of NADH have been used will have a slope of $\frac{1}{K_D}$, whence K_D may be determined, and an intercept on the ordinate axis of $[E]_o$, the concentration of binding sites (Gutfreund, 1972). Linearity of this plot may be taken as evidence for independence of the binding sites.

3.1.3 Application to Fluorescence Titration

The fluorescence difference observed in the emission spectra of free NADH and the enzyme-NADH complex (see results) allows the enzyme present in solution to be "titrated" with NADH, that is NADH may be added to the solution until the fluorescence change between aliquots is the same as if no enzyme was present. In the control sample in which NADH is added to a buffer solution, the following relationship holds;

$$Q_1 [NADH]_o = F_1 \qquad (6)$$

where Q_1 is the molar emission of the coenzyme
$[NADH]_o$ is the total concentration of NADH
and F_1 is the observed fluorescence of NADH

This expression was found to hold as long as $[NADH]_o$ was less than about 10μM. Above this value deviations from the linear relationship occurred due to absorption of the

incident light by the NADH solution. When enzyme and NADH are mixed in solution two species contribute towards the fluorescence

$$Q_1 [\underline{NADH}] + Q_2 [\underline{E\text{-}NADH}] = F_2 \qquad (7)$$

where $[\underline{NADH}]$ is the concentration of unbound coenzyme
$[\underline{E\text{-}NADH}]$ is the concentration of bound coenzyme
Q_2 is the molar emission of the bound coenzyme
and F_2 is the observed fluorescence.
However, as $[\underline{NADH}] = [\underline{NADH}]_o - [\underline{E\text{-}NADH}]$
then equation (7) becomes

$$Q_1 [\underline{NADH}]_o + (Q_2 - Q_1)[\underline{E\text{-}NADH}] = F_2 \qquad (8)$$

equations (6) and (8) being applicable to the control titration and the enzyme titration respectively. The E-NADH concentration is found to be proportional to the difference in fluorescence between the enzyme and control solutions, that is equation (8) minus equation (6).

$$(Q_2 - Q_1)[\underline{E\text{-}NADH}] = F_2 - F_1 = \Delta F \qquad (9)$$

On titration of all the enzyme in solution, the concentration of occupied binding sites reaches a maximum, equivalent to the total binding site concentration, $[\underline{E}]_o$, and hence ΔF of equation (9) reaches a maximum value, ΔF_{max}. In defining the fraction of binding sites occupied by NADH as R, where $R = \frac{\Delta F}{\Delta F_{max}}$, we have enough information to be able to use equation (5) as values for R and $[\underline{NADH}]_o$ are known. A plot of $\frac{1}{(1 - R)}$ against $\frac{[\underline{NADH}]_o}{R}$ may be constructed from which values for K_D and $[\underline{E}]_o$ are obtainable.

3.1.4 Thiol Determinations

The amino acids cysteine and cystine contain a thiol group and a disulphide group respectively, so these groups are also features of the enzymes containing these amino acids. However, thiol groups present in a single enzyme molecule may have measurably different properties and these differences are of interest in determining the various functions of these thiol groups in the enzyme. Many enzymes

have been studied in this regard (Jocelyn, 1972).

Several studies have been reported relating the sulphydryl character of aldehyde dehydrogenase from various sources (Stoppani and Milstein, 1957; Deitrich, 1967; Duncan and Tipton, 1971a). Deitrich (1967) found that the bovine liver enzyme was inhibited by a series of sulphydryl reagents, arsenite, γ-(p-arsenophenyl)-n-butyrate, o-iodosobenzoate and p-chloromercuribenzoate and that the inhibition was reduced in the presence of NAD^+. A reaction mechanism has been postulated by Feldman and Weiner (1972b) for the action of horse liver aldehyde dehydrogenase in which the aldehyde substrate reacts with an enzyme sulphydryl group, but no direct evidence for the presence of the group was presented.

It is known that alcoholics drinking alcohol while under treatment with disulfiram (tetraethylthiuram disulphide) suffer from very unpleasant symptoms including nausea, vomiting, respiratory distress and headaches and hence are discouraged from consuming alcohol (aversion therapy). The action of the disulphide, disulfiram, is attributed to inhibition of aldehyde dehydrogenase and subsequent accumulation of acetaldehyde (Truitt and Duritz, 1967). Kitson (1975) in this laboratory found with cytoplasmic sheep liver aldehyde dehydrogenase that disulfiram produced an initial inhibition of the enzyme followed by a time dependant loss of activity. 2-Mercaptoethanol used in all the buffers during the purification procedure was found to increase the stability of the sheep liver enzyme and from these observations it appeared to be of interest to examine the relationship between the number of sulphydryl groups and the enzyme activity. 5,5'-Dithio-bis(2-nitrobenzoic acid)(Nbs_2) was used in the study as its absorbance change on reaction meant that the thiol reaction and the enzyme activity could be studied simultaneously.

3.2 MATERIAL AND METHODS

3.2.1 U.V. Spectra

The ultraviolet spectra of the coenzymes were studied on a Shimadzu MPS-500 Spectrophotometer. The instrument had

an 8 point range control switch which allowed scale expansion down to ± 0.0125 absorbance units, a feature which was useful in difference experiments. By doubling the NADH concentration of a buffered solution and halving the sensitivity the two absorbance curves could be superimposed. Repeated addition of NADH to a solution of aldehyde dehydrogenase using this method meant that the standard NADH peak height was constant and any change in the shape of the peak must be due to the presence of the enzyme. NADH was added by a Hamilton syringe in aliquots, one of which made a 2μM solution in the pH 7.6 phosphate buffer.

3.2.2 Fluorimeter

Fluorescence measurements were made with a Turner 430 Spectrofluorimeter with a 150W Xenon light source. Excitation and emission spectra were recorded on a $3cm^3$ solution contained in a jacketed cell holder through which water at 25°C was circulated. The slit widths for the excitation and emission monochromators were 15 nm and 60 nm respectively. The change in fluorescence between a quinine sulphate solution (25μg/litre in 0.05M sulphuric acid) and 0.05M sulphuric acid was used as a standard and arbitrarily set to 1.0. All other fluorescence changes were related to this value so all results could be directly compared. These fluorescence changes could be related to the actual concentration of the NADH by construction of standard curves with NADH of known concentration.

3.2.3 Titration Reagents

The phosphate buffer used was a solution of potassium dihydrogen phosphate (0.025M) containing sufficient sodium hydroxide to bring the pH to 7.6. The buffer solution was filtered slowly through sintered glass to remove any particulate material which might interfere with the fluorescence measurements by causing scattering of the incident radiation. The NADH was obtained in preweighed vials (Sigma Grade III). The concentration of the NADH could be checked by measuring the absorption at 340 nm assuming a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}cm^{-1}$ (Horecker and Kornberg, 1948).

3.2.4 Titration Method

Fluorescence titrations were carried out in quartz cells in which NADH was excited at 340 nm and the emission measured at 435 nm. The NADH was added to the enzyme solution by a Hamilton syringe attached to a micrometer which delivered a $0.01cm^3$ aliquot for each revolution. Titrations were performed by addition of fifteen to twenty aliquots of $0.01cm^3$ NADH solution (1.28 nmoles/aliquot) to a $3cm^3$ enzyme solution, the fluorescence being measured after each addition. As the maximum dilution of the enzyme during the titration was only 7%, the enzyme concentration was assumed to be constant. The total NADH concentration was, however, corrected at each step for the volume increase. A control titration was always carried out, in conjunction with the sample titration, in which the fluorescence of NADH added to a buffer solution was measured.

3.2.5 Treatment of Data

From the titration, the difference in fluorescence between the sample and control solutions (that is, ΔF), was found after each aliquot addition, that is for each value of $[NADH]_o$. The titration was continued until there was no further fluorescence enhancement and so the limiting value of the fluorescence difference was reached. An estimate of ΔF_{max} was obtained from the titration curve and this value varied to give a straight line when the data was plotted according to equation (5). Estimates of ΔF_{max} either 5% too low or too high produced marked deviations from a straight line, at high values of

$$\frac{[NADH]_o}{R}$$

(Stinson and Holbrook, 1973), and the ΔF_{max} value selected in this way was always reasonable with respect to the original titration curve.

It was found that the apparent ΔF_{max} obtained from the experimental titration curve was 10% too low due to a number of factors. Since the dissociation constant is of the same order of magnitude as the enzyme concentration, the

fluorescence difference only slowly approaches an assymptotic value making it difficult to estimate whether the maximum has been reached. The titration could not be extended as the NADH fluorescence becomes non-linear after about 10μM NADH and if the enzyme concentration is reduced, the results are less accurate due to lack of instrument sensitivity.

3.2.6 Enzyme Assay

An enzyme aliquot ($0.1cm^3$) was assayed spectrofluorimetrically (excitation wavelength 345 nm and emission wavelength 455 nm) with an assay mixture consisting of pH 7.6 phosphate buffer ($2.15cm^3$), NAD^+ (420μM) and propionaldehyde (95μM). For the enzyme assay used in the thiol determinations NAD^+ (1.7mM) was used.

3.2.7 Zinc Content

An enzyme sample ($2cm^3$) was dialysed for four days against frequent changes of pH 7.4, 0.05M phosphate buffer, which was made up in deionized water and contained 0.01 ppm zinc. The zinc concentration of the dialysed sample was determined by atomic absorption spectroscopy, a method which has a sensitivity of 0.01 ppm. Measurements were made using a Varian-Techtron AA-5 at a wavelength of 213.9 nm and the zinc concentration was determined from a standard curve prepared by using standard zinc solutions of known concentration. The possibility of some of the observed absorption being non-atomic in origin was checked by measuring the absorption signal from a hydrogen continuum source.

3.2.8 Thiol Determinations

Phosphate buffer pH 8.0, $\mu = 0.1$, was used in all the thiol procedures unless otherwise stated, and in later runs EDTA (1mM) was added to the buffer to help stabilize the colour of 2-nitro-5-thiobenzoate, which slowly fades due to autooxidation (Jocelyn, 1972). The reagent used in the thiol determinations was Ellman's reagent (Ellman, 1959). The Ellman's reagent consisted of 5,5'-dithio -bis(2-nitrobenzoic acid), (Nbs_2), (2 - 8mM) in pH 7.2 phosphate buffer

(μ = 0.1) in which the compound is stable. The reaction with thiol groups was followed at room temperature in a U.V. cell containing enzyme solution ($1cm^3$), Ellman's reagent ($0.1cm^3$) and buffer ($2cm^3$). Sodium dodecylsulphate was .3% (0.3g in $100cm^3$) when used in assays. The reaction of an enzyme thiol group with Ellman's reagent is as follows:

$$E - s^- + Nbs_2 \longrightarrow E\text{-}s\text{-}s\text{-}Nb + \text{2-nitro-5-thiobenzoate } (Nbs^-)$$

The release of 2-nitro-5-thiobenzoate was followed spectrophotometrically at 412 nm against a blank without enzyme. The number of thiol groups was determined assuming a molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$ for 2-nitro-5-thiobenzoate (Ellman, 1959). Addition of an excess of reducing agent (for example 2-mercaptoethanol) cleaves the mixed disulphide bond and liberates further 2-nitro-5-thiobenzoate (Butterworth et al., 1967).

As 2-mercaptoethanol (14mM), which was present in the buffers used in the purification and hence in the enzyme sample, also reacts with Nbs_2 it must be removed before the determination of thiols. An enzyme solution ($1cm^3$) was dialysed against pH 8.0 phosphate buffer for 2 hours at 4°C. The dialysed enzyme solution was added to a Sephadex G-25-80 gel filtration column (1.75cm(dia) x 7cm) and eluted with buffer. The elutant was collected with a fraction collector and analysed at 280 nm for protein absorption to detect the unretarded enzyme peak. The peak fractions were bulked to give $3cm^3$ of enzyme solution. The fractions which followed were treated with $0.1cm^3$ of Nbs_2 stock solution and from the absorption at 412 nm the elution pattern of mercaptoethanol (which is reactive towards Nbs_2) was found and the separation between the enzyme and mercaptoethanol could be observed (Fig. 3.1). All thiol determinations were carried out as soon as possible after the removal of mercaptoethanol as the enzyme solutions were only stable for a few hours in the absence of mercaptoethanol.

3.3 RESULTS

3.3.1 U.V. Spectra

The coenzyme NADH has an ultraviolet absorption maximum at 340 nm and it was found that aldehyde dehydrogenase did not alter the spectrum. When NADH (aliquots of 6 nmoles in .01cm^3) was added to a solution of enzyme (2μM in 3cm^3) against an enzyme blank and using the sensitivity scale as outlined in the methods section, the peak remained at 340 nm with the height being that expected for native NADH using an extinction coefficient of 6.22 x $10^3 M^{-1} cm^{-1}$. Even a ten-fold increase in enzyme concentration produced no detectable difference. However, it must be noted that in carrying out NADH displacement experiments which are detailed in Section 5, there was found to be a decrease in extinction coefficient on binding of NADH to the enzyme, ($\Delta\varepsilon$ = 0.66 x $10^3 M^{-1} cm^{-1}$) at 340 nm.

The spectrum of NAD^+ (13μM) showed no change in the presence of aldehyde dehydrogenase (1μM) and chloral hydrate, a strong inhibitor of the enzyme, produced no change when added to the enzyme, NAD^+ mixture.

3.3.2 Fluorescence Spectra

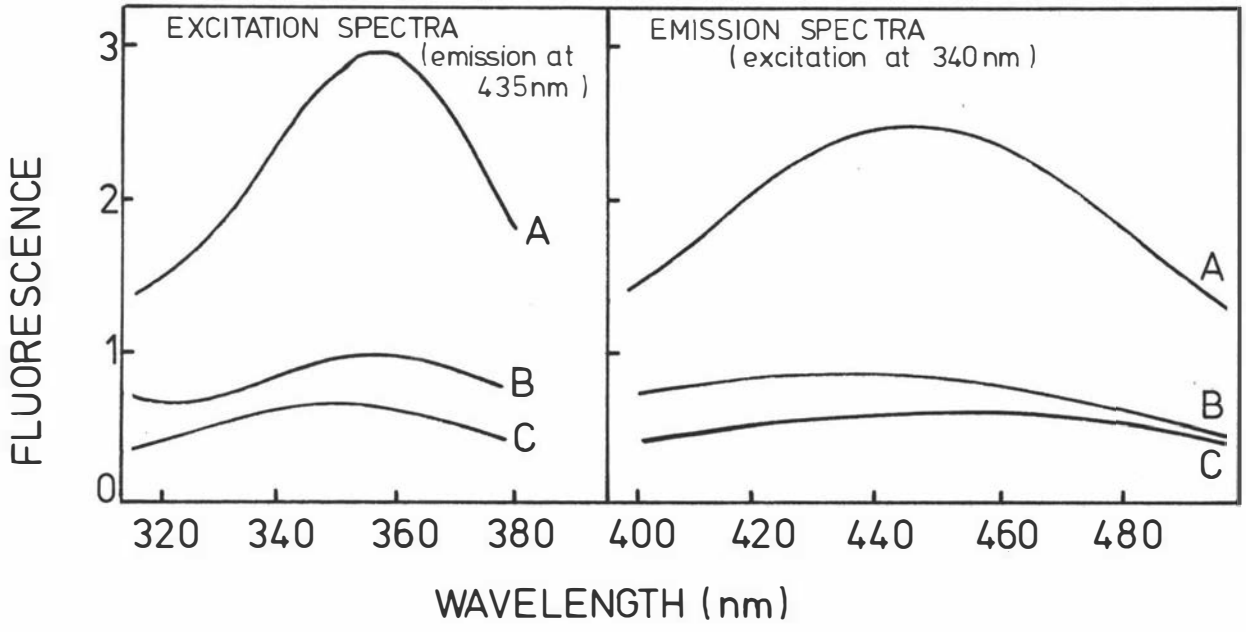
Fig. 3.2a shows the effect of enzyme complex formation on the excitation and emission spectrum of NADH. The addition of aldehyde dehydrogenase to the NADH solution results in a significant enhancement of the fluorescence emission and a shift in the emission maximum from 460 nm to about 445 nm. The protein fluorescence of aldehyde dehydrogenase was quenched on the addition of NADH (Fig. 3.2b) and it was found that NAD^+ was just as effective as NADH in its quenching properties.

3.3.3 NADH Titration

The enhancement of NADH fluorescence on binding to the enzyme was utilized in the titration experiments to determine the number of binding sites in solution as shown in Fig. 3.3a. The ΔF_{max} value chosen can be seen to be

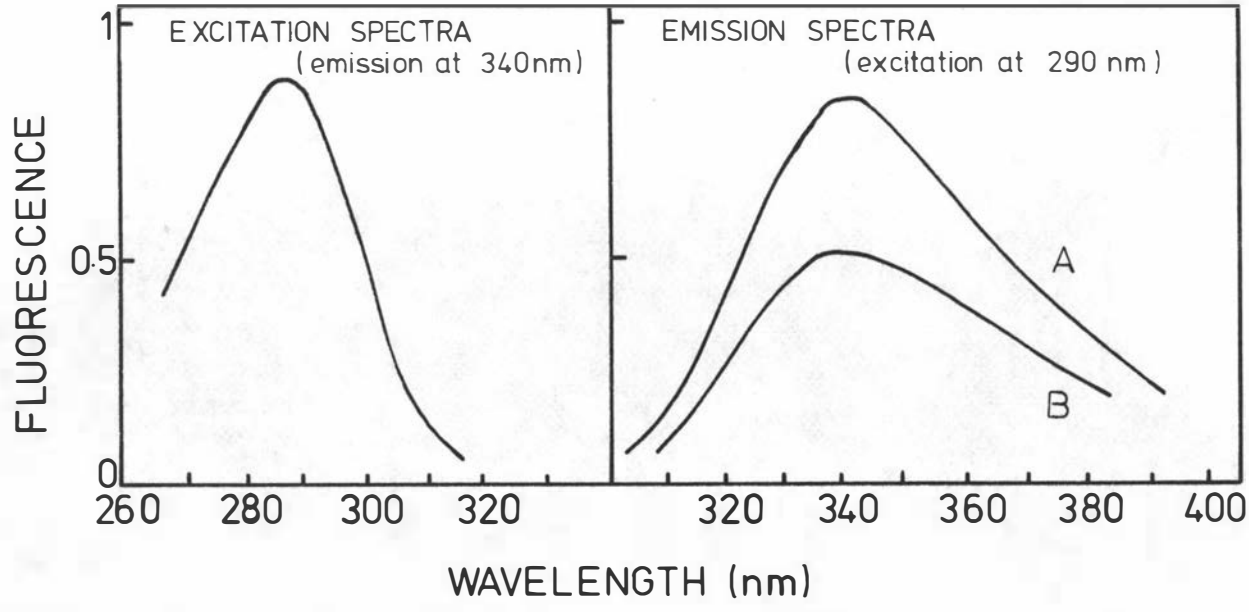
FIG. 3.2 FLUORESCENCE SPECTRA

(a) NUCLEOTIDE FLUORESCENCE



The nucleotide fluorescence spectra of aldehyde dehydrogenase (0.6 mg/cm^3) and NADH (1μM) in pH 7.6 phosphate buffer is shown in curve A. Curve B and C are enzyme and NADH alone, respectively.

(b) PROTEIN FLUORESCENCE



The protein fluorescence spectra of aldehyde dehydrogenase (0.1 mg/cm^3) in pH 7.6 phosophate buffer is shown in curve A. Curve B shows the quenching of fluorescence on addition of NADH (13μM).

consistent with the curve. A plot of $\frac{1}{(1 - R)}$ against

$$\frac{[NADH]_o}{R}$$

(equation (5)) shown in Fig. 3.3b was linear, the dissociation constant was 1.1μM and the concentration of NADH binding sites was 1.5μM. Hill plots of these results (Brown and Hill, 1922-23) gave straight lines with slopes of 1.0 ± .03 (Fig. 3.4).

3.3.4 Fluorescence Enhancement (Q)

Attempts were made to calculate the fluorescence enhancement of bound NADH by titration of a fixed NADH concentration with enzyme until the fluorescence reached a maximum value. However, the high protein concentrations required produced quenching of the fluorescence which made analysis difficult. The enhancement was, therefore, calculated from the titration of a fixed concentration of enzyme with NADH. The titration produced a value for ΔF_{max}, the maximum difference in fluorescence used in graphical analysis, and the number of binding sites. Since

$$\frac{\Delta F_{max}}{[E]_o} = (Q_2 - Q_1)$$

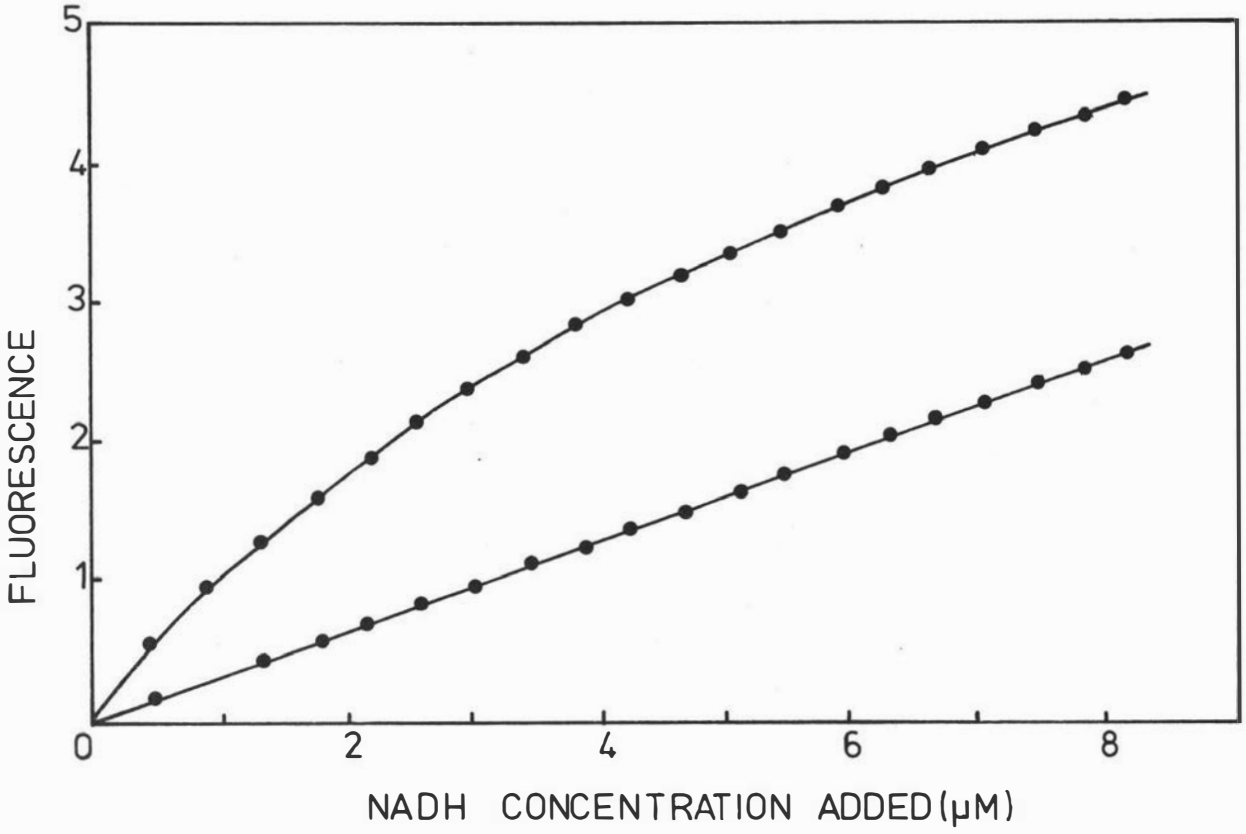
from equation (9), a value for $(Q_2 - Q_1)$ may be obtained from the titration curve. As Q_1, the molar emission of NADH, was simply the slope of the control titration then a value for Q_2, the molar emission of the enzyme bound NADH, could be calculated. The average value for Q_1 was 0.34 (fluorescence units). μM^{-1} and for Q_2 1.9 (fluorescence units) μM^{-1} both with respect to the fluorescence standard. The fluorescence enhancement Q

$$(Q = \frac{Q_2}{Q_1})$$

was therefore 5.6. These constants are really only applicable to the instrument and conditions used. However, the fluorescence enhancement, which depends only on the wavelengths and slit widths employed would be reasonably consistent.

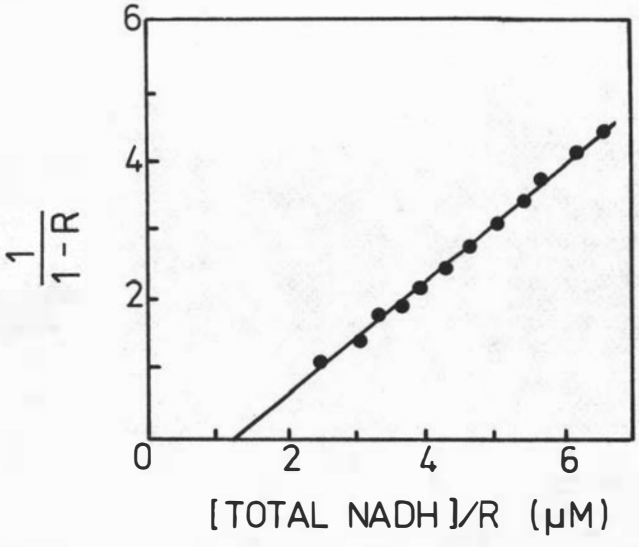
FIG. 3.3 FLUORESCENCE TITRATION OF ALDEHYDE DEHYDROGENASE

(a) FLUORESCENCE INTENSITIES



The upper curve shows the fluorescence observed when NADH is added to an enzyme solution while in the lower line NADH is added to buffer. Excitation at 340 nm and Emission at 435 nm.

(b) REPLOT



This plot represents the data shown in Fig. 3.3 (a) analysed by equation (5). The intercept shows the binding site concentration of the enzyme solution to be 1.4 μM. ΔF_{max} was taken as 2.2

3.3.5 Comparison of the Enzyme Binding Sites with Enzyme Activity

The estimate of the active enzyme concentration from the concentration of binding sites involves the assumption that the enzyme preparation consists of active enzyme which binds NADH and inactive enzyme which does not. To test this proposition, the effect of partial heat inactivation of the enzyme on the binding site concentration was measured. An enzyme sample was incubated at 45°C and both the enzyme activity and the binding site concentration was measured at several time intervals.

TABLE 3.1 EFFECT OF ENZYME ACTIVITY ON CONCENTRATION OF NADH BINDING SITES

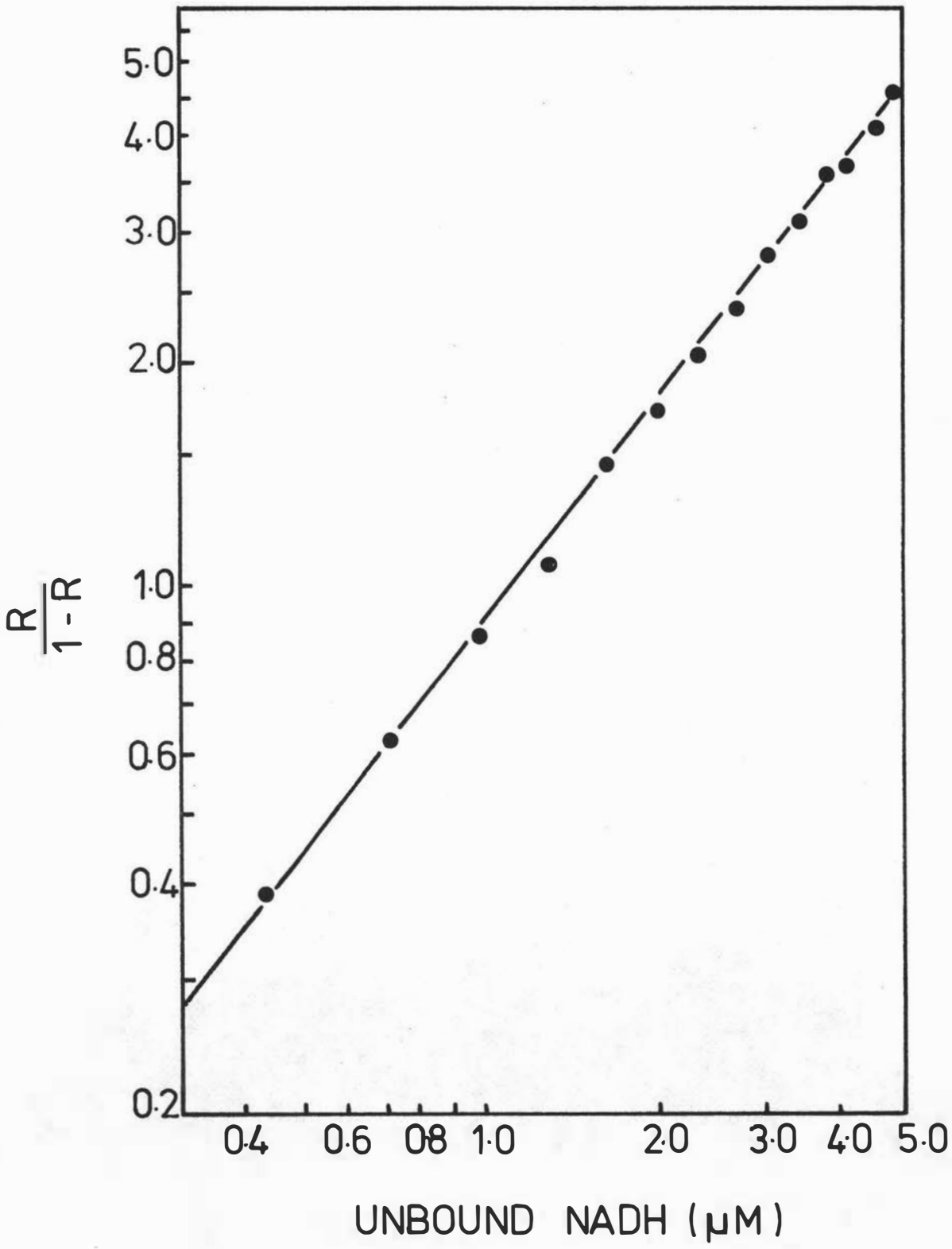
Incubation time (min)	[E] (μM)	% [E] at zero time	% initial rate at zero time
0	1.4	100	100
30	0.9	64	63
70	0.8	57	52

Table 3.1 shows that there was good agreement between the amount of enzyme which could be titrated with NADH and the initial reaction rate while the enzyme was being denatured.

3.3.6 Effect of Additional Compounds on the Titration of the Enzyme

The standard titration procedure was carried out in the presence of various compounds to observe their effect on the dissociation constant and the binding site concentration. Propionaldehyde (1.9mM) a substrate of the enzyme, chloral hydrate (.86mM) a potent inhibitor (competitive with respect to aldehydes) and 2-bromo-2-phenylacetic acid (.4mM) another enzyme inhibitor all failed to produce any change in the dissociation constant or the concentration of binding sites. Three zinc complexing agents were tested and a change was observed for 1,10 phenanthroline but none for EDTA or 2,2'-dipyridyl (see Table 3.2). Some variation was found in the dissociation constant values but they are reasonably consistent.

FIG. 3.4 HILL PLOT OF NADH BINDING TO ALDEHYDE DEHYDROGENASE



R is the fractional saturation

TABLE 3.2 EFFECT OF CHELATING AGENTS ON NADH TITRATION

The chelating agents were added to a standard enzyme solution and titrated with NADH

Reagent	K_D (μM)	[E] (μM)
Control	.9	1.8
EDTA (920μM)	1.2	1.9
2 , 2'-Dipyridyl (430μM)	1.0	2.0
1,10-phenanthroline (250μM)	0.9	0.3

1,10-phenathroline was found to reduce the NADH binding site concentration to 17% of its original value and it therefore appears that 1,10-phenanthroline is binding tightly to the enzyme in such a way as to block the NADH binding site on the enzyme.

3.3.7 Zinc Content

When the zinc concentrations of the dialysed solutions were compared with the concentration of protein and binding sites it was found that zinc was present in less than stoichiometric amounts.

TABLE 3.3 ZINC CONCENTRATION OF TWO DIALYSED ENZYME SAMPLES

[Zinc] (μM)	[Protein] (μg.(212,000g.litre)$^{-1}$)	[Binding Sites] (μM)	$\frac{[Zinc]}{[Binding\ Site]}$
7.7	-	62	.12
10	33	54	.19

Table 3.3 shows that the ratio of zinc atoms to NADH binding sites is less than 0.2 which indicates that though the zinc concentration in the enzyme solution is much greater than in the dialysate (0.01 ppm) there is still not enough zinc to be an integral part of the molecule. As the solution was dialysed for four days, the enzyme may have lost activity and so the binding site concentration after dialysis is a better estimate of the amount of active enzyme than the protein determination. An assay of the enzyme solution gave a catalytic rate constant of $0.1 s^{-1}$, using the determined

binding site concentration and the maximum velocity of the enzymic assay utilizing propionaldehyde, which is in good agreement with the results from the steady-state using non-dialysed enzyme (see Section 4).

3.3.8 Thiol Determinations

The reactivity of the thiol groups of aldehyde dehydrogenase were examined with a Nbs_2/enzyme ratio of 100. In the presence of sodium dodecylsulphate, a denaturating agent, which exposes previously masked groups, the thiol groups reacted in a few minutes but with the native enzyme, however, the profile curve of the liberation of 2-nitro-5-thiobenzoate was in two phases, a fast change followed by a slow change (see Fig. 3.5). The results of four separate determinations are shown in Table 3.4 in terms of the enzyme concentration expressed as moles of 2-nitro-5-thiobenzoate per 212,000g protein. The binding site concentration was known for the first result in Table 3.4, giving 3.6 moles 2-nitro-5-thiobenzoate/binding site for the initial phase and 13.2 moles 2-nitro-5-thiobenzoate/binding site for the total change in the presence of sodium dodecylsulphate. Since the initial fast change was a variable proportion of the total change, it was feared that trace amounts of 2-mercaptoethanol may have been present. From the elution pattern of the Sephadex G-25 column (Fig. 3.1) this concentration of contamination could not result from

TABLE 3.4 REACTION OF ALDEHYDE DEHYDROGENASE WITH 5,5'-DITHIOBIS(2-NITROBENZOIC ACID) IN THE PRESENCE AND ABSENCE OF SODIUM DODECYLSULPHATE

Results are expressed as moles of 2-nitro-5-thiobenzoate per 212,000g protein

Initial fast change in absence of SDS	Total change in presence of SDS
13	49
6	35
1.4[1]	38[1]
.7[1,2]	39[1,2]

1 Contained 1mM EDTA in buffer

2 Using 12.5 cm column

leakage from the column. When the entire reaction mixture was reapplied to a Sephadex G-25 column towards the end of the reaction (the number of thiol groups reacted at this stage was still less than with sodium dodecylsulphate) the Nbs-protein complex was unhindered on the column while the coloured 2-nitro-5-thiobenzoate was retarded. On addition of 2-mercaptoethanol (0.2M) to the protein complex, the amount of 2-nitro-5-thiobenzoate released was measured. The two experiments which were carried out showed differing results (Table 3.5). The results show that recovery from the column was poor in each case, but as the two experiments

TABLE 3.5 APPLICATION OF THIOL REACTION MIXTURE TO COLUMN

The reaction mixture, which contained Nbs_2 and enzyme, was applied to a Sephadex G-25 column after most of the thiol groups had reacted.

	Run 1	Run 2[1]
Protein concentration (ng/212,000g)	6.8	5.6
2-nitro-5-thiobenzoate added to column (nmoles)	180	94
2-nitro-5-thiobenzoate retarded (nmoles)	104	51
2-nitro-5-thiobenzoate liberated from enzyme (nmoles)	28	45
2-nitro-5-thiobenzoate liberated from enzyme/212,000g protein	4.1	8

1 Buffer included EDTA

were conducted on the first and third reaction mixtures of Table 3.3 respectively, the results may be compared. In the experiment containing EDTA, the 2-nitro-5-thiobenzoate regenerated from the protein was similar to the amount of 2-nitro-5-thiobenzoate retarded, showing that the original 2-nitro-5-thiobenzoate was produced by reaction with protein. Run 1, however, shows a large difference in the two values which suggests a non-enzymatic reaction and it will be noted that this run also had a much longer initial fast reaction phase in the original mixture. The reaction of the initial fast phase was further investigated using stopped-flow spectrophotometry, a method which is described in Section 5.

FIG. 3.5 REACTION OF ENZYME THIOL GROUPS WITH ELLMANS REAGENT

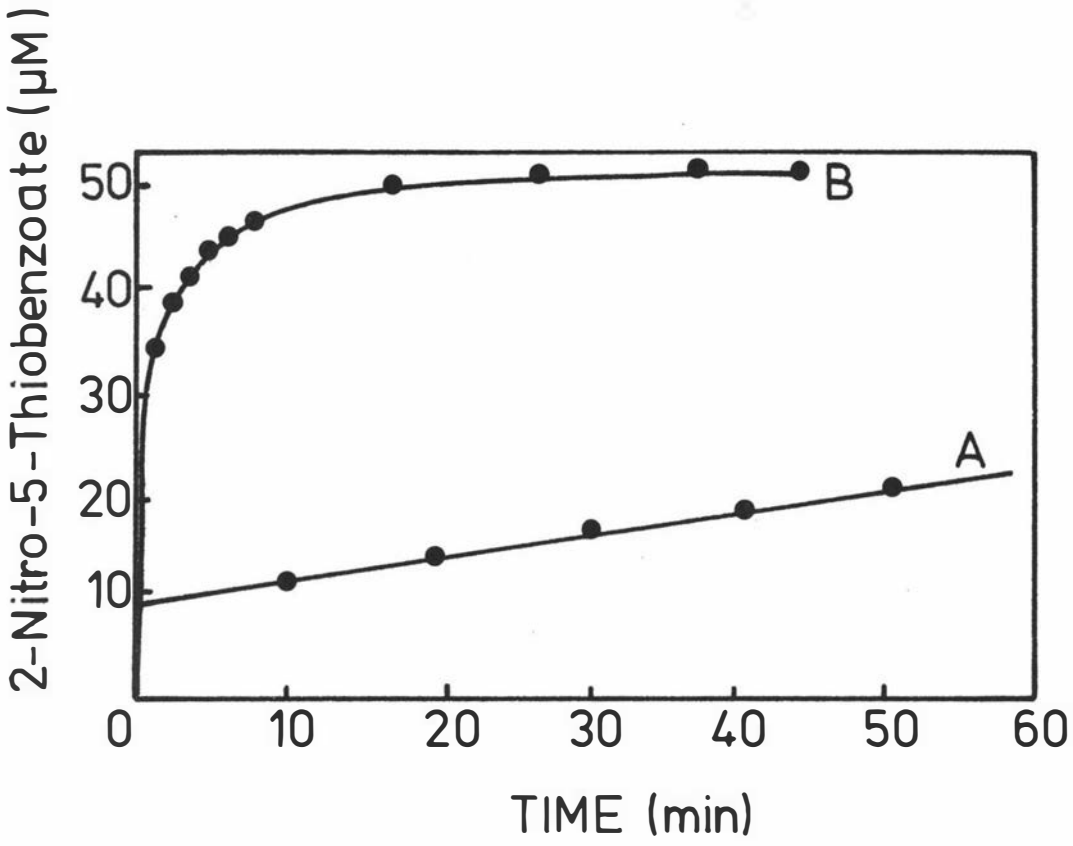
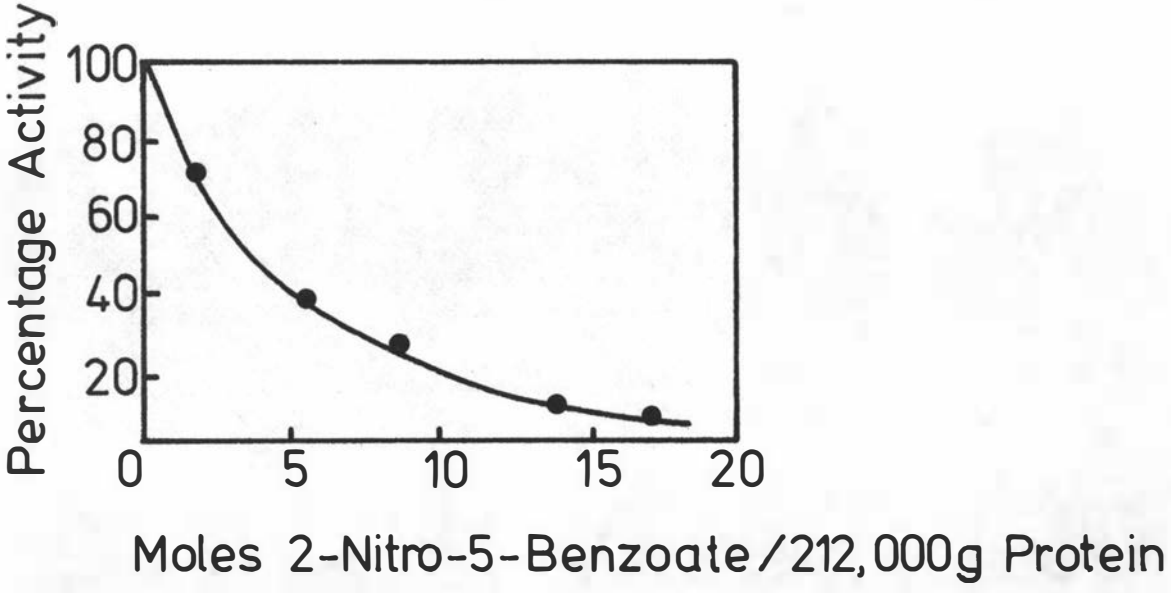


Fig 3.5 shows the reaction of Ellmans reagent (240 μM) with aldehyde dehydrogenase (1.5 μM from protein concentration) in pH 8.0 phosphate buffer.

Curve A, production of 2-nitro-5-thiobenzoate in the absence of sodium dodecysulphate.

Curve B, production in the presence of sodium dodecysulphate (0.3% w/w).

FIG. 3.6 RELATIONSHIP BETWEEN ENZYME ACTIVITY AND THIOL REACTION



Study of an enzyme solution (.21mg/cm^3) reacting with Nbs_2 (84μM) showed a biphasic trace at 412nm, a fast reaction followed by a zero order reaction, on the 1 minute per sweep time scale. Extrapolation of the slow phase (see Section 5) gave an apparent first order rate constant of $0.11s^{-1}$ which, with the excess concentration of Nbs_2 used, produced a second order rate constant of $1400 \pm 300M^{-1}s^{-1}$. The reaction of 2-mercaptoethanol and Nbs_2 on the stopped flow was found to have a second order rate constant of $1750 \pm 200M^{-1}s^{-1}$.

When the activity of the enzyme was monitored during the reaction, it was found that little activity was lost during the initial fast phase and that 10% of the activity remained after 13 moles of 2-nitro-5-thiobenzoate per 212,000g protein had been released (see Fig. 3.6). When 2-mercaptoethanol (0.2M) was added to the assay mixture containing enzyme with 6% activity, 80% of the original activity was restored. The results show no distinct proportionality between activity and thiol groups reacted, suggesting that the loss of activity in the course of mercaptide formation was probably due to progressive disorientation of the enzyme rather than a direct chemical change at the active site. This view was confirmed when NAD^+ (800μM) failed to give the enzyme any protection from Nbs_2, even though it binds at the active site.

3.4 DISCUSSION

Though no change was found in the NADH spectrum, it was later found using stopped-flow displacement, that the absorbance at 340 nm of the enzyme-NADH complex was smaller than that of unbound NADH ($\Delta\varepsilon = 0.66 \times 10^3 M^{-1}cm^{-1}$). For alcohol dehydrogenase, the addition of a strong inhibitor, pyrazole, to a solution of enzyme and NAD^+ produced a change in the spectrum with a maximum difference at 300 nm (Theorell and Yonetani, 1963). However, attempts to produce a change in physical properties by forming a ternary complex of sheep liver aldehyde dehydrogenase using chloral hydrate, which is a strong inhibitor of the enzyme, failed to produce a change in the spectrum of NAD^+ bound to the

enzyme.

The fluorescence properties of nucleotides bound to aldehyde dehydrogenase from sheep liver show many similarities to other dehydrogenases, which have already been studied. A blue shift in the maximum fluorescence of the coenzyme on binding to the enzyme is observed with an enhancement in fluorescence at 435 nm of 5.6. The altered fluorescence on binding may be due to a change to a more polar molecular environment for NADH (Scott et al., 1970). Recently Takio et al. (1974) found similar properties for horse liver aldehyde dehydrogenase.

The quenching of protein fluorescence by coenzymes seems to be a common feature of dehydrogenases. In pig heart lactate dehydrogenase (Holbrook, 1972) the protein fluorescence is quenched by NADH but not by NAD^+ while in horse liver alcohol dehydrogenase (Theorell and Tatemoto, 1971) not only do NADH and NAD^+ quench fluorescence but also 1,10-phenanthroline. Luisi and Favilla (1970) working on the same enzyme found that the quenching of tryptophan fluorescence (the residue involved in protein fluorescence) was due to a specific interaction between the coenzyme and the enzyme active site. Aldehyde dehydrogenase exhibits quenching with both NAD^+ and NADH. There are three mechanisms by which this quenching could take place. The first is energy transfer from the fluorescent residues of the enzyme to the bound coenzyme, a mechanism which is possible for NADH, but the NAD^+ absorption peak at 260 nm precludes it acting as an acceptor (Shore et al., 1975) and so quenching with NAD^+ would not take place. Secondly, a molecular complex could be formed between the residue and the coenzyme and, thirdly, a conformational change altering the environment of the tryptophan may occur and hence a change in the fluorescence may be produced by coenzyme binding. Which of the latter two possible mechanisms is taking place cannot be distinguished until the amino acids involved at the coenzyme binding site are identified. X-ray studies on alcohol dehydrogenase have shown that the tryptophans are not near the active site of that enzyme and hence conformation changes must be responsible for the quenching of protein fluorescence (Shore et al., 1975).

The changes in nucleotide fluorescence on binding to aldehyde dehydrogenase show that NADH is able to bind to the free enzyme and the hyperbolic nature of the titration curves with NADH and the resultant linearity of the replot show that the NADH binding sites of the enzyme are all equivalent. This independance of binding sites is borne out by the linear Hill plot with a slope of unity.

The major application of the NADH titration was in the evaluation of the number of binding sites in an enzyme sample. As the binding site concentration by NADH titration was found to be proportional to the activity of the enzyme by following the heat denaturation and the fact that only one class of binding site was observed, it seemed reasonable to equate the binding site concentration with the active site concentration. This active site concentration is required to determine the rate of enzyme action (see Section 4). The advantage of this determination over using protein concentration is that only active enzyme is measured. Unfortunately both protein and active site concentrations were not determined on enough samples for the number of active sites in the tetrameric aldehyde dehydrogenase to be determined accurately, but results tend to favour two rather than four active sites in the molecule though the concentration of inactive protein could have a large effect on this value. Winer et al. (1974) in results published after this work was completed, found that fluorescence studies gave two moles of NADH binding per mole of tetrameric enzyme with a dissociation constant of 2μM. Using equilibrium dialysis they found two classes of binding sites, one with a dissociation constant of 6μM and the other 50μM. The binding of only two moles of coenzyme per mole of enzyme was also found for yeast aldehyde dehydrogenase (Bradbury and Jacoby, 1971b).

The formation of dead end ternary complexes from the enzyme-coenzyme complex by the addition of a third compound has been noted for several dehydrogenases. Rabbit muscle lactate dehydrogenase (Fromm, 1970), yeast alcohol dehydrogenase (Dickenson, 1970) and octopine dehydrogenase (Luisi et al., 1973) show formation of enzyme ternary products with reaction products, substrates or inhibitors. Luisi et al. (1973) showed that octopine dehydrogenase had a dissociation

constant of 0.9μM for the ternary complex enzyme-NADH-octopine, where octopine is a product of the reaction of substrates with enzyme-NADH, which is twenty times less than the dissociation constant for the binary enzyme-NADH complex. Decreases in the dissociation constant make the NADH titration more accurate and easier to carry out as the binding to the enzyme is much tighter, however, attempts to form ternary complexes with aldehyde dehydrogenase were unsuccessful. Propionaldehyde, chloral hydrate and 2-bromo-2-phenylacetic acid respectively a substrate, a substrate inhibitor and a product analogue, all failed to change the NADH dissociation constant as determined by NADH titration.

Inhibition of aldehyde dehydrogenase from bovine liver by zinc complexing agents has been reported by Stoppani *et al*. (1966). When zinc complexing agents EDTA, 2 , 2'-dipyridyl and 1,10-phenanthroline were present in the NADH titration only 1,10-phenanthroline had any effect on the results. The dissociation constant remained unchanged but the binding site concentration was reduced to 17% of the original suggesting that 1,10-phenanthroline is binding tightly to the enzyme in such a way as to block the NADH binding site.

Though several dehydrogenases have been reported to be zinc metallo-enzymes, only for alcohol dehydrogenase had the presence of zinc been thoroughly documented (Eichhorn, 1973). Horse liver alcohol dehydrogenase has been found to contain four zinc atoms per molecule, two atoms functioning in catalysis and two further atoms which appear to have a structural role (Branden *et al*., 1973). Stoppani *et al*. (1966) in their study of yeast and liver aldehyde dehydrogenase found that the metal complexing agents, such as 1,10-phenanthroline, 8-hydroxyquinoline, diethyldithiocarbamate and 2 : 2'-dipyridyl, produced instantaneous reversible inhibition which was competitive between chelator and coenzyme and mostly non-competitive with respect to substrate. As the effectiveness of the inhibitors were in agreement with the stability constants of their respective zinc complexes and traces of zinc were found in preparation of yeast aldehyde dehydrogenase, Stoppani *et al*. (1966) were led to conclude that the results were consistent with the presence of zinc as an intrinsic

constituent of both enzymes.

The effect of 1,10-phenanthroline on the titration of sheep liver aldehyde dehydrogenase made it of interest to study the zinc content of the enzyme. As the binding site concentration and the molecular weight of the enzyme were known, not only could the presence of the metal be detected, but also the stoichiometry with respect to the enzyme molecule determined. Results of zinc analysis by atomic absorption spectroscopy showed that the zinc concentration was less than a fifth of the binding site concentration and so could not possibly be an intrinsic constituent of sheep liver aldehyde dehydrogenase. Further evidence was furnished by the fact that the dialysed enzyme with the low zinc content had a catalytic-centre activity which was the same as non-dialysed enzyme showing that zinc was not required for enzyme activity. Though sheep liver aldehyde dehydrogenase was found to contain no essential zinc atoms, inhibition with zinc chelating agents was observed. Steady-state kinetics (see Section 4) showed that 1,10-phenanthroline was a competitive inhibitor with respect to NAD^+ with an inhibition constant of 75μM. Use of this inhibition constant would lead to the prediction that the presence of 1,10-phenanthroline in the reported NADH titration would result in a reduction in the binding site concentration to 0.4μM which is in good agreement with the observed 0.3μM and suggests that 1,10-phenanthroline binds at the coenzyme binding site on the enzyme. Further evidence was provided from the stopped-flow displacement experiments (see Section 5) in which NADH was found to be displaced from the binary enzyme-NADH complex by high concentrations of 1,10-phenanthroline. A possible explanation of the binding of 1,10-phenanthroline to the coenzyme binding site in the absence of zinc is that the binding is stabilized through hydrophobic interactions rather than by zinc chelation. Anderson *et al.* (1966) found that the compounds 1,5-phenanthroline and 2,9-dimethyl-1,10-phenanthroline were more effective inhibitors of yeast alcohol dehydrogenase than 1,10-phenanthroline even though their metal chelating power was much weaker due to steric hindrance.

The work on sheep liver aldehyde dehydrogenase has recently been corroborated by Sidhu and Blair (1975), studying human liver aldehyde dehydrogenase, who found that non-chelating analogues of 1,10-phenanthroline and 2,2'-dipyridyl were just as effective inhibitors of the enzyme as chelating compounds and that the enzyme contained no significant amount of zinc.

It has been suggested that all dehydrogenases have a common coenzyme binding domain (Ohlssen et al., 1974) as lactate, alcohol and glyceraldehyde-3-phosphate dehydrogenases all have a narrow hydrophobic pocket in which the adenosine end of the coenzyme molecule binds. Thus, a planar molecule such as 1,10-phenanthroline could conceivably bind in this hydrophobic pocket whereas EDTA and the non-planar 2,2'-dipyridyl molecule could not. In agreement with this idea is the observation that 2,2'-dipyridyl does not inhibit the steady state initial velocity but 8-hydroxyquinoline another planar rigid molecule does.

With regard to the thiol determinations, it would appear that the initial fast reaction with Nbs_2 was due to 2-mercaptoethanol contamination of the sample. This change was a variable proportion of the total number of thiol groups and when rechromatographed, the proportion of Nbs_2 complexed protein eluted was less in samples that produced a large initial phase. The fact that the loss of enzyme activity was only gradual during which time a large number of thiol groups had been titrated showed that the initial fast phase was not directly involved in the enzyme activity. Further evidence that 2-mercaptoethanol was involved, was found from stopped-flow experiments in which the rate constant for the reaction of 2-mercaptoethanol with Nbs_2 was found to be similar to that for the fast phase. Though the evidence points to the fact that 2-mercaptoethanol is present in the sample, the process by which such a large amount passes through the gel filtration column with the high molecular weight enzyme, is not known.

Considering the initial fast reaction to be non-enzymic, the total number of thiol groups per 212,000g protein is the difference between the total change and initial change, which is reasonably consistent at about 36 thiol groups. For

comparison, yeast alcohol dehydrogenase (MW 140,000) contains 36 thiol groups (Whitehead and Rabin, 1964) and horse liver alcohol dehydrogenase (MW 80,000) contains 24 thiol groups (Li and Vallee, 1965) while it has recently been reported by Eckfeldt and Yonetani (1976) that the two isozymes of horse liver aldehyde dehydrogenase (F_1 and F_2) contain 23 and 19 thiol groups using molecular weights of 230,000 and 240,000 respectively. As the enzyme activity was not completely inhibited until a large number of thiol groups have been reacted and NAD^+ has no effect on this inhibition, Nbs_2 cannot be attacking the active site. NAD^+ protection of enzyme reaction with other thiol reagents suggest that bovine liver aldehyde dehydrogenase (Deitrich, 1967) and pig brain aldehyde dehydrogenase (Duncan and Tipton, 1971a) do contain a thiol group in the active site. It seems possible that either Nbs_2 is too bulky a reagent or that the negative charge on the molecule is repelled by the active site. Deitrich (1967) found that for the bovine liver enzyme, cationic and neutral reagents were more effective, as did Kitson (1975). Experiments with other thiol reagents may, therefore, provide information about the enzyme active site.

SECTION 4

STEADY-STATE KINETICS

4.1 INTRODUCTION

As the characteristic property and function of enzymes is the catalysis of chemical reactions, the eventual elucidation of detailed enzyme mechanisms demands the understanding of both the chemistry of the proteins and the behaviour of the catalysed reactions. An understanding of the behaviour of the reactions may be gained by study of the rate of enzyme catalysis and the effect of changes in the reaction conditions on this rate. These changes may involve alteration of the substrate concentration, pH, ionic strength or temperature. When the reaction proceeds under steady-state conditions the kinetic data obtained from the study of the enzyme reaction may be compared with the predicted kinetics for various plausible mechanisms and those which do not fit the experimental data may be discarded.

While extensive kinetic studies have been carried out on alcohol dehydrogenase, aldehyde dehydrogenase has had relatively little attention. Though the existence of the enzyme was first shown by Racker (1949), the difficulties involved in the purification have meant that little work on the kinetics of the enzyme catalysed reaction have been carried out. Studies involving partially purified enzyme were carried out on bovine liver aldehyde dehydrogenase (Deitrich et al., 1962; Freda and Stoppani, 1970) and pig brain aldehyde dehydrogenase (Duncan and Tipton, 1971a). However, it was not until 1970 that an aldehyde dehydrogenase was purified to homogeneity, when Clark and Jakoby isolated aldehyde dehydrogenase from yeast. The first mammalian aldehyde dehydrogenase to be purified to homogeneity was from horse liver (Feldman and Weiner, 1972a).

Recently the aldehyde dehydrogenases (cytoplasmic and mitochondrial) from sheep liver have been purified to homogeneity in this laboratory (Crow et al., 1974). Steady-state studies on sheep liver aldehyde dehydrogenase were undertaken for

three reasons. To allow a comparison of the kinetic properties of aldehyde dehydrogenases from different species, as well as to compare the results from the cytoplasmic enzyme with those of the mitochondrial sheep liver aldehyde dehydrogenase prepared in the same laboratory (Crow *et al.*, 1974). Also, the parameters of the overall reaction in the steady-state are necessary when interpreting the results of the presteady-state kinetics study of the enzyme.

Steady-State Kinetics

Enzyme-catalysed reactions involving the conversion of substrate to product proceed via an intermediate: the enzyme-substrate complex.

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + P$$

Michaelis and Menten (1913) formulated a rate equation based on the above mechanism making the assumption that the second reaction does not disturb the first equilibrium. A more general formulation was given by Briggs and Haldane (1925) on the basis of the steady-state hypothesis that the net rate of change of $[EA]$, the concentration of the complex, is zero. The equation describing the relationship between velocity of the reaction and the substrate concentration is known as the Michaelis-Menten equation -

$$v = \frac{V[A]}{K_m + [A]}$$

Where v is the reaction velocity, $[A]$ is the substrate concentration and V and K_m are constants, known as the maximum velocity and the Michaelis constant respectively.

It should be noted that when $[A]$ is equal to K_m then

$$v = \frac{V}{2}$$

The concentration of substrate which is found experimentally to give half the maximum velocity, is called the Michaelis constant (K_m). A Michaelis constant may be determined for

any reaction but the physical significance of the constant, or relationship to individual rate constants, depends on the mechanism by which the reaction takes place.

The Michaelis-Menten equation, when v is plotted against $/\!A\!/$, is a rectangular hyperbola through the origin, with asymptotes $v = V$ and $/\!A\!/ = -K_m$. Two features which may be noted are, firstly that at concentrations of A much less than K_m, $v = \frac{V/\!A\!/}{K_m}$, i.e. first order in A and secondly at concentrations of A much greater than K_m, $v = V$ that is the reaction is zero order in A. As physical necessity restricts measurements of v to finite positive values of $/\!A\!/$, it is not possible to measure V and K_m accurately from such a plot, because the asymptotes cannot be approached closely enough.

Several transformations of the equation may be used to get around this difficulty -

(i) $\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V} \cdot \frac{1}{/\!A\!/}$ Lineweaver and Burk

(ii) $\frac{/\!A\!/}{v} = \frac{K_m}{V} + \frac{1}{V} \cdot /\!A\!/$ Hanes

(iii) $v = V - K_m \cdot \frac{v}{/\!A\!/}$ Eadie and Hofstee

The Lineweaver-Burk plot is the most commonly used, while the Eadie and Hofstee plot has the advantage that the dependent variable v is linear in form and deviations from linearity show up more readily. However, in computer evaluation of the parameters, the method of plotting data is not critical.

Because of the inhibition of the reaction by products and further, because of possible partial denaturation of the enzyme during the prolonged course of a reaction, it is usually advantageous to evaluate kinetic behaviour on the basis of initial rates. This initial value should then reflect the reaction velocity under the known conditions of enzyme and substrate concentration and zero product concentration.

Two Substrate Reactions

Most enzymes catalyse reactions with two or more substrates. Cleland (1963) has described a general equation

for two substrate reactions

$$v = \frac{V[A][B]}{[A].[B] + K_a[B] + K_b[A] + K_{ia}K_b} \qquad (6)$$

in which there are four constants V, K_a, K_b, K_{ia} compared with only two parameters required for the single substrate equation and where $[A]$ and $[B]$ are the concentrations of the two substrates. When $[B]$ becomes very large equation (6) reduces to

$$v = \frac{V.[A]}{K_a + [A]}$$

It will be noted that this is of the form of the Michaelis-Menten equation for single substrate enzyme reactions and K_a is the Michaelis constant for A at high concentrations of B. Similarly at high $[A]$

$$v = \frac{V.[B]}{K_b + [B]}$$

with K_b the Michaelis constant for B at high concentrations of A. The increasing complexity of the two substrate reactions means that more parameters are needed to describe the Michaelis constants and the maximal velocity.

Considering equation (6) in double reciprocal form

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{ia}K_b}{[A].[B]}\right)$$

A plot of $\frac{1}{v}$ against $\frac{1}{[A]}$ at constant $[B]$ will, therefore, be linear with a slope of

$$\frac{K_a + \frac{K_{ia}K_b}{[B]}}{V}$$

and an intercept on the $\frac{1}{v}$ axis of

$$\frac{1 + \frac{K_b}{[B]}}{V}$$

When several concentrations of B are used, a series of lines

are produced with differing slope and intercept, and from replots of those slopes and intercepts the four parameters K_a, K_{ia}, K_b, V may be evaluated (see Fig. 4.1). Using constant concentrations of A, a similar series of equations may be produced and the results of the two graphs checked for internal consistency.

Though equation (6) holds for many types of two substrate mechanisms, it is formulated in terms of parameters which are themselves functions of individual rate constants. These functions are different with different enzyme mechanisms and so the physical significance of the parameters differs between mechanisms. A convenient method for the solution of rate equations in terms of individual rate constants has been devised by King and Altman (1956).

A large number of possible mechanisms of two substrate enzymic reactions have the same overall rate equations and so to distinguish the mechanism from the experimental initial velocity data is difficult. Cleland (1963) has defined enzyme reactions involving two substrates and two products as Bi Bi mechanisms. The three main categories of enzyme mechanisms are defined by the relationship between the two substrates, whether the first product is released before the second substrate binds (Ping Pong mechanism), or both substrates must bind before any reaction takes place and any products are released (sequential mechanism).

1) Ping-Pong Mechanism

The above notation of Cleland, where the addition and release arrows designate reversible steps, shows that the product (P) is formed prior to the reaction with the second substrate at the expense of conversion of the enzyme from a form E to a form E'.

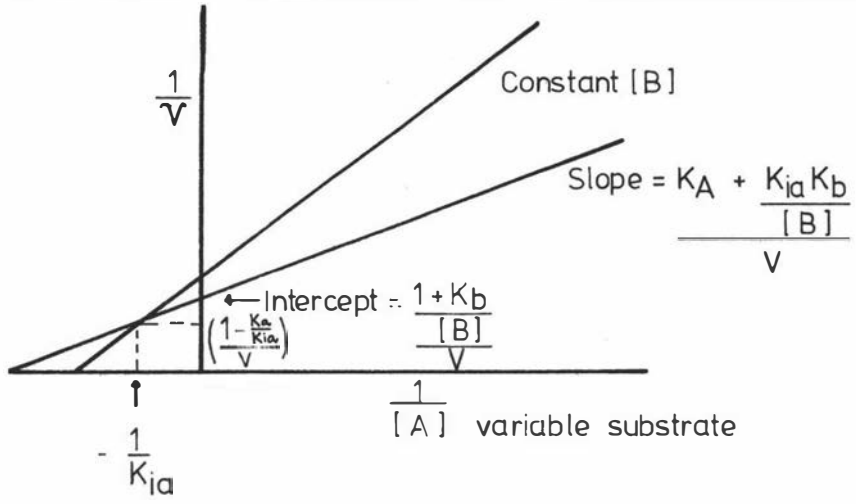
Kinetic analysis shows that the rate equation is

$$\frac{V}{v} = 1 + \frac{K_a}{/A/} + \frac{K_b}{/B/}$$

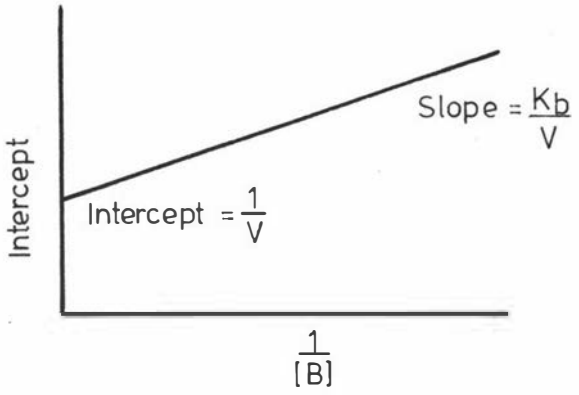
which compared with equation (6) lacks a term in $/A/\,/B/$ and

FIG. 4.1 DOUBLE RECIPROCAL PLOTS FOR BISUBSTRATE REACTIONS

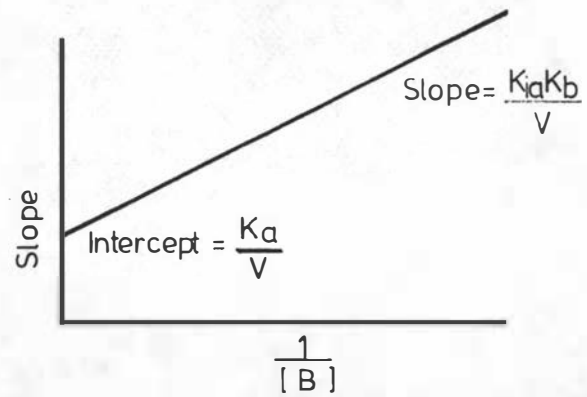
(a) Primary Plot reciprocal initial velocity versus reciprocal variable substrate A.



(b) Secondary Plot intercepts from primary plot versus reciprocal of constant substrate

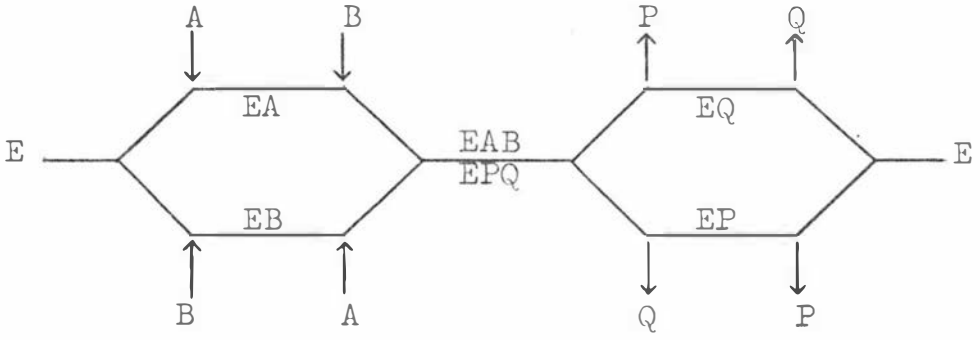


(c) Secondary Plot slope from primary plot versus reciprocal of constant substrate



hence a double reciprocal plot of $\frac{1}{v}$ versus either $\frac{1}{[A]}$ or $\frac{1}{[B]}$ as variable substrates is a series of parallel lines independent of the concentration of the fixed substrate.

2) Random Mechanism



The general sequential mechanism is called the random mechanism and either substrate may bind to the free enzyme. This mechanism gives rise to rate equations too complicated to analyse. However, if the interconversion of ternary complexes is slow enough that EA, EB and EAB are in equilibrium, then the mechanism is known as a rapid equilibrium random mechanism and obeys equation (6).

3) Ordered Mechanism

This sequential mechanism is a special case of the random mechanism in which only one of the substrates may bind to the free enzyme, that is, substrate B has an infinite dissociation constant with the free enzyme. The overall rate equation may be expressed as equation (6) so it is indistinguishable from the rapid equilibrium random mechanism. Because of the symmetry of equation (6) it is not obvious which of the two substrates is the "leading-substrate" described as A.

Although initial velocity studies alone can only distinguish ping pong mechanisms from sequential mechanisms, the results of other studies, such as inhibition patterns, may allow the enzyme mechanism to be determined.

Enzyme Inhibition

Substances which bring about a reduction in the rate of reaction are called inhibitors and studies of the effect of these inhibitors can help elucidate the mechanism by which the reaction occurs. The terminology used for types of inhibition originated with the simple Michaelis-Menten single intermediate mechanism, so while the terms are applied to two substrate reactions, the conclusions they imply may not be justified in the more complex case.

There are three main types of reversible inhibition for two substrate reactions (Fig. 4.2) -

1) Competitive Inhibition

Inhibition is said to be competitive when only the slope of a double reciprocal plot is affected. This situation exists when the inhibitor reacts with the same enzyme form as does the variable substrate. Since the same form of the enzyme is involved, high concentrations of the variable substrate removes the inhibition, by saturating that form of the enzyme.

If the constant substrate is in excess, the inhibition equation is

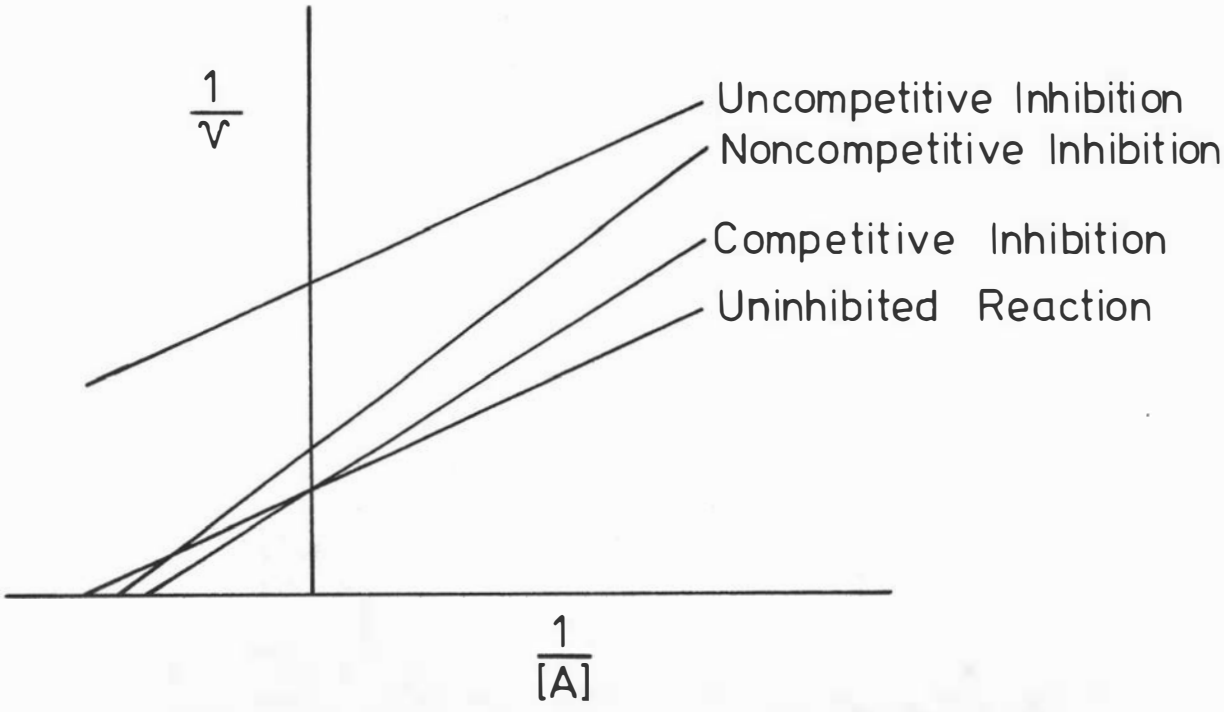
$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{[I]}{K_{is}}\right)\left(\frac{1}{[A]}\right) + \frac{1}{V}$$

where K_{is} is the graphical slope inhibition constant and $[I]$ is the inhibitor concentration (nomenclature of Cleland). The inhibition factor only appears in the slope term of the expression.

2) Uncompetitive Inhibition

If the inhibitor has the effect of only changing the ordinate intercept of the double reciprocal plot, the inhibition is called uncompetitive. This type of inhibition occurs when the inhibitor associates with an enzyme form other than the one with which the variable substrate combines, thus lowering the amount of the total enzyme available for distribution among the usual enzyme forms in a manner which cannot be overcome by saturation with substrate. At high concentrations of fixed substrate the inhibition equation is

FIG. 4.2 EFFECT OF INHIBITION PATTERNS ON THE DOUBLE RECIPROCAL PLOT



$$\frac{1}{v} = \frac{K_a}{V}\left(\frac{1}{[A]}\right) + \frac{1}{V}\left(1 + \frac{[I]}{K_{ii}}\right)$$

where K_{ii} is the graphical intercept inhibition constant of I.

3) Noncompetitive Inhibition

If the inhibitor affects both the slope and the intercept of a double reciprocal plot the inhibition is said to be noncompetitive. This type of inhibition occurs when the inhibitor associates with a form of the enzyme which is connected by a series of reversible steps to the form with which the variable substrate combines. The equation at high concentrations of fixed substrate (B) is

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{[I]}{K_{is}}\right)\frac{1}{[A]} + \frac{1}{V}\left(1 + \frac{[I]}{K_{ii}}\right)$$

If the inhibitor associates with more than one enzyme form, or more than one inhibitor molecule binds to the enzyme, or the inhibitor is such that it forms part of a new reaction sequence, the inhibition plots may be non-linear.

A special class of inhibitors, the products of the enzyme reaction, are of particular interest because they provide a method of testing proposed reaction mechanisms. From the proposed mechanism the enzyme form with which the products associate is known and hence the type of inhibition with the two substrates may be predicted. If these predictions are not verified experimentally, the mechanism may be discarded. Cleland (1963) has produced a table, based on the inhibition equations, predicting the product inhibition patterns of a series of mechanisms. Some of these patterns are shown in Table 4.1 and illustrate the way in which product inhibition patterns may be used to differentiate mechanisms.

When product inhibition does not give unequivocal answers, use of inhibitors, which reversibly bind to the enzyme to form a complex which cannot undergo any further reaction (so called dead-end inhibitors), can give further information about the order of substrate binding and the enzyme mechanism.

TABLE 4.1

PRODUCT INHIBITION PATTERNS FOR SEQUENTIAL BI BI MECHANISMS

Mechanism	Inhibitory Product	Variable Substrate A: Unsaturated	A: Saturated with B	B: Unsaturated	B: Saturated with A
Ordered Bi Bi	P	NC	UC	NC	NC
	Q	Comp	Comp	NC	-
Theorell-Chance	P	NC	-	NC	-
	Q	Comp	Comp	NC	NC
Iso Ordered Bi Bi	P	NC	UC	NC	NC
	Q	NC	NC	NC	UC
Iso Theorell-Chance	P	NC	-	Comp	Comp
	Q	NC	NC	NC	NC
Rapid Equilibrium Random Bi Bi	P or Q	Comp	-	Comp	-
Rapid Equilibrium Random Bi Bi with Dead End EBQ complex	P	Comp	-	Comp	-
	Q	Comp	-	NC	-
Random Bi Bi	P or Q	NC[1]	NC[2]	NC[1]	NC[2]

Mechanisms are defined by Cleland (1963).

Abbreviations used are: Comp, competitive; UC, uncompetitive; NC, noncompetitive; —, no inhibition.

1. Reciprocal plots are theoretically nonlinear although curvature may be difficult to see.
2. Reciprocal plots are linear, but slope and intercept replots are hyperbolic functions of the product inhibitor concentration.

4.2 METHODS

4.2.1 Spectrophotometric Assays at pH 9.3

4.2.1.1 Assay of aldehyde dehydrogenase

The steady state kinetic studies at pH 9.3 were carried out in 33mM sodium pyrophosphate, and the data collected on a Beckman Acta III recording spectrophotometer by following the appearance of NADH at 340 nm. The assay mixture was made up as follows:

1.0cm^3 sodium pyrophosphate (100mM)
1.2cm^3 distilled water
0.5cm^3 NAD^+ solution (Sigma Grade AA)
0.1cm^3 enzyme solution
0.2cm^3 aldehyde solution

to make a final volume of 3cm^3. All the reagents bar the aldehyde, were mixed together and a check for endogenous activity was made by monitoring the absorbance change at 340 nm. A few samples did exhibit endogenous activity due to contamination by alcohol dehydrogenase which utilized the traces of alcohol in the NAD^+ used. These samples were not used for kinetic studies. The K_m values for glyceraldehyde were determined using a constant NAD^+ concentration (1.7mM) while varying the glyceraldehyde concentration. The K_m values for NAD^+ were determined by varying the NAD^+ concentration in the presence of a constant concentration of acetaldehyde (1.7mM).

4.2.1.2 Buffers for pH profile

Veronal sodium-HCl buffers were prepared by adding hydrochloric acid (0.02M) to 100cm^3 of veronal sodium solution (0.04M) until the required pH was reached and the solution was made up to a final volume of 120cm^3 with distilled water. Veronal sodium-HCl buffers were used as they are effective in the range pH 7-10. When added to the enzyme assay the concentration of veronal sodium was 0.024M and the pH values were 7.1, 7.6, 8.0, 8.5, 9.0.

4.2.1.3 Esterase activity

The rate of hydrolysis of p-nitrophenyl acetate was determined spectrophotometrically at 400 nm (following the production of p-nitrophenol). Reaction mixtures contained 0.1M tris buffer (pH 8.0), p-nitrophenyl acetate (1mM) and

enzyme solution. A molar extinction coefficient of 16×10^3 at 400 nm for nitrophenol was used (Kezdy and Bender, 1962).

4.2.2 Fluorimetry

4.2.2.1 Instrumentation

The fluorimeter described in Section 3 was also used for kinetic measurements. The fluorescence of free NADH was monitored by exciting the assay solution at 345 nm and measuring the emission at 455 nm (in the equilibrium studies the fluorescence of the enzyme-NADH complex was monitored). All experiments were carried out at 25°C and changes were related to the quinine sulphate standard.

4.2.2.2 Linearity of NADH fluorescence

As NADH fluorescence was proportional to NADH concentration up to about 10μM (as observed in NADH titrations) a standard curve could be constructed and fluorescence changes could be related to NADH production. Above 10μM NADH deviations from linearity occurred due to the absorption of the exciting light by the NADH solution. If the NADH concentration was further increased, a point is reached, above 150μM, where the fluorescence of NADH actually decreases with increasing concentration (Fig. 4.3).

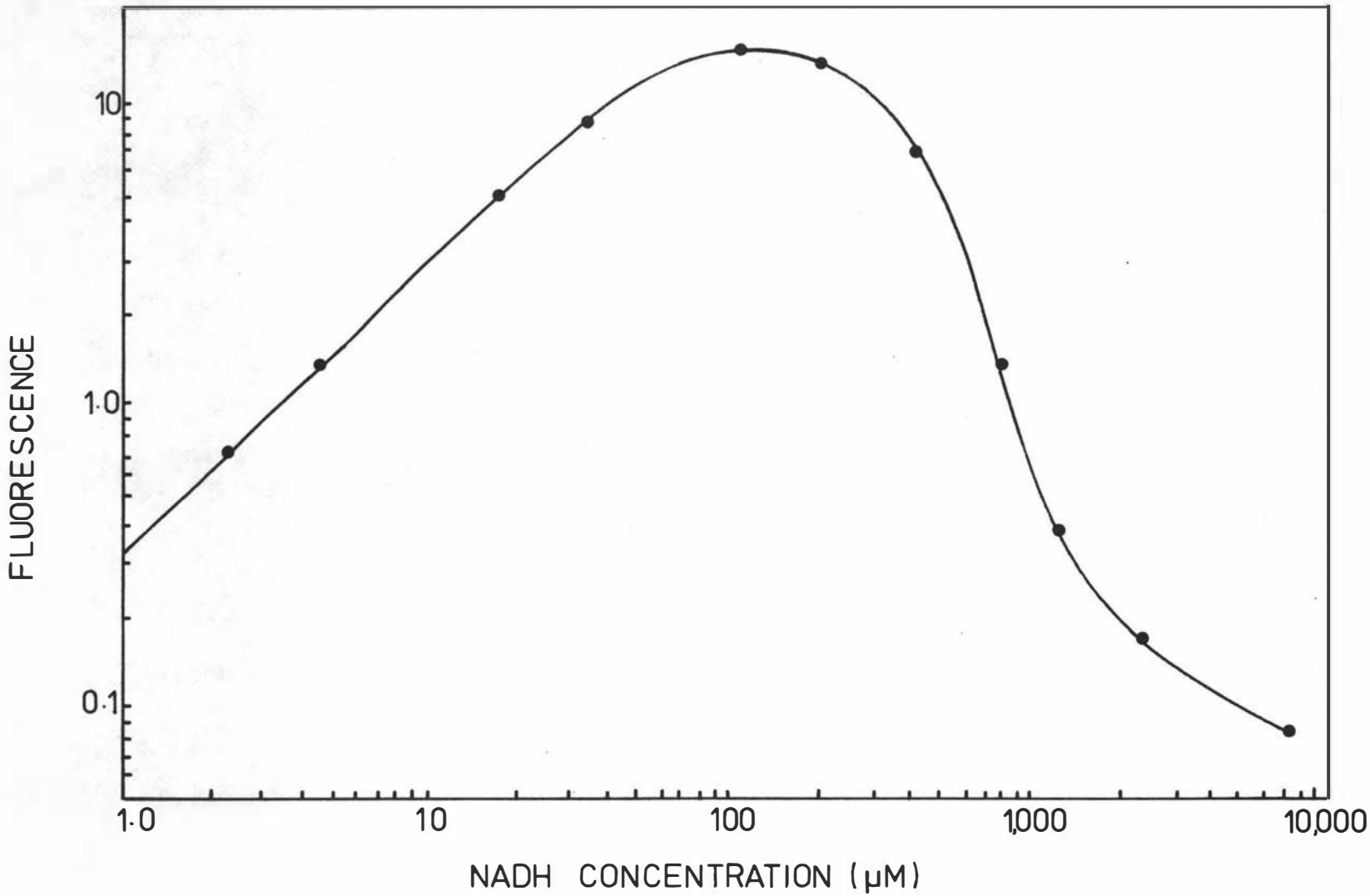
4.2.2.3 Influence of pH on NADH fluorescence

NADH (4.25μM) was made up in veronal sodium buffers of varying pH and the change in fluorescence with respect to a buffer blank was measured for each solution. The results (Table 4.2) show little change in fluorescence with pH, thus fluorescence changes at different pH values in a pH profile may be compared directly.

TABLE 4.2 VARIATION OF NADH FLUORESCENCE WITH pH

pH	Fluorescence
9.6	2.0
9.18	1.98
8.8	1.93
8.4	1.92
8.0	1.95
7.7	1.91
7.24	1.92
6.9	1.90

FIG. 4.3 FLUORESCENCE OF NADH SOLUTIONS



Excitation wavelength 340 nm Emission wavelength 435 nm NADH solutions contained pH 7.6 phosphate buffer

4.2.2.4 Fluorimetric assay at pH 9.3

The assay was the same as used in the spectrophotometric assay, however, the pyrophosphate buffer solution was filtered through a sintered glass funnel to remove any large particles as the fluorescence technique is susceptible to light scattering effects.

4.2.2.5 Distillation of propionaldehyde

Propionaldehyde was distilled under nitrogen from a 250cm^3 three necked flask into a collection flask immersed in ice. Prior to use the collection flask was filled with water and heated to boiling on a hot plate to remove any acid on the glass which may act as a catalyst in polymerization of propionaldehyde. The same flask was used for storage of the aldehyde. All equipment was thoroughly dried before use and the distillation apparatus when assembled was flushed with dry nitrogen. Silica gel was placed in the gas outlet to prevent moisture entering the system. Only 50% of the 100cm^3 propionaldehyde was distilled so there was no danger of concentrating any peroxides that may have been present. The temperature of distillation could not be determined as the nitrogen flow cooled the thermometer but after distillation, the refractive index of the propionaldehyde was determined and the value always agreed with the literature value of 1.3636 at 20°C (Handbook of Chemistry and Physics, 1971).

4.2.2.6 Determination of aldehyde concentration

Semicarbazide hydrochloride readily reacts with carbonyl compounds to form semicarbazones which have absorption maxima at about 220 nm (Burbridge *et al.*, 1950). Semicarbazide hydrochloride (0.186g), sodium dihydrogen phosphate (2.337g) and di-sodium hydrogen phosphate (4.975g) were dissolved and made up to 250cm^3 with water, to act as a buffer with a pH of 7.0. This reagent solution (3cm^3) was added to a standard aldehyde solution (3cm^3), a sample solution (3cm^3) or water for blank, and made up to 10cm^3 with water. The mixture was left for 15 minutes before reading. For propionaldehyde the semicarbazone derivative had a maximum at 223 nm, the response being linear in the range of aldehyde concentration used (up to 120μM). Results

could be determined from the standard curve. Aqueous solutions of propionaldehyde which had been stored at 4°C for 3 weeks were found to give the same reading as freshly prepared samples.

It is well known that in aqueous solutions aldehydes exist as a mixture of the free aldehyde, RCHO, and the hydrate, $RCH(OH)_2$, the exact ratio being determined by the gem-diol equilibrium constant (Bell, 1966). Bodley and Blair (1971) were able to show that human liver aldehyde dehydrogenase solely utilized the free aldehyde as a substrate. The hydrate of aldehydes in solution, therefore, has the effect of lowering the concentration of actual substrate present such that the substrate concentration is equal to the total aldehyde concentration multiplied by the fraction of the total aldehyde which is present in the free form. While the expression of substrate concentrations in terms of free aldehyde is more correct, the total aldehyde concentration may be used as long as both the kinetic parameters and the substrate concentration are expressed in this way. Due to the simplicity of using the total concentration and the fact that the gem-diol equilibrium constant for some aldehydes are unknown, all results and kinetic parameters reported here have been expressed in terms of total aldehyde concentration. Care must be taken, however, when comparing different substrates as the fraction of free aldehyde present will differ. So, for instance, the apparent K_m of a compound with a low fraction of free aldehyde would appear higher than the apparent K_m of a compound with a high fraction of free aldehyde even though their actual K_m values (in terms of free aldehyde concentration) were the same.

4.2.2.7 Fluorimetric assay at pH 7.6

The assay was conducted as for pH 9.3 with the use of a phosphate buffer. The buffer consisted of 0.1M NaOH (approx. $150cm^3$) added to potassium dihydrogen phosphate (2.380g) to bring the pH to 7.6 and made up to $500cm^3$. The buffer was filtered as for pH 9.3 pyrophosphate buffer and $2.15cm^3$ of phosphate buffer was added to the assay to make the buffer .025M in phosphate, all the other concentrations were as for the spectrophotometric assay.

4.2.2.8 Attempts to initiate the reverse reaction

A strong phosphate buffer (0.75M in phosphate) was made up by adding sodium hydroxide (2M) to a solution of potassium dihydrogen phosphate (13.6g in 100cm^3) to adjust to pH 7.7 and made up with distilled water to 150cm^3. The stock solutions of carboxylic acids made up to 10cm^3 with buffer were acetic acid (100mM), propionic acid (95mM) and 2-bromo-2-phenylacetic acid (12mM). A 512µM NADH stock solution was prepared from a Sigma preweighed vial. The assay was:

0.1cm^3 enzyme (assay concentration 0.4µM)
0.5cm^3 NADH (85µM)
0.3cm^3 carboxylic acid
2.1cm^3 Buffer

The absorbance of NADH at 340nm was followed on a Unicam SP500 spectrophotometer thermostated at 25°C. Buffer was used in the reference cell and a control cell of carboxylic acid and NADH was used to follow any nonenzymic changes in absorbance over 3 hours.

4.2.3 Treatment of Data

The initial rates of reaction were generally linear for at least 3 minutes and were always determined in duplicate. Results were plotted as reciprocal of initial velocity versus reciprocal of substrate concentration, lines being fitted to the points initially by eye to check for linearity and then fitted by linear least squares analysis to an Eadie plot (Eadie, 1942) and a Lineweaver-Burk plot (Lineweaver and Burk, 1934) on an IBM 1130 computer (Youden, 1951). Intercepts and slopes obtained from the primary plots were then replotted against reciprocal of the nonvaried substrate concentration.

4.3 RESULTS

4.3.1 Steady-State Kinetics at pH 9.3 using Spectrophotometry

4.3.1.1 Specificity of aldehyde dehydrogenase

Sheep liver aldehyde dehydrogenase is capable of oxidizing a wide range of aldehydes including both aliphatic and aromatic compounds (see Table 4.3a). The rate at which these aldehydes were oxidized varied widely demonstrating that the maximal velocity is dependant on the substrate structure.

TABLE 4.3a SUBSTRATE SPECIFICITY OF ALDEHYDE DEHYDROGENASE

Maximal velocities for various aldehydes with sheep liver aldehyde dehydrogenase at pH 9.3

Substrate	Concentration (mM)	Velocity (μmoles.min^{-1}. (cm^3 enzyme solution)$^{-1}$)
Acetaldehyde	1.67	1.04
Propionaldehyde	1.67	0.83
Butyraldehyde	1.67	0.55
D,L-Glyceraldehyde	1.67	0.86
Pyruvic aldehyde	1.67	0.27
Benzaldehyde	1.67	0.12

The velocity of NADH production was followed spectrophotometrically in an assay containing NAD^+ (1.67mM) in pH 9.3 pyrophosphate buffer.

4.3.1.2 Esterase activity

Sheep liver aldehyde dehydrogenase was found to be capable of catalyzing the hydrolysis of p-nitrophenyl acetate at pH 8.0. Though the enzyme solution contained 2-mercaptoethanol, the rate of reaction was more than twice that found for the hydrolysis of p-nitrophenyl acetate in the presence of 2-mercaptoethanol at the same concentration (Table 4.3b).

TABLE 4.3b ESTERASE ACTIVITY OF SHEEP LIVER ALDEHYDE DEHYDROGENASE

Spontaneous rate of hydrolysis of p-nitrophenyl acetate	10 nmoles/min
Rate of hydrolysis in the presence of 2-mercaptoethanol	51 nmoles/min
Rate of hydrolysis in the presence of enzyme solution	123 nmoles/min

4.3.1.3 Initial velocity studies with glyceraldehyde at pH 9.3

Initial velocity studies on aldehyde dehydrogenase were carried out in pH 9.3 pyrophosphate buffer with glyceraldehyde as the aldehyde substrate. Glyceraldehyde was chosen because Freda and Stoppani (1970) in their work using bovine liver aldehyde dehydrogenase found that this substrate, unlike acetaldehyde, gave linear double reciprocal plots. It was also reported that glyceraldehyde had a high Michaelis constant which, if true for the sheep liver enzyme, would mean that higher and hence more accurately determinable concentrations of substrate could be used. The substrate used in the experimental work was D,L-glyceraldehyde, both isomers of which are oxidized by liver aldehyde dehydrogenase (Holldorf et al., 1959). However, experimental problems were encountered with the glyceraldehyde initial rates as the points on the reciprocal plots were either scattered or non-linear. Since either the reagents used or the enzyme sample may have caused the irregularities, these possibilities were checked. The enzyme sample did contain endogenous activity which was removed by better separation techniques in the later preparations and coupled with a change in the quality of NAD^+ used (from Sigma Grade AA (90-96%) to Sigma Grade III (98%)) brought about a great improvement in the consistency of the data and the linearity of the double reciprocal plots. Solid glyceraldehyde is known to exist mainly as a dimer and to take several days to depolymerize in solution (Beck, 1957). However, the same K_m result was obtained whether the glyceraldehyde solution had been made up immediately prior to use or had been left standing for nine days and thus it was concluded

that polymerization of glyceraldehyde was not a significant factor in the kinetics. The Michaelis constant for glyceraldehyde, the concentration required to give half the maximum reaction velocity, was found to be 160μM ± 15μM. 2-Mercaptoethanol was present in all buffers used during the purification procedure in order to stabilise the enzyme, and was, therefore, also present in the assay mixture. However, steady-state kinetics carried out after the complete removal of 2-mercaptoethanol gave the same value of K_m for NAD^+ and glyceraldehyde and the same maximum velocity, demonstrating that the 2-mercaptoethanol had no effect on the enzyme catalysed reaction.

Using high concentrations of glyceraldehyde (.5mM to 20mM) in the same conditions as used for the K_m determinations, it was found that at concentrations of glyceraldehyde above 5mM, there was apparent substrate activation but in the intermediate range (1mM to 2mM) there appeared to be substrate inhibition. The latter feature was also observed in double reciprocal plots used to determine K_m, at the highest glyceraldehyde concentration.

4.3.1.4 Effect of pH on K_m for glyceraldehyde

The pH profile of the aldehyde dehydrogenase catalysed reaction determined with veronal sodium-HCl buffers showed that as the pH was increased from pH 7 to pH 9 the K_m for glyceraldehyde decreased by three fold (350μM to 130μM) while in the same range, the maximum velocity increased by only 25%. The change in pH caused little effect on the maximum velocity.

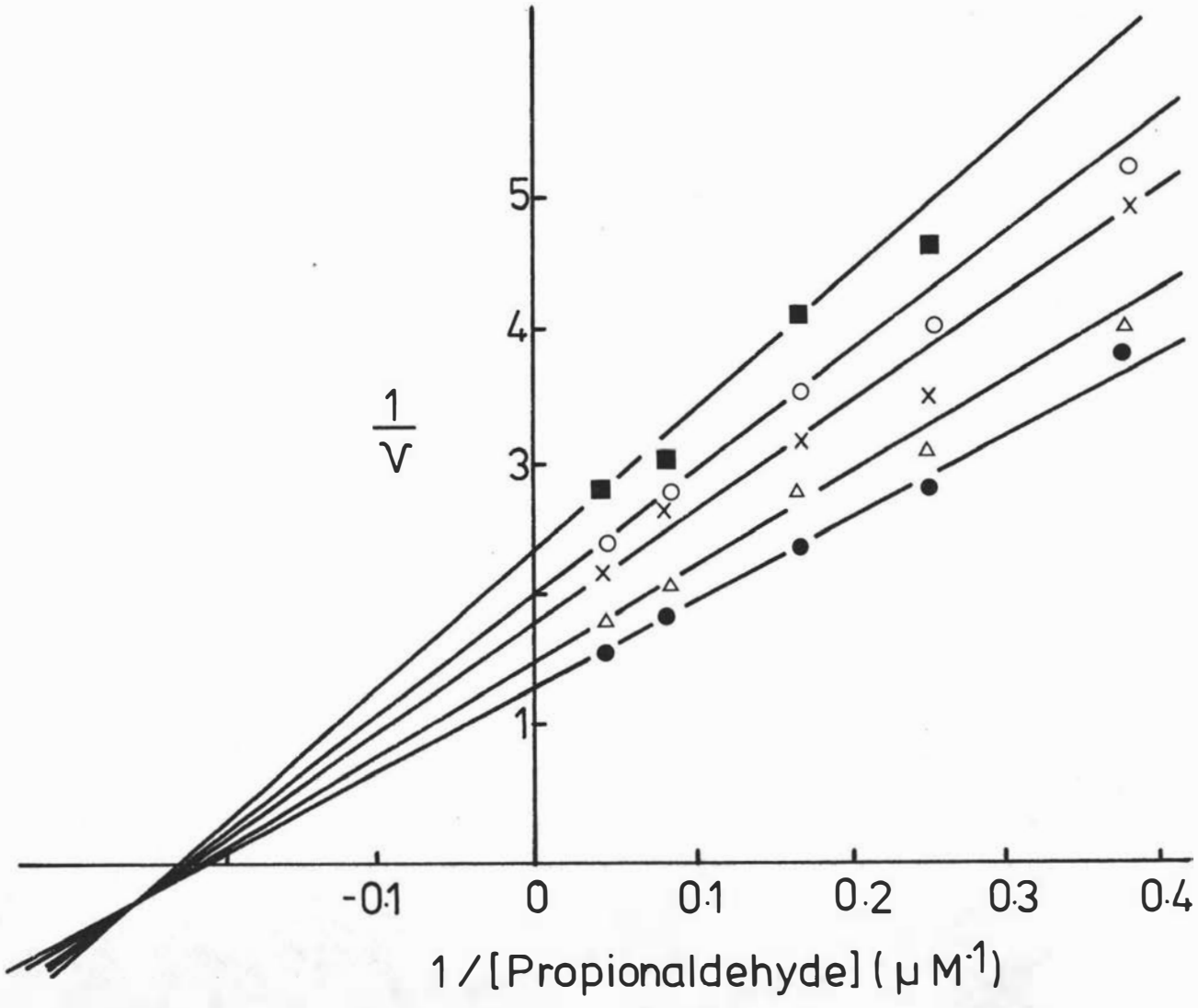
4.3.1.5 Initial velocity studies with NAD^+ at pH 9.3

The Michaelis constant for NAD^+ was determined with much less difficulty than was experienced with glyceraldehyde. The value was determined using acetaldehyde (1.67mM) as the aldehyde substrate and the Michaelis constant for NAD^+ was found to be 12μM ± 2μM, a value of the order of ten times less than the K_m for glyceraldehyde.

4.3.1.6 Difficulties in initial velocity studies

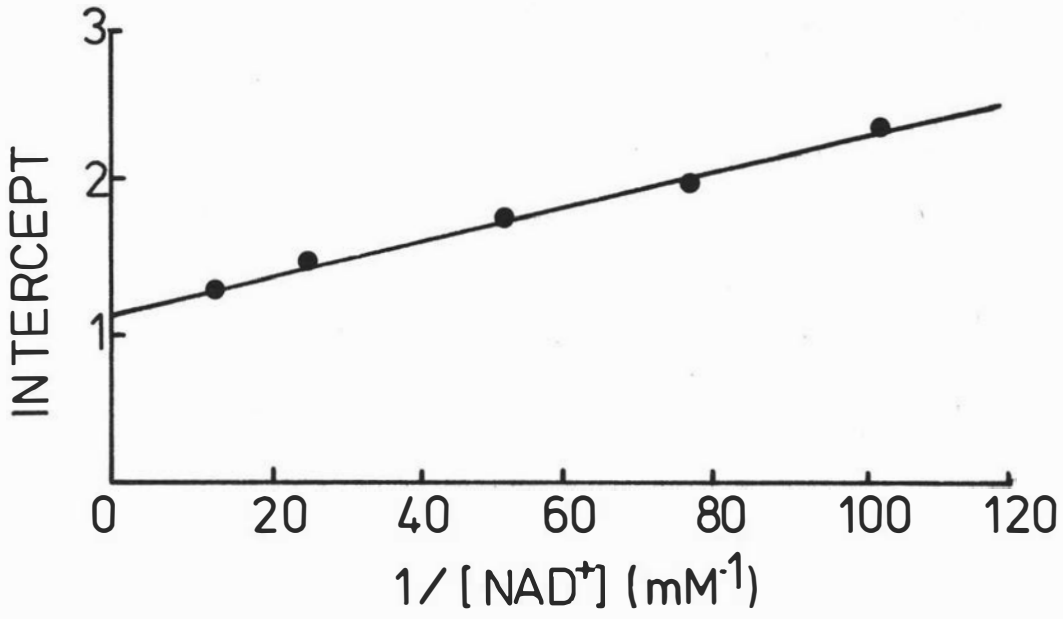
Even using saturating concentrations of substrate, the slow rate of the enzyme catalysed reaction gave only very small absorbance changes after reasonable assay times;

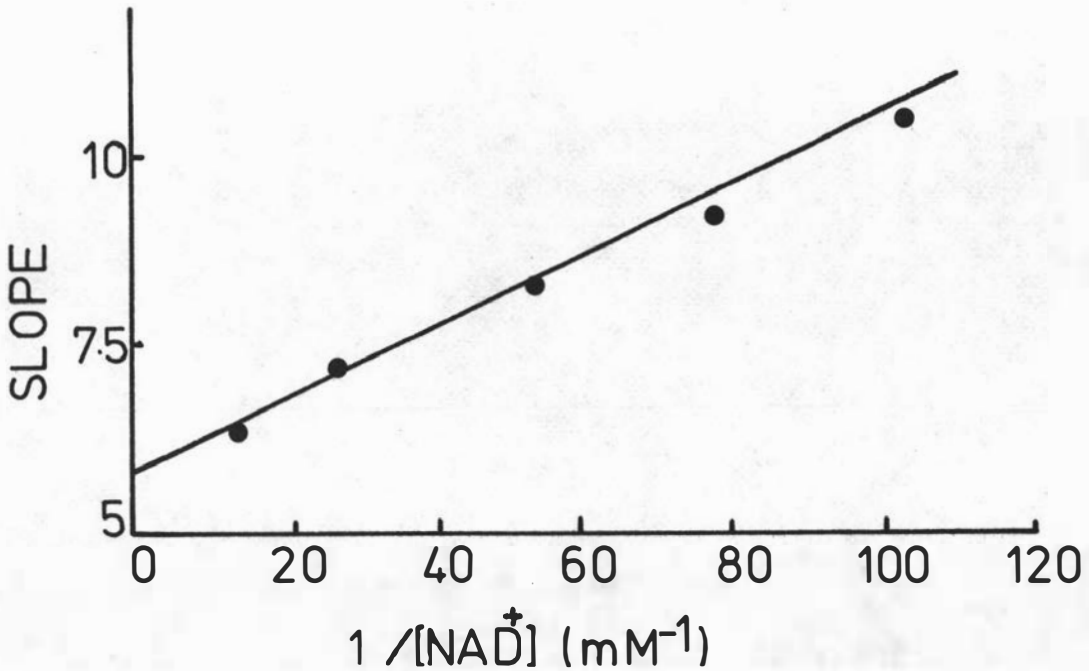
FIG. 4.4 DOUBLE RECIPROCAL PLOT OF INITIAL VELOCITY WITH RESPECT TO PROPIONALDEHYDE AT FIXED CONCENTRATIONS OF NAD^+ AT pH 9.3



The concentration of propionaldehyde was varied while the NAD^+ concentration was held constant at the following values; ■, 9.8 μM; ○, 13 μM; x, 18.5 μM; △, 39 μM; ●, 78 μM.
The enzyme concentration was 10 ng/cm^3.

FIG. 4.5 SECONDARY PLOTS OF DATA FROM FIG. 4.4





and for less than saturating concentrations of fixed substrate, precise determination of rates was extremely difficult. It was decided that reliable data necessary to carry out the double reciprocal plots of initial velocity against variable substrate concentration at different concentrations of fixed substrate, could not be obtained by this method.

For this reason, the more sensitive technique of fluorescence was adopted for following NADH production. Glyceraldehyde as a substrate was found to have several disadvantages. It was difficult to check the purity as the melting point was not distinct and the structure of glyceraldehyde was very different from acetaldehyde, the aldehyde substrate of the enzyme during alcohol metabolism. Finally glyceraldehyde, an α-hydroxy carbonyl compound, was reported to react with NAD^+ at pH 10 (Burton and Kaplan, 1953).

Propionaldehyde was chosen as a more suitable substrate as it more closely resembled acetaldehyde; it was not an α-hydroxyl carbonyl compound and was easy to purify. For these reasons propionaldehyde was used as the experimental substrate in all further kinetics.

4.3.2 Steady State Kinetics at pH 9.3 using Fluorimetry

4.3.2.1 Initial velocity studies with propionaldehyde and NAD^+ as substrates

Initial velocity studies were carried out with propionaldehyde as the variable substrate in the presence of several fixed concentrations of NAD^+. Double reciprocal plots (Fig. 4.4) gave a series of intersecting lines indicating that both substrates bound before a product was released and hence that the enzyme mechanism must be ordered or random (but not Ping Pong). Secondary plots of the slopes and intercepts on the ordinate axes of these plots are shown in Fig. 4.5. The constants which may be derived from these figures are shown in Table 4.4 assuming the reaction was sequential and either propionaldehyde or NAD^+ may bind first to the enzyme. In the presence of high propionaldehyde concentrations (up to 2mM) there was no substrate activation as seen for glyceraldehyde. Using high NAD^+ concentrations, at constant propionaldehyde concentration (48μM), there was only

slight deviation from linear behaviour above 200μM showing insignificant inhibition.

TABLE 4.4

KINETIC PARAMETERS OF ALDEHYDE DEHYDROGENASE REACTION AT pH 9.3

	K_a	K_b	K_{ia}
A = NAD^+	10.4μM		8.4μM
B = propionaldehyde		5.0μM	
A = propionaldehyde	5.0μM		4.0μM
B = NAD^+		10.4μM	

The kinetic parameters were calculated from equation (6) for sequential mechanisms with the nomenclature of Cleland (1963).

4.3.2.2 Inhibition studies

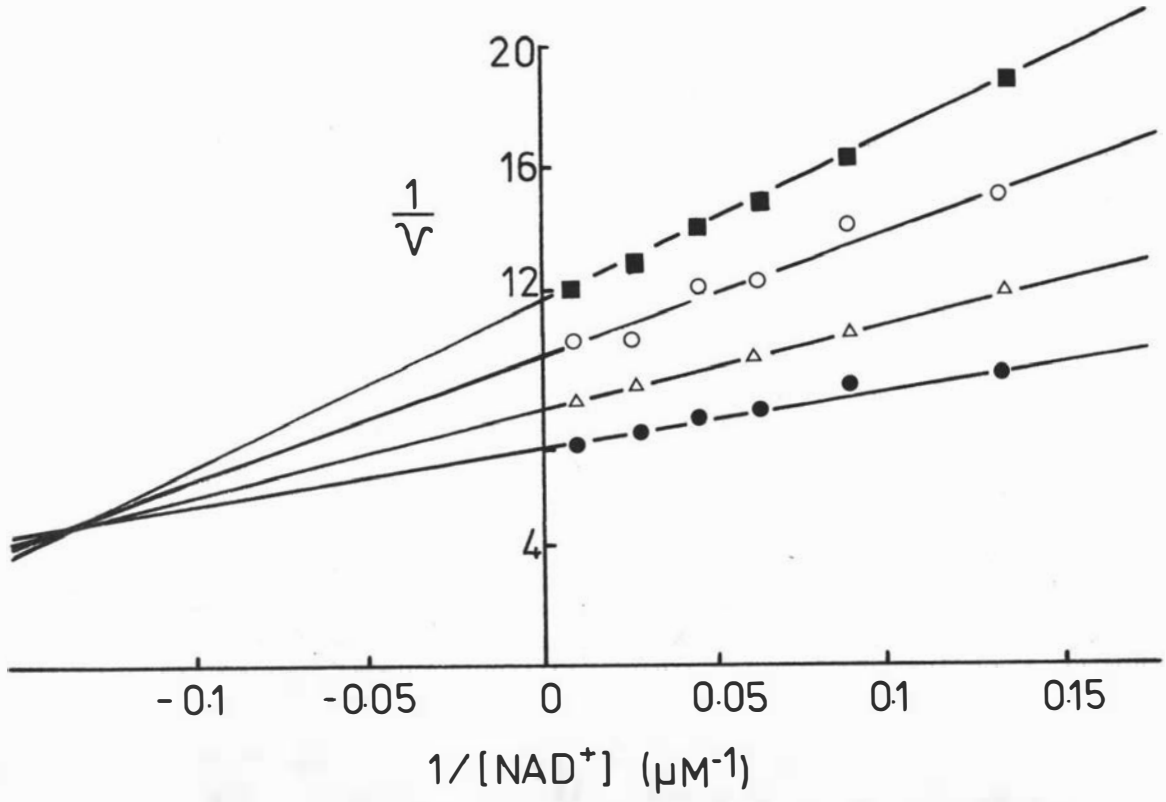
Inhibition by NADH was found to be competitive with NAD^+ in pyrophosphate buffer at pH 9.3, giving an inhibition constant K_{is} of 4.8μM. As propionaldehyde was saturating (480μM) it may be concluded that the enzyme mechanism was not rapid equilibrium random BiBi for which no inhibition would be expected at high aldehyde concentrations.

4.3.3 Steady State Kinetics at pH 7.6

4.3.3.1 Initial velocity studies at pH 7.6

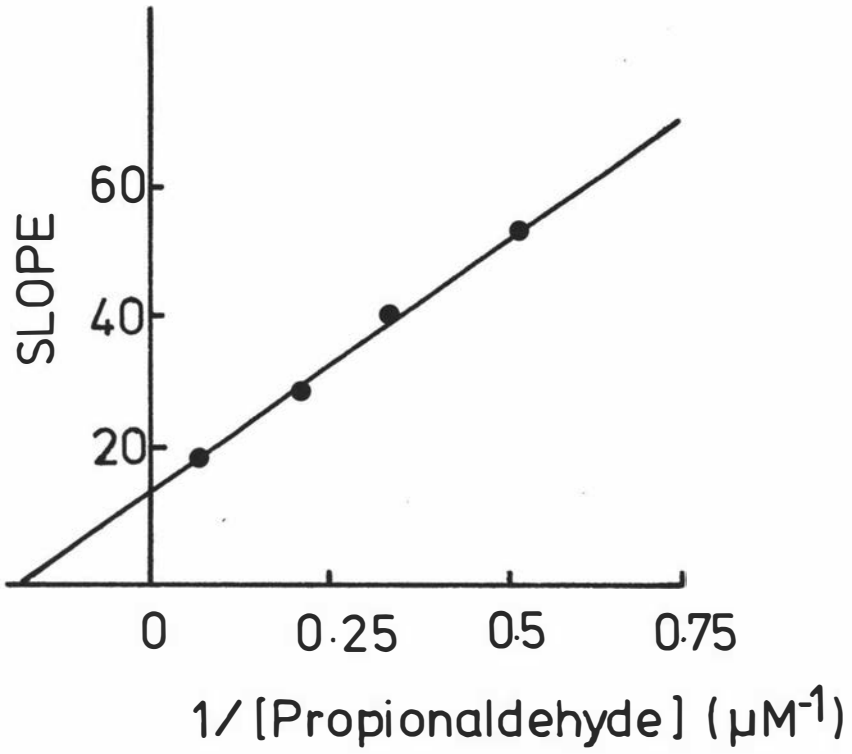
Steady state experiments were repeated at pH 7.6 so the results could be used in conjunction with the equilibrium studies results of active site concentration and NADH dissociation constant. In addition, study of the reaction at the lower pH means that it is closer to physiological pH and would perhaps give results that more closely resemble the reaction taking place *in vivo*. The initial velocity data at pH 7.6 using propionaldehyde and NAD^+ as substrates was found to give a double reciprocal plot of a set of straight lines converging above the x axis (Fig. 4.6), consistent with a sequential mechanism as was proposed for the enzyme at pH 9.3. Secondary plots of the slopes and intercepts are shown in Fig. 4.7 and in Table 4.5 the kinetic constants based on a sequential mechanism are presented.

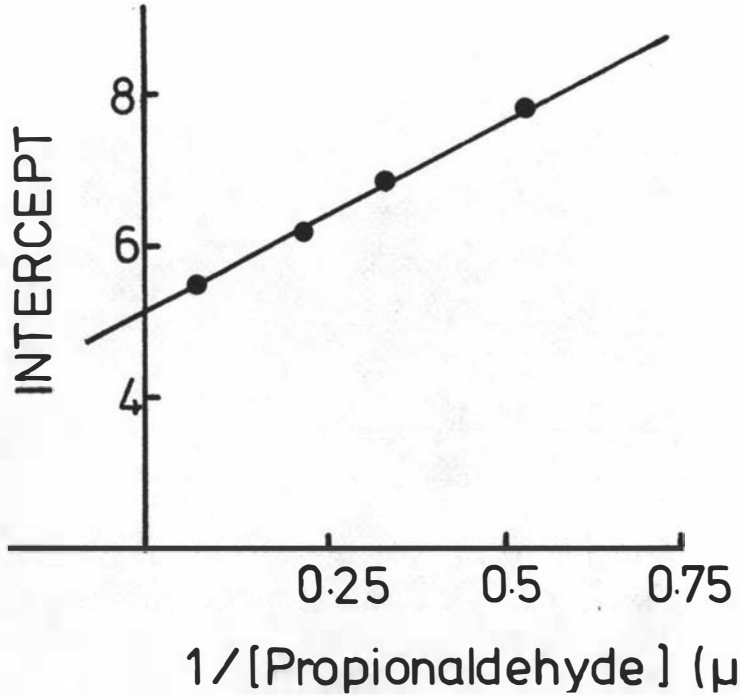
FIG. 4.6 DOUBLE RECIPROCAL PLOT OF INITIAL VELOCITY WITH RESPECT TO NAD^+ AT FIXED CONCENTRATIONS OF PROPIONALDEHYDE AT pH 7.6



The concentration of NAD^+ was varied while the propionaldehyde concentration was held constant at the following values: ■, 1.9 μM; ○, 3.2 μM; △, 4.8 μM; ●, 19.1 μM. The enzyme concentration was 9.3 ng/cm^3.

FIG. 4.7 SECONDARY PLOTS OF DATA FROM FIG. 4.6





Constants derived from two separate experiments are presented in this table and show the excellent agreement between results. On plotting the data with the other substrate as the variable substrate very similar results were obtained.

TABLE 4.5 KINETIC CONSTANTS FOR ALDEHYDE DEHYDROGENASE IN pH 7.6 PHOSPHATE BUFFER

Calculations of the constants were based on the equation for a sequential mechanism. The two values for each constant represent values obtained in two separate experiments.

Substrate	K_a	K_b	K_{ia}
A = NAD^+ B = propionaldehyde	2.2, 2.4μM	1.5, 1.2μM	8.0, 8.0μM
A = propionaldehyde B = NAD^+	1.5, 1.2μM	2.2, 2.4μM	5.5, 4.0μM

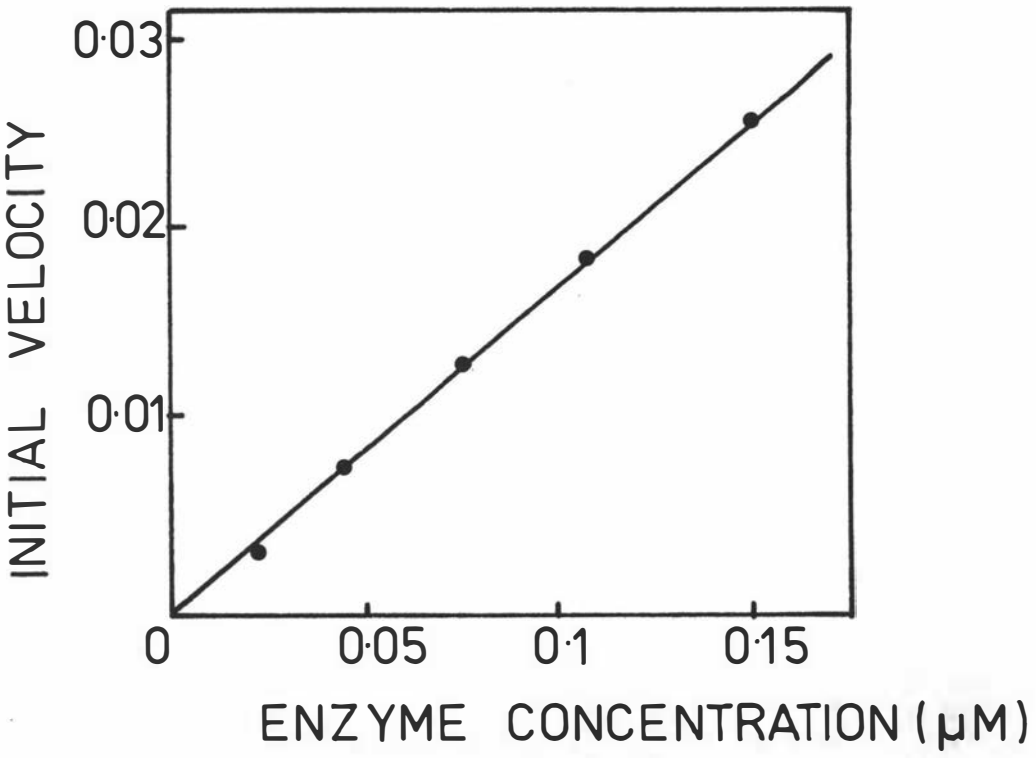
4.3.3.2 Relationship between enzyme concentration and initial velocity

The enzyme reaction velocity using high substrate concentrations was proportional to the active site concentration of the enzyme (Fig. 4.8) with a slope of 0.083 moles NADH.(mole active sites.sec)$^{-1}$ representing the catalytic-centre activity or the turnover number. The linear relationship indicates equivalence of the active sites because, in the range used, twice the active site concentration produced twice as much NADH in a minute.

4.3.3.3 Effect of pH on K_m for propionaldehyde

A two fold decrease in the K_m for propionaldehyde was found as the pH of veronal sodium-HCl buffers was increased in the range pH 6.8 to pH 9.6. In the same range there was a less than two fold increase in V, however, the maximum velocity at pH 7.6 in sodium veronal buffer was less than a third the velocity in phosphate buffer. Comparing the K_m for propionaldehyde in pH 7.6 phosphate buffer and pH 9.3 pyrophosphate buffer the value increased from 1.2μM to 5.0μM. This is in conflict with the results from the profile using veronal sodium-HCl buffers. One

FIG. 4.8 RELATIONSHIP BETWEEN INITIAL VELOCITY AND THE ENZYME CONCENTRATION



The enzyme concentration is expressed in terms of the active site concentration determined by NADH titration. Units of initial velocity are fluorescence units/minute (where a 2.92 μM NADH solution has unit fluorescence).

possibility is that the veronal sodium-HCl buffer is having an affect on the enzyme reaction, as it has been shown that barbital is capable of inducing increases in enzyme activity in one of the aldehyde dehydrogenases found in genetically responsive rats (Deitrich and Siew, 1974). However, a far more likely explanation is that the change in buffers and ionic strength, phosphate buffer (.025M in phosphate) at pH 7.6 to pyrophosphate buffer (.033M in pyrophosphate) at pH 9.3, is the cause of the apparent anomaly.

4.3.3.4 Product inhibition

Product inhibition studies were carried out to help establish the order of substrate addition to aldehyde dehydrogenase. NADH was found to be a competitive inhibitor with respect to NAD^+ in the presence of excess propionaldehyde with an inhibition constant $K_{is} = 1.2 \pm 0.2\mu M$. With propionaldehyde, NADH gave non-competitive inhibition at low NAD^+ concentration (16μM) giving K_{is} of 6μM and K_{ii} of 5μM while at high concentrations of NAD^+ (790μM), NADH had no inhibitory effect. High concentrations of the acid product propionic acid were found to alter the pH of the assay system but using a stronger buffer (0.7M) the propionic acid (3.2mM) was found to have no effect on the enzyme reaction. As NADH is the only product to inhibit the enzymic reaction, not enough information is available to define the mechanism since from Table 4.1 the ordered Bi Bi and rapid equilibrium random Bi Bi plus dead end EBQ complex both with NAD^+ binding first and Theorell-Chance mechanism with aldehyde binding first are consistent with the results.

4.3.3.5 Dead end inhibition

An acid, 2-bromo-2-phenylacetic acid, was found to be a reversible inhibitor of aldehyde dehydrogenase even though propionic acid was not. 2-Bromo-2-phenylacetic acid (180μM) was found to be an uncompetitive inhibitor with respect to propionaldehyde at fixed NAD concentration (200μM) with an inhibition constant K_{ii} of 1.2mM. However, as the inhibition constant is so high the inhibition found was small, and so it was difficult to distinguish the uncompetitive inhibition from non-competitive inhibition. This was also the case with NAD^+ as the variable substrate, the two

forms of 2-bromo-2-phenylacetic acid inhibition being difficult to distinguish.

The inhibition pattern suggests that 2-bromo-2-phenylacetic acid is acting as an acid product analogue and and is consistent with an ordered Bi Bi mechanism, while a rapid equilibrium random mechanism with the EBQ complex would be expected to exhibit no inhibition at saturating concentrations of fixed substrate and competitive inhibition at unsaturating concentrations.

Chloral hydrate, a potent inhibitor of aldehyde dehydrogenase is an aldehyde which is almost entirely in its hydrated form (Bell 1966). This compound gave linear competitive inhibition (K_{is} of 19μM) with propionaldehyde. The variable substrate at fixed NAD^+ concentration (227μM) 1,10-phenanthroline, a zinc chelating agent, was found to be a competitive inhibitor of NAD^+ with an inhibition constant of 75μM. The inhibition results are summarised in Table 4.6.

TABLE 4.6 INHIBITION OF SHEEP LIVER CYTOPLASMIC ALDEHYDE DEHYDROGENASE

The values were determined in pH 7.6 phosphate buffer.

Inhibitor	Varied substrate	Inhibition	Inhibition constants K_{is}	K_{ii}
NADH	NAD^+	Competitive	1.2μM	
	propionaldehyde (low NAD^+)	Non-competitive	6 μM	5μM
	propionaldehyde (high NAD^+)	No inhibition	-	-
2-Bromo-2-phenylacetic acid	NAD^+ (high prop)	Uncompetitive		570μM
	propionaldehyde (high NAD^+)	Uncompetitive		1200μM
Chloral hydrate	propionaldehyde (high NAD^+)	Competitive	19 μM	
o-phenanthroline	NAD^+ (high prop)	Competitive	75 μM	

4.3.3.6 Attempts to initiate the reverse reaction

Using high concentration buffer to reduce the effect of high concentrations of carboxylic acids on the pH (a change from pH 7.7 to pH 7.6 being observed) attempts were

made to observe the utilization of NADH and carboxylic acids by the enzyme. On following the absorbance at 340 nm there was no difference between the sample cells with carboxylic acids and the control cell which contained NADH and acetic acid but no enzyme, though all values decreased 15% over a 3 hour period. This decrease must have been due to non-enzymic destruction of NADH or instability in the spectrophotometer over the time period. It was demonstrated in this way that acetic acid (10mM) and propionic acid (9.5mM) both products of enzyme reaction are not substrates for the reverse reaction and 2-bromo-2-phenylacetic acid (1.2mM), an inhibitor of the enzyme is also unable to initiate the reverse reaction.

With p-nitrobenzaldehyde it was shown that the expected stoichiometric amount of NADH was produced from solutions containing accurately known weights of aldehyde in the presence of excess NAD^+ (4.6mM), Table 4.7.

TABLE 4.7 QUANTITATIVE PRODUCTION OF PRODUCT

Initial substrate (p-nitrobenzaldehyde)	Product produced
102μM	100μM
41μM	43μM

4.3.3.7 Effect of high propionaldehyde concentration

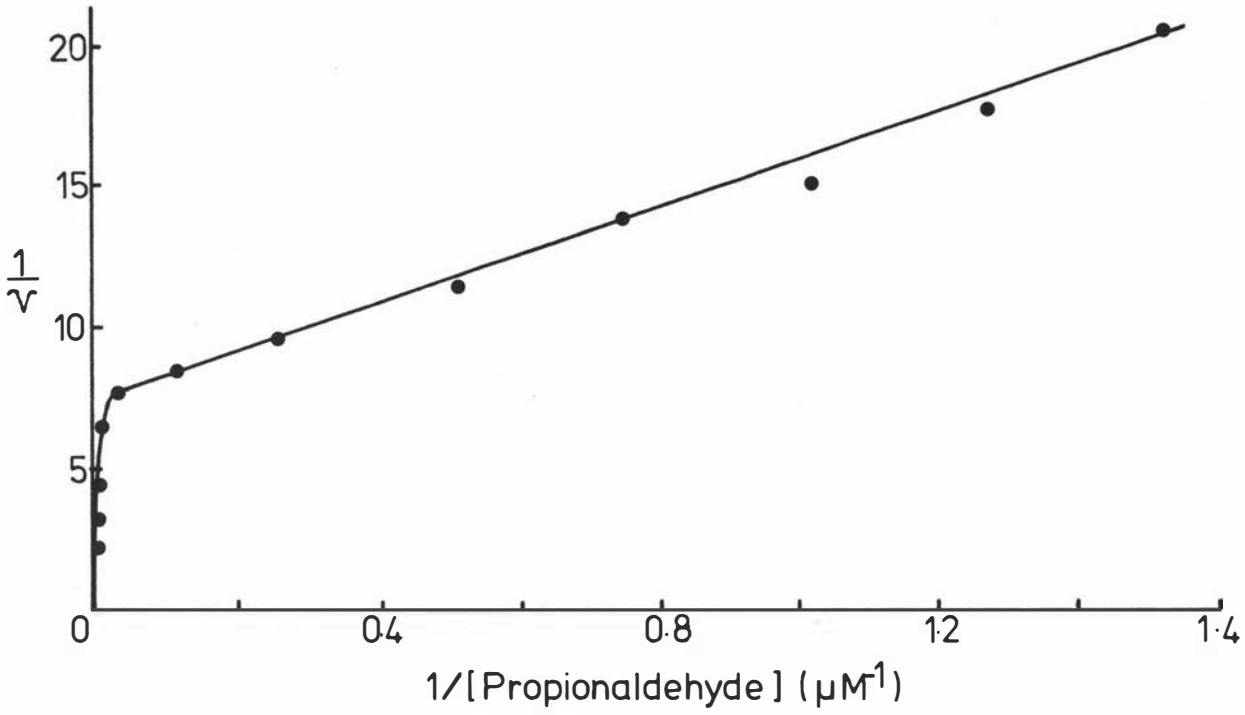
When a wide range of propionaldehyde concentrations was used it was found that the double reciprocal plots were biphasic (see Fig. 4.9) being linear to 50μM and deviating to faster rates above this value. The maximum velocity for the linear portion of the graph gave a catalytic-centre activity ($V/[E]$) of $0.082s^{-1}$ using an active site concentration of 0.08μM. At very high propionaldehyde concentrations the catalytic-centre activity was $0.27s^{-1}$.

4.3.3.8 Effect of high acetaldehyde concentration

Acetaldehyde was also studied over a wide concentration range. As can be seen in Fig. 4.8 the double reciprocal plot deviated from linearity over almost the entire concentration range rather than the abrupt change between the two phases observed for propionaldehyde. At low concentrations

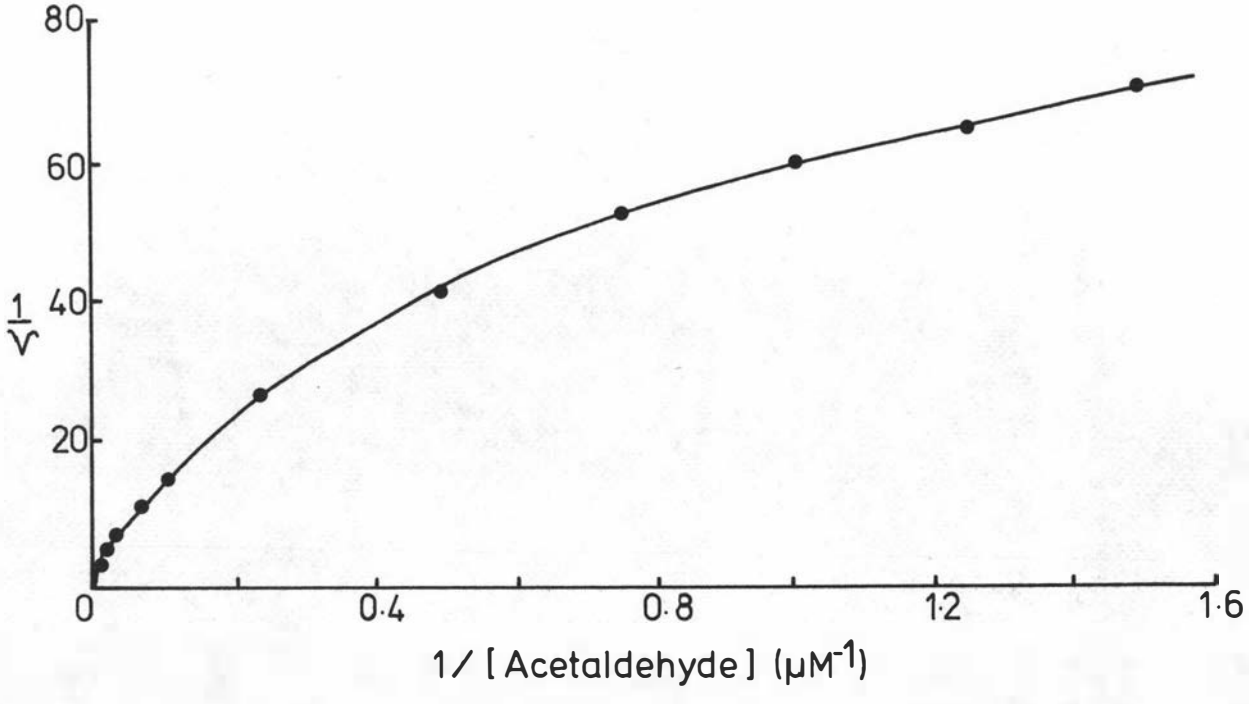
FIG. 4.9 EFFECT OF WIDE RANGE OF ALDEHYDE CONCENTRATIONS OF THE INITIAL VELOCITY AT pH 7.6

(a) PROPIONALDEHYDE



Enzyme active site concentration, 0.08 μM and NAD^+ concentration, 360 μM.

(b) ACETALDEHYDE



Enzyme active site concentration, 0.08 μM and NAD^+ concentration, 360 μM.

the line appears linear with a K_m for acetaldehyde of 0.67μM and a turnover number of $0.015s^{-1}$. When the acetaldehyde concentration was increased deviations from linearity occurred with a maximum velocity giving a turnover number of $0.25s^{-1}$, a value 16 times that obtained from the linear portion of the graph.

4.3.3.9 Substrate specificity

At pH 7.6 the substrate structure is also seen to have an effect on the maximum velocity. As the enzyme concentration is known, the catalytic centre activity (μmoles NADH produced per sec per μmoles of active sites) may be determined for the various substrates (Table 4.8). At

TABLE 4.8 SUBSTRATE SPECIFICITY AT pH 7.6

The NADH production was followed fluorimetrically in an assay containing aldehyde dehydrogenase (0.2μM), NAD^+ (2mM) in pH 7.6 phosphate buffer.

Substrate	Concentration (mM)	Catalytic Centre Activity (s^{-1})
Propionaldehyde	.054	.03
	5.0	.2
Butyraldehyde	2	.14
	4.6	.13
	9.8	.13
Benzaldehyde	2.4	.04
	120	.024
p-nitrobenzaldehyde	0.64	.02
	1.2	.01
m-nitrobenzaldehyde	0.4	.04
	0.8	.04
p-methoxybenzaldehyde	0.5	.04
	1.0	.04
Formaldehyde	1	.06
	11	.11

concentrations of substrate much greater than their respective K_m's a change in concentration should have no effect on the catalytic rate constant. In cases where this constant increases or decreases with larger concentrations, substrate activation or inhibition is occurring. Results show that the aldehyde structure has an effect on the catalytic rate constant of the enzyme, the aromatic aldehydes reacting

slower than the aliphatic aldehydes. This effect would mean that the substrate or product formed was involved in the rate limiting step, a step of almost equivalent rate or that there is a change in rate determining step on changing substrates.

Aldehyde dehydrogenase was at first thought to be capable of using $NADP^+$ as a coenzyme substrate since an absorption increase was observed when $NADP^+$ and propionaldehyde were added to the enzyme. However, the absorption change was less than 1% of that expected if all the $NADP^+$ was reduced and the change was reversed on the addition of yeast alcohol dehydrogenase, an enzyme which cannot utilize NADPH as a cofactor (Barman, 1969). An explanation of this result is that only NAD^+ impurities of $NADP^+$ were reduced by aldehyde dehydrogenase and the NADH produced was reoxidized by alcohol dehydrogenase using propionaldehyde as the substrate. Hence $NADP^+$ is not a coenzyme for aldehyde dehydrogenase.

4.4 DISCUSSION

The low specificity shown by sheep liver aldehyde dehydrogenase with respect to aldehyde substrates appears to be a feature common to all aldehyde dehydrogenases (for instance, Duncan and Tipton (1971b), Freda and Stoppani (1970)). Aldehyde dehydrogenases have been reported to be capable of utilizing substrates as different as formaldehyde and 3-pyridinealdehyde-NAD^+. The wide range of aldehyde substrates which bind to the enzyme but the lack of any inhibition by structurally similar compounds such as alcohols and ketones suggest a binding site specific for the $-CH=O$ group but which is large enough to accommodate bulky molecules.

Although aldehyde dehydrogenases capable of using $NADP^+$ as a coenzyme have been found in some species (Jakoby, 1958; Horton and Barrett, 1975) no activity was observed with $NADP^+$ in the case of the sheep liver enzyme or the horse liver enzyme (Feldman and Weiner, 1972a). Analogues of NAD^+ have been shown to substitute for NAD^+ with the beef liver enzyme (Freda and Stoppani, 1970) and presteady-state studies (see Section 5) have shown that the sheep liver

enzyme may also act on NAD^+ analogues. A feature which also appears to be common to all aldehyde dehydrogenases is the irreversibility of the enzyme reaction, carboxylic acids and NADH being unable to act as substrates in the reverse reaction. The initial velocity studies at pH 9.3 with the intersecting double reciprocal plots show the mechanism to be sequential. The low values for the Michaelis constants are not unusual for aldehyde dehydrogenases as Deitrich et al. (1962) found the constants for NAD^+ and propionaldehyde from bovine liver to be 16μM and 3.8μM respectively at pH 9.6 and the pig brain enzyme has corresponding K_m's of 7.8μM and 0.6μM at pH 7.2 (Duncan and Tipton, 1971b). Generally the kinetic constants are found to be lower than for alcohol dehydrogenase. For instance the horse liver aldehyde dehydrogenase has K_m's of NAD^+ and acetaldehyde of 2.9μM and 60μM respectively at pH 7 (Eckfeldt and Yonetani, 1976) while the alcohol dehydrogenase from the same origin has K_m values of 17.4μM, 550μM, 244μM and 26.8μM for NAD^+, ethanol, acetaldehyde and NADH respectively at pH 7.15 (Bamman, 1969).

The esterase activity shown for the sheep liver enzyme is consistent with observations from other aldehyde dehydrogenases. Feldman and Weiner (1972b) found that the horse liver enzyme was capable of hydrolyzing p-nitrophenyl acetate and were able to show that the ester bound in the aldehyde binding site. As the inhibition constant was found to be over 1000 times greater than that calculated for the aldehyde binding to the free enzyme from the dehydrogenase reaction data, it was suggested that the dehydrogenase mechanism was ordered with NAD^+ binding prior to the aldehyde. Feldman and Weiner went so far as to suggest that the esterase and dehydrogenase reactions have a common thiohemiacetal intermediate formed from either the oxidation of aldehyde or the hydrolysis of a nitrophenyl ester in the presence of NADH. Whether the intermediate is in fact common may be shown when the presence of a thiol group in the aldehyde binding site of dehydrogenases is demonstrated.

The steady state kinetics at pH 7.6 with the intersecting initial velocity patterns also indicate a sequential mechanism, and the micro-molar range of the derived parameters

mean that the enzyme can operate at very low substrate concentrations. From product inhibition studies a dissociation constant of 1.2μM for NADH was obtained, a value the same as the dissociation constant for NADH found in Section 3 by titration of the free enzyme with NADH. The fact that this steady state dissociation constant agrees with the constant found from the NADH titration in the absence of any aldehyde substrate suggests that during the enzyme reaction NAD^+ does not bind as the second substrate in an ordered mechanism. The product inhibition patterns for NADH are consistent with an ordered Bi Bi (with NAD^+ as leading substrate) mechanism or a rapid equilibrium random Bi Bi with dead end EBQ complex mechanism. These are the only mechanisms in which the last product to be released (Q), in Table 4.1, gives inhibition patterns of the type observed for NADH inhibition. As the Theorell-Chance mechanism gives the observed inhibition pattern for NADH only for the first product released (P), a product which cannot bind to the free enzyme, this mechanism cannot be operating and hence a significant amount of a ternary complex must be formed. The fact that the acid products do not bind means that product inhibition cannot distinguish the two possible mechanisms. However, the uncompetitive inhibition of propionaldehyde and NAD^+ at saturating concentrations of fixed substrate with the compound 2-bromo-2-phenylacetic acid is consistent with an ordered Bi Bi mechanism in which the acid analogue does not react and hence there can be no reversible sequence between the analogue and the aldehyde binding step. (It has already been shown that 2-bromo-2-phenylacetic acid fails to initiate the reverse reaction with NADH.) The inhibition patterns are not consistent with a rapid equilibrium random Bi Bi with dead end complex EBQ for which one would expect competitive inhibition with respect to both substrates at low concentrations of fixed substrate and no inhibition at all at saturating concentrations of fixed substrate. Even if it was possible to form both an EBQ and an EAP dead end complex, the rapid equilibrium random mechanism should exhibit competitive inhibition with respect to propionaldehyde at all concentrations of NAD^+, a situation which does not agree with the data. It is of interest to note that in Section 3 the dissociation constant

of NADH from the enzyme complex was unchanged in the presence of propionaldehyde, suggesting that the EBQ complex is not formed.

The ordered mechanism with ternary complexes postulated for aldehyde dehydrogenase is shown in Fig. 4.10 with NAD^+ bound prior to the aldehyde followed by the irreversible release of the acid product and finally the dissociation of NADH. The irreversible release of acids is proposed because of the overall irreversibility of the reaction and the failure of normal acid products to inhibit. The ordered mechanism with the oxidized and reduced coenzymes binding to the free enzyme seems to be quite common among dehydrogenases, for instance alcohol dehydrogenase, lactate dehydrogenase and malic dehydrogenase (Schwert and Winer, 1970). Horse liver aldehyde dehydrogenase (Feldman and Weiner, 1972b), pig brain aldehyde dehydrogenase (Duncan and Tipton, 1971b), bovine liver aldehyde dehydrogenase (Freda and Stoppani, 1970) also postulated an ordered mechanism with NAD^+ binding first while Bradbury and Jakoby (1971b) found that yeast aldehyde dehydrogenase followed an ordered mechanism in which the aldehyde bound first. The only non-ordered mechanism to have been postulated is for human liver, where product and dead end inhibition results required that a random mechanism be proposed (Sidhu and Blair, 1975b).

Use of the King-Altman method for deriving steady-state equations (King and Altman, 1956) enables the kinetic equation for the pathway in Fig. 4.10 to be calculated.

$$\frac{V}{v} = 1 + \frac{K_a}{[NAD^+]}\left(1 + \frac{[NADH]}{K_i}\right) + \frac{K_b}{[ald]} + \frac{K_{ia}K_b}{[NAD^+][ald]}\left(1 + \frac{[NADH]}{K_i}\right)$$

where $$V = \frac{k_3 k_4 k_5 [E]}{(k_{-3}k_5 + k_4k_5 + k_3k_5 + k_3k_4)}$$

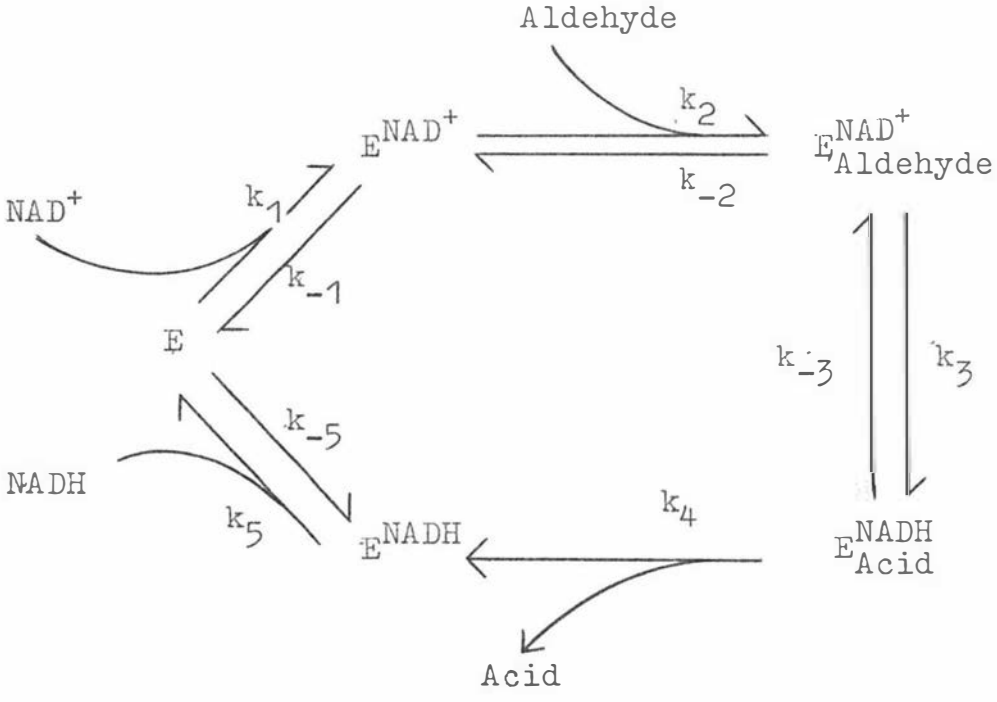
$$K_a = \frac{k_3 k_4 k_5}{k_1(k_{-3}k_5 + k_4k_5 + k_3k_5 + k_3k_4)}$$

$$K_{ia} = \frac{k_{-1}}{k_1}$$

$$K_b = \frac{k_5(k_{-2}k_4 + k_{-2}k_{-3} + k_3k_4)}{k_2(k_{-3}k_5 + k_4k_5 + k_3k_5 + k_3k_4)}$$

$$K_i = \frac{k_5}{k_{-5}}$$

FIG. 4.10 POSSIBLE MECHANISM CONSISTENT WITH STEADY-STATE KINETIC RESULTS



As there are so many individual rate constants concerned with each kinetic parameter the steady-state kinetics do not provide enough information to be able to determine each rate constant. However $\frac{V}{K_a} = k_1[E]$ and since the active site concentration is known, $k_1 = 5 \times 10^4\ M^{-1}\ s^{-1}$ and as $k_{-1} = K_{ia} \cdot k_1$, then $k_{-1} = 0.4s^{-1}$. Study of the reverse reaction of the enzyme usually allows further reaction parameters to be obtained, however, since the reaction of aldehyde dehydrogenase is irreversible these additional parameters cannot be obtained and hence fewer individual rate constants can be resolved. Presteady-state kinetics is required to determine further rate constants.

The catalytic-centre activity of sheep liver aldehyde dehydrogenase is $0.083s^{-1}$ (from Fig. 4.8) which is a very low rate and may have important implications in the *in vivo* situation as far as removal of aldehydes present in the liver are concerned. Alcohol dehydrogenase has a catalytic centre activity of $3s^{-1}$ for the utilization of ethanol (Shore and Gutfreund, 1970), a rate which is a factor of 30 that found for aldehyde dehydrogenase. Recently Eckfeldt and Yonetani (1976) reported that the F_1 isozyme of horse liver aldehyde dehydrogenase had a site turnover number of $0.3s^{-1}$, a rate which is of the same order as that found for the sheep liver enzyme.

The reason for the deviation of the double reciprocal plot at high aldehyde concentrations could be due to a number of factors. Two enzymes with different K_m and V values may give rise to plots of this form; there may be interactions between subunits; or a random order of addition of substrates may be operating at high aldehyde concentrations. Apparent substrate activation has been noted previously by various workers. Duncan and Tipton (1971b) found that propionaldehyde and acetaldehyde gave biphasic double reciprocal plots but only one phase was found for other aldehydes studied. Similar behaviour for acetaldehyde has been reported for other aldehyde dehydrogenases (Horton and Barnett, 1975; Tottmar *et al.*, 1973; Erwin and Deitrich, 1966).

With regard to the possibility of two enzymes acting on the same substrate, an attempt was made to fit two values of V and K_m to the experimental curve. K_m^1 and V_1 were

estimated from the linear portion of the plot at low substrate concentrations, referring to postulated enzyme (1). The assumption that postulated enzyme (2) contributed little to the velocity at low substrate concentrations was reasonable as the K_m's were widely different and the maximum velocities much more similar. From the total maximum velocity of the system, the maximum velocity of enzyme (2) could be estimated as $(V_{TOTAL} - V_1)$ and K_m^2 as the concentration which gave half V_{TOTAL} (a reasonable approximation if $V_2 > V_1$, while the value may be refined if this does not hold). The velocity of the reaction with both enzymes was found by adding the individual velocities of each enzyme found by substituting the derived parameters in the Michaelis-Menten equation

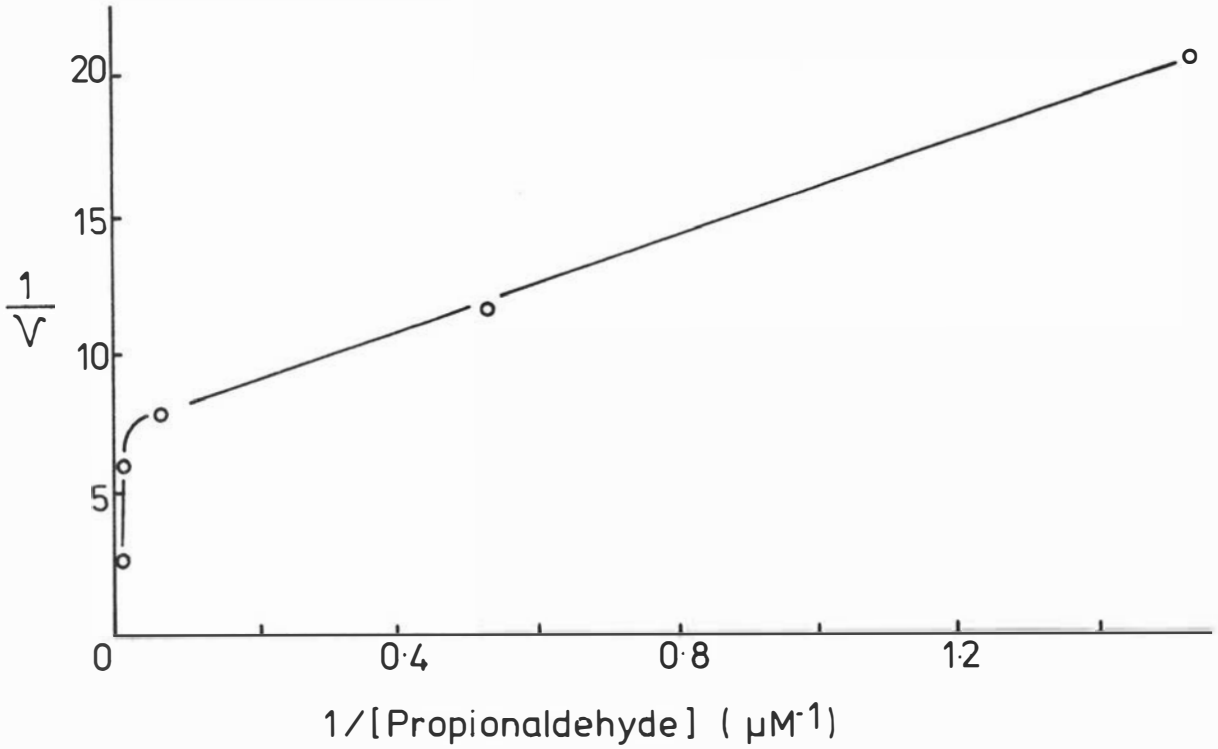
$$v = v_1 + v_2 = \frac{V_1[B]}{K_m^1 + [B]} + \frac{V_2[B]}{K_m^2 + [B]}$$

The estimated parameters (especially K_m^2) could be refined by comparison of the results with the experimental data.

For propionaldehyde it was found that the experimental data could be fitted to a two enzyme system, enzyme (1) with a K_m of 1.1μM and V_1 of 1.17 nmoles NADH per minute and enzyme (2) with a K_m of 3.5mM (3000 times K_m^1) and a maximum velocity of 3.21 nmoles NADH produced per minute (Fig. 4.11). However, in attempting to fit the acetaldehyde experimental data to two enzymes (K_m^1 = 0.67μM, K_m^2 = 2mM, V_1 = .22 nmol.min^{-1}, V_2 = 3.6 nmol.min^{-1}), it was found that two enzymes acting on acetaldehyde would give a much more abrupt transition between the two phases as the values for K_m and V are so different (see Fig. 4.11). Of course, there could be the possibility that there are more than two enzymes acting on acetaldehyde but this would require that the additional enzymes do not act on propionaldehyde or at least act with the same constants as previous enzymes. The double reciprocal plots for NAD^+ show no activation at high concentration and only one apparent value for K_m for NAD^+. The NADH titration of cytoplasmic aldehyde dehydrogenase demonstrates the equivalence of the NADH binding sites at the concentrations used and so it appears that there is no change in the coenzyme parameters.

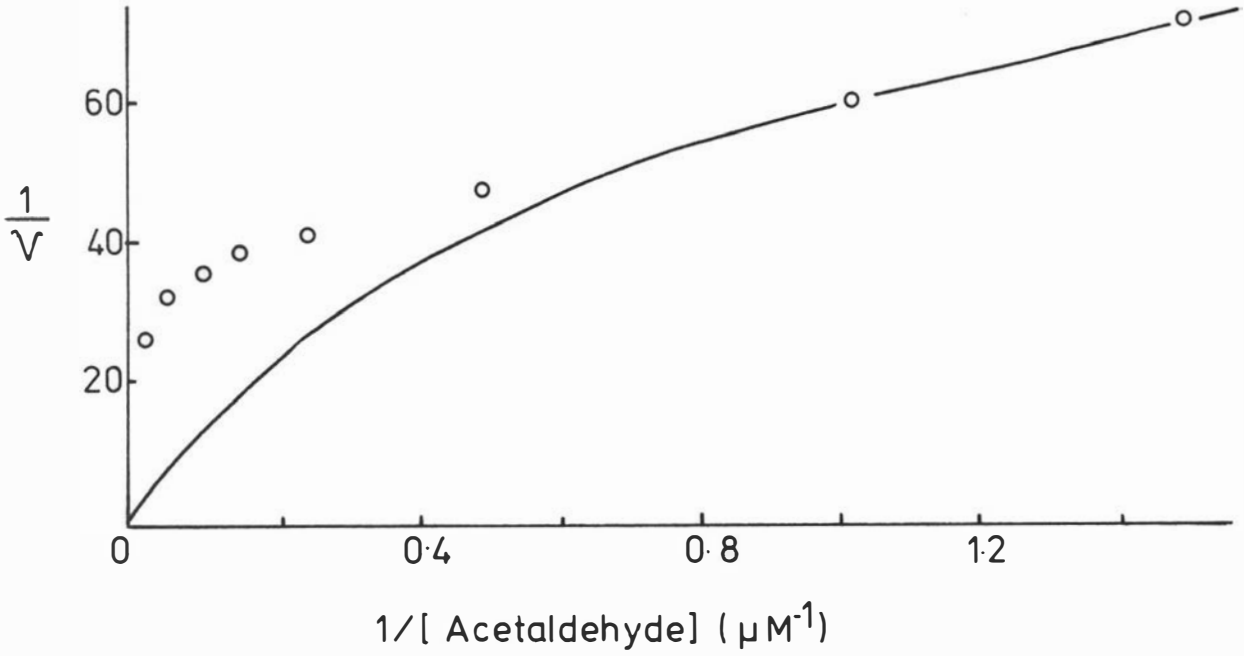
FIG. 4.11 ATTEMPT TO FIT THE BIPHASIC DOUBLE RECIPROCAL PLOTS FOR ALDEHYDES TO A TWO ENZYME SYSTEM

(a) PROPIONALDEHYDE



Fitting of data to a two enzyme system in which enzyme (1) has parameters K_m = 1.1 μM and V_1 = 1.17 nmoles NADH/min while for enzyme (2) K_m = 3.5 mM and V_2 = 3.21 nmoles NADH/min. The circles represent calculated points using the parameters above while the solid line is the experimental data of Fig. 4.9(a).

(b) ACETALDEHYDE



Circles represent calculated points while the solid line represents experimental data of Fig. 4.9(b).

Enzyme (1) (K_m = 0.67 μM, V_1 = 0.22 nmol. min^{-1})

Enzyme (2) (K_m = 2 mM, V_2 = 3.6 nmol. min^{-1})

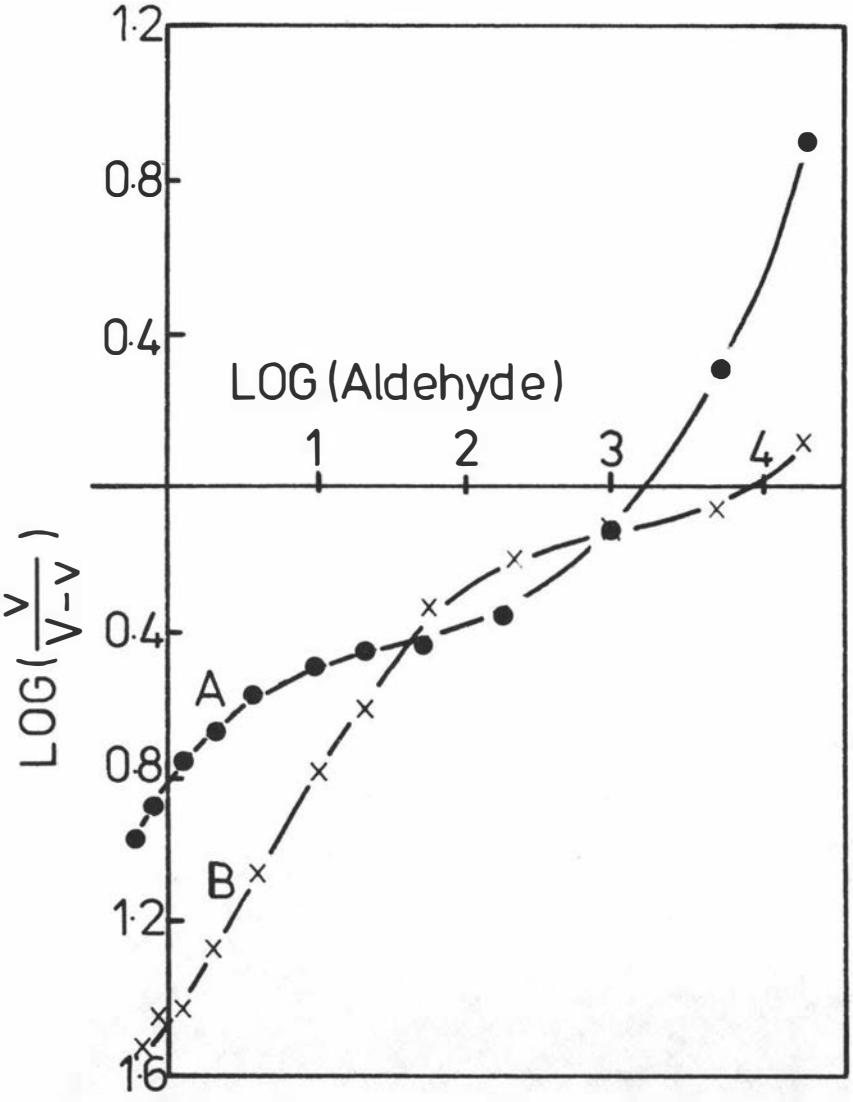
In the purification of cytoplasmic sheep liver aldehyde dehydrogenase only one activity band was observed on polyacrylamide gels and only one band, with an isoelectric point at pH 5.25, was found when the enzyme was added to a glass column isoelectric focusing apparatus in the pH range 4.5 to 5.5 (Crow, 1975). The reports of multiple enzyme forms in preparations of aldehyde dehydrogenase from different sources has been discussed previously (Section 2), however the majority were separated by column chromatography during the general enzyme purification. Only Feldman and Weiner (1972a) in isolating the enzyme from horse liver have separated isozymes from a single chromatographic peak. The separation was affected by isoelectric focusing, but the kinetic properties of the isozymes were found to be identical (Weiner et al., 1974). These latter workers found that isoelectric focusing on polyacrylamide gels on the crude enzyme samples during the purification showed five cytoplasmic and two mitochondrial isozymes in all, and of the cytoplasmic forms all stained heavily in an activity stain with acetaldehyde and p-nitrobenzaldehyde but only one stained heavily for propionaldehyde and butyraldehyde. In rat liver, the same workers found at least 17 forms of aldehyde dehydrogenase in the cytosol and at least 3 forms in the mitochondria. These latter results contradict Tottmar et al. (1974) who could find no enzyme activity present in the rat liver cytosol. Though the reports of aldehyde dehydrogenase isozymes are confusing, there does appear to be several forms found for most species, three forms are known for the sheep liver enzyme, but no evidence has been found for multiple enzyme forms in the purified samples of cytoplasmic sheep liver aldehyde dehydrogenase though the possible existence of such forms should not be overlooked.

Another possibility for the observed plots is interaction between subunits of the enzyme molecule, such that the active sites are no longer independent. Though the Hill plot was derived for the fractional saturation of binding sites, the use of kinetic data in terms of $\frac{V}{V}$ has also been applied to the plot. A graph of $\frac{V}{V-v}$ against log $[B]$ is linear with a slope of 1 only when the active sites are independent.

Cornish-Bowden and Koshland Jr. (1975) exhibited the shapes of curves for various forms of subunit interaction where a slope of greater than, or less than one indicates positive or negative cooperativity respectively. If the propionaldehyde or acetaldehyde data is plotted as a Hill plot the shape of the graph is curved but the slope is always less than 1 (Fig. 4.12) indicating that if a subunit effect is occurring it is a negative cooperative effect, in which the subsequent substrates are bound less tightly and hence the substrate concentration range over which the enzyme would operate is greatly increased. Cooperative effects have been found by Sheppard et al. (1970) for mice liver aldehyde dehydrogenase in which one strain has a positive cooperative effect (n = 2) while the other has no cooperative effect with respect to acetaldehyde. The fact that for the sheep liver enzyme at $\frac{v}{V - v}$ equal to 1 (that is when $v = \frac{V}{2}$) the slope of the Hill plot is close to 1 in the case of both aldehyde substrates demonstrates that the effect is not symmetrical and hence there are either more than two sites interacting (for example, a four site system in which only the binding of the second substrate is negatively cooperative) or there is no cooperativity at all and the observed effect is due to some other factor.

Another interesting possibility is that the hydrated form of the aldehyde may be producing an effect on the enzyme reaction at high total aldehyde concentrations by inhibition. It has been shown that where an inhibitor is in constant proportion to the variable substrate, as is the case of hydrated aldehyde and the free aldehyde substrate, then downward deviation of double reciprocal plots occurs at high substrate concentration when the inhibition is hyperbolic competitive inhibition (Cleland et al., 1973). However, hyperbolic inhibition would involve the hydrated aldehyde being able to bind to a complex, which seems unlikely. Also benzaldehyde, a compound which is virtually all in the free aldehyde form, should then have no inhibition and hence have a higher apparent V, where in fact specificity studies show that the catalytic-centre activity is much less than some significantly hydrated aldehydes, such as propionaldehyde. This possibility can, therefore, be discounted.

FIG. 4.12 HILL PLOT OF PROIONALDEHYDE AND ACETALDEHYDE DATA OF FIG. 4.9



Curve A is data from reaction involving propionaldehyde while curve B is for acetaldehyde.

The rapid equilibrium assumption was applied to the initial rate experiments since equations for the steady state random mechanism are not available. However Segel (1975) has shown that random Bi Bi systems which are not at equilibrium and in which one route to the ternary complex is significantly more favourable than the other, give rise to a variety of nonhyperbolic velocity curves. Under certain conditions a double reciprocal plot may curve downwards as it approaches the $\frac{1}{v}$ axis, to resemble the experimental data at high aldehyde concentrations. The aldehyde substrate may therefore have the ability to bind to the free enzyme at high aldehyde concentrations producing a significant second pathway in the reaction scheme which is inoperative or insignificant at lower concentrations. It is interesting that though Feldman and Weiner (1972b) have postulated an ordered Bi Bi mechanism for the reaction of their homogeneous horse liver aldehyde dehydrogenase they found that high concentrations of propionaldehyde (20mM) inhibited the esterase activity. This must mean that at that concentration propionaldehyde was binding to the free enzyme, as no coenzyme was present, and perhaps suggests that at high aldehyde concentrations a random mechanism is possible for the horse liver enzyme.

Though several different explanations have been proposed for the apparent substrate activation, they are similar in that they all involve binding sites for which the aldehyde has a low affinity. The difference is a matter of whether these sites are on a different enzyme, the same enzyme, or the same location on the enzyme, as the site with a high aldehyde affinity. As to which explanation is the most likely, it would seem that the operation of a partially random mechanism at high aldehyde concentrations would be the most probable but the possibility of multiple isozymes cannot be discounted. However, though propionaldehyde does produce biphasic plots, because the transition is so abrupt the two portions are virtually independent and especially in the lower concentration region kinetic studies may be carried out without worry of interference from the other region and hence an understanding of the cause of the biphasic plot is not essential if the substrate concentrations are low.

SECTION 5

PRESTEADY-STATE KINETICS

5.1 INTRODUCTION

In the steady-state experiments the overall rate of reaction is studied and information is inferred indirectly about the enzyme intermediates which are involved. However, if the reaction is followed during the short period of the first turnover of the enzyme-substrate reaction, before the reaction has reached a steady-state (that is presteady-state), then more detailed information about the enzyme mechanism may be obtained. The advantage of a presteady-state kinetic study lies in the fact that it is often possible to study steps other than the rate determining step. If the enzyme is in sufficiently high concentration, the reaction intermediates may be observable under suitable experimental conditions and direct measurement of their rates of formation and decomposition may be made. An instrument which may be used for the presteady-state kinetic study is the stopped-flow spectrophotometer first described by Gibson and Milnes (1964). The reaction is initiated within times as short as 1-2 milliseconds by forced mixing of the reactants, after which changes in the physical properties of the solution (such as absorbance or fluorescence) may be monitored. The technique and instrumentation have been reviewed by Gibson (1969).

For the presteady-state phase of an enzyme catalysed reaction (which is sometimes described as the burst) the general equations describing the whole time course of the transient appearance of product (enzyme bound and free) are complex and cannot be easily used for the evaluation of data (for instance see Darvey, 1968). Since the number of exponential terms in the transient phase is equal to the number of enzyme containing intermediates in the mechanism, not counting the free enzyme (Maguire *et al.*, 1974), the observed behaviour can in principle be very complicated. However, a number of factors may simplify the actual situation. No information may be gained about steps occurring

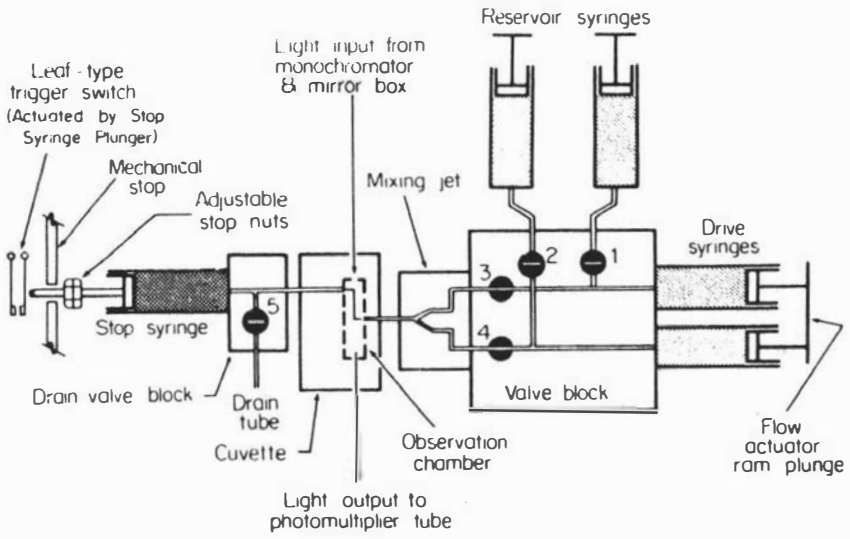
after the rate determining step in the steady-state and nothing will be observed until the step with the first physical change occurs, although the preceding steps may affect this change. Also the amplitude of the exponential terms will finally determine whether steps will be observed or not. So while theoretically the analysis of the pre-steady-state phase may be rather complex, the actual presteady-state phase produced may be much more simple. Laidler and Bunting (1973) have described the derivation of equations describing the presteady-state phase and the approximations involved to produce terms which may be usefully applied to experimental data.

Several dehydrogenases have been extensively studied by stopped-flow techniques. Iwatsubo and Pantaloni (1967) carried out the first rapid kinetic measurements on L-glutamate dehydrogenase, which showed a rapid presteady-state phase, with a rate constant of $250s^{-1}$, corresponding to the reduction of the coenzyme bound to the enzyme followed by a slow steady-state phase which was similar in its rate to the one measured under classical steady-state conditions. Heck *et al.* (1968) using stopped-flow methods found that for lactate dehydrogenase at pH 8.0 the reduction of the first mole equivalent of NAD^+ per mole of enzyme sites is much more rapid than the steady-state rate of NADH production and that dissociation of the enzyme-NADH complex is rate-determining for the steady-state oxidation of lactate. Probably the most intensively investigated dehydrogenase has been alcohol dehydrogenase. Theorell *et al.* (1967) first studied this enzyme by stopped-flow methods using fluorescence enhancement of NADH on binding to follow the association of NADH with the enzyme. Although binding of NADH was postulated as a two step process this was later refuted (Geraci and Gibson, 1967). Study of the transient kinetics of alcohol dehydrogenase using chromophoric aldehydes (Bernhard *et al.*, 1970) showed an initial burst due to oxidation of NADH and reduction of the aldehyde. When the reaction was followed in the opposite direction (Brooks and Shore, 1971) an initial burst was again observed and by demonstrating an isotope effect of 6.2 between ethanol

and deuterated ethanol it was possible to relate the observed burst rate constant to the actual hydride transfer step.

To try and determine more about the mechanism of action of aldehyde dehydrogenase and to compare the mechanism and rates of reaction with that of other dehydrogenases, it was decided to study the enzyme under presteady-state conditions.

FIG. 5.1 STOPPED-FLOW APPARATUS



Schematic representation of the stopped-flow apparatus (from Gutfreund (1972)). In the fluorescence mode the light output to the photomultiplier tube is at right angles to the light source.

Consecutive Reactions

The solution to a reaction scheme consisting of two consecutive reversible first-order reactions has been discussed in detail by Frost and Pearson (1961).

$$E_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_3 \qquad \text{(mechanism (1))}$$

The three solutions for the decay constant λ are

$$\lambda_1 = 0$$

$$\lambda_2 = \tfrac{1}{2}(p + q) \qquad (1)$$

$$\lambda_3 = \tfrac{1}{2}(p - q) \qquad (2)$$

where $p = (k_1 + k_{-1} + k_2 + k_{-2})$

and $q = (p^2 - 4(k_1 \cdot k_2 + k_{-1} \cdot k_{-2} + k_1 \cdot k_{-2}))^{\frac{1}{2}}$

It will be noted that $\lambda_2 + \lambda_3 = p = k_1 + k_{-1} + k_2 + k_{-2}$ (3)

$$\lambda_2 \cdot \lambda_3 = k_1 \cdot k_2 + k_{-1} \cdot k_{-2} + k_1 \cdot k_{-2} \qquad (4)$$

If at time zero all the molecules are in the form E_1, that is $E_1 = E_1^\circ$ and $E_2 = E_3 = 0$ at time zero, then the concentrations of E_1, E_2 and E_3 are given by the expressions:

$$E_1 = E_1^\circ \left(\frac{k_1 k_{-2}}{\lambda_2 \lambda_3} + \frac{k_1(\lambda_2 - k_2 - k_{-2})}{\lambda_2(\lambda_2 - \lambda_3)} \cdot e^{-\lambda_2 t} + \frac{k_1(k_2 + k_{-2} - \lambda_3)}{\lambda_3(\lambda_2 - \lambda_3)} \cdot e^{-\lambda_3 t}\right) \qquad (5)$$

$$E_2 = E_1^\circ \left(\frac{k_1 k_{-2}}{\lambda_2 \lambda_3} + \frac{k_1(k_{-2} - \lambda_2)}{\lambda_2(\lambda_2 - \lambda_3)} \cdot e^{-\lambda_2 t} + \frac{k_1(\lambda_3 - k_{-2})}{\lambda_3(\lambda_2 - \lambda_3)} \cdot e^{-\lambda_3 t}\right) \qquad (6)$$

$$E_3 = E_1^\circ \left(\frac{k_1 k_2}{\lambda_2 \lambda_3} + \frac{k_1 k_2}{\lambda_2(\lambda_2 - \lambda_3)} \cdot e^{-\lambda_2 t} - \frac{k_1 k_2}{\lambda_3(\lambda_2 - \lambda_3)} \cdot e^{-\lambda_3 t}\right) \qquad (7)$$

These general equations have been utilized for various situations in the fast reaction experiments;

(1) In the biphasic NADH displacement experiments E_3 is the free enzyme and E_1 and E_2 are enzyme-coenzyme complexes, all of which are present at time zero. Since the NADH

displacement is irreversible under the experimental conditions, k_{-2} is equal to zero.

(2) In the NADH association experiments k_{-2} is a pseudo first-order rate constant dependent on NADH concentration and at time zero all the enzyme is in the form E_3 (though equations (5), (6) and (7) are all in terms of E_1° the symmetry of mechanism (1) allows these equations to be expressed in terms of E_3° if required).

(3) In the burst experiments the proposed mechanism

$$E \underset{k_{-1}}{\overset{k_1[NAD^+]}{\rightleftharpoons}} E^{NAD^+} \underset{k_{-2}}{\overset{k_2[Ald]}{\rightleftharpoons}} E^{NAD^+}_{Ald} \xrightarrow{k_3} E^{NADH} \xrightarrow[\text{slow displacement}]{k_4} E$$

... (scheme 1)

in which the enzyme is preincubated with excess NAD^+ and hence all in the E^{NAD^+} form and as the step k_4 is small may be ignored. Scheme (1) under these conditions is the same as mechanism (1) with k_{-2} (of mechanism (1)) equal to zero. At time zero all the enzyme is in the form E_1 and hence the equations for the concentrations of E_1, E_2, and E_3 at any time t, (equations (5), (6) and (7)) may be used.

5.2 METHODS

5.2.1 Stopped-flow Apparatus

Stopped-flow experiments were carried out using a Durrum-Gibson D110 Stopped-Flow Spectrophotometer (Durrum Instrument Corp., Palo Alto, California, U.S.A.) and a Hewlett-Packard model 141B storage oscilloscope. Fluorescence measurements were taken with the photomultiplier at a 90° angle to the incident light with the apparatus set in the transmission mode (Fig. 5.1). The stopped-flow spectrophotometer allows rapid mixing of reactant solutions. The reactants are placed in separate syringes and an air-actuated plunger pushes the two solutions through a mixing jet into the observation chamber. When the stopping syringe hits the trigger, the flow stops and the oscilloscope is activated allowing changes in the observation chamber to be monitored. The mixing time is about 2 milliseconds and $0.15 cm^3$ of each reactant solution is used in each run. The length of the

observation chamber is 1.7cm. The trace produced on the storage oscilloscope was photographed using a single reflex Asahi Pentax SP500 camera with FP4 panchromatic film. After developing, the negatives were projected onto graph paper, by use of an enlarger, such that the grids on each matched. The curves were then traced to provide a record on graph paper from which measurements could be made.

For nucleotide fluorescence measurements the excitation monochromator was set on 340nm, with a tungsten light source and a 9nm bond width, and the fluorescence was observed, at 90° to the exciting light, through Wratten 47B and 2B filters sandwiched between glass discs made from emission spectrograph plates. These filters together have a maximum transmission at 435nm.

Protein fluorescence was studied by exciting the enzyme solution at 290nm and measuring the fluorescence at 90° transmitted through a Wratten 18A filter (maximum transmission at 335nm).

5.2.2. Standardization of Fluorescence Signal

As fluorescence has no inherent calibration system, before the beginning of an experiment a known amount of NADH was added and the increase in fluorescence was noted. The procedure was as follows: the photomultiplier voltage was set to 950V and the oscilloscope was zeroed with no input signal, then phosphate buffer was forced into the observation chamber and the oscilloscope was rezeroed on this signal using the stopped-flow offset control. A standard NADH solution (in the same buffer) was added to one syringe, and mixed with buffer from the other syringe in the stopped flow to produce a fluorescence change. Experimental fluorescence changes could then be related to this standard. It was found convenient to use NADH in preweighed (.2mg) vials obtained from Sigma, and made up to give a final concentration of about 5μM.

5.2. Buffers and Reagents

The standard buffer used was the pH 7.6 phosphate buffer used in the steady-state experiments (Section 4). By diluting the stock buffer ($50cm^3$) with water ($20cm^3$) the same

concentration as used in the steady-state assays was obtained. Before use the buffer solutions were degassed, by attachment to a water pump for 20 minutes. This procedure was necessary to eliminate air bubbles since it was found that their presence in the observation chamber of the stopped-flow instrument caused fluctuations and base line drift in the oscilloscope trace. All reagents used were made up in the degassed buffer solution. The concentrations of all reagents are quoted as the concentration in the observation chamber after mixing unless otherwise stated (that is one half the concentration of the reagents in the syringes). The effect of pH on the displacement of NADH was studied with pyrophosphate buffers (0.05M in pyrophosphate in the stopped-flow) which were brought to the required pH with concentrated hydrochloric acid. NAD^+ (Sigma Grade III) was used without purification unless otherwise stated. [1 - ^{2}H] propionaldehyde (CH_3CH_2CDO) was prepared by the Nef reaction (Leitch, 1955) on [1,1 - ^{2}H] nitropropane.

5.2.4 NAD^+ Purification

The purification procedure follows that of Whitaker *et al.* (1974). NAD^+ (0.25g), Sigma Grade III, was dissolved in water ($3cm^3$) and neutralized to pH 6.4 with sodium carbonate (2M). This solution was added to a DEAE Sephadex A-25-120 column (2cm x 10cm), the column washed thoroughly with water and then the NAD^+ was eluted from the column with 0.1M HCl. Monitoring of the absorbance of the eluant at 260nm, with a continuous flow Uvicord U.V. monitor, showed a large band followed by two smaller bands. The peak fractions of the large band were bulked and neutralized to pH 6.5 with sodium carbonate (2M). The concentration of NAD^+, assuming an extinction coefficient of 1.8×10^4 M^{-1} cm^{-1} was about 30mM for the peak fraction. On dilution of the bulked solutions to obtain the required NAD^+ concentration the ionic strength and buffering capacity of the sodium carbonate became small. The solutions were used either on the day of preparation or the following day.

5.2.5 Esterase Activity

The rate of hydrolysis of p-nitrophenyl acetate by aldehyde dehydrogenase was determined in the stopped-flow by following the increase in absorbance at 400nm, due to the production of the p-nitrophenolate ion ($\varepsilon = 16 \times 10^3$ $M^{-1}cm^{-1}$, Feldman and Weiner, 1972b). The p-nitrophenyl acetate was dissolved in acetone and then diluted with pH 7.6 buffer to a concentration of 50μM when mixed in the stopped-flow (final acetone concentration, 0.8% v/v) with aldehyde dehydrogenase solution (4.2μM mixed) containing NADH (9.6μM), which is reported to stimulate the esterase activity (Feldman and Weiner, 1972b). The enzyme solution contained 2-mercaptoethanol (280μM), present in buffers throughout the purification scheme, which may react with p-nitrophenyl acetate. The contribution of the nonenzymic p-nitrophenyl acetate hydrolysis to the observed absorbance change for the enzyme solution was measured by running a blank, which contained 2-mercaptoethanol (280μM) but no enzyme.

5.2.6 Enzyme Concentration

The enzyme concentration was always expressed in terms of the active site concentration determined from titration of the enzyme with NADH (Section 3).

5.2.7 Displacement Experiments

When E-NADH complex in one syringe is mixed with X, a ligand capable of binding to the same enzyme site as NADH, from another syringe then some NADH will be displaced from the enzyme.

$$E - NADH \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} E + NADH \qquad \text{Mechanism (2)}$$

$$E + X \xrightarrow{k_X} EX$$

If the concentrations of X are such that $k_X[X]$ is much greater than k_{off} and also much greater than $k_{on}[NADH]$ then NADH will be completely displaced and the fluorescence change produced by the conversion of the E - NADH complex to the less fluorescent free NADH species may be interpreted

in terms of the rate constant k_{off}. To ensure that the conditions requiring $k_X[X]$ to be large are upheld, the experiment was conducted at various concentrations of X, all of which should proceed at the same rate.

5.2.8 Treatment of Displacement Data

After the displacement reaction was recorded on the oscilloscope, the instrument was retriggered to obtain the infinity reading of the fluorescence at the end of the displacement. From the record produced on graph paper of the oscilloscope trace, the difference between the voltage at each time interval and the voltage at the end of the displacement (infinity reading) was measured. It was usually more convenient to measure the difference with a ruler graduated in millimetres as no subtractions were then necessary allowing the values to be plotted directly. The value in volts could be calculated by simple conversion of grid sizes if required.

For a simple first order process of A producing B and C

$$A \xrightarrow{k} B + C \qquad \text{Mechanism (3)}$$

then $$-\frac{d[A]}{dt} = k[A]$$

$$\frac{d[A]}{[A]} = -k.dt$$

and on integration $\ln[A] = -kt + c$

with the initial conditions that $[A] = [A]_o$ at time $t = 0$

then $$\ln \frac{[A]_o}{[A]} = kt$$

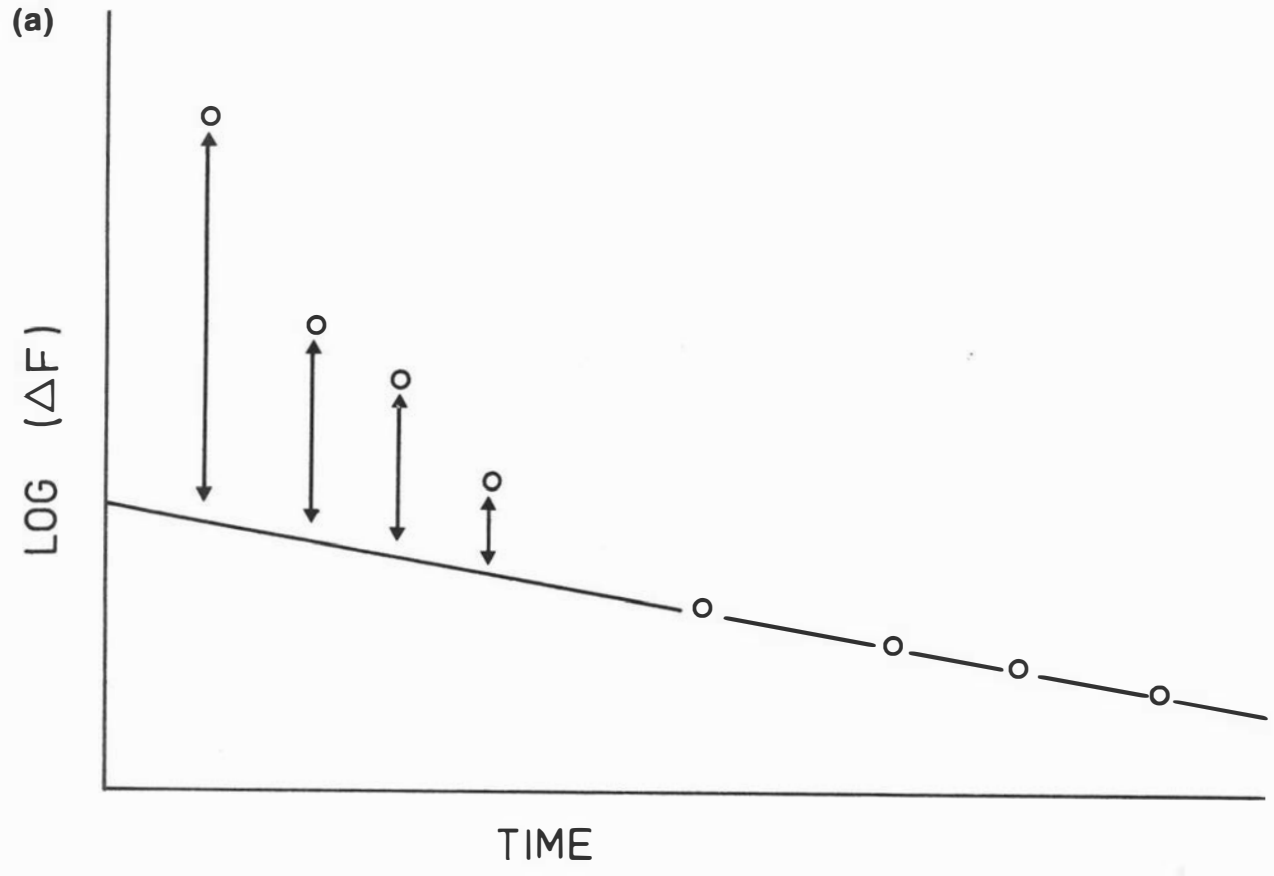
$$\ln [A] = -kt + \ln[A]_o$$

or $$\log [A] = \left(\frac{-k}{2.303}\right)t + \log [A]_o \qquad (8)$$

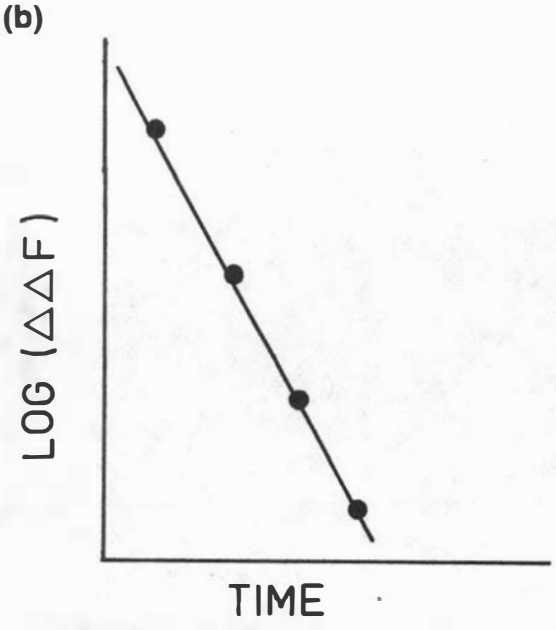
From equation (8) a plot of $\log [A]$ vs t will give a straight line of slope $-k/2.303$ from which the first order rate constant k may be obtained.

However, in all cases biphasic plots were observed for the displacement of NADH and the treatment of the data had to be adapted in the following way. The data was plotted

FIG. 5.2 GRAPHICAL DERIVATION OF RATE CONSTANTS FROM A BIPHASIC PLOT



If the logarithm of the difference between the fluorescence at time t and the fluorescence at time infinity (that is ΔF) is plotted against time t, then the rate constant of the slow process may be obtained from the slope of the extrapolated line (rate constant = -2.303 x slope).



By plotting the logarithm of the actual difference between the experimental ΔF values and the extrapolated values of ΔF (that is $\Delta\Delta F$) against time, then from the slope of such a plot the rate constant for the fast process may be obtained. The amplitudes of the fast and slow processes are the extrapolated values for $\Delta\Delta F$ and ΔF respectively, at time zero.

as for a single first order process, and a straight line drawn through the later points, to obtain the slow rate, was extrapolated back to time zero. The difference between this extrapolated line and the actual experimental points was then replotted at each time interval. The rate constant for the fast process was derived from the slope of this line (Blackwell et al., 1973). This process is only useful if there is a large enough linear portion to extrapolate accurately, a condition which will not be met if the rate constants of the two processes are similar (Fig. 5.2).

5.2.9 Treatment of Burst Data

To obtain the burst rate constant from the pre-steady-state traces the following procedure was used. The steady-state rate was extrapolated back to zero time and the differences between the fluorescence at any time and the extrapolated fluorescence value at that same time was recorded. From a plot of the logarithm of this difference against time the burst rate constant could be determined (Laidler, 1973).

Derivation of Dependence of k_{obs} on Aldehyde Concentration

When NAD^+ is premixed with enzyme before reaction with an aldehyde then the sequence involves only a single substrate addition and may be represented as

$$EA \underset{k_{-2}}{\overset{k_2[B]}{\rightleftharpoons}} EAB \xrightarrow{k_3} EQ \qquad \text{Mechanism (4)}$$

where A, B, Q are NAD^+, aldehyde and NADH respectively and where the rate of dissociation of NADH is very slow and may be ignored (Shore and Gutfreund, 1970). Since the aldehyde concentration is much greater than $[E - NAD^+]$ then the aldehyde addition becomes pseudo-first-order. Assuming the fluorescence increase is observed in the step with the rate constant k_3 and the concentration of EAB is low then

$$\frac{d[EAB]}{dt} = 0 = k_2[B][EA] - (k_3 + k_{-2})[EAB]$$

$$[EAB] = \frac{k_2[B][EA]}{(k_3 + k_{-2})}$$

But the total enzyme concentration is equal to the sum of the enzyme species

$$/E/_o = /EA/ + /EAB/ + /EQ/$$

$$= /EAB/ \left(1 + \frac{(k_3 + k_{-2})}{k_2/B/}\right) + /EQ/$$

$$\frac{d/EQ/}{dt} = k_3/EAB/ = \frac{k_2k_3/B/(/E/_o - /EQ/)}{k_2/B/ + (k_3 + k_{-2})}$$

$$\frac{d/EQ/}{/E/_o - /EQ/} = \frac{k_2k_3/B/}{k_2/B/ + (k_3 + k_{-2})} \cdot dt$$

On Integration

$$\ln (/E/_o - /EQ/) = \frac{k_2k_3/B/}{k_2/B/ + k_3 + k_{-2}} \cdot t + \ln /E/_o$$

where the observed first order rate constant for the production of EQ is

$$k_{obs} = \frac{k_2k_3/B/}{k_2/B/ + k_3 + k_{-2}}$$

In reciprocal form

$$\frac{1}{k_{obs}} = \frac{1}{k_3} + \frac{(k_3 + k_{-2})}{k_2k_3/B/} \qquad (9)$$

This equation indicates that the intercept of a double reciprocal plot should provide $\frac{1}{k_3}$ and the concentration of B which produces half the maximum burst rate is

$$\frac{(k_3 + k_{-2})}{k_2}$$

that is when $\frac{1}{k_{obs}} = 0$, $\quad -/B/ = \frac{k_3 + k_{-2}}{k_2}$ (10)

It is interesting to note that study of the solutions of the consecutive first-order reactions (mechanism (1), equations (3) and (4)) shows that using the nomenclature of mechanism (3)

$$\lambda_2 + \lambda_3 = k_2/B/ + k_{-2} + k_3$$

$$\lambda_2 \cdot \lambda_3 = k_2 \cdot k_3 \cdot /B/$$

On substitution for λ_2 (which is assumed fast and of small amplitude)

$$\frac{1}{\lambda_3} = \frac{1}{k_3} + \frac{(k_3 + k_{-2} - \lambda 3)}{k_2k_3/B/} \qquad (11)$$

which resembles the equation (9) derived from the steady-state treatment and produces a double reciprocal relationship as long as $k_3 + k_{-2}$ is much greater than λ_3.

Burst Amplitude

According to Laidler (1973) the burst amplitude of scheme (1), where k_4 is an irreversible NADH displacement step, at zero free NADH concentration is given by the following equation (nomenclature of scheme (1))

$$\text{Burst amplitude, } B_a = [E]_o \left(\frac{k_2k_3[Ald]}{k_2[Ald](k_3 + k_4) + (k_3 + k_{-2})k_4}\right)^2 \quad (12)$$

as long as the condition $k_2k_3[Ald]$ greater than $(k_4)^2$ is satisfied.

Which means that

$$\left(\frac{[E]_o}{B_a}\right)^{\frac{1}{2}} = \frac{(k_3 + k_4)}{k_3} + \frac{(k_3 + k_{-2})k_4}{k_2k_3[Ald]} \quad (13)$$

So a plot of $\left(\frac{1}{B_a}\right)^{\frac{1}{2}}$ against $\frac{1}{[Ald]}$ should have a value for (slope/intercept) equal to

$$\frac{(k_3 + k_{-2})k_4}{k_2(k_3 + k_4)}$$

which is the expression for the Michalis constant for the aldehyde for the mechanism in scheme (1) (see discussion).

5.2.10 Computer Simulations

Computer simulations were carried out using the "Continuous System Modelling Program" (CSMP73) designed to facilitate the digital simulation of continuous processes on the Burroughs B6700 computer. The modelling programme accepts as input a list of differential equations defining the model, parameter definitions and control statements which it translates into a FORTRAN programme. This programme solves the differential equations as a function of time at specified integration intervals (see discussion). The burst was simulated using scheme (1) as the model. The programme produced concentrations of all intermediates, products, substrates and in addition a term consisting of the sum of 5.6 times the concentration of E^{NADH} plus the concentration of NADH, that is an approximation to the observed burst

FIG. 5.3 OBSERVATION OF BIPHASIC DISPLACEMENT FROM DEHYDROGENASES BY NUCLEOTIDE FLUORESCENCE AND ABSORBANCE

(a) ALDEHYDE DEHYDROGENASE

Photograph A
Nucleotide fluorescence

The reaction mixture contained enzyme (0.25mg/cm^3) and NADH (4.2μM) mixed with NAD^+ (4.3mM). Vertical scale, 0.2V/div; horizontal scale, 2 s/div.

Photograph B
Absorbance at 340nm

The reaction mixture contained enzyme (5μM) and NADH (3.4μM) mixed with NAD^+ (10mM). Vertical scale, 0.001 absorbancy/div; horizontal scale, 1 s/div.

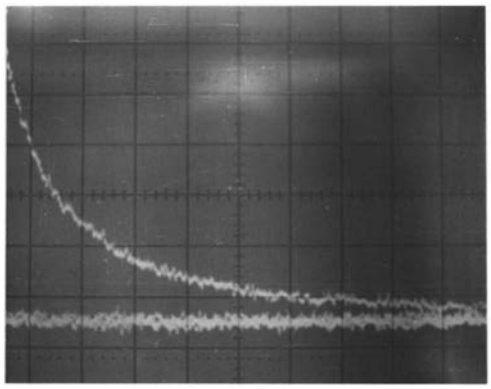
(b) ALCOHOL DEHYDROGENASE

Photograph C
Nucleotide fluorescence
Vertical scale, 0.5V/div; horizontal scale, 0.1 s/div.

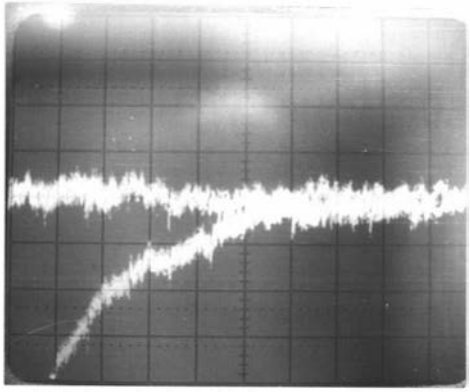
Photograph D
Absorbance at 340nm
Vertical scale, 0.005 absorbancy/div; horizontal scale, 0.05 s/div.

The reaction mixture, in photographs C and D, contained alcohol dehydrogenase (5μM) and NADH (5μM) mixed with NAD^+ (3.3mM) and pyrazole (5mM).

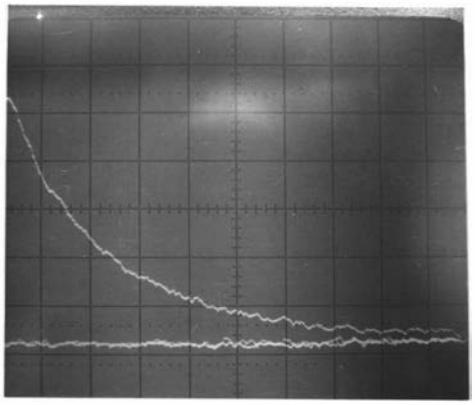
A



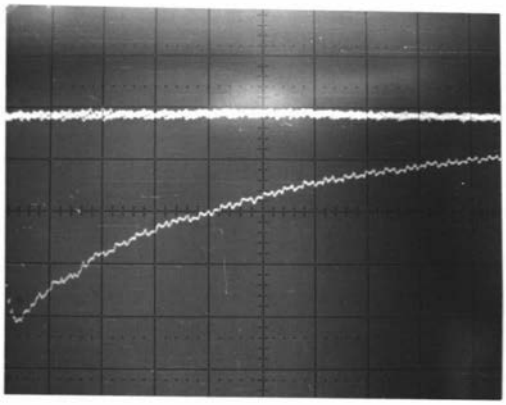
B



C



D



amplitude. The simulation was run long enough to obtain a maximum value for the concentration of the intermediate E^{NADH} and the burst rate constant was then obtained manually using the same graphical technique used for the experimental data.

5.3 RESULTS

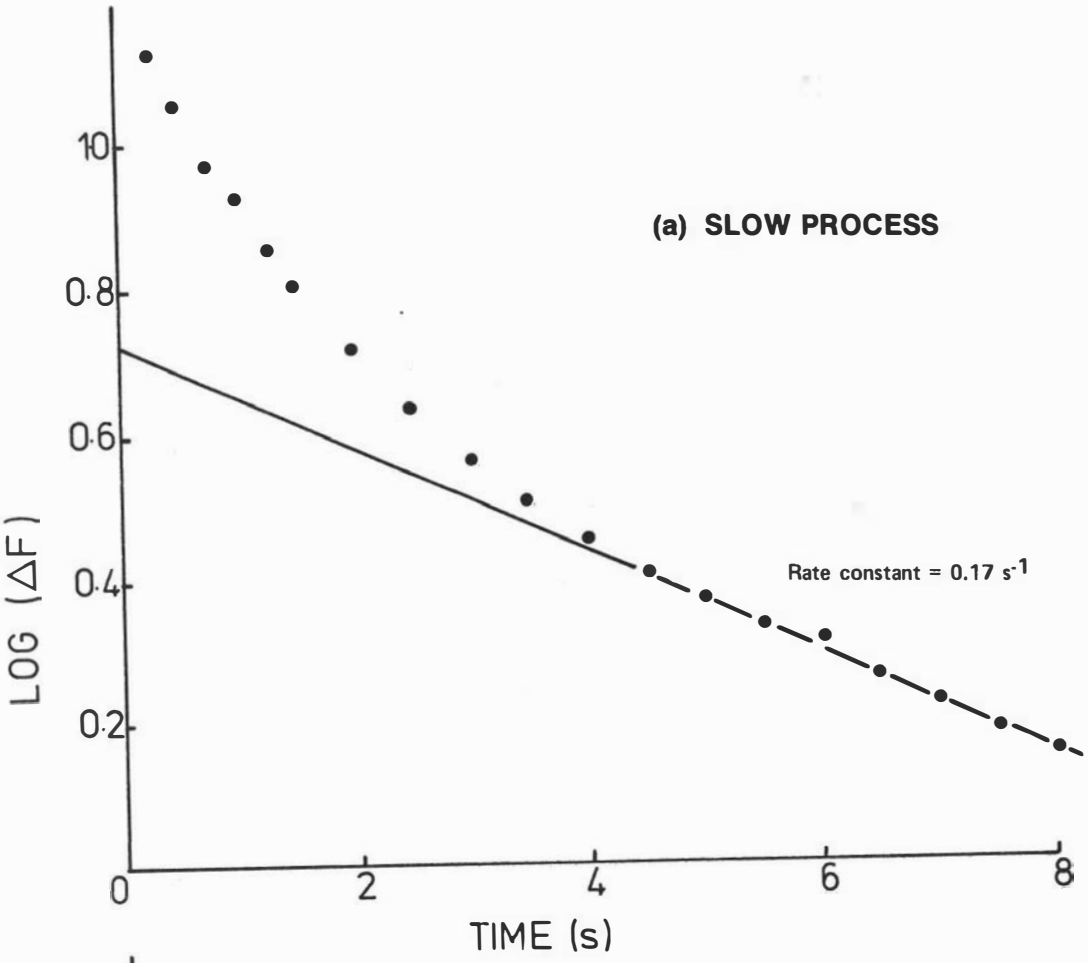
5.3.1 Displacement Experiments

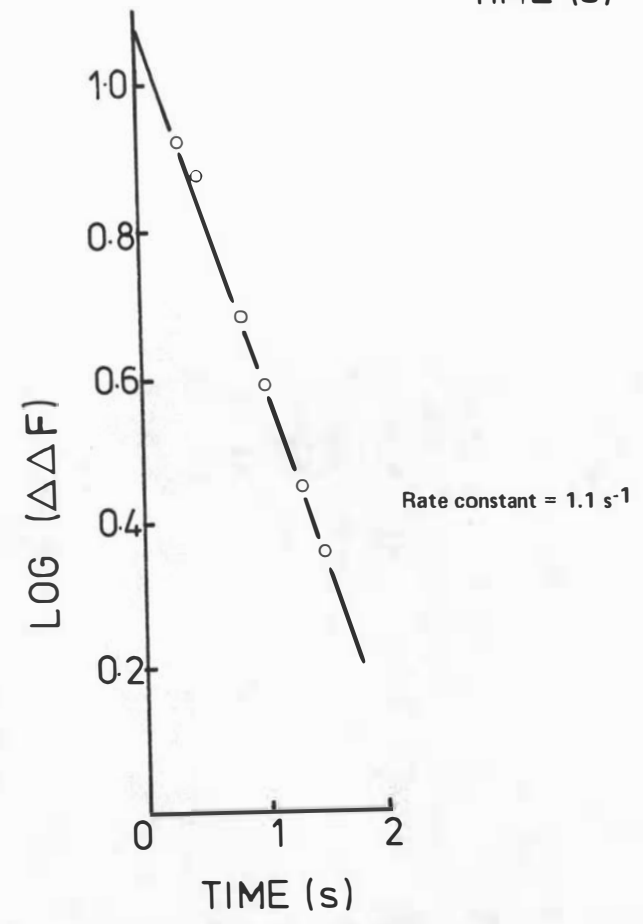
5.3.1.1 Displacement of NADH

Displacement of NADH from aldehyde dehydrogenase with excess NAD^+ gave biphasic fluorescence plots, a fast change followed by a slower change for the conversion of the highly fluorescent enzyme-NADH complex to the less fluorescent free NADH (Fig. 5.3). To ensure that this was not an artefact of the fluorescence measurement the displacement was repeated using the absorbance mode. It was found that there was a small absorbance difference on displacement which was also biphasic (Fig. 5.3). To check the linearity of the stopped-flow instrument, in case the biphasic nature of the plots was an instrumental feature, a study was made of the reaction between α-chymotrypsin and proflavin. This reaction has been reported by Fersht and Requena (1971) to be a single phase process. α-Chymotrypsin (10.4μM) and proflavin (110μM), both in pH 7.3 phosphate buffer (0.05M in phosphate), were mixed in the stopped-flow apparatus and the change in absorbance at 465nm was followed. The trace produced was a single exponential which gave a first order rate constant of $3.4s^{-1}$, in good agreement with the literature value. As a single exponential is produced in this case, it appears that the biphasic nature of aldehyde dehydrogenase displacement is in fact a real phenomenon.

Results from the displacement of NADH from aldehyde dehydrogenase by NAD^+, followed by fluorescence, showed a fast reaction with a rate constant of $0.85s^{-1} \pm 0.20s^{-1}$ followed by a slower reaction with a rate constant of $0.22s^{-1} \pm 0.05s^{-1}$. NAD^+ concentrations of 0.52mM to 10mM all gave results in this range and a graphical analysis is shown in Fig. 5.4 The amplitude of the fluorescence change for the fast process was 50% to 70% of the total change. Similar results were found when purified

FIG. 5.4 TYPICAL GRAPHICAL DERIVATION OF RATE CONSTANTS FROM THE DISPLACEMENT EXPERIMENT





NAD^+ was used as the displacing agent. Table 5.1 shows other displacing agents which were used to displace NADH and it can be seen that they almost all involved two processes with rate constants in the same range as those found for NAD^+. The only exception was 3-pyridinealdehyde-NAD^+ which had a very low rate constant of $0.05s^{-1}$ for the slow process.

In an attempt to observe the effect of any possible ternary complexes a mixture of enzyme (2.2μM), NAD^+ (2.8μM) and propionaldehyde (9.6mM) from one syringe was displaced with 1,10-phenanthroline (0.28mM). The resulting trace was biphasic with rate constants of $1.1s^{-1}$ and $0.3s^{-1}$, similar to the rate constants in the absence of propionaldehyde. A similar experiment using 2-bromo-2-phenylacetic acid (0.3mM), a product analogue, and again displacing with 1,10-phenanthroline (0.28mM) produced rate constants of $1.0s^{-1}$ and $0.25s^{-1}$. The fact that both propionaldehyde and 2-bromo-2-phenylacetic acid have no effect on the rate constants suggests that neither forms a significant ternary complex with the binary enzyme-NADH complex and hence ternary complex formation cannot be used to further elucidate the enzyme behaviour.

TABLE 5.1 DISPLACEMENT RATE CONSTANTS FOR THE ENZYME-NADH COMPLEX

Displacing Agent	Rate Constant for the Fast Process	Rate Constant for the Slow Process
NAD^+ (0.52mM-10mM)	$0.85s^{-1} \pm 0.20$	$0.22s^{-1} \pm 0.05$
1,10-phenathroline (0.045mM-0.71mM)	$1.0s^{-1}$	$0.25s^{-1}$
deamino-NAD^+ (.6mM-1.6mM)	$1.0s^{-1}$	$0.24s^{-1}$
ADP-ribose (.36mM-1.8mM)	$1.1s^{-1}$	$0.29s^{-1}$
3-pyridinealdehyde-NAD^+ (1.9mM)	$0.9s^{-1}$	$0.053s^{-1}$

5.3.1.2 Displacement of NADH Analogue

As no NADH analogues were available they were prepared from NAD^+ analogues. By reacting limited amounts of aldehyde and NAD^+ analogues with the enzyme, significant amounts of the enzyme-NADH analogue binary complex could be produced and subsequently displaced by NAD^+ in a typical displacement

experiment. The remaining NAD^+ analogue should be in such small amounts as to not interfere in the displacement. The scheme was tested by reacting NAD^+ (8μM) and propionaldehyde (5.4μM) with enzyme (1.7μM). After allowing the system to react for a few minutes the mixture was added to one syringe of the stopped flow instrument and the NADH formed displaced by mixing with NAD^+ (4.3mM) from the other syringe. The displacement was biphasic with a rate constant for the fast phase of $0.6s^{-1}$ and a rate constant of $0.15s^{-1}$ for the slow phase, in good agreement with the values found for NADH displacement. When the experiment was repeated using deamino-NAD^+ (8μM), propionaldehyde (5.4μM) and enzyme (1.7μM) using NAD^+ (4.3mM) as the displacing agent it was found that only a slow process with a rate constant of $0.16s^{-1}$ could be observed, even using a sweep time of 5 milliseconds per division.

5.3.1.3 Effect of pH on the Displacement of NADH

The pH dependence of the displacement was determined using a range of pyrophosphate buffers. The NAD^+ displacing agent was made up in pyrophosphate buffer and placed in one syringe while the other syringe contained $1cm^3$ enzyme and $1.5cm^3$ NADH in pH 7.6 phosphate buffer and $7.5cm^3$ of water, so that this second solution had little buffering capacity. Table 5.2 shows that a reaction mixture of NAD^+ (2.2mM), enzyme (3μM) and NADH (5μM) showed little variation in displacement rate with pH, though in pyrophosphate buffer the slow process had a smaller rate constant than in phosphate buffer.

TABLE 5.2 EFFECT OF pH ON NADH DISPLACEMENT

pH[1]	Fast Rate Constant	Slow Rate Constant
6.3	0.79	0.073
9.0	0.6, 0.64	0.053, 0.055
9.9	0.68, 0.50	0.061, 0.053
7.6 phosphate[2]	0.85 ± 0.2	0.22 ± 0.05

[1] pH value after mixing, determined by collecting the reacted mixture and measuring its pH.

[2] Standard phosphate buffer.

5.3.1.4 Limits to the Use of the Guggenheim Plot

The Guggenheim method for evaluating rate coefficients of first order reactions does not require a knowledge of the initial or final concentrations of the reacting species (Guggenheim, 1926). If time t_1, t_2, t_3 etc. and $t_1 + \Delta$, $t_2 + \Delta$, $t_3 + \Delta$ are selected where Δ is a constant increment (usually two or three times as great as the half-life period of the reaction, for accuracy) and r_i and r_i' are readings of a suitable physical property at t_i and $t_i + \Delta$, respectively, then $\log (r_i - r_i') = \text{constant} - kt_i/2.303$, and a plot of $\log (r_i - r_i')$ vs t_i gives a slope of $-k/2.303$.

The Guggenheim method was used in an attempt to overcome a problem of drifting infinities which occurred in some earlier runs. However, on analysis, the Guggenheim plots of the displacement experiment were linear. When NAD^+ displaced NADH in an experiment with a stable infinity value, the trace was found by the usual analysis to be biphasic with rate constants of $1.0s^{-1}$ and $0.21s^{-1}$ while a Guggenheim plot on the same data, using $\Delta = 3s$, gave a linear plot with a rate constant of $0.54s^{-1}$, intermediate between the two values derived from the biphasic plot. Similar results were obtained using deamino-NAD^+ as the displacing agent, producing rate constants of $1.1s^{-1}$ and $0.27s^{-1}$ from the biphasic plots and $0.67s^{-1}$ from a Guggenheim plot. It is therefore obvious that care must be taken to ensure that a reaction is a true single first order process before analysing data in terms of a Guggenheim plot (see Frost and Pearson, 1961).

5.3.1.5 Note on NADH Displacement from Alcohol Dehydrogenase

As the displacement of NADH from aldehyde dehydrogenase was found to be biphasic it was decided to observe the displacement from horse liver alcohol dehydrogenase. Shore and Gutfreund (1970) reported that the NADH displacement from horse liver alcohol dehydrogenase was a single exponential with a rate constant of $2.8s^{-1}$ to $3.4s^{-1}$.

When NAD^+ (4.3mM) was used to displace NADH (4.2μM) from alcohol dehydrogenase (3μM) in pH 7.6 phosphate buffer, with pyrazole in the NAD^+ syringe (since NAD^+ contains traces

of alcohol, which may produce a slow steady-state reaction) and using the same filters as for the previous displacements, a biphasic nucleotide fluorescence trace was observed. The rate constant for the fast process was $11s^{-1}$ to $17s^{-1}$ and the slow process $3s^{-1}$ to $4s^{-1}$ with about 30% of the total fluorescence change associated with the fast process (the proportion of the fast process is much less than found for aldehyde dehydrogenase). When the displacement was repeated in the absorbance mode at 355nm (wavelength used by Shore and Gutfreund (1972)) for their displacement experiments with this enzyme and corresponding to the maximum difference in absorption between free and bound NADH (Shore, 1969)), similar rate constants of about $20s^{-1}$ and 3.4 to $4.3s^{-1}$ were obtained demonstrating that the biphasic nature of the displacement was not just a feature of fluorescence. It will be noted that the slow rate constant obtained is similar to the rate constant of the single exponential of Shore and Gutfreund (1970). They also reported a value of about $8s^{-1}$ (determined by Guggenheim plot) when 50mM sodium chloride was added, with a corresponding increase in the catalytic-centre activity from $3.0s^{-1}$ to $5.0s^{-1}$. However when the displacement was repeated with 50mM NaCl added, the process was again biphasic with rate constants of $15\text{-}20s^{-1}$ and $5\text{-}6s^{-1}$. Thus sodium chloride increases the rate of the slow process to about the same extent as the catalytic-centre activity and increases the amplitude of the fast phase to approximately 50% of the total change. Thus the increase in catalytic-centre activity observed by Theorell *et al.* (1955) when sodium chloride is added can be explained by an increase in the rate of the slow process.

5.3.1.6 Concentration Jump

Aldehyde dehydrogenase and NADH in one syringe were mixed with an equal volume of buffer from the other syringe and the return of the system to equilibrium was followed by nucleotide fluorescence. A decrease in fluorescence was observed due to the dissociation of the enzyme-NADH complex. Biphasic plots were again obtained. A typical experiment involving aldehyde dehydrogenase (3μM) and NADH (4.8μM) produced rate constants for the fast and slow processes of $3.0s^{-1}$ and $0.7s^{-1}$ respectively.

FIG. 5.5 NADH ANALOGUE DISPLACEMENT AND NADH ASSOCIATION TO ALDEHYDE DEHYDROGENASE

Photograph A
Deamino-NADH Displacement

Nucleotide fluorescence. The reaction mixture contained premixed cytoplasmic aldehyde dehydrogenase (1.7μM), deamino-NAD^+ (8μM) and propionaldehyde (5.2μM) mixed with displacing agent NAD^+ (4.3mM). Vertical scale, 0.1V/div; horizontal scale, 2 s/div.

Photograph B
Mitochondrial NADH Displacement (see section 6)

Nucleotide fluorescence. The reaction mixture contained mitochondrial aldehyde dehydrogenase and NADH (5.2μM) displaced with NAD^+ (2.9mM). Vertical scale, 0.2V/div; horizontal scale, 5 s/div.

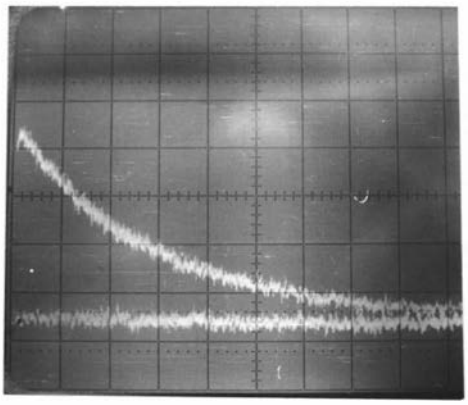
Photograph C
Concentration Jump

Nucleotide fluorescence. The reaction mixture contained cytoplasmic enzyme (0.4mg/cm^3) and NADH (4.8μM) displaced with pH 7.6 phosphate buffer. Vertical scale, 0.2V/div; horizontal scale, 0.5 s/div.

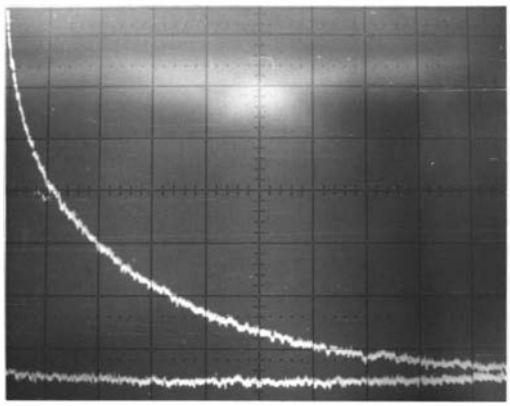
Photograph D
NADH Association

Nucleotide fluorescence. The reaction mixture contained cytoplasmic enzyme (0.9μM) and NADH (8μM). Vertical scale, 0.2V/div; horizontal scale, 0.2 s/div.

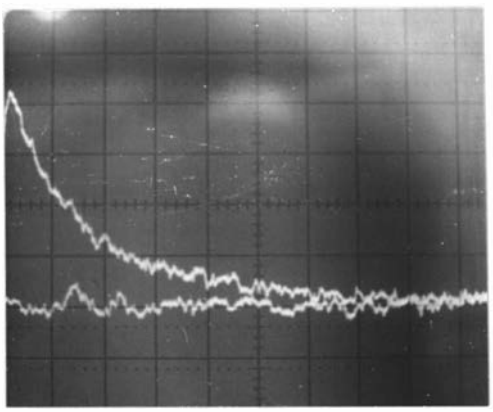
A



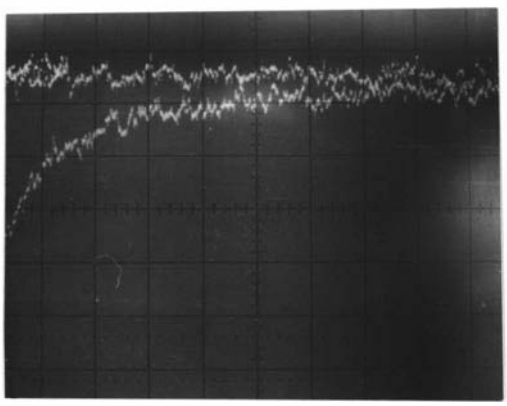
B



C



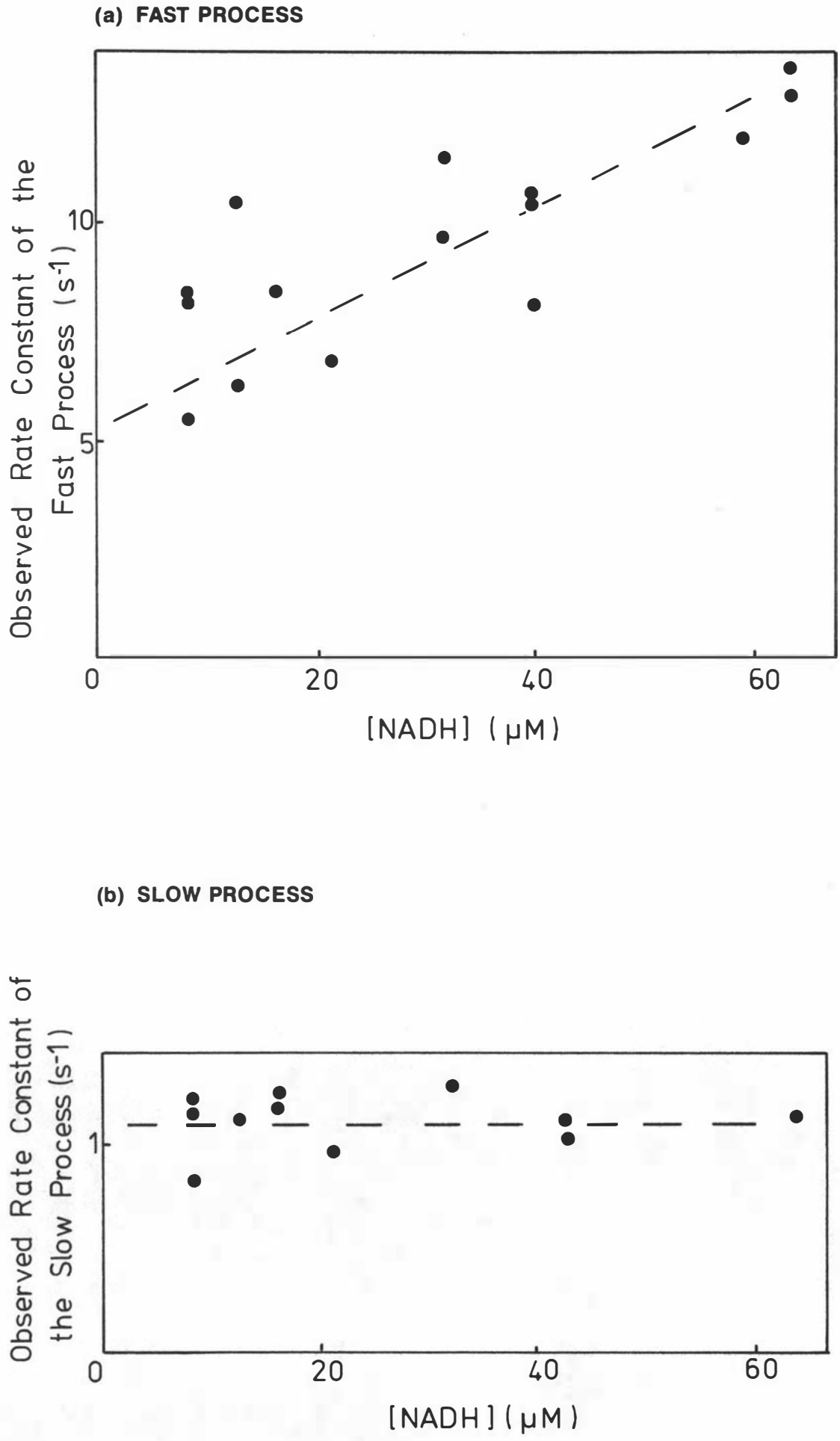
D



5.3.1.7 Association of Enzyme and NADH

When aldehyde dehydrogenase was mixed with a larger concentration of NADH in the stopped-flow apparatus, an increase in fluorescence was observed (Fig. 5.5) due to the association of the enzyme and NADH to form a more fluorescent species. Analysis of the traces showed that the association was biphasic, a feature which had already been noted for NADH dissociation and the concentration jump experiments. Though the NADH concentrations used were often outside the linear fluorescence range, it was thought that linearity within the fluorescence change may still be assumed as the change was only a small fraction of the total NADH fluorescence. As with the displacement experiment the accuracy of the rate constant for the fast process depended on the reliability of the extrapolation of the slower process. When the NADH concentration was varied from 8μM to 64μM, there was little change in the slow process while larger changes were seen for the faster process (Fig. 5.6). When the association was repeated in pH 6.0 phosphate buffer similar results were obtained.

FIG. 5.6 NADH ASSOCIATION TO ALDEHYDE DEHYDROGENASE FOLLOWED BY NUCLEOTIDE FLUORESCENCE



The plots show the NADH dependence of the two processes observed on NADH associating with the enzyme. The final solution contained enzyme (0.9 μM) and various NADH concentrations.

5.3.2 Burst Analysis

5.3.2.1 Observation of the Presteady-State Phase

The mixing of aldehyde dehydrogenase with NAD^+ and propionaldehyde in the stopped-flow spectrophotometer produced a rapid transient absorbance change when followed at 340nm. This transient was followed by a linear increase in absorbance representing the steady-state production of NADH. On following the reaction in the fluorescence mode the same two phases were observed (Fig. 5.7). A comparison of the rates of appearance of the transient and subsequent steady-state rate of production of NADH was made, by mixing the same solution of enzyme with saturating concentrations of NAD^+ and propionaldehyde, and following the reaction by changes in the nucleotide fluorescence, absorbance and transmittance (the latter two at 328nm). Care was needed in separating the presteady-state and steady-state phases in the absorbance mode because of the small change in the molar extinction coefficient of the NADH bound to the enzyme and free NADH. It was necessary to retrigger the oscilloscope several times after the completion of the first trace to obtain reasonable estimates of the steady-state rate. However, the separation was much easier in the fluorescence mode where there is a pronounced enhancement of fluorescence when NADH is bound to the enzyme, relative to free NADH. Results showed that the slow change, equivalent to the steady-state rate, was identical for all modes and similarly the rate of production of the fast transient was first-order in each case and analysis gave rate constants of $11.7s^{-1}$, $12.2s^{-1}$, $10.9s^{-1}$ from the data obtained in the fluorescence, absorbance and transmittance modes respectively.

5.3.2.2 Effect of NAD^+ Concentration on the Burst

When aldehyde dehydrogenase was mixed with a solution containing saturating propionaldehyde and various concentrations of purified NAD^+, it was found that at low concentrations the rate constant of the presteady-state phase (burst) was proportional to the NAD^+ concentration, with a

FIG. 5.7 OBSERVATION OF A BURST IN NUCLEOTIDE FLUORESCENCE FOR THE REACTION OF ALDEHYDE DEHYDROGENASE

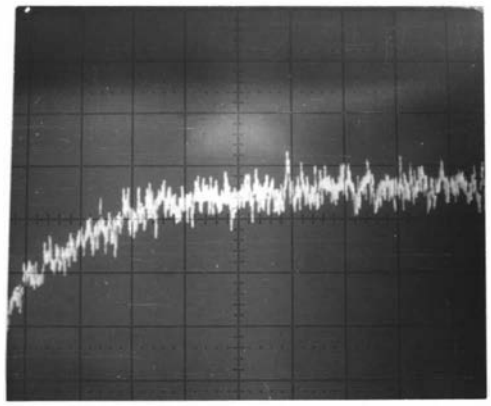
Photograph A
Burst with Propionaldehyde as the Substrate

The reaction mixture contained enzyme (1.8μM) and NAD^+ (420μM) mixed with propionaldehyde (5mM). Vertical scale, 0.1V/div; horizontal scale, 50 ms/div.

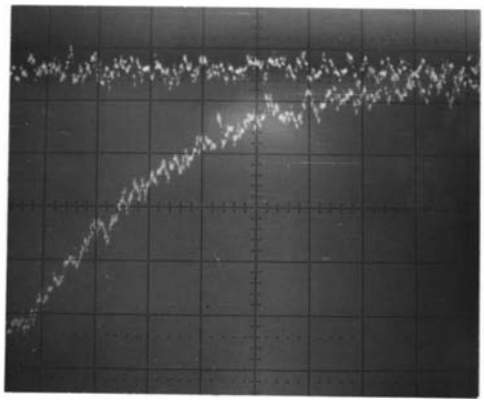
Photograph B
Burst with Acetaldehyde as the Substrate

The reaction mixture contained enzyme (1.8μM) and NAD^+ (390μM) mixed with acetaldehyde (18mM). Vertical scale, 0.5V/div; horizontal scale, 20 ms/div.

A



B



slope of $2.3 \times 10^{+5}$ $M^{-1}s^{-1}$, but at high concentrations reaches a maximum value (Fig. 5.8). NAD^+ which had not been purified was found to give exactly the same values as the purified NAD^+. At low NAD^+ concentrations there was a noticeable lag phase in the fluorescence burst, which has been shown by computer simulation to result from NAD^+ having to bind to the enzyme before the reaction can take place.

5.3.2.3 Effect of Propionaldehyde Concentration on the Burst

The effect of mixing various concentrations of propionaldehyde with a solution of aldehyde dehydrogenase and saturating NAD^+ is shown in Fig. 5.9 , where the data is plotted as

$$1/k_{obs}$$

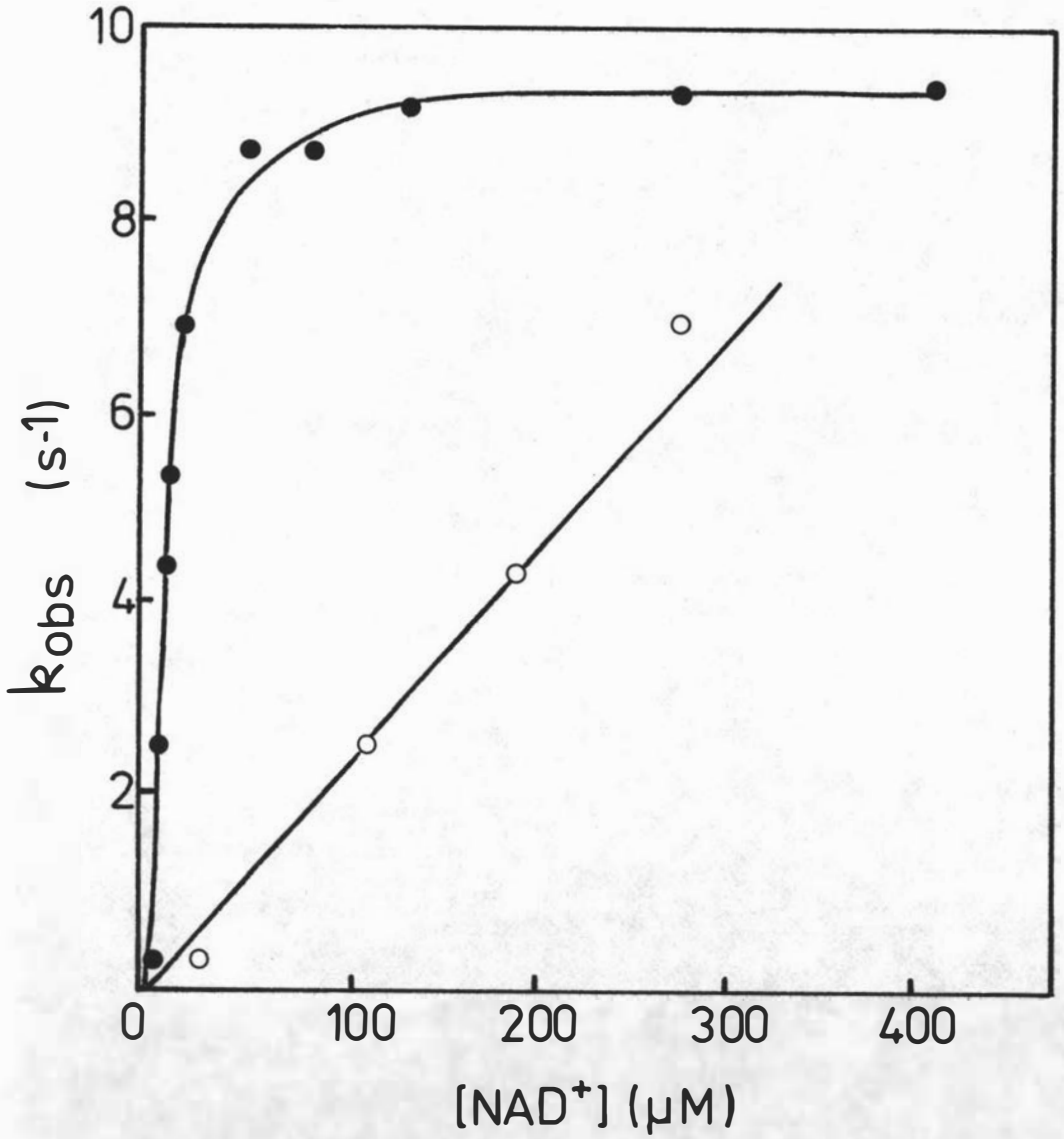
against the inverse of the propionaldehyde concentration, k_{obs} being the observed rate constant of the presteady-state phase. From Fig. 5.9 the slope is 4.5×10^{-6} M s, the half saturating concentration of propionaldehyde is 50μM and the transient rate constant at infinite substrate concentration is $11s^{-1}$, The variation of the transient rate constant was also studied with deuterated propionaldehyde [1-^{2}H] propionaldehyde (CH_3CH_2CDO), and results showed a similar hyperbolic plot with a comparable half-saturating concentration of 57μM and a rate constant at infinite propionaldehyde concentration of $8.3s^{-1}$. A small kinetic isotope effect of 1.3 was observed, however, at much higher propionaldehyde concentrations (1mM to 20mM) no isotope effect was discernible.

Varying concentrations of aldehyde dehydrogenase (0.47μM to 3.8μM) when preincubated with NAD^+ (337μM) and mixed with propionaldehyde (410μM) gave a burst rate constant which was independent of the enzyme concentration.

5.3.2.4 Effect of pH on the Burst

Using saturating concentrations of both substrates (300μM) the burst rate constant was measured in .05M pyrophosphate buffers of varying pH. The steady-state rate

FIG. 5.8 DEPENDENCE OF OBSERVED BURST RATE CONSTANT ON NAD+ CONCENTRATION



Reaction mixture in stopped-flow apparatus contained NAD^+, propionaldehyde (415 μM), enzyme (3 μM). The open circles are a replot of the data at low NAD^+ concentration, where the $[NAD^+]$ is one tenth of that shown on the scale. The slope of this line is $2.3 \times 10^5\ M^{-1}s^{-1}$.

could also be measured at the same time by using a long sweep time, since the instrument had been calibrated with known concentrations of NADH. Results in Table 5.3 show that the burst rate constant increases about 8 fold in the range pH 6.3 to pH 9.8. The catalytic-centre activity increased only three fold over the same pH range which is consistent with the steady-state pH data.

When benzaldehyde was used as a substrate in the place of propionaldehyde (Table 5.4) the burst rate constant was much smaller at low pH but increased with pH, to approach the value for propionaldehyde at pH 9.8.

Table 5.3 Effect of pH on the Burst

Reaction mixtures contained AlDH (5.2μM) and NAD (350μM) in one syringe, and propionaldehyde (280μM) and the appropriate 0.05M pyrophosphate buffer in another

Final pH	Burst rate constant	Catalytic centre activity[1]
9.8	$20.5s^{-1}$	$.39s^{-1}$
9.3	$15.8s^{-1}$	$.33s^{-1}$
8.3	$9.9s^{-1}$	$.29s^{-1}$
7.3	$6.2s^{-1}$	$.16s^{-1}$
6.3	$2.6s^{-1}$	$.14s^{-1}$

[1] Catalytic centre activity determined with .52μM enzyme.

Table 5.4 Effect of pH with Benzaldehyde as substrate

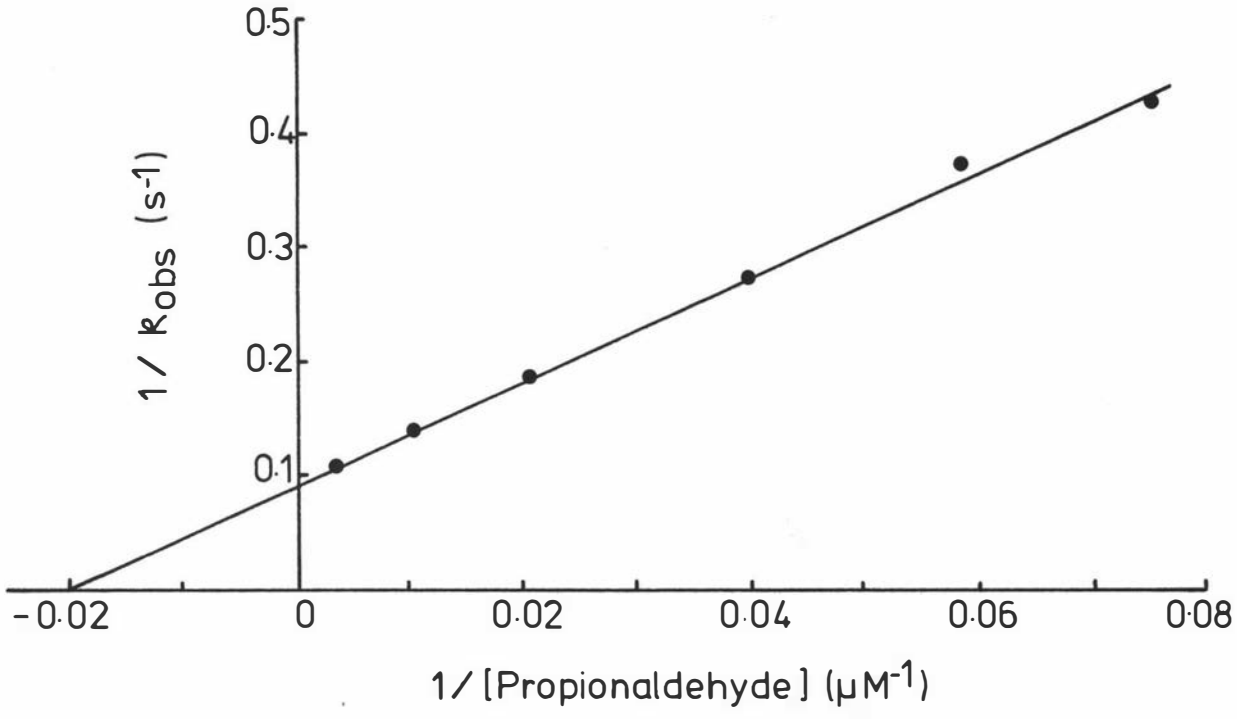
AlDH (5.8μM) and NAD (542μM) were mixed with Benzaldehyde (2.3mM) in pyrophosphate buffer of varying pH

Final pH	Burst rate constant
9.8	$17.5s^{-1}$
9.3	8.1
8.3	2.8
7.3	2.4
6.3	1.7

5.3.2.5 NAD^+ Analogues

A burst was detected using propionaldehyde and deamino-NAD^+ as substrates in the enzymic reaction. A study,

FIG. 5.9 DOUBLE RECIPROCAL DEPENDENCE OF THE OBSERVED BURST RATE CONSTANT ON PROPIONALDEHYDE CONCENTRATION



The reaction mixture contained premixed enzyme (4.8 μM) and NAD^+ (240 μM). The slope of the line is 4.54×10^{-6} s^{-1} and the concentration of propionaldehyde at one half the maximum rate constant is 50 μM.

similar to Fig. 5.8 for NAD^+, of the effect of mixing varying concentrations of propionaldehyde with a premixed solution of aldehyde dehydrogenase (1μM) and deamino-NAD^+ (950μM) gave a maximum rate constant of $26s^{-1}$ ($\pm 6s^{-1}$) and a half-saturating propionaldehyde concentration of 230μM. By premixing the deamino-NAD^+ most of the enzyme will be in a binary complex with the coenzyme and hence the burst rate constant only depends on the aldehyde binding and subsequent steps. The maximum rate constant is about twice that obtained for NAD^+ but the half-saturating propionaldehyde concentration is also higher by about four fold. It therefore appears that the deamino-NAD^+ does not allow the propionaldehyde to bind as tightly as does NAD^+, but having bound the aldehyde the reaction proceeds faster. Studies with [1-^{2}H] propionaldehyde showed a similar small isotope effect (1.3) with deamino-NAD^+ as the coenzyme, as had been found for NAD^+, and the steady-state rate showed no isotope effect.

When deamino-NAD^+ (0.6mM and 1.35mM) was mixed with a preincubated solution of enzyme (1.8μM) and propionaldehyde (0.9mM) the observed rate constant for the burst were $2.6s^{-1}$ and $6s^{-1}$ respectively. On the basis of results observed when enzyme-deamino-NAD^+ complex were mixed with the same propionaldehyde concentration (0.9mM) it would be expected that saturating concentrations of deamino-NAD^+ would produce a rate constant of $20s^{-1}$. This result appears to demonstrate that deamino-NAD^+ has less affinity for the enzyme than NAD^+.

When acetylpyridine adenine dinucleotide (2.7mM) was preincubated with enzyme (1.0μM) and mixed with propionaldehyde there was no observable burst. The reaction either occurring within the mixing time of the stopped-flow, which is extremely unlikely as the reaction of the propionaldehyde with the other binary complexes is slow, or there is no burst at all.

5.3.2.6 Inhibition of the Burst

A series of compounds were tested for their effect on the transient rate constant. However acetone (8.6mM), methylethylketone (6.9mM), methylacetate (6.7mM), ethyl-

acetate (5.7mM) had no effect on the rate constant when premixed with propionaldehyde (90μM) and mixed with AlDH (1.8μM) and NAD^+ (413μM). α-Bromophenylacetic acid (1.6mM) slightly inhibited the transient rate constant when added with unsaturating propionaldehyde (51μM) and mixed with enzyme (3μM) and NAD^+ (180μM) by reducing the observed rate constant from $6.5s^{-1}$ to $5.4s^{-1}$.

5.3.2.7 Effect of Acetaldehyde on the Burst

It was found that when acetaldehyde was mixed with a preincubated mixture of aldehyde dehydrogenase and NAD^+, a hyperbolic relationship existed between the burst rate constant and the acetaldehyde concentration (Fig. 5.10) similar to that found for propionaldehyde. However the maximum burst rate constant is found to be $23s^{-1}$, which is about double that for propionaldehyde, and the half-saturating acetaldehyde concentration is 2.3mM, a factor of 50 times the value for propionaldehyde.

When the relationship between acetaldehyde and [1,2-^{2}H] acetaldehyde was studied it was found that at low acetaldehyde concentrations (less than 1mM) there was a kinetic isotope

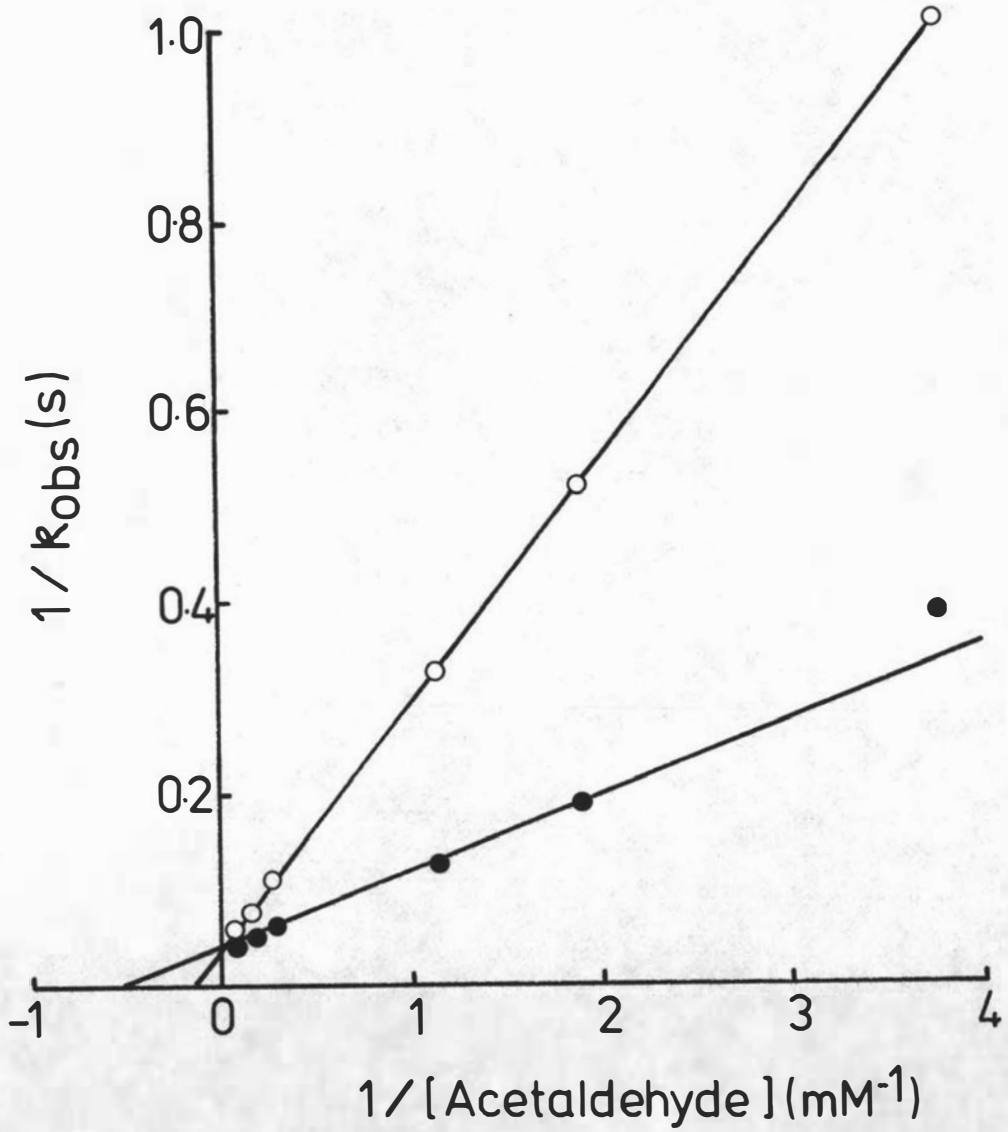
$$(k^H_{obs}/k^D_{obs})$$

effect of 2.6, which was reduced to unity at saturating concentrations. A double reciprocal plot of the burst rate constant and acetaldehyde concentrations gave the same maximum rate constant ($23s^{-1}$) for each isotope but the half-saturating concentrations were 2mM and 6.5mM for the acetaldehyde and [1,2-^{2}H] acetaldehyde respectively.

5.3.2.8 Other Substrates

The chromophore trans-4-N,N-dimethylaminocinnamaldehyde, described by Dunn and Hutchison (1973), has been found to react rapidly with aldehyde dehydrogenase and NAD^+. When the trans-4-N,N-dimethylaminocinnamaldehyde (36μM) was mixed with enzyme (4.5μM) and NAD^+ (560μM) there was found to be a decrease in absorbance at 400nm due to formation of the acid with a rate constant of $8s^{-1}$ which is comparable with

FIG. 5.10 KINETIC ISOTOPE EFFECT ON THE OBSERVED BURST RATE CONSTANT WITH ACETALDEHYDE



The reaction mixture contained premixed enzyme (1.8 μM) and NAD^+ (390 μM).

Results for acetaldehyde are shown as closed circles and deuterated acetaldehyde (CD_3CDO) as open circles.

the nucleotide absorbance increase at 340nm of $13.9s^{-1}$ for the same reaction conditions. It does appear that the same step of the reaction sequence was being observed in each case. The enzyme also oxidized the chromophore, 4-(2'-imidazolylazo)benzaldehyde (prepared by the method of Bernhard et al. (1970)) to the corresponding acid with an absorbance decrease at 410nm. The rate constant obtained when the aldehyde (6.5μM) was mixed with enzyme and NAD^+ (concentrations as above) was about $4s^{-1}$.

Other aldehydes which produced a presteady-state phase are shown in Table 5.5.

Table 5.5 Various Aldehydes which Produce a Presteady-State Phase at pH 7.6

Aldehyde	Concentration	Burst rate constant (s^{-1})
Furfuraldehyde	11.1mM	3.0
Formaldehyde	0.39M	3.0
	0.039M	3.2
p-Methoxybenzaldehyde	67μM-1mM	1.0
p-Methylbenzaldehyde	3.7mM	2.5
Benzaldehyde	2mM-5mM	3.0
p-Nitrobenzaldehyde	50μM-500μM	No observable burst

5.3.2.9 Burst Amplitude

When the burst amplitudes for the mixing of various concentrations of propionaldehyde (over the range 17-280μM) with enzyme-NAD^+ were plotted according to equation (13) it was found that while the error in the amplitudes was quite large the points appeared to obey the relationship and a Michaelis constant of 3.2μM was found. This is to be compared with the Michaelis constant from the steady-state of 1.1μM indicating the value is of the right order. However when mM concentrations were used the amplitudes were greater than predicted. When acetaldehyde amplitudes were plotted (acetaldehyde concentration range 90μM-20mM) the low acetaldehyde concentrations appeared linear with a Michaelis constant of 52μM, a value which cannot be compared with the steady-state as those lines were nonlinear,

while at higher concentrations the curved deviated to higher amplitudes than predicted.

5.3.2.10 Esterase Activity

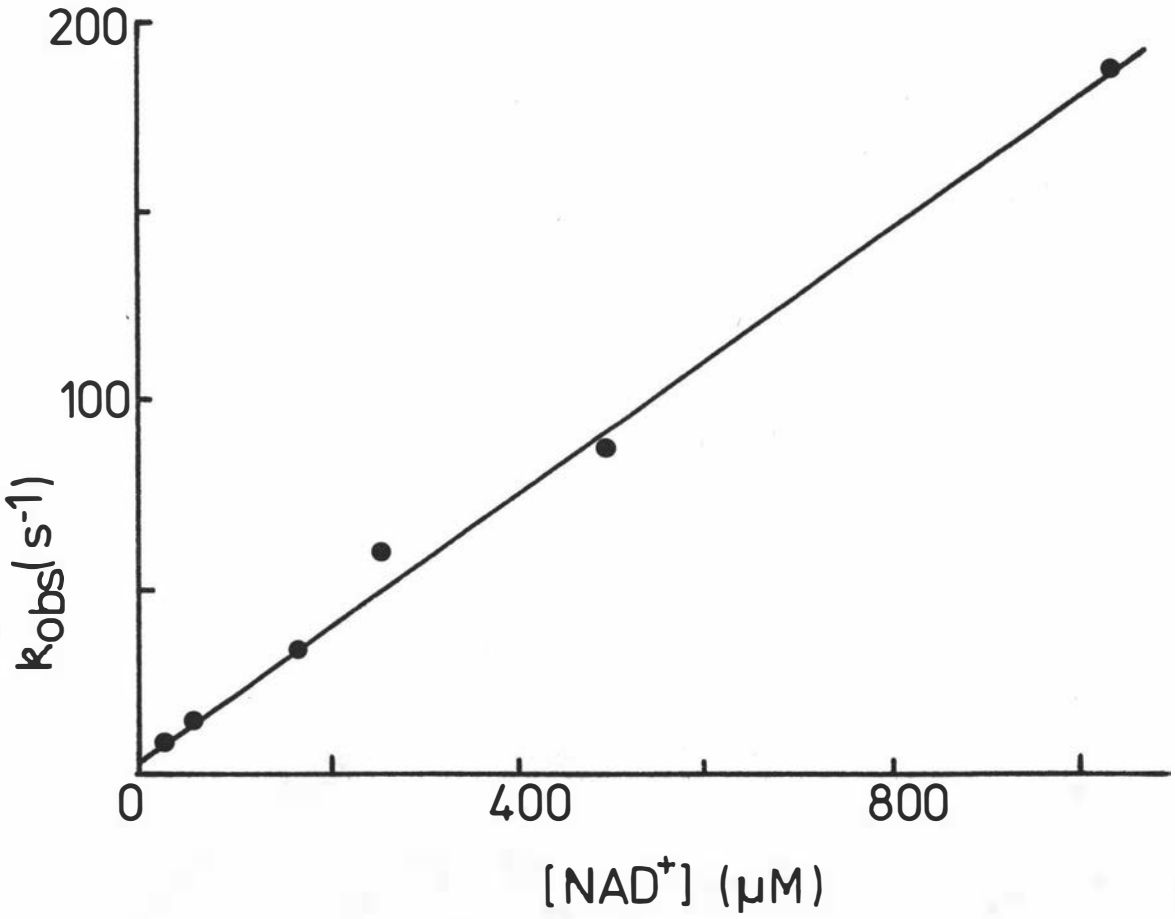
Stopped-flow study of the esterase activity showed a burst with a rate constant of $10s^{-1}$ followed by a slow linear production of p-nitrophenolate with a rate of $0.64\mu M\, s^{-1}$. The catalytic-centre activity of the slow phase was $0.15s^{-1}$. The amplitude of the burst was only 10% of that expected for the enzyme concentration used. However the p-nitrophenyl acetate may not have been saturating in which case the burst amplitude and rate constant will be less than the maximum.

When p-nitrophenylacetate was mixed with 2-mercaptoethanol there was no burst, only a slow production of the p-nitrophenolate ion of $0.01\mu M\, s^{-1}$. It therefore appears that the observed esterase activity is due to the enzyme, and not to the mercaptoethanol present in the enzyme solutions, and it is of particular interest that the observed rates of reaction are similar to the rates found for dehydrogenase activity (even though NADH is not directly involved in the esterase reaction).

5.3.2.11 Protein Fluorescence

When a range of NAD^+ concentrations were mixed with enzyme in the stopped flow and the protein fluorescence studied it was found that a plot of k_{obs} against NAD^+ concentration was linear with a slope of $1.8 \times 10^5\ M^{-1}s^{-1}$ (Fig. 5.11). Though protein fluorescence quenching may exhibit a non-linear relationship with the increase in the fraction of coenzyme-binding sites occupied (Holbrook, 1972), a correction not made in this work, the value for the apparent NAD^+ binding rate constant (k_1) of $1.8 \times 10^{+5}\ M^{-1}s^{-1}$ is in close agreement with the value of $2.3 \times 10^{+5}\ M^{-1}s^{-1}$ found from nucleotide fluorescence experiments. The intercept (k_{-1}) is of the order of $2s^{-1}$ which would give a K_{ia} of about $11\mu M$ which is in good agreement with the steady-state result.

FIG. 5.11 NAD+ ASSOCIATION TO ALDEHYDE DEHYDROGENASE FOLLOWED BY PROTEIN FLUORESCENCE



The reaction mixture consisted of enzyme (2 μM) and various concentrations of purified NAD^+. The slope is $1.8 \times 10^5\ M^{-1}s^{-1}$.

On mixing enzyme with NAD^+ and propionaldehyde the protein fluorescence change was found to be the same as for the mixing of enzyme and NAD^+. That is, the major change was the quenching of protein fluorescence on NAD^+ binding. When premixed NAD^+ (1mM) and enzyme (1.9μM) were mixed with propionaldehyde (772μM) an increase in protein fluorescence was observed with a rate constant about the same as that obtained from nucleotide fluorescence. As NAD^+ quenches the protein fluorescence more than NADH, the conversion of NAD^+ to NADH on the enzyme produces an increase in the protein fluorescence.

The quenching of protein fluorescence on binding of NADH (64μM) to the enzyme was also studied but the traces were not single exponentials, appearing biphasic with rates constants of about $14s^{-1}$ and $2.5s^{-1}$. The reason for this could be two binding steps and, or, non-linearity of the quenching of protein fluorescence by NADH.

5.3.2.12 Computer Simulations of the Burst

It was of interest to determine whether the rate constants which were used in the model produced a burst similar to the experimental results. Since the simulated model, scheme (1), is formed by numerical analysis not involving approximations (other than arithmetical) it is a good test of the derived equations.

Table 5.6 Rate Constants for Scheme (1) Used in Burst Simulation

$k_1 = 2 \times 10^5\ M^{-1}s^{-1}$	$k_3 = 11s^{-1}$
$k_{-1} = 1.6s^{-1}$	$k_4 = 0.2s^{-1}$
$k_2 = 10^{+6}\ M^{-1}s^{-1}$	$k_{-4} = 2 \times 10^5\ M^{-1}s^{-1}$
$k_{-2} = 50s^{-1}$	

Since distinct values have not been obtained for k_2 and k_{-2} the values are estimated and although NADH dissociation is thought to be biphasic, for simplicity it was considered as one step.

Only the species E^{NADH} and NADH were considered to be fluorescent in the simulation, with a fluorescence ratio of

5.6 : 1 respectively.

When the reaction at low NAD^+ concentrations and saturating propionaldehyde concentration was simulated it was found that the slope of a plot of k_{obs} against NAD^+ concentration was the rate constant for NAD^+ binding used in the simulation, showing that it is probable that the plot involving the experimental data also produces the NAD^+ binding rate constant, k_1. An interesting feature of the simulated plots was that at low concentrations of NAD^+ a lag phase was present in the burst, a phenomenon which was observed in the experimental data, suggesting that at these concentrations build up of E^{NAD^+} is slow. This means that the burst trace cannot be extrapolated back to time zero as this would give an overestimation of the burst amplitude.

Naturally simulation of varying propionaldehyde concentrations gave data similar to the experimental data as these were the major experiments used to estimate the rate constants. However simulation of a single turnover experiment was interesting. The conditions simulated were premixed enzyme (6.7μM) and NAD^+ (500μM) reacting with propionaldehyde (1.1μM) and the burst trace produced was an exponential increase followed by a decrease, which could be rationalized in terms of formation of E^{NADH} and then displacement of NADH by NAD^+. From the simulation a burst rate constant of $1.9s^{-1}$ and decay rate constant of $0.18s^{-1}$ were produced. These results may be compared with the experimental data under the same conditions which produced rate constants of $2.1s^{-1}$ and $0.28s^{-1}$ respectively, showing excellent agreement.

5.4 DISCUSSION

5.4.1 Displacement

There appear to be three possible explanations of the biphasic nature of the NADH displacement.

(1) Two isozymes of aldehyde dehydrogenase are present which have different rates of NADH displacement.

(2) A single enzyme is present possessing two different classes of NADH binding sites, and hence two different NADH displacement rates. These sites may be independent or the displacement of NADH from one may affect the displacement from the other.

(3) All the binding sites on a single enzyme are identical, but the NADH may be displaced in two steps. A first step involving either a change in conformation of NADH in the binding site, or a conformational change of the enzyme-NADH complex, which is followed in each case by the actual displacement of the NADH.

The NADH titrations (Section 3) showed that the binding sites of aldehyde dehydrogenase appeared to be independent and equivalent over a wide range of NADH concentrations. The fact that the displacement experiment produced biphasic plots, under conditions of enzyme and NADH concentration similar to those pertaining to the NADH titration, means that explanation (2) may be discounted. For the same reason, and furthermore because of the inability to produce any evidence for the presence of isozymes (Section 4.4), explanation (1) may be similarly discounted. Thus on the basis of experimental observations explanation (3) is the most likely reason for the biphasic decrease in fluorescence in the displacement of NADH from aldehyde dehydrogenase.

Using the model proposed in explanation (3), the displacement reaction may be represented as follows:

$$E^{*}_{NADH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{NADH} \xrightarrow{k_2} E + NADH \qquad \text{(Mechanism 5)}$$

The displacement reaction represented by mechanism (5) is identical to mechanism (1) for two consecutive first order reactions (with $k_{-1} = 0$) and will in general be biphasic with measured first-order decay constants λ_2 and λ_3 (see

equations (1) and (2)). The amplitudes of the two phases will depend on the values of k_1, k_{-1} and k_2 and on the initial concentrations of the species present. These amplitudes for various experimental conditions were obtained by computer simulation of mechanism (5). The initial concentrations of the enzyme species were derived by first determining the total concentration of enzyme-NADH complexes and free enzyme and NADH from the known dissociation constant and then the individual concentration values for E^*_{NADH} and E_{NADH} were obtained from the relative magnitude of the rate constants k_1 and k_{-1} to be used in the simulation. The programme produced concentrations of all the enzyme species and NADH, and in addition supplied a graphical representation of the logarithm of the change in fluorescence with time (the change of the sum of the concentrations of E^*_{NADH} and E_{NADH}) which showed the amplitudes of the fast and slow processes.

Since the replot of the NADH titration curves is linear (Section 3) then the consistency of the NADH titration data with mechanism (5) requires that either

(a) One of the enzyme-NADH complexes is present in negligibly small concentrations throughout the NADH titration, or

(b) Only two of the three fluorescing species present (E^*_{NADH}, E_{NADH}, NADH) have different fluorescence properties.

Considering explanation (a), if the E_{NADH} complex is present in much larger concentrations than E^*_{NADH} at equilibrium, then, as may have been expected, and confirmed by computer simulation, only a single process is observed, with a characteristic decay constant equal to k_2, the rate constant for NADH release. On the other hand, if E^*_{NADH} is the complex present in much larger concentrations at equilibrium then k_{-1} must be larger than k_1. As shown by equation (3) the sum of the decay constants, which is about $1.2s^{-1}$ (0.22 + 0.85), should be the sum of the individual rate constants and hence the maximum possible value for k_{-1} would be about $1.2s^{-1}$ while k_1 and k_2 would have to be much smaller (of the order of $0.1s^{-1}$). However when such small k_1 and k_2 values are used the calculated decay constant for the slow process, λ_3, is much smaller than the experimentally observed value. For smaller values of k_{-1}, k_1 must be made corresponding smaller and although larger k_2 values may now be used,

equations (3) and (4) must still be satisfied with respect to the experimentally observed decay constants (that is $\lambda_2 + \lambda_3$ is about 1.2 and $\lambda_2 . \lambda_3$ is about 0.19). It will be appreciated that when k_2 is large, say of the order $.9s^{-1}$, then k_1 must be of the order of $.2s^{-1}$ (to satisfy equation (4)) which means that k_{-1} is small (to satisfy equation (3)) and hence E^*_{NADH} is no longer the major species. It is therefore not possible to choose parameters that simultaneously meet the thermodynamic requirement that the equilibrium concentration of E^*_{NADH} is much greater than E_{NADH} and which also give reasonable estimates of the experimental decay constants, λ_2 and λ_3.

Thus, no matter whether E^*_{NADH} or E_{NADH} is assumed to be the complex present in very small amounts, explanation (a) does not accommodate the experimental observations.

Explanation (b) allows the possibility that either

(i) E_{NADH} and NADH

or (ii) E^*_{NADH} and NADH

or (iii) E^*_{NADH} and E_{NADH}

have virtually the same fluorescence properties. With respect to the NADH titration, the dissociation constant derived for a consecutive process would be:

$$K_D = \frac{[E][NADH]}{[E^*_{NADH}] + [E_{NADH}]}$$

The change in fluorescence, ΔF, between the sample and the blank in the titration would then be proportional in

case (i) to $[E^*_{NADH}]$

case (ii) to $[E_{NADH}]$

case (iii) to $([E^*_{NADH}] + [E_{NADH}])$

These three different cases would all give an apparent single titration curve. Since the decay constants of mechanism (1) for consecutive reactions are not dependent on the species being observed (equations (1) and (2)) then the three possibilities could not be distinguished by decay constants alone. However, the amplitude and the sign of the decay does depend on the observed species and so amplitude studies, involving computer simulation, may be used to distinguish the three cases.

When simulations were carried out over the widest possible allowable range of the parameters k_1, k_{-1} and k_2 consistent with the rate constants found from the experiments, it is found that the decay of E^*_{NADH}, that is case (i), proceeds as a single exponential or else displays a pronounced lag phase. Both types of behaviour are inconsistent with the biphasic traces found experimentally.

From these same simulations it was found that for suitable choices of the parameters k_1, k_{-1} and k_2 the decay of E_{NADH} with time, case (ii), gave biphasic plots, with rate constants and relative amplitudes which agree well with the experimental displacement data. However, when association experiments were simulated it was found that the concentration of E_{NADH} passed through a maximum. As can be seen from Fig. 5.5 the fluorescence actually increased throughout the association reaction and hence on the basis of these simulations case (ii) may also be excluded.

Attempts were then made to obtain the best estimates of k_1, k_{-1} and k_2, assuming case (iii) in which E^*_{NADH} and E_{NADH} have the same fluorescence enhancement relative to free NADH. Firstly the relative values of k_1, k_{-1} and k_2 were systematically varied to find those values for which the calculated decay constants (equations (1) and (2)) agreed within experimental error to the experimentally determined rate constants for the displacement reaction ($0.85s^{-1}$ and $0.22s^{-1}$). These values were then used in computer simulations to determine the relative amplitudes of the fast and slow processes and compare these with the experimental amplitudes to further restrict the possibilities.

It was found that the following combination of rate constants produced the best fit to the data:

$$E^*_{NADH} \underset{0.05}{\overset{0.2}{\rightleftharpoons}} E_{NADH} \xrightarrow{0.8} E + NADH$$

in which the concentration of the species, E_{NADH}, is relatively high and the fast process can be visualised in terms of the dissociation of NADH from this species, while the significant step in the slower process is interconversion of enzyme-NADH complexes. Obviously there is some lattitude in the values of the rate constants owing to experimental error in the original experimental data and to a certain

amount of insensitivity to small changes in the values.

The apparent dissociation constant for NADH may be expressed in terms of the rate constant for the individual steps:

$$K_D = \frac{k_2}{k_{-2}} \left(\frac{1}{1 + \frac{k_{-1}}{k_1}} \right)$$

Since values for K_D (1.2μM, from Section 3), k_1, k_{-1} and k_2 have been derived, an estimate of the rate constant k_{-2}, the binding of NADH to the enzyme, may be calculated. This value is found to be 5×10^5 $M^{-1}s^{-1}$. It is interesting to use these values to predict the rate constants for the concentration jump experiment. The calculated constants for the reported experiment (see results) are $2.6s^{-1}$ and $0.24s^{-1}$ which are in fairly good agreement with the experimental values of $3s^{-1}$ and $0.7s^{-1}$.

In considering the association experiments it is found that one of the decay constants is dependent on NADH while the other is independent (Fig. 5.6). This is consistent with the postulate of two step binding, since if parallel binding on different NADH binding sites was occurring both the processes would be dependent on NADH. Using the same constants as for the concentration jump it is possible to calculate the decay constants expected for the two processes. Under the same conditions as Fig. 5.6, at 10μM NADH the two processes would be $5.8s^{-1}$ and $0.25s^{-1}$ while at 20μM NADH they would be $10.8s^{-1}$ and $0.25s^{-1}$. It will be noted that qualitatively the decay constants give results in good agreement with the experimental data, that is a fast NADH dependent process and a slow NADH independent process. The calculated results show a lower value for the slow process and a greater NADH dependence for the fast process, however, the experimental errors are very large, as demonstrated by the scatter in the points of Fig. 5.6. The association data is then in general agreement with the displacement data but difficulty in determining accurate association decay constants means that the data does not significantly aid the refinement of the rate constants.

The insensitivity of the observed displacement rate constants for aldehyde dehydrogenase to changes in displacing

agent show that the rate constants are truely associated with the release of NADH. The only exception was 3-pyridine-aldehyde-NAD^+ which was interesting as it was found that this coenzyme analogue did not act as a coenzyme with propionaldehyde in the steady-state reaction, but rather, acted as an aldehyde substrate with NAD^+. This analogue may not then be expected to act as a suitable displacing agent. In contrast to NADH, when deamino-NADH was displaced from the enzyme only a single phase process was observed at a rate similar to the slow process of NADH. This lack of a second phase could be interpreted in several ways. There may be an absence of the factor producing the additional phase in NADH displacement or the extremely unlikely possibility that the process is too fast to be observed (requiring a very large increase over the NADH rate). However, from the work on computer simulations to determine amplitudes of biphasic displacement processes it is found that if the rate constant for the interconversion of enzyme complexes, k_1, is equal to or greater than, k_2, the rate constant of the dissociation step, then only a single phase will be observed. So this additional possibility for the observation of a single process should be considered.

The effect of pH on the displacement of NADH was shown to be negligible and that neither the fast nor the slow rate constant appeared to be dependent on pH. The association experiment also showed no variation in the rates with pH. This would argue against the postulation of a proton release step as one of the two processes, as has been suggested for malate dehydrogenase (Czerlinski and Schreck, 1964b).

Perhaps the most significant aspect of the NADH displacement is the fact that the slow phase of $0.22s^{-1}$ is so similar to the catalytic-centre activity found for propionaldehyde and acetaldehyde in the steady-state of $0.25s^{-1}$ and $0.27s^{-1}$ respectively at very high aldehyde concentrations (Section 4). It is even close to the catalytic centre activity for the linear portion of the Lineweaver-Burk plot of $0.08s^{-1}$. This relation to the catalytic-centre activity demonstrates that the displacement of NADH contributes significantly to the limitation of the enzyme catalytic velocity. Further confirmation of this relationship has been presented by

Eckfeldt and Yonetani (1976) who observed that horse liver aldehyde dehydrogenase possessed an NADH displacement rate of $0.7s^{-1}$, a value close to the catalytic-centre activity of $0.3s^{-1}$, for acetaldehyde. Eckfeldt and Yonetani (1976) have only reported a single phase displacement of NADH, however the study was followed in absorbance where the changes are small so any biphasic nature may not have been apparent.

Since a two step process on NADH displacement has been observed for both sheep liver aldehyde dehydrogenase and horse liver alcohol dehydrogenase, a two step displacement, and perhaps association of NADH may be a more common feature of dehydrogenases than has been suspected. Czerlinski and Schreck (1964a) showed that NADH binds to rabbit muscle M_4 lactate dehydrogenase in two distinct steps. A two phase process was also found by the same authors for malate dehydrogenase, which was rationalized in terms of a slow binding of NADH to the enzyme followed by protonation of the binary complex (Czerlinski and Schreck, 1964b). Geraci and Gibson (1967) refuted the postulation of Theorell *et al.* (1967) that NADH association to alcohol dehydrogenase was a two step process, suggesting that the lag phase observed was a mixing artifact. Certainly the results for aldehyde dehydrogenase exhibit no lag phase, but rather a fast process followed by a slower process. While no previous biphasic displacement data has been reported for horse liver alcohol dehydrogenase, the presence of a conformational change, or some similar step has often been postulated to explain the difference between the catalytic-centre activity and the NADH dissociation rate. This 'step' is reputed to have a rate constant of $11s^{-1}$ (Shore and Gutfreund, 1970). This step could be related to the faster process in the biphasic NADH displacement from alcohol dehydrogenase reported in this work.

As aldehyde dehydrogenase and alcohol dehydrogenase exhibit two step binding of NADH and X-ray crystallographic studies have shown striking similarities among the coenzyme binding domains of dehydrogenases (Ohlsson *et al.*, 1974) it is possible that this is a general feature of dehydrogenases but the observation of the phenomenon may be obscured by the relative sizes of the rate constants of the two processes.

5.4.2 Burst

The observation of a burst in the kinetics of aldehyde dehydrogenase indicates that the first appearance of enzyme-intermediates containing NADH occurs before the rate determining step in the enzymic reaction. This is consistent with the displacement experiments which indicate that NADH release contributes significantly to the limiting step. The presteady-state burst rate constant of $11s^{-1}$ for aldehyde dehydrogenase acting on NAD^+ and propionaldehyde has been observed in absorbance, nucleotide fluorescence and protein fluorescence. The lack of a significant isotope effect (greater than 1.4) on the burst rate constant when using $[1-{}^2H]$ propionaldehyde in place of propionaldehyde demonstrated that the hydride transfer cannot contribute significantly to the burst, therefore the data has been examined in terms of the following reaction sequence:

$$E \underset{k_{-1}}{\overset{k_1[NAD^+]}{\rightleftharpoons}} E^{NAD^+} \underset{k_{-2}}{\overset{k_2[Ald]}{\rightleftharpoons}} E^{NAD^+}_{Ald} \xrightarrow{k_3} E^{NADH} \xrightarrow[\text{displacement}]{\text{slow}} E$$

(scheme 1)

where it is assumed that $E^{NAD^+}_{Ald}$ and E^{NADH}_{Acid} are rapidly interconverted and the concentration of

$$E^{NADH}_{Acid}$$

is low so that no burst due to this complex is seen. The complex

$$E^{NAD^+}_{Ald}$$

in the scheme could be replaced by

$$E^{NADH}_{Acid}$$

if the concentrations of E^{NADH}_{Acid} was small and $E^{NAD^+}_{Ald}$ was not significant.

The presence of three kinetic intermediates in scheme (1) would be expected to give a triphasic exponential rise to the steady state (Maguire *et al.*, 1974). However, as only a single exponential was observed this indicates that two of the steps in the above scheme were too fast

and, or, of too small an amplitude to be observed. It is recognized that the mechanism represents an oversimplification and that other steps could have been included. However, the inclusion of additional steps would have made the interpretation of the results very difficult. These limitations must be recognized when confronting data which is inconsistent with the proposed mechanism.

From Fig. 5.8 the rate constant for the NAD^+ binding, k_1, is 2.3×10^5 $M^{-1}s^{-1}$ which agrees well with the value obtained from protein fluorescence of 1.8×10^5 $M^{-1}s^{-1}$. From the dissociation constant found in the steady-state (Section 4) K_{ia} = 8μM, then the dissociation rate constant, k_{-1}, can be calculated as $1.6s^{-1}$ which is similar to the intercept of Fig. 5.11 , where $k_{obs} = k_1[NAD^+] + k_{-1}$. The same intercept is not found from Fig. 5.8 , for nucleotide fluorescence because the enzyme and enzyme-NAD^+ binary complex are not necessarily in equilibrium, as is the case in the protein fluorescence experiment. A value for k_1 derived from the steady-state analysis (Section 4) was 5×10^4 $M^{-1}s^{-1}$ which is lower than that found by stopped-flow experiments. However, if the k_{cat} value obtained at very high propionaldehyde concentrations is used in estimating k_1, a value of 1.2×10^5 $M^{-1}s^{-1}$ is obtained, which is in much better agreement. The quenching of protein fluorescence by NAD^+ suggests the possibility of a conformational change on binding (see Section 3). As has been suggested for lactate dehydrogenase (Everse et al., 1972), such a conformational change may be a prerequisite for the binding of aldehydes and thus may be an explanation for the compulsory order of binding in aldehyde dehydrogenase.

In considering the relative rate of binding of aldehyde to the enzyme-NAD^+ complex compared to the burst rate there are three possible hypotheses: (1) addition of aldehyde is much slower than the following step ($k_2[Ald]$ much less than k_3); (2) addition of aldehyde is comparable to the following step ($k_2[Ald] = k_3$); (3) addition of aldehyde is much faster than the following step ($k_2[Ald]$ much greater than k_3). If the first hypothesis was true the relationship between the observed burst rate constant and the aldehyde concentration would be linear and even if at high concentrations hypothesis (2) was approached the relationship would

not be hyperbolic. If the second hypothesis was true a biphasic plot would result, unless k_{-2} was much larger than $k_2[Ald]$ in which case probably only a single phase would be observed, since the other phase would be very fast and the amplitude small (equation (3)). Also to reach a maximum burst at high aldehyde concentrations $k_2[Ald]$ would become large and hypothesis (2) may no longer apply. The third hypothesis predicts a double reciprocal relationship between k_{obs} and the aldehyde concentration (equation (11)), a relationship shown by the experimental data. It would therefore appear that the rate determining step in the burst reaction sequence is k_3 (which of course is a composite rate constant). Fitting the data using equation (11) a value of k_3 equal to $11s^{-1}$ is obtained from the intercept and

$$k_{-2} = \left(\frac{\text{slope}}{\text{intercept}}\right) k_2 - k_3,$$

that is $k_{-2} = (50 \times 10^{-6})k_2 - 11$. Although individual values for k_2 and k_{-2} cannot be obtained, the smallest values the rate constants may have are $2.2 \times 10^5\ M^{-1}s^{-1}$ and $0.0s^{-1}$ for k_2 and k_{-2} respectively and the highest values would be related to a diffusion controlled rate for k_2. In comparing the data with the steady-state predictions of the same mechanism in which

$$K_m \text{ (propionaldehyde)} = \frac{k_4(k_3 + k_{-2})}{k_2(k_3 + k_4)} = 1.1\mu M$$

(derived by method of King and Altman (1956) and Cleland (1963)), it is found that on substituting the values for k_3 and $(k_3 + k_{-2}).(k_2)^{-1}$, a value for k_4 of $0.24s^{-1}$ is derived. This value is similar to the slow NADH displacement rate constant and the catalytic-centre activity, hence the pre-steady-state and steady-state data appear to be consistent.

From the presteady-state behaviour of propionaldehyde and NAD^+ some of the rate constants of scheme (1) may be estimated.

$$k_1 = 2 \times 10^5\ M^{-1}s^{-1}$$
$$k_{-1} = 1.6s^{-1}$$
$$k_{-2} = (50 \times 10^{-6}).k_2 - 11$$
$$k_3 = 11s^{-1}$$

While absolute values for the propionaldehyde binding and dissociation rate constants (k_2 and k_{-2}) are not known, the relationship between k_2 and k_{-2} is known. As the reaction of aldehyde dehydrogenase is irreversible, presteady-state studies in the reverse direction cannot be undertaken to help elucidate further steps in the mechanism.

The k_{obs} pH profile showed that the step involved in the burst was pH dependent with the greatest rate constants occurring at high pH's, a trend that was shown even more dramatically by benzaldehyde. Either the removal of a proton is directly involved in this step or indirectly through the ionization of an adjacent group.

Studies with enzyme-deamino-NAD^+ complexes and varying propionaldehyde concentrations showed a maximum burst rate constant, k_3, of $26s^{-1}$ and $k_{-2} = (230 \times 10^{-6}).k_2 - 26$. Comparison with the results for NAD^+ show that k_3, the burst rate constant, cannot be the only value to change with variation in the coenzyme (since the two expressions cannot be solved in terms of identical k_2 and k_{-2}). Hence the coenzyme must in some way be affecting the rate of association or dissociation of aldehyde from the enzyme-coenzyme complex.

When acetaldehyde was used as the aldehyde substrate in the burst studies it was found that the maximum burst rate constant was greater than for propionaldehyde and had no kinetic isotope effect. However, at lower acetaldehyde concentrations there was an isotope effect of 2.6. With respect to the mechanism suggested for propionaldehyde, this would mean that there was no isotope effect on k_3 but there was an isotope effect on $(k_3 + k_{-2}).(k_2)^{-1}$, that is k_2 and k_{-2} are affected suggesting a kinetic isotope effect on the association and dissociation of acetaldehyde from the enzyme. Unlike the deuterated propionaldehyde, the deuterated acetaldehyde used in the isotope experiments was fully deuterated (CD_3CDO). However any secondary isotope effects that this may produce would only account for a k_H/k_D ratio of about 1.3 (Laidler and Bunting, 1973) and so could not produce the observed isotope effect. Similarly the fraction of unhydrated aldehyde in sodium phosphate buffer (pH 7.0, $\mu = 0.1$) at 25°C is 0.48 for acetaldehyde and 0.40 for deuterated acetaldehyde (Eckfeldt and Yonetani, 1976) and

since the unhydrated aldehyde is the probable substrate (Bodley and Blair, 1971) an apparent $^{k_H}/_{k_D}$ ratio of 1.2 would result which also cannot explain the observed result.

It is possible that some sort of covalent binding of the aldehyde on attachment to the enzyme is occurring or removal of the hydride ion may have already proceeded to some extent in the Michaelis complex.

With regard to the mechanism in scheme (1) it is possible that the hydride transfer step is involved in the steps designated k_2 and k_{-2}, a suggestion which did not need to be considered in the case of propionaldehyde since there was no significant isotope effect on any of the steps and hence it was not possible to know with which step the hydride transfer rate was associated. For acetaldehyde it is possible that aldehyde binding and transfer of the hydride occurs in a concerted sequence so that the reaction quickly proceeds to E^{NADH}_{Acid}. The observed burst rate is the same no matter whether it is followed in absorbance, nucleotide fluorescence or protein fluorescence. If E^{NADH}_{Acid} is regarded as a significant intermediate and yet does not produce an observed spectral change then either the intermediate has no absorbance or fluorescence properties, or the amplitude of these changes are so small as not to be observed. It seems unlikely that an intermediate containing such a strongly absorbing, fluorescing and protein fluorescence quenching group as NADH does not show any of these characteristics, so it is probably more likely that the intermediate does absorb and fluoresce.

If this is the case then such a complex would be observed unless its concentration was very small, that is k_{-2} was very large. However, from the proposed mechanism we have a restriction from the data that $k_{-2} = (2.3 \times 10^{-3})k_2 - 23$ which requires that $k_2/\underline{Ald}/$ be greater than k_{-2} except at low concentrations of aldehyde, producing a significant concentration of E^{NADH}_{Acid} and hence a biphasic trace would be expected with the contribution of the faster phase becoming greater as the aldehyde concentration increased. This means that even if the fast phase was completed in the mixing time of the instrument the amplitude of the slower phase should decrease with increasing aldehyde

concentration (see equation (7)). From this discussion it does not seem possible to explain the isotope effect at low aldehyde concentrations in terms of a fluorescent E^{NADH}_{Acid} species and it would seem unlikely, but possible, that E^{NADH}_{Acid} is not fluorescent suggesting that the scheme would have to contain additional steps in order to explain the isotope effect. However, it is not obvious whether additional steps would be able to accommodate both the isotope effect and the other observed results, that is a single exponential burst and the hyperbolic aldehyde dependence. Some method of observing single transient intermediates, for instance study of proton release (Shore et al., 1974) or blocking of segments of the pathway by inhibitors, if possible, would elicit more information.

Recently Eckfeldt and Yonetani (1976) studied the presteady-state kinetics of horse liver aldehyde dehydrogenase with acetaldehyde by following the burst at 350nm utilizing a small change in absorbance between free and bound NADH, similar to that found for sheep liver aldehyde dehydrogenase (Section 3). They found a kinetic isotope effect with acetaldehyde and deuterated acetaldehyde (CH_3CDO) and because of the low value, 2.8, concluded that k_3 was a hydride transfer step, and that k_{-2} was of the same order of magnitude as k_3. However, from a double reciprocal plot of k_{obs} and aldehyde concentration the isotope effect on k_3 was found to be 1.9 ± 1.5 while the isotope effect on the slope, which is $(k_3 + k_{-2}).(k_3k_2)^{-1}$, was 2.8 ± 0.1. While these results were interpreted in terms of k_3 being the hydride transfer step the fact that the isotope effect onthe isolated hydride transfer step, k_3, is much less than on the more complex slope term except at the very high end of the k_3 error scale when in fact it should be much greater as it was stated that k_3 and k_{-2} were of the same magnitude and because the magnitude of the isotope effect is much less than the ratio of about 7 expected if k_3 was solely the hydride transfer step suggest that the interpretation may not be correct. The same results would better fit the situation found for sheep liver aldehyde dehydrogenase where the hydride transfer step is not observed directly but affects the steps

designated k_2 and k_{-2} in scheme (1). This agrees with the thought that chemical steps in catalysis are usually not the major rate determining ones (Cleland, 1975) and in fact alcohol dehydrogenase is one of the few dehydrogenases to show an isotope effect (6.0) in the burst (Shore and Gutfreund, 1972).

It is interesting that a similar maximum burst rate constant with acetaldehyde for the enzyme from horse liver and sheep liver is found ($33s^{-1}$ and $23s^{-1}$ respectively) and the apparent similarity of enzyme action may well be extended to other species.

At this point it is worthwhile to consider the esterase results. p-Nitrophenylacetate binds to the enzyme (acylation of the nitrophenyl ester) and then hydrolysis produces the chromophore, p-nitrophenolate ion, and an acetyl-enzyme complex which then proceeds by deacylation to form acetate and free enzyme. The burst observed indicates that the acetyl-enzyme complex is quickly formed and the rate determining step is the deacylation, which is in contrast to the results of Feldman and Weiner (1972b) who from steady-state comparison with dehydrogenase activity suggested that the acylation was rate limiting. The interest in the acetyl-enzyme complex is due to the fact that when NADH is bound to the complex as well it should resemble the acetyl intermediate from the dehydrogenase reaction with acetaldehyde and hence the deacylation rates should be the same for both the esterase and the dehydrogenase reactions. It is therefore interesting that the catalytic-centre activity for the esterase reaction is about $0.15s^{-1}$ since a step of the same order of magnitude as the NADH dissociation step is required to explain the different catalytic-centre activities for various aldehydes in the dehydrogenase reaction. This step may be the deacylation step.

In comparing the steady-state results with the pre-steady-state results the most interesting is the behaviour of the burst at aldehyde concentrations which give the apparent substrate activation in the steady-state. Analysis of the burst rate constants show no deviations from the expected behaviour for propionaldehyde and the same is true for acetaldehyde (Fig. 5.9 and Fig. 5.10) over wide

concentration ranges so the steady-state effect does not reflect itself in the burst rate constant, as it may have been expected to. However, when the amplitudes of the burst are analysed it is found that at high aldehyde concentrations the amplitude is greater than would be predicted and it is possible that this behaviour may be associated with the non-linear steady-state. It would appear from the values for the NADH displacement from the enzyme, of $.22s^{-1}$, that this step is solely rate determining at high aldehyde concentrations (for both propionaldehyde and acetaldehyde, see section 4) and at lower concentrations some additional step has a significant effect on the overall rate of reaction. Eckfeldt and Yonetani (1976) also postulated such a step, for horse liver aldehyde dehydrogenase, to explain the difference between NADH dissociation rate and the catalytic-centre activity. This additional step must be after the burst producing step, which is over $5s^{-1}$ at 50μM propionaldehyde, but before the NADH dissociation step. The step may well be deacylation, as suggested from the esterase reaction.

In summary it has been shown that scheme (1) is a good approximation to the kinetic behaviour in the presteady-state using NAD^+ and propionaldehyde as substrates but that it does not explain some facets of the steady-state behaviour. However for acetaldehyde even the burst kinetics cannot be explained fully by scheme (1) and a more complex mechanism has to be considered.

SECTION 6

COMPARISON OF MITOCHONDRIAL AND CYTOPLASMIC ALDEHYDE DEHYDROGENASES

6.1 INTRODUCTION

While it has been generally recognized that aldehyde dehydrogenase is the major enzyme in the oxidation of acetaldehyde produced from the metabolism of ethanol (Richert and Westerfield, 1957) there has been some confusion in the literature over the localization of the enzyme activity. The most extensively studied aldehyde dehydrogenase has been that from rat liver. Dietrich (1966) reported that 87% of the total liver aldehyde oxidizing capacity was located in the cytoplasm, however, Tottmar *et al.* (1973) found aldehyde dehydrogenase activity in the mitochondria and the microsomes but virtually none in the cytoplasm. This activity was attributed to a single enzyme existing solely in the mitochondria and another distributed between the mitochondria and the microsomes. These findings were supported by Horton and Barrett (1975) who found a similar enzyme distribution, 80% in the mitochondria and 20% in the microsomes. However Marjanen (1972) reported that 80% of the total enzyme activity found was in the mitochondrial fraction, 20% in the cytoplasm, but no activity was observed in the microsomes. While much of this information is contradictory and may depend on the efficiency of the cellular fractionation procedures, it would appear that for rat liver aldehyde dehydrogenase most of the enzyme activity is found in the mitochondria. This view is supported by the *in vivo* studies of Eriksson *et al.* (1975).

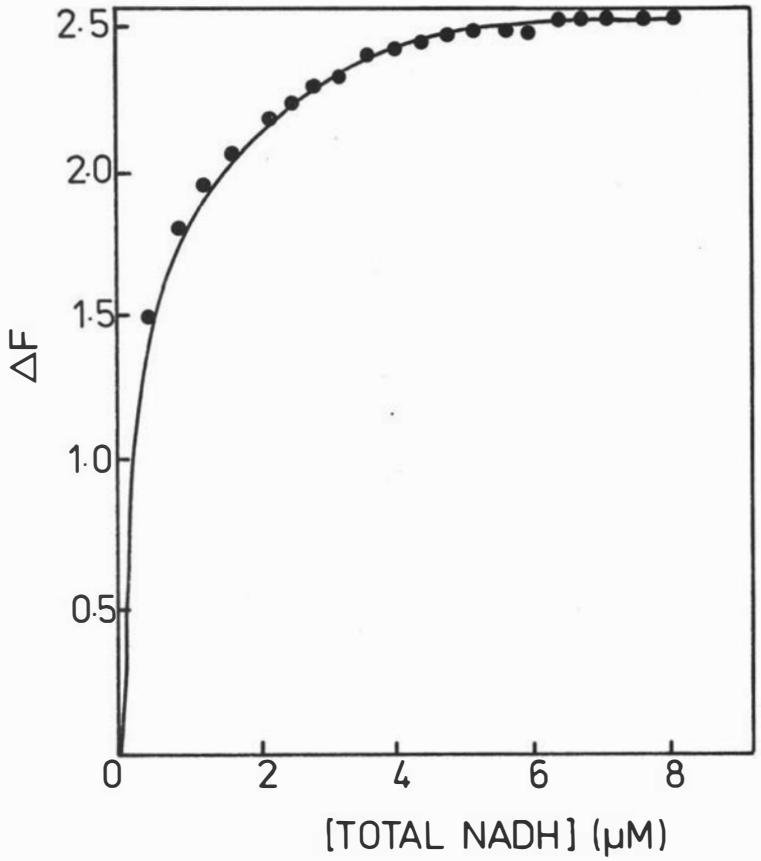
In this laboratory a study of the intracellular localization of sheep liver aldehyde dehydrogenase has been carried out and it was observed that 37% of the enzyme activity was in the cytoplasm, 34% in the mitochondria, 10% in the microsomes and 19% in the 500 x g centrifuge sediment, which consisted of whole cells and nuclei (Crow *et al.*, 1974). Enzymes of known subcellular distribution were used as markers to detect any possible cross-contamination of the

subcellular fractions. The enzymes from both the cytoplasmic and mitochondrial fractions have been purified and their properties investigated. The most significant difference in kinetic properties between the two enzymes was the difference in the Michaelis constant for NAD^+ at pH 9.3 using acetaldehyde as a substrate, Km's of 12μM and 77μM being observed for the cytoplasmic and mitochondrial enzymes respectively. As found for aldehyde dehydrogenase from other sources (Maxwell, 1962) the sheep liver enzymes were both affected by steroidal hormones, however, while progesterone lowered the activity and diethylstilbestrol raised it in both cases the quantitative effect was much greater for the cytoplasmic enzyme. Differences in substrate specificity between the cytoplasmic and mitochondrial enzymes were also observed. Kitson (1975) showed that the cytoplasmic enzyme was much more sensitive to the thiol reagent disulfiram than the mitochondrial enzyme in that 0.01mM disulfiram reduced the initial activity of the former to 5% of the control while the latter was only reduced to 96%. Similar differences in sensitivity to disulfiram have been shown by the horse liver aldehyde dehydrogenase isozymes F_1 and F_2, which are of cytoplasmic and mitochondrial origin respectively (Eckfeldt et al., 1976). Although some of the kinetic properties of the two sheep liver enzymes were different, the physical properties were very similar. The molecular weights were almost identical, as determined by gel filtration (212,000 and 205,000 for the cytoplasmic and mitochondrial enzymes respectively) and the subunit molecular weights as determined by sodium dodecylsulphate-polyacrylamide gel electrophoresis were 53,100 $\pm$ 2,000 in each case (Crow, 1975).

To aid in the understanding of the function of the two similar but distinct enzymes in separate intracellular compartments, it was of interest to compare more of the kinetic properties of the two enzymes. In this regard some of the kinetic experiments carried out on the cytoplasmic enzyme were repeated using the mitochondrial enzyme.

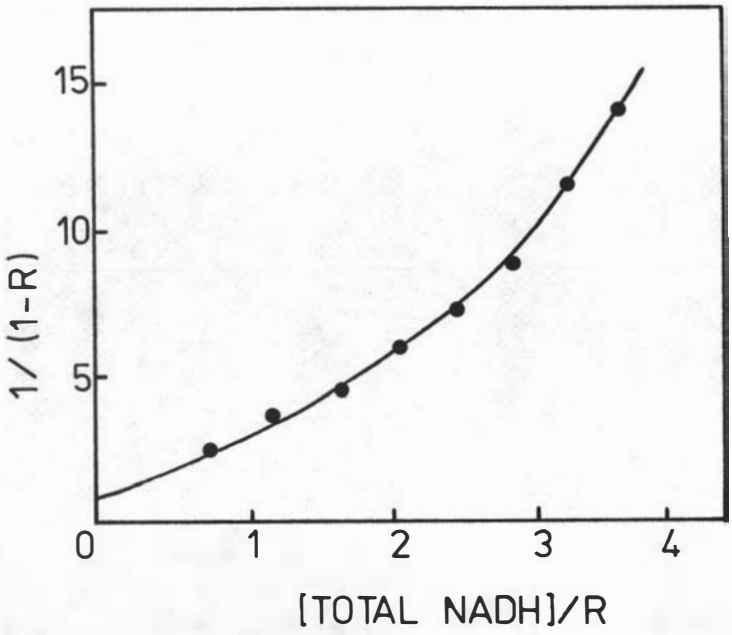
FIG. 6.1 ATTEMPT AT NADH TITRATION FOR MITOCHONDRIAL ALDEHYDE DEHYDROGENASE

(a) NADH TITRATION



ΔF is the difference in fluorescence between the enzyme and buffer solutions.

(b) REPLOT OF NADAH TITRATION DATA



ΔF_{max} was taken as 2.5 fluorescence units

6.2 METHODS

The mitochondrial sheep liver aldehyde dehydrogenase was supplied by Dr. T.M. Kitson and the methods and reagents used for the experiments involving the mitochondrial enzyme were identical to those previously described for the corresponding cytoplasmic enzyme experiments. Phosphate buffer, pH 7.6, was used throughout.

6.3 RESULTS

6.3.1 Equilibrium Studies

An attempt was made to titrate the mitochondrial enzyme with NADH, in an identical manner to the cytoplasmic enzyme, in order to determine the catalytic-centre activity. However, as shown in Fig. 6.1(a), there was an initial sharp rise in fluorescence on addition of the first aliquots of NADH but continued addition of NADH produced smaller increases in fluorescence, until a maximum value was reached. The result of this behaviour was that the replot was not linear (Fig. 6.1(b)) and any extrapolated enzyme concentration would appear to be negative, a situation which was obviously not physically meaningful.

6.3.2 Steady-State Studies

The effect of a wide range of acetaldehyde concentrations on the initial velocity of the mitochondrial enzyme was studied (Fig. 6.2). The double reciprocal plot was linear up to 10μM acetaldehyde and a Michaelis constant of 0.2μM was derived. However at higher acetaldehyde concentrations the line deviates from linearity towards higher initial velocities. It was found that at 20mM acetaldehyde the initial velocity was nine times that predicted by extrapolation of the linear portion of the graph to a supposed maximum velocity. In fact the overall shape of the plot is very similar to that found for the cytoplasmic enzyme (Section 4.3.3.8) although the plot is linear over a wider range at low acetaldehyde concentrations.

6.3.3 Presteady-State Studies

Displacement of NADH (5μM) from the mitochondrial

aldehyde dehydrogenase by various displacing agents was observed to be a biphasic process (Table 6.1). It would appear from the consistency of these results that saturating

TABLE 6.1 NADH DISPLACEMENT FROM MITOCHONDRIAL ALDEHYDE DEHYDROGENASE

Displacing Agent	Rate Constant for the Fast Process	Rate Constant for the Slow Process
NAD^+ (1mM)	$0.54s^{-1}$	$0.09s^{-1}$
NAD^+ (2.9mM)	$0.7s^{-1}$	$0.08s^{-1}$
ADP-ribose (1.7mM)	$0.57s^{-1}$	$0.09s^{-1}$

concentrations of NAD^+ and ADP-ribose were used. When these results are compared with those of the cytoplasmic enzyme (Section 5.3.1.1) which has rate constants of $0.85s^{-1}$ and $0.22s^{-1}$ the striking similarities are immediately apparent. However the proportion of fluorescence change attributable to the slow process was about 60%, significantly greater than the 30-50% seen for the cytoplasmic enzyme (Fig. 5.5).

When the mitochondrial enzyme, premixed with NAD^+ (2.7mM) was mixed with propionaldehyde (780μM) the burst observed in nucleotide fluorescence had a rate constant of about $10s^{-1}$. However it was noted that the size of the fluorescence change in the burst for this enzyme solution was about one sixth of the change observed for NADH displacement from an enzyme solution of the same concentration. This suggested that the concentration of the enzyme species being observed in the burst was much less than the total enzyme concentration, and that other nonfluorescent enzyme species must be in appreciable concentration. Supporting evidence for this statement was the fact that a range of propionaldehyde concentrations (0.4mM to 20mM) all produced about the same observed rate constant of $12s^{-1} \pm 3$ (with large scatter due to the small burst size) but the amplitude of the fluorescence change in the burst increased by over a factor of three. When the maximum burst rate constants with propionaldehyde for the mitochondrial and cytoplasmic enzymes are compared, $12s^{-1}$ and $11s^{-1}$ respectively, great similarity in the two enzymes is apparent.

On following NAD^+ binding to the enzyme by quenching of protein fluorescence it was found that the observed rate constant varied with the NAD^+ concentration (Table 6.2).

TABLE 6.2 NAD+ BINDING TO THE MITOCHONDRIAL ENZYME FOLLOWED BY QUENCHING OF PROTEIN FLUORESCENCE

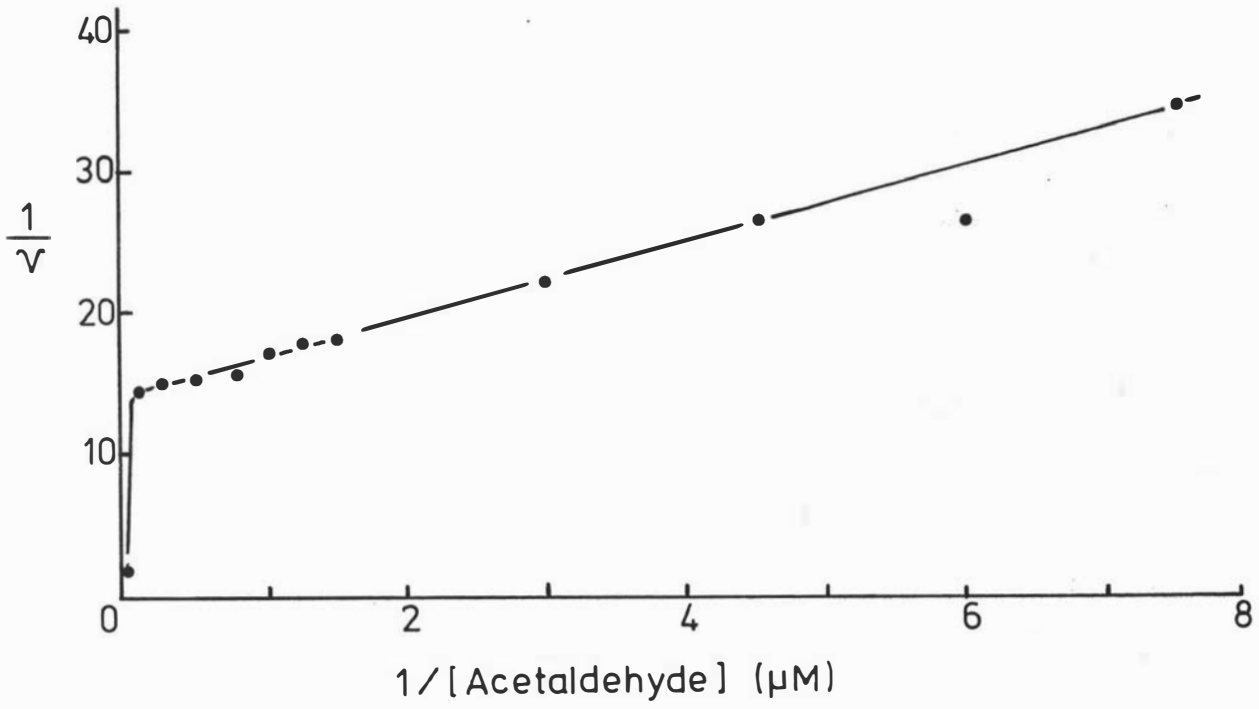
$[NAD^+]$ (μM)	Observed Rate Constant (s^{-1})
25	7.4
50	9.5
150	7.4
250	9.1
500	12.5
1000	23

While the scatter in values from Table 6.2 is obvious, it is possible to estimate a value for the NAD^+ binding rate constant, k_1, as 2×10^4 $M^{-1}s^{-1}$ and k_{-1} as $4s^{-1}$. These results may be compared with the cytoplasmic enzyme values of 1.8×10^5 $M^{-1}s^{-1}$ and $2s^{-1}$ for k_1 and k_{-1} respectively from protein fluorescence. It should be remembered however that in the case of the cytoplasmic enzyme corroborative evidence was available from nucleotide fluorescence so the results were more reliable. However if a comparison is made of the binding rates it is seen that the rate constant for the cytoplasmic enzyme is a factor of ten faster while the dissociation rate constants are fairly similar, only differing by a factor of two. The difference in NAD^+ binding rate constants is perhaps not unexpected since the Michaelis constant of NAD^+ for the mitochondrial enzyme is six times that of the cytoplasmic enzyme (Crow et al., 1974). The Michaelis constant for the four intermediate reaction schemes shown in Fig. 4.10 is

$$K_a = \frac{k_3 k_4 k_5}{k_1(k_{-3}k_5 + k_4 k_5 + k_3 k_5 + k_3 k_4)}$$

and hence any changes in k_1 would be expected to be reflected in the value of K_a.

FIG. 6.2 DOUBLE RECIPROCAL PLOT OF INITIAL VELOCITY AGAINST ACETALDEHYDE CONCENTRATION FOR THE MITOCHONDRIAL ALDEHYDE DEHYDROGENASE



The concentration of NAD^+ was held at 1.35 mM. K_m for acetaldehyde (at low concentrations) is 0.2 μM and V_{max} at these concentrations is 0.07 fluorescence units per minute, while at 20 mM the initial velocity is 0.66 fluorescence units per minute. (Excitation 345 nm, emission 455 nm).

6.4 DISCUSSION

The nonlinear nature of the NADH titration plots would suggest that NADH binding sites of the mitochondrial enzyme are not equivalent. Either there are multiple classes of NADH binding sites or there is a single binding site but a two step binding process. As discussed in Section 5.4.1 a two step process for NADH binding in which the fluorescence of each of the enzyme-coenzyme complexes are different would also lead to nonlinear NADH titration plots. While in isolation there is no compelling reason to favour either possibility, the fact that the steady-state and presteady-state results are so similar to the results for the cytoplasmic enzyme makes it perhaps more probable that a two step binding process is taking place in which the two enzyme-coenzyme complexes have different fluorescent properties. The displacement experiment for the mitochondrial enzyme produced two rate constants of the same order of magnitude as those obtained for the cytoplasmic enzyme. The rate constant for the slower process of $0.08s^{-1}$ is somewhat less (compared with $0.22s^{-1}$) while that for the faster one is about the same in both cases. It would therefore appear that the catalytic-centre activity of the mitochondrial enzyme should be less than for the cytoplasmic enzyme, as the mitochondrial enzyme reaction velocity cannot be greater than the NADH displacement rate but may be less if this step is not rate limiting.

The steady-state data for the mitochondrial enzyme showed the same deviations from linearity as in the data for the cytoplasmic enzyme. It is interesting that over these same concentration ranges the amplitudes of both the bursts in the presteady-state experiments changed drastically. It is unfortunate that no estimate of the mitochondrial enzyme active site concentration could be made since it may have been expected that the catalytic-centre activity of the enzyme would be the same as the NADH dissociation rate.

It appears that the general features of the kinetics of mitochondrial and cytoplasmic sheep liver aldehyde dehydrogenases are very similar, the only obvious difference being the NAD^+ binding rate constants. Since the proportion of

aldehyde dehydrogenase activity in the mitochondria and the cytoplasm are similar (Crow et al., 1974) and the NADH displacement rates about the same, then the actual concentrations of the enzymes would be expected to be similar in the two compartments. Therefore it would appear that in the in vivo situation any differences in the activities or importance of the two enzymes would be related to the concentrations of the substrates and the products in the two compartments rather than any kinetic factor.

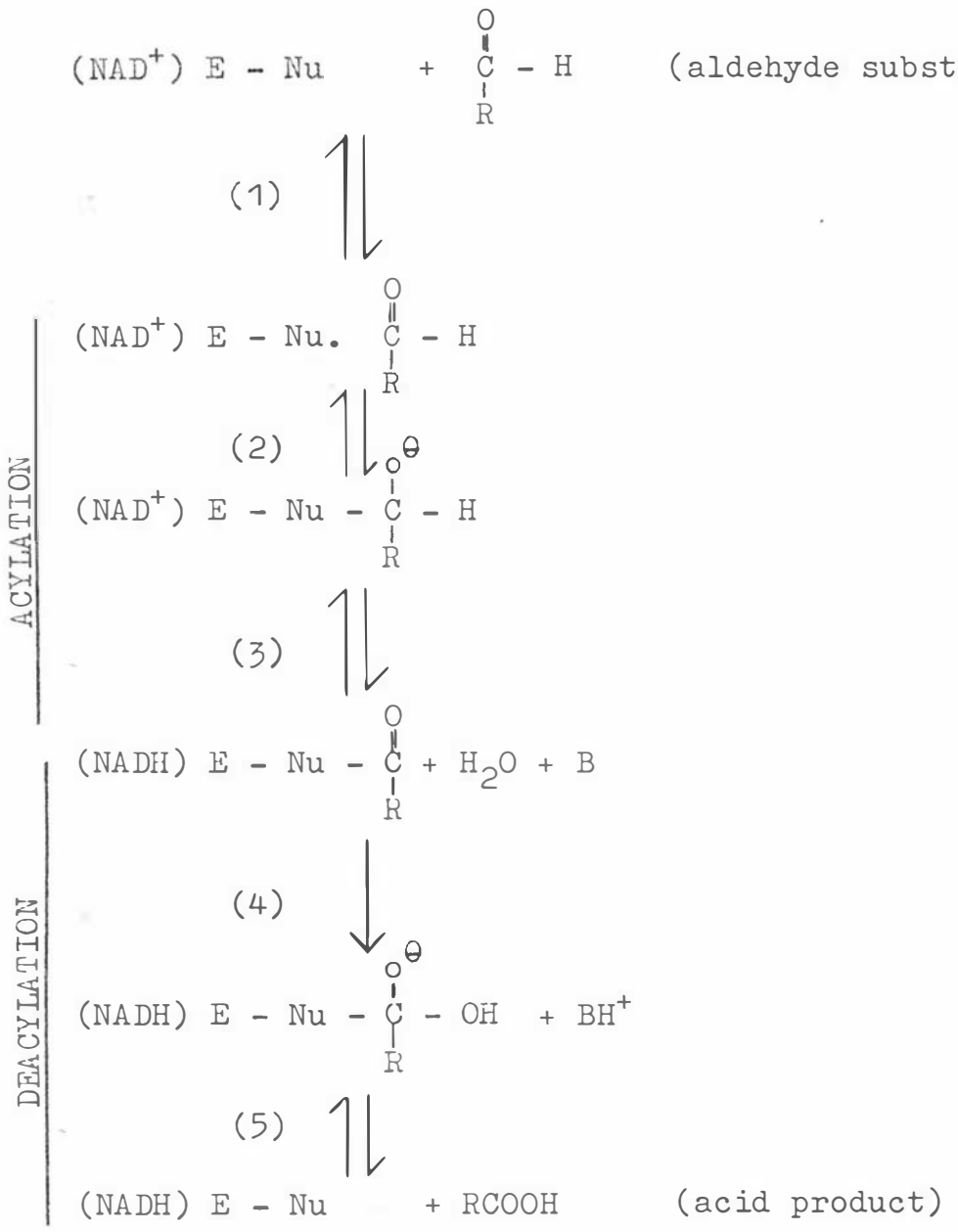
SECTION 7

CONCLUSION

In the belief that the interpretation of experimental data must be solidly based on what is observed, and that the simpler is to be preferred to the more complicated explanation, in the analysis of the presteady-state data (Section 5) the simplest reaction scheme consistent with the experimental observations was considered. The scheme used was sufficiently simple to allow a reasonably detailed analysis of the data in terms of rate constants for individual steps. While, except indirectly from the acetaldehyde isotope effect studies, evidence was not obtained for other steps in the mechanism, it is possible to speculate about other transformations which our rationalization of chemical changes in similar systems may lead us to believe are taking place.

It is possible to postulate a series of reaction steps which may be involved in the oxidation of aldehydes to carboxylic acids by the enzyme aldehyde dehydrogenase from sheep liver (Fig. 7.1). Since the reaction is ordered, NAD^+ binds to the enzyme first after which the aldehyde may associate with the enzyme at the active site (step (1)). Subsequently it is proposed that a nucleophile in the enzyme active site reacts with the aldehyde to form a covalently bonded intermediate with a tetrahedral carbon atom. The hydride transfer reaction then occurs with the formation of an acyl intermediate (step (3)) and the production of NADH. This is followed by a general base catalysed nucleophilic attack by water on the acyl intermediate to form a tetra hedral intermediate. The breakdown of this tetrahedral intermediate leads to the release of the carboxylic acid product (step (5)) and finally NADH dissociates in a two step reaction. It is reasonable to assume that the covalent reaction of the enzyme and aldehydes involves a nucleophilic group on the enzyme. Although it has been postulated by other workers that aldehyde dehydrogenase contains a thiol group in the active site (Stoppani and Milstein, 1957; Feldman and Weiner, 1972b) as the nucleophile, the existence of such a group could not be shown for the sheep liver enzyme

FIG. 7.1 POSTULATED MECHANISM OF ENZYMATIC OXIDATION OF ALDEHYDES



E enzyme (aldehyde dehydrogenase)
Nu enzyme nucleophilic group
(NAD^+) NAD^+ bound to the enzyme
B basic group on the enzyme close to the active site

using Ellman's reagent. However the reaction with the drug disulfiram (Kitson, 1975) suggests that the sheep liver enzyme may in fact contain an essential thiol group in the active site. It has been found that the enzyme glyceraldehyde 3-phosphate dehydrogenase, which catalyses the oxidative phosphorylation of D-glyceraldehyde-3-phosphate from glyceraldehyde, involves a thiol group (Harris et al., 1963). This dehydrogenase is of interest since glyceraldehyde combines with the thiol group to produce a thiol ester intermediate, and so it is possible that aldehyde dehydrogenase reacts with aldehydes in a similar manner That a nucleophile is involved in the aldehyde dehydrogenase reaction is shown by the fact that the same enzyme may act as an esterase (Section 5), catalyzing the hydrolysis of esters. The two reactions have been shown to occur at the same site on the enzyme (Feldman and Weiner, 1972b) and so any dehydrogenase sequence must be able to accommodate ester hydrolysis as well. Mechanism (1) can accommodate esterase activity since a covalent reaction of the enzyme nucleophile and an ester would form the same acyl intermediate and the subsequent reaction would be identical to the dehydrogenase reaction. Since for propionaldehyde the maximum burst rate had no isotope effect a step such as step (4) may be rate limiting in the burst. This seems reasonable as the burst showed a pH dependence (Section 5), a greater rate constant being observed at high pH, an effect which could be due to the presence of a weak acid in the enzyme active site (BH^+) which only at a high pH will be completely deprotonated and hence be able to carry out its full catalytic effect. The catalytic effect is envisaged as a general base catalysis of the hydrolysis of the acyl enzyme intermediate. The overall enzyme reaction is irreversible and in mechanism (1) it has been postulated that this irreversible step is the formation of the tetrahedral intermediate (step (4)) during deacylation of the acyl-enzyme intermediate. This postulate would seem reasonable since the hydroxyl group of the carboxylic acid is such a poor leaving group. The breakdown of this tetrahedral intermediate, by comparison of the aldehyde dehydrogenase and esterase bursts, is probably quite slow and of the same order of magnitude as the NADH displacement.

Step (5) in the reverse direction, the reaction of the carboxylic acid with the enzyme nucleophilic group, would not be expected to proceed to any significant extent since the carboxylate group does not normally undergo nucleophilic substitution reactions.

It is well recognized that oxidation of ethanol by the liver *in vivo* and *in vitro* is associated with an increase in the ratio of NADH/NAD^+ in both the cytoplasmic and mitochondrial spaces (Forsander, 1970). The perturbation of the normal NADH/NAD^+ ratio in these compartments is in turn thought to be responsible for the abnormalities of carbohydrate and fat metabolism observed after ethanol administration (Arky, 1971). Conversely the hepatic rate of ethanol oxidation is considered to be determined by the rate of reoxidation of cytosolic NADH. According to the kinetic mechanism in which the removal of NADH from the enzyme complex is the rate determining step, a high cytosolic NADH/NAD^+ ratio should decrease the flux through alcohol dehydrogenase.

This study of sheep liver cytoplasmic aldehyde dehydrogenase shows that the NADH dissociation step for this enzyme also has an effect on the overall rate of reaction and hence the activity of the enzyme may be expected to reduce when the NADH/NAD^+ ratio increases in the liver. The same would be true of the mitochondrial aldehyde dehydrogenase (Section 6). From the results for sheep liver an estimate can be made of the cytoplasmic aldehyde dehydrogenase concentration. The cytoplasmic enzyme activity of 200,000 nmoles of NADH produced per minute (Table 2.1) may be converted to 3,500 nmoles per second. Using a catalytic-centre activity of $0.2s^{-1}$ this activity would correspond to 17,500 nmoles of enzyme active sites. The weight of liver from which the cytoplasmic enzyme was extracted was 500g, and so assuming a cytoplasmic water volume of about $0.5cm^3$ per g, an enzyme concentration of 70μM is obtained. It has been reported that the concentration of alcohol dehydrogenase in horse liver is about 40μM (Reynolds, 1970). Since the catalytic-centre activity of alcohol dehydrogenase is greater than that of aldehyde dehydrogenase it is perhaps not surprising that the concentration of aldehyde dehydrogenase

is greater. It is also apparent that since the coenzyme dissociation constants of aldehyde dehydrogenase are so small (8μM and 1.2μM for NAD^+ and NADH respectively) that a large proportion of the coenzymes in the cell are going to be bound to the enzyme.

Acetaldehyde concentrations are difficult to measure since the concentrations are so low and artefacts may affect results (Truitt and Walsh, 1971). However Korsten et al. (1975) reported a blood acetaldehyde level of 20μM in patients after alcohol consumption. The level in the liver would be expected to be at least as high as the blood level and at these levels, the cytoplasmic aldehyde dehydrogenase from sheep's liver would not be functioning at its maximum rate. The Michaelis constant for the horse liver alcohol dehydrogenase catalyzing the reduction of acetaldehyde to ethanol is 100μM and this may explain why the acetaldehyde concentrations never approach the original ethanol concentrations.

Since the enzymes alcohol dehydrogenase and aldehyde dehydrogenase both require the coenzyme NAD^+ there will be competition between the enzymes in the cytoplasm for NAD^+. it has been suggested that in rat liver most of the acetaldehyde is transported to the mitochondria, where the $NADH/NAD^+$ ratio is lower, and oxidized by the mitochondrial aldehyde dehydrogenase. This is supported by the fact that most of the rat liver enzyme activity is in the mitochondria and the rat liver mitochondrial enzyme has a much lower Michaelis constant for acetaldehyde than the rat liver cytoplasmic enzyme. Neither of these observations are true for sheep liver dehydrogenase and hence it is not known which is the major acetaldehyde oxidizing compartment. The cytoplasmic enzyme has a much lower Michaelis constant for NAD^+ (Section 6) and this may negate the effect of the higher $NADH/NAD^+$ ratio in the cytoplasmic compartment on ethanol consumption. Also the cytoplasmic enzyme is more sensitive to disulfiram (Kitson, 1975) which suggests that it may have an important role in alcohol metabolism.

For the obvious reason of lack of availability of fresh livers, little work has been reported on the human liver aldehyde dehydrogenase, but it is unfortunate that no study of the subcellular distributing of the enzyme has been made.

However, the work which has been carried out (Blair and Bodley, 1969; Sidhu and Blair, 1975a) shows similarities with the sheep liver enzyme. Since remarkable similarities have been shown among the enzyme from various mammalian species (horse liver, rat liver, bovine liver and sheep liver) this suggests that the sheep liver enzyme may be a good model for investigations into human alcohol metabolism. Work has already been carried out in this department on computer simulation of the alcohol metabolism in the liver utilizing some of the kinetic parameters derived in this study.

APPENDIX I

ABBREVIATIONS

$[A]$	concentration of species A
ADP-ribose	adenosine 5'-diphosphoribose
CM	carboxymethyl
DEAE	diethylaminoethyl
Deamino-NAD^+	deamino diphosphopyridine nucleotide
E	enzyme
E_{NADH}	enzyme with NADH bound
E^{*}_{NADH}	enzyme with NADH bound (distinct from E_{NADH})
E^{NADH}_{Acid}	enzyme with NADH and acid bound
ΔF	fluorescence difference
ΔF_{max}	maximum fluorescence difference
g	gram
g	unit of gravitational field (in centrifugation)
k_{cat}	catalytic-centre activity (also known as the turnover number)
k_{obs}	observed rate constant
K_a, K_b	Michaelis constants for A, B
K_D	dissociation constant
K_{ia}	dissociation constant for A
K_{ii}	graphical intercept inhibition constant
K_{is}	graphical slope inhibition constant
λ	observed decay constant
NAD^+	nicotinamide adenine dinucleotide
NADH	dihydronicotinamide adenine dinucleotide

$NADP^+$	nicotinamide adenine dinucleotide phosphate
Nbs_2	5,5'-dithiobis(2-nitrobenzoic acid)
3-pyridinealdehyde-NAD^+	pyridine-3-aldehyde adenine dinucleotide
Q	fluorescence enhancement
R	fractional saturation of NADH binding sites
s	seconds
v	velocity
V	maximum velocity

APPENDIX II

CHEMICALS

Acetaldehyde	British Drug Houses (BDH), Poole, England
[1,2,2,2-^{2}H] Acetaldehyde	Bio-Rad Laboratories, Richmond, California
ADP-ribose	Sigma Chemical Company, Saint Louis, Missouri
Barbitone-sodium	Hopkin and Williams Ltd., Essex, England
α-Bromophenylacetic acid	Chemical Procurement Laboratories, New York
p-Carboxybenzaldehyde	Chemical Procurement Laboratories
Chloral hydrate	BDH
1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide methyo-p-toluene sulphonate	Aldrich Chemical Company, Milwaukee, Wisconsin
Deamino-NAD^{+}	Sigma Chemical Company
3,3'-Diaminodipropylamine	Aldrich Chemical Company
p-Dimethylaminocinnamaldehyde	Grade II, Sigma Chemical Company
2,2'Dipyridyl	BDH
5,5'-Dithiobis(2-nitrobenzoic acid)	Sigma Chemical Company
EDTA	BDH
2-Mercaptoethanol	Fluka AG, Buchs, Switzerland
NAD^{+}	Grade III, Sigma Chemical Company
NADH	Grade III, Sigma Chemical Company
$NADP^{+}$	Sigma Chemical Company
1,10-Phenanthroline	Hopkin and Williams Ltd.

Propionaldehyde	Koch-Light Laboratories, Buckinghamshire, England
Protion resin	Tasman Vaccine Laboratories, Wellington, New Zealand
Pyrazole	gift from Dr. M.R. Grimmet, University of Otago
3-Pyridinealdehyde-NAD^+	Sigma Chemical Company
Quinine sulphate	BDH
Semicarbazide hydrochloride	May and Baker, Dagenham, Essex, England
Sodium pyrophosphate	BDH

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