STRUCTURAL AND MECHANISTIC STUDIES OF SHEEP LIVER ALDEHYDE DEHYDROGENASE

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fulfilment of the requirements
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Rosemary Lynne MOTION
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ABSTRACT

Studies of NADH displacement in the presence of excess aldehyde dehydrogenase confirmed that a conformational change of the enzyme.NADH binary complex occurs as an essential step in the reaction mechanism.

Modification of sulphydryl groups on the enzyme by the thiol reagent p-(chloromercuri)benzoate (PCMB) produced either activation or inhibition of the enzyme activity, depending upon the relative concentrations of PCMB and aldehyde dehydrogenase, the mixing conditions, and on the concentration of the aldehyde substrate. There was no direct evidence to support the widely held view that a sulphydryl group is catalytically essential.

Studies of the pH dependence of the steady state and presteady state phases of the reaction indicated that there was a change in the rate limiting step as the pH was increased from acyl-enzyme hydrolysis at low pH to release of NADH from the enzyme at high pH. At low pH the release of NADH may occur before acyl-enzyme hydrolysis. Activation by high concentrations of propionaldehyde was shown to occur over the entire pH range (5 to 10).

The reaction could be reversed when acid anhydrides were used to acylate the enzyme.NADH complex but the binding of the substrates for the reverse reaction did not appear to be ordered. Under these conditions other groups on the enzyme were acylated with resultant inhibition or activation of the dehydrogenase activity of the enzyme depending on the relative concentrations of the substrates and reactants, and on the mixing conditions.

The enzyme catalysed the hydrolysis of p-nitrophenylacetate in the presence of NADH but with no significant production of acetaldehyde. It was concluded that ester hydrolysis does not occur at the site of aldehyde oxidation.

Preliminary studies on the reaction of the enzyme with diethylpyrocarbonate indicated that the enzyme may contain a catalytically essential histidine residue.

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INTRODUCTION

At least three enzymes are thought to be involved in the oxidation of acetaldehyde in the body, the flavoproteins xanthine oxidase (EC 1.2.3.1) and aldehyde oxidase (EC 1.2.3.2) and the NAD^+-dependent aldehyde dehydrogenase (EC 1.2.1.3). The first two of these enzymes have been shown to have much lower affinities for aldehydes (Rajogopalan & Handler, 1964, Mackler et al., 1954) than aldehyde dehydrogenase (Buttner, 1965, Feldman & Weiner, 1972, Crow et al., 1974), which is therefore considered to be the most important enzyme involved in the metabolism of acetaldehyde in mammals.

Aldehyde dehydrogenase was first isolated from bovine liver by Racker (1949), who reported that the enzyme was capable of oxidising a wide variety of aldehydes. It has been demonstrated that, in addition to oxidation of acetaldehyde produced from ethanol following alcohol consumption, aldehyde dehydrogenase is involved in the oxidation of aldehydes arising from biogenic amines (Erwin & Dietrich, 1966). The probable importance of aldehyde dehydrogenase in the oxidation of aldehydes arising from metabolic processes other than those following alcohol consumption is indicated by the distribution of this enzyme in the body. Whereas alcohol dehydrogenase (EC 1.1.1.1), the major enzyme responsible for the conversion of ethanol to acetaldehyde, is found mainly in the liver, aldehyde dehydrogenase has been shown to be widely distributed throughout the body, with 70% in the liver, 10% in the kidneys, 7% in the adrenal glands, 4% in the small intestine, and 2% in the brain (Dietrich, 1966).

Aldehyde dehydrogenases have now been isolated and purified to homogeneity from a number of mammalian sources, including horse liver (Feldman & Weiner, 1972, Eckfeldt & Yonetani, 1976), bovine liver (Sugimoto et al., 1976, Leicht et al., 1978), rat liver (Shum & Blair, 1972, Tottmar et al., 1973), rabbit liver (Duncan, 1977), sheep liver (Crow et al., 1974, MacGibbon et al., 1979, Hart & Dickinson, 1977) and human liver (Greenfield & Pietruszko, 1977, Kraemer & Dietrich, 1968).

Sheep liver aldehyde dehydrogenase has been extensively studied since it was first isolated by Crow et al. (1974). The enzyme has been shown to be present in sheep liver as three distinct isoenzymes, one located in the mitochondria, one in the cytoplasm, and the other in the microsomes (Crow et al., 1974). Most of the enzyme is present, in

approximately equal proportions, in the mitochondria and the cytoplasm.

Extensive studies of the kinetics of cytoplasmic aldehyde dehydrogenase have been carried out by MacGibbon et al. (1977a,b,c, 1978b), Bennett et al. (1982, 1983), Blackwell et al. (1983a,b), Buckley et al. (1982), and Deady et al. (1985) in this laboratory, and also by Hart & Dickinson (1978a, 1982, 1983), Dickinson and Hart (1982), Dickinson (1985), and Buckley & Dunn (1982, 1985). MacGibbon et al. (1977a,b,c) demonstrated that the enzyme operates by an ordered mechanism with NAD^+ binding before aldehyde and the steady state appears to be at least partly controlled by the rate of dissociation of NADH from the binary E.NADH complex. From stopped flow studies of the enzyme catalysed reaction Bennett et al. (1982) showed that a conformational change of the enzyme, which follows aldehyde binding (with simultaneous release of a proton) is rate limiting in the presteady state phase of the reaction. A more detailed summary of the established work is given in Chapter 2.

Cytoplasmic aldehyde dehydrogenase is considered to be a good model for studies of human aldehyde metabolism. Kitson (1975, 1978, 1981) has shown that the enzyme is very sensitive to disulphiram (antabuse), a drug which is used in the "aversion therapy" treatment of alcoholism. Administration of this drug has been shown to result in elevated acetaldehyde levels if alcohol is consumed, producing symptoms very similar to those of a hangover. In addition the enzyme is stable and easily isolated from sheep livers which are always in plentiful supply in New Zealand.

Despite very extensive studies of the kinetics of aldehyde dehydrogenases from mammalian sources, the nature of the reactive group in the active site has not yet been identified. A proposal by Jakoby (1963), based on supposed similarities between aldehyde dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, that a sulphydryl group may be involved at the active site, has not yet been verified. However, despite a lack of unequivocal evidence to support this suggestion, it has frequently been tacitly assumed (Hart & Dickinson, 1978a, Dickinson, 1985, Duncan, 1979, 1985) that oxidation of aldehydes proceeds by means of an enzyme-thiol-acyl complex.

Oxidation of aldehydes by aldehyde dehydrogenase is normally irreversible. However, Hart & Dickinson (1978a) demonstrated that it was possible to reverse at least a part of the process by using simple acid anhydrides instead of acetic acid, and showed that oxidation of

NADH occurred.

In this study, the mechanism of aldehyde dehydrogenase catalysed oxidation of aldehydes is investigated by an examination of the pH and buffer system dependence of the steady state and presteady state phases of the reaction, and by an in-depth investigation of the reverse reaction using a number of anhydrides. In addition, the effect of modification by thiol reagents of reactive sulphydryl groups on the enzyme activity is examined to elucidate the role (if any) of sulphydryl groups in the enzyme catalytic activity.

CHAPTER 1

PURIFICATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE FROM SHEEPS LIVER

1.1 INTRODUCTION

Aldehyde dehydrogenase (EC 1.2.1.3) was first isolated by Racker (1949) from bovine liver. Most early studies utilized crude enzyme preparations from acetone powders (Dietrich et al., 1962, Freda and Stoppani, 1970, Duncan & Tipton, 1971). Aldehyde dehydrogenase from yeast was purified to homogeneity by Clark & Jakoby (1970) and the first homogeneous preparation of a mammalian aldehyde dehydrogenase was reported by Feldman & Weiner (1972), utilising ammonium sulphate precipitation and ion exchange chromatography. Dramatic improvements in the yield, purity and stability of aldehyde dehydrogenase resulted from the introduction to the purification procedure of more extensive column chromatography techniques, first reported by Shum & Blair (1972)

In typical purification procedures, the tissue from which the enzyme is to be extracted is first homogenised. Centrifugation to remove insoluble cellular debris follows, and then ammonium sulphate precipitation is used to isolate a fraction containing the aldehyde dehydrogenase activity. Finally a variety of chromatographic columns, generally including both ion exchange and gel filtration methods, are used to complete the purification of the enzyme.

The utilization of improved purification procedures led to the discovery of at least two isoenzymes in mammalian livers, one located in the mitochondria, and the other in the cytoplasm. These isoenzymes have subsequently been shown to have somewhat different kinetic properties (MacGibbon et al., 1978a Hart & Dickinson, 1977, 1978b) and different susceptibility to certain modifiers, such as tetraethylthiuram disulphide (disulphiram) so that separation of the isoenzymes is a necessary prerequisite for meaningful kinetic studies.

Methods used for the separation of the isoenzymes vary, depending on the species from which the aldehyde dehydrogenase is obtained. The two isoenzymes usually have different affinities for DEAE cellulose resins and separation methods based on this difference have been utilized by Feldman & Weiner (1972) and Eckfeldt et al. (1976) for the isolation of aldehyde dehydrogenases from horse liver. Sugimoto et al. (1976) used differential centrifugation to separate the isoenzymes from beef liver and affinity chromatography has been used by Greenfield &

Pietruszko (1977) to separate the isoenzymes of human liver aldehyde dehydrogenase.

Crow et al. (1974) have isolated two isoenzymes of aldehyde dehydrogenase from sheep liver using similar procedures and more recently purification procedures for the cytoplasmic enzyme, using improved column techniques, have been reported by MacGibbon et al. (1979) and by Dickinson et al. (1981).

Cytoplasmic aldehyde dehydrogenase from sheep liver was prepared for use in this study initially by the method of MacGibbon et al. (1979), but some alterations and refinements of this method were made, in conjunction with co-workers in this laboratory, during the course of this study. However, the basic principles involved remain the same and both the initial method and all subsequent alterations are described in this chapter.

Separation of the cytoplasmic enzyme from the mitochondrial isoenzyme is achieved by removal of the mitochondria by centrifugation, following homogenisation methods chosen to cause as little mitochondrial rupture as possible and with the addition of sucrose to the homogenising buffer to prevent mitochondrial swelling.

1.2 METHODS

1.2.1 Buffers

Phosphate buffers were prepared from A.R. grade potassium dihydrogen phosphate and adjusted to the appropriate pH using NaOH, as described by Dawson et al. (1969). Buffer strengths are expressed in terms of phosphate concentration and all buffers used in the preparative procedures contained 0.1% vol/vol 2-mercaptoethanol.

1.2.2 Enzyme Assays

Assays for aldehyde dehydrogenase and various contaminant enzymes were performed as follows: Initial reagent concentrations in the cuvette at the start of the assay are given in parentheses, and the total volume was 3.0 cm^3 in each case.

(1) Aldehyde Dehydrogenase

2.20 cm^3 of 35 mM phosphate buffer, pH 7.6	(25 mM)
0.45 cm^3 of 16 mM NAD^+ solution	(1.0 mM)
0.25 cm^3 of 240 mM propionaldehyde solution	(20 mM)
0.10 cm^3 of enzyme solution	

Reaction was initiated by addition of propionaldehyde to a cuvette containing the other components which had been thermally equilibrated at 25°C. Reaction progress was followed by monitoring the production of NADH, observed as an increase in absorbance at 340 nm.

In cases in which the sample was suspected to contain alcohol dehydrogenase, pyrazole (0.5 mM), a potent inhibitor of alcohol dehydrogenase (but which does not affect aldehyde dehydrogenase activity) was added to the assay mixture.

(2) Lactate Dehydrogenase

2.2 cm^3 of 35 mM phosphate buffer, pH 7.6	(28 mM)
0.2 cm^3 of 2.4 mM NADH solution	(160 µM)
0.5 cm^3 of 5.0 mM sodium pyruvate solution	(83 µM)
0.1 cm^3 of sample	

The NADH solution was prepared in 0.035 M phosphate buffer pH 7.6 and the reaction was initiated by the addition of the pyruvate solution. The reaction was followed by monitoring the decrease in NADH absorbance at 340 nm.

(3) Alcohol Dehydrogenase

2.5 cm^3 of 35 mM phosphate buffer, pH 7.6	(31 mM)
0.2 cm^3 of 2.4 mM NADH solution (as above)	(160 µM)
0.2 cm^3 of 32 mM acetaldehyde solution	(12 µM)
0.1 cm^3 of sample	

The reaction was initiated by the addition of aldehyde, and was monitored by following the disappearance of NADH at 340 nm.

(4) Glutamate Dehydrogenase

Glutamate dehydrogenase was assayed according to the method of Robins et al. (1956).

2.0 cm^3 of 50 mM pH 8.0 triethanolamine-HCl buffer	(33 mM)
0.15 cm^3 of 3.0mM NADH in buffer as above	(150µM)
0.03 cm^3 of 10% w/v EDTA	(0.1%)
0.10 cm^3 of 3.0 M ammonium acetate	(0.1 M)
40 µl of 0.4 M α-ketoglutarate	(533 µM)
10 µl of crude enzyme sample	
water to a total volume of 3.0 cm^3	

Reaction was initiated by the addition of α-ketoglutarate and the disappearance of NADH absorbance at 340 nm was monitored.

1.2.3 Enzyme Purification Procedure: Method 1

Livers obtained from freshly slaughtered sheep were placed directly in ice and the purification was commenced within one hour. All preparative procedures were carried out at 4°C. 500 g of sliced liver was homogenised in 1.8 l of 0.005 M pH 7.3 phosphate buffer containing 0.25 M sucrose, using a glass-teflon Potter-Elvehjem homogenizer fitted to an electric motor. The homogenate was then centrifuged at 13,800x**g** for one hour using a Sorvall RC2B Centrifuge. The precipitate was discarded and the supernatant was strained through glass wool to remove fat, then recentrifuged for two hours at 34,000x**g** to sediment the mitochondria. The precipitate was again discarded.

Finely powdered ammonium sulphate (low in heavy metals) was added to the supernatant over 30 minutes with stirring to give 45% saturation (258 g/l at 4°C). The mixture was equilibrated with further stirring for 30 minutes, then centrifuged for 15 minutes at 12,500x**g** and the precipitate discarded. Further ammonium sulphate was added to give 70% saturation (156 g/l) and after a further 30 minutes equilibration the mixture was again centrifuged for 15 minutes at 12,500x**g**.

The precipitate was then dissolved in 250 cm^3 0.005 M pH 7.3 phosphate buffer with a conductivity of 360 $\mu\Omega^{-1}$ at 4°C and dialysed against successive 20 litre changes of the same buffer until the conductivity reached 360 $\mu\Omega^{-1}$. Normally about 36 hours were required for this, with buffer changes every two hours during the day. After 30

hours the pH of the crude enzyme solution was adjusted to pH 7.3 with 0.1 M NaOH.

When the conductivity reached 360 $\mu\Omega^{-1}$, any precipitate was removed by centrifugation (5 minutes at 12,000x**g**), and the enzyme sample was loaded on to a Whatman DE 52 cellulose column (20 x 5 cm) pre-equilbrated with dialysis buffer. The column was washed with 0.005 M pH 7.3 phosphate buffer until the absorbance at 254 nm was less than 0.1 (measured by connecting the column outlet to an LKB 8300 uvicord ultraviolet monitor). It was then eluted with 0.022 M pH 7.3 phosphate buffer at an approximate rate of 1 cm^3/min. and collected on an LKB ultrorac 7000 fraction collector. Aldehyde dehydrogenase first appeared after about two hours and about four hours were required to completely elute the enzyme. The fractions were assayed for aldehyde dehydrogenase and lactate dehydrogenase activity and those fractions containing significant quantities of lactate dehydrogenase and those of a greenish colour (indicating the presence of catalase) were discarded. The fractions containing aldehyde dehydrogenase were combined and the volume reduced from approximately 300 cm^3 to 6-10 cm^3 over a period of 4-8 hours using a diaflo ultrafiltration apparatus with an Amicon XM 100 filter. This filter retains all species with a molecular weight greater than 100,000.

The concentrated sample was centrifuged for five minutes at 6,000x**g** to remove solid material and then loaded onto a Biogel 0.5 M gel filtration column (5 x 35 cm) and eluted with 0.022 M pH 7.3 phosphate buffer. The eluate was collected on the LKB fraction collector described previously, at a rate of about 8-10 cm^3 per hour. Enzyme was normally detected after about 30 hours and completely eluted after a further 5-6 hours. The enzyme samples were then sealed with parafilm and stored at 4°C until required.

If longer storage was required samples were either stored under nitrogen in sealed containers or frozen. Enzyme could only be successfully frozen if the aldehyde dehydrogenase concentration was greater than about 90 μM or for enzyme of lower concentration if 50% v/v glycerol was added.

1.2.4 Enzyme Purification Procedure: Method 2

The following alternative method was developed as new equipment and different chromatographic materials became available. Several changes to the original method were made in order to reduce the time required for the purification procedure. These included:

(a) Homogenization by a sonication technique to replace the Potter homogenizer.
(b) Some shortening of the initial centrifugation times.
(c) Use of polyethylene glycol as the precipitating agent.
(d) Use of a Sephacryl S 300 gel filtration column to replace the Biogel column.
(e) Use of additional optional chromatographic methods (affinity chromatography and pH-gradient ion exchange chromatography) to further purify the enzyme after gel filtration, when necessary.

1.2.4.1 Homogenisation Procedure

(C. Bishop, unpublished results)

500 g sliced liver was divided into 3 batches and each was homogenized by sonication for 30 seconds in 300 cm^3 of 0.005 M phosphate buffer containing 0.25 M sucrose and 0.1% 2-mercaptoethanol using a Janke & Kunkel Ultra Turrax T45 sonicator.

1.2.4.2 Centrifugation

The homogenate was centrifuged for 15 minutes at 13,800x**g** to remove cell wall material, whole cells and connective tissue, and the precipitate was discarded. The supernatant was strained through glass wool as described previously to remove fat, and then recentrifuged at 34,000x**g** for one hour to sediment the mitochondria. The precipitate was discarded.

1.2.4.3 Precipitation of Enzyme using Polyethylene Glycol

(C. Bishop and L. Myers, unpublished results)

After the centrifugation procedures outlined above, the supernatant volume was made up to 1500 cm^3 with 0.005 M phosphate buffer containing 0.25 M sucrose and powdered A.R. grade polyethylene glycol 6000 was added over 30 minutes with stirring to give a mixture containing 12% weight by volume (120 g/l) polyethylene glycol. Stirring was continued for a further 30 minutes to allow equilibration,

followed by centrifugation for 15 minutes at 12,500x**g**. The precipitate was discarded. Further polyethylene glycol was added to the supernatant as before, to increase the concentration to 20% w/v (a further 80 g/l) and the mixture equilibrated for 30 minutes. Following centrifugation for 15 minutes at 12,500x**g**, the precipitate was redissolved in 300 cm^3 of 0.005 M pH 7.3 phosphate buffer with a conductivity of 360 $\mu\Omega^{-1}$. This solution was loaded directly on to a 5 x 20 cm Whatman DE 52 or DE 32 column pre-equilibrated with the same buffer which was then washed and eluted as described above.

1.2.4.4 Gel Filtration using a Sephacryl S-200 or S-300 Column

Enzyme solution was concentrated to a volume of 6-10 cm^3 by ultrafiltration as described above, or in a few cases by a 0-70% ammonium sulphate precipitation, and then loaded on to a 5 x 35 cm column of Sephacryl S-300 Superfine (Pharmacia Chemicals, Uppsala, Sweden) or alternatively a 3 x 50 cm column of Sephacryl S-200 Superfine. S-200 separates proteins of molecular weights in the range 5,000-250,000, whereas S-300 is used for the molecular weight range 10,000-1,500,000. The enzyme was eluted with 0.022 M pH 7.3 phosphate buffer at a rate of 10-12 cm^3 per hour. Fractions were collected as described previously and assayed for aldehyde dehydrogenase, lactate dehydrogenase and alcohol dehydrogenase activity.

1.2.4.5 pH-Gradient Ion Exchange Chromatography
(Dickinson et al., 1981)

A solution of enzyme in 0.010 M pH 6.5 bistris (2-[2-bis(hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol) buffer was prepared, either by dialysis of the enzyme sample against this buffer, or by the addition of a large volume of the buffer (approx. 200 cm^3) to a smaller volume (about 30 cm^3) of enzyme solution to lower the conductivity to that of the bistris buffer. The enzyme was then loaded on a 2 x 16 cm column of Pharmacia DEAE Sephacel which had been equilibrated with the same buffer. All buffers contained 0.1% 2-mercaptoethanol.

The enzyme was eluted using a pH gradient produced by running 400 cm^3 of 0.010 M pH 4.6 sodium acetate buffer into 400 cm^3 of pH 6.5 bistris buffer. Fractions of 8-10 cm^3 were collected using the apparatus described previously and the pH of each fraction measured using a Radiometer 28 pH meter. Fractions were assayed for aldehyde

and lactate dehydrogenase activity and those samples containing aldehyde dehydrogenase were adjusted to pH 7.3 for storage.

1.2.4.6 Affinity Chromatography

(A. Bennett, unpublished results)

The enzyme sample was first dialysed against 0.030 M pH 6.0 phosphate buffer. Any material which precipitated was removed by centrifugation and the sample was loaded on a 1 x 20 cm Pharmacia 5'AMP-Sepharose 4B column pre-equilibrated with the same buffer. This column has an affinity for enzymes requiring NAD^+ or ATP as cofactors, including most NAD^+-dependent dehydrogenases. The column was washed with 100-200 cm^3 0.030 M pH 6.4 phosphate buffer, then eluted with 0.030 M pH 8.0 phosphate buffer containing 3 mM NAD^+ (normally 50 cm^3 was required).

Alternatively, 0.030 M pH 7.0 phosphate buffer containing 0.2 M sodium oxalate was used for the dialysis, equilibration and washing stages. The enzyme was then eluted with the same buffer containing 3 mM NAD^+.

In each case, the column was recycled by washing with 6.0 M guanidine hydrochloride, followed by distilled water.

1.2.5 SDS Gel Electrophoresis

Slab gel electrophoresis was carried out using an Ortec 4200 Electrophoresis System. A single layer 15% acrylamide gel containing 1% sodium dodecylsulphate (SDS) was run in 0.1 M pH 8.9 tris glycine buffer containing 1% SDS. Enzyme samples were prepared by incubation of a sample containing enzyme, SDS (1%) and 2-mercaptoethanol (5%) at 100°C for 5 minutes. Bromophenol blue was added as a dye marker.

Following electrophoresis (2-3 h at 150-200 V, 8 mA) the gel was stained using a Coomassie Blue stain prepared by the method of Blakesley & Boezi (1977). This stained only the protein material and therefore the gel did not require destaining.

1.2.6 Protein Determinations

The determination of the protein concentration in the purified enzyme sample was by measurement of the absorbance at 280 nm, using a value of 11.3 for $A^{1\%}_{1cm}$ (Dickinson et al., 1981). Prior to the publication of this value, protein determinations were by the method of Lowry (1951), or by the Coomassie blue protein estimation method of Bradford (1976).

1.3 RESULTS

1.3.1 Homogenisation and Centrifugation

A smooth homogenate of sheep liver was produced by the Ultra-Turrax, within 30 seconds, running at half speed. Following two centrifugation steps as described above (1.2.4.2), the supernatant was shown to contain not more than 2% of the mitochondrial marker enzyme, glutamate dehydrogenase, as compared with the original homogenate.

Shortening of the centrifugation times had no detectable effect on the quality of the enzyme product. Glutamate dehydrogenase activity in the resulting supernatant was not significantly higher than previously observed for longer spin times.

1.3.2 Precipitation using Polyethylene Glycol (PEG)

Precipitation of protein material using 12% PEG produced a red-coloured supernatant after centrifugation. Aldehyde dehydrogenase activity in the precipitate was negligible and catalase was removed by this precipitation, since no catalase activity was detected in the supernatant.

When the PEG concentration was increased to 20%, most of the aldehyde dehydrogenase was precipitated, and a viscous red-coloured precipitate and straw-coloured supernatant were obtained after centrifugation. This indicated a different precipitation pattern to that observed for ammonium sulphate precipitations, in which case a greenish precipitate containing catalase and aldehyde dehydrogenase and a red coloured supernatant were obtained.

Although some aldehyde dehydrogenase activity was sometimes

detected in the supernatant, attempts to recover more enzyme by increasing the PEG concentration in the second precipitation step to 21.5% were unsuccessful, because of problems encountered with the DEAE cellulose column as a result of the high viscosity of the redissolved precipitate. As the supernatant volume was large, (1.5 litre), even a low residual activity of aldehyde dehydrogenase in the supernatant represented loss of considerable amounts of enzyme at this stage. To minimise this loss, attempts were made to keep the PEG precipitation steps as consistent as possible between different preparations. The initial 12% PEG precipitation step was always carried out at constant volume (1.5 l) and the times taken for the addition of PEG and the subsequent equilibration were always the same. Despite these precautions, there was some variation in the results for the 20% precipitation. It is considered that some of the variability in the final yield of enzyme is a direct result of this precipitation step.

1.3.3 DEAE Cellulose Column

When ammonium sulphate was used as the precipitating agent, protein material (which included catalase and aldehyde, lactate and alcohol dehydrogenases) was observed to bind readily to the top 2-4 cm of the column throughout the washing procedure. Red coloured protein material (presumably haeme-containing proteins) was washed off the column with the 0.005 M buffer.

The increase in buffer concentration used was sufficient to readily elute the aldehyde dehydrogenase, with the green catalase-containing band being slightly retarded. As catalase could not be separated from aldehyde dehydrogenase by gel filtration (MacGibbon, 1976), any fractions of a greenish colour were discarded and as a result some active fractions were lost. Figure 1.1a shows the elution profile of enzyme on the DEAE cellulose column and the point at which the green band started to appear off the column.

When PEG was used as the precipitating agent, and the redissolved enzyme extract was loaded directly on to the DEAE cellulose column without prior dialysis, the enzyme was again observed to bind readily to the top of the column, together with other protein material. The bulk of the red coloured material was again washed off with 0.005 M buffer but, quite frequently, a slow-moving band of bright red material was observed. This usually formed a sharp band which was only eluted

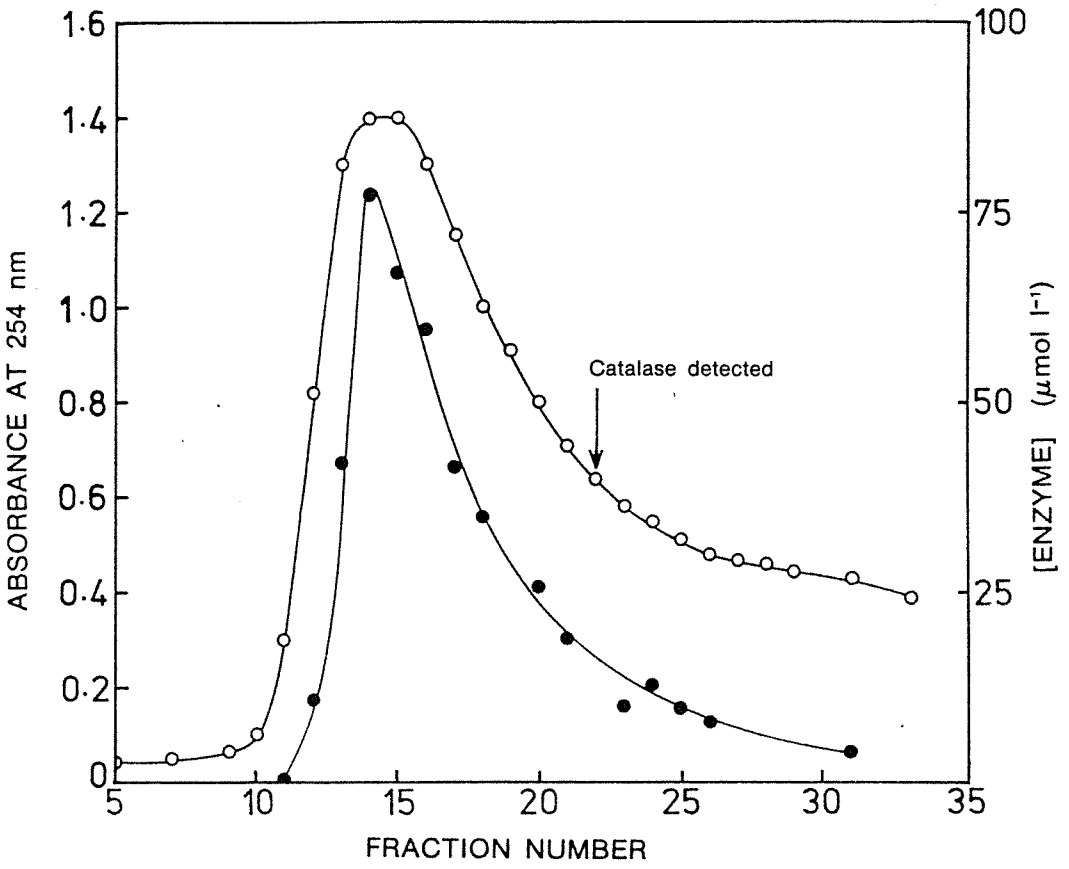
Figure 1.1 Approximately 250 g DE 52 was used in each column. After loading the crude enzyme sample, unwanted protein material was eluted with 5 mM pH 7.3 phosphate buffer for 16-20 hours at a flow rate of 1-2 cm^3/min. The buffer concentration was then increased to 22 mM. Fractions were collected following the increase in buffer concentration in each case.
(o) Absorbance at 254 nm (arbitrary units) obtained from the uvicord output trace.
(●) Concentration of aldehyde dehydrogenase (μmoles active sites/l).

1.1(a) 450 cm^3 of crude enzyme sample was applied to the column and 8 cm^3 fractions were collected. Catalase was first detected in the marked fraction.

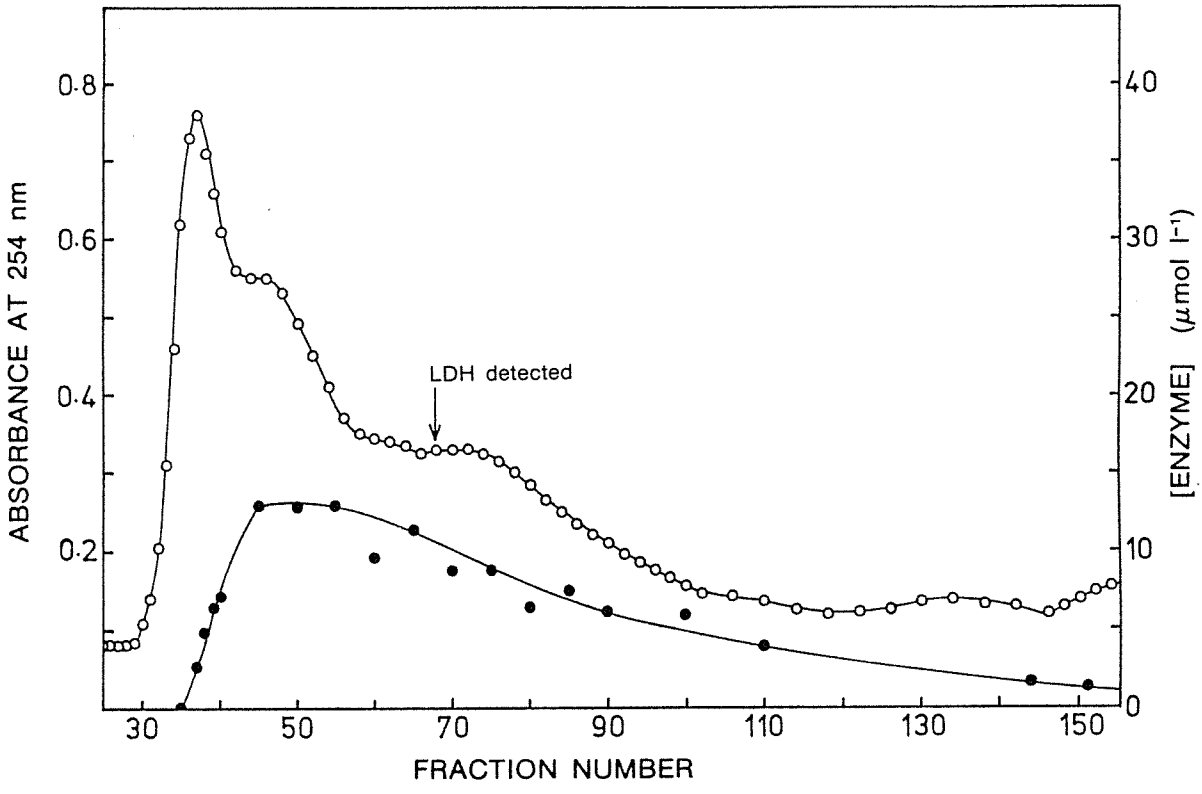
1.1(b) 400 cm^3 of crude sample was applied to the column and 8 cm^3 fractions were collected. Lactate dehydrogenase (LDH) was first detected in the marked fraction.

Figure 1:1 ELUTION PROFILE OF ALDEHYDE DEHYDROGENASE ON DEAE CELLULOSE COLUMN

(a) AMMONIUM SULPHATE PRECIPITATION



(b) POLYETHYLENE GLYCOL PRECIPITATION



very slowly, considerably increasing the time required for this washing procedure.

As catalase was removed during the PEG precipitation stages, the green band was absent from the material bound to the column, which in this case was a brownish orange colour. When the buffer concentration was increased to elute the enzyme, this coloured material was eluted with the aldehyde dehydrogenase.

The elution profile for enzyme obtained by PEG precipitation is shown in Figure 1.1b. A considerable broadening of the elution profile was observed when PEG was used. Elution of all the enzyme took about twice as long as previously, and a greater volume of enzyme solution was obtained, which increased the time required for ultrafiltration. MacGibbon (1976) reported that lactate and alcohol dehydrogenases were slightly retarded on the column, moving with the catalase band and eluting just after most of the aldehyde dehydrogenase. Similar elution patterns were observed when PEG was used, thus it was not advantageous to increase the rate at which the enzyme eluted from the column by increasing the concentration of the elution buffer, as this resulted in significant contamination of the enzyme by lactate and alcohol dehydrogenases. However, it must be noted that in a few cases, the enzyme did not elute with 0.022 M buffer and a small increase in buffer concentration to 0.025 M was required.

In most cases, lactate dehydrogenase activity was observed close to the point marked on the profile (Figure 1.1b), and fractions containing lactate dehydrogenase were discarded. When more significant contamination occurred, the fractions were not discarded and attempts were made to remove the lactate dehydrogenase at a later stage.

When a 21.5% precipitation was attempted, much of the enzyme was washed off the column, presumably because of overloading. This appeared to be due to the high viscosity of the enzyme extract, rather than the total amount of protein, as normally all enzyme bound to the top 10-15% of the column, indicating that the column capacity should have been quite sufficient, even for a two or three fold increase in the total amount of protein.

1.3.4 Gel Filtration

Elution profiles for the Biogel column are shown in Figure 1.2 for the two precipitation methods. When PEG precipitation was used (Figure 1.2b) extra protein material was observed, preceding the aldehyde dehydrogenase. This was the brownish-orange material which eluted together with the enzyme from the DEAE cellulose column. Poor separation of the two species was achieved.

When a Sephacryl S-200 column was used, separation was improved considerably (Figure 1.3) but aldehyde dehydrogenase fractions still retained some colour. The molecular weight of aldehyde dehydrogenase of 212,000 ± 8000 (MacGibbon et al., 1979) is close to the maximum (250,000) for this gel, so S-300 was used once it became available. Figure 1.4a shows the elution profile for enzyme (PEG precipitation) on S-300 and it is apparent that separation of the coloured material was considerably improved. However, it was observed that separation deteriorated unless the column was repacked regularly (after every 2-3 runs). Generally the active fractions obtained from the S-300 column contained very little coloured material, the peak fractions being only a very pale straw colour, as for enzyme prepared by ammonium sulphate precipitation.

The separation of lactate and alcohol dehydrogenases from aldehyde dehydrogenase on the S-300 column was not greatly improved over that reported by MacGibbon (1976) for the Biogel column. Figure 1.4b shows the elution profile for an enzyme sample from which these contaminating enzymes were not removed during elution of the DEAE cellulose column as described previously. Only the early fractions were not contaminated by other dehydrogenases. However, it must be noted that although the activity of the contaminating enzymes was high, they both react much faster than aldehyde dehydrogenase and thus the actual protein concentrations of these species was in fact small. As only the most active enzyme fractions were used for kinetic experiments, contamination by alcohol dehydrogenase was not a problem. Fractions contaminated with lactate dehydrogenase were purified using the AMP Sepharose column.

Elution of aldehyde dehydrogenase from the Sephacryl S-300 column was usually completed within 24 hours.

Figure 1.2 Enzyme samples were concentrated by ultrafiltration, centrifuged to remove sediment, and applied to the column which was then eluted with 22 mM pH 7.3 phosphate buffer at a rate of 10-12 cm^3/hour.

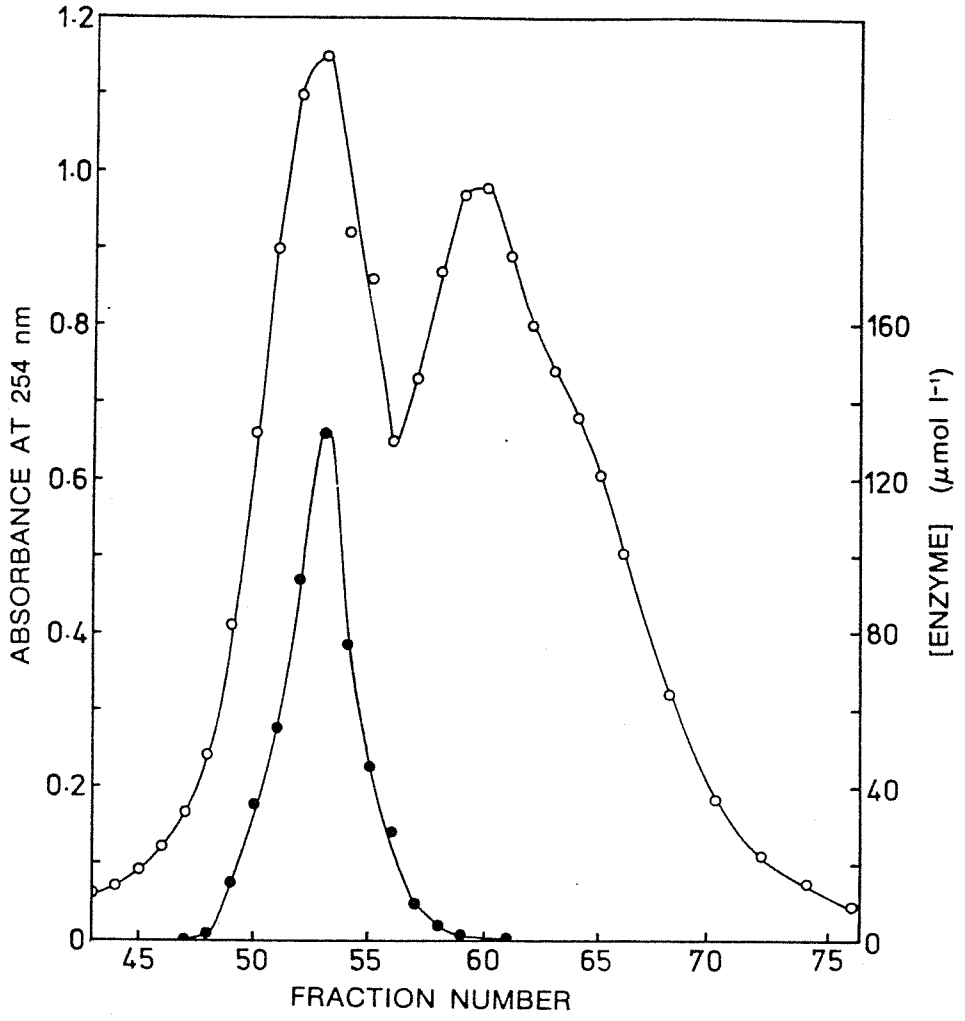
(o) Absorbance at 254 nm (arbitrary units) obtained from the uvicord output trace.

(●) Concentration of aldehyde dehydrogenase (μmoles active sites/l).

In both cases a sample of 15 cm^3 was loaded and approximately 8 cm^3 fractions were collected.

Figure 1:2 ELUTION PROFILE OF ALDEHYDE DEHYDROGENASE ON BIOGEL FILTRATION COLUMN

(a) AMMONIUM SULPHATE PRECIPITATION



(b) POLYETHYLENE GLYCOL PRECIPITATION

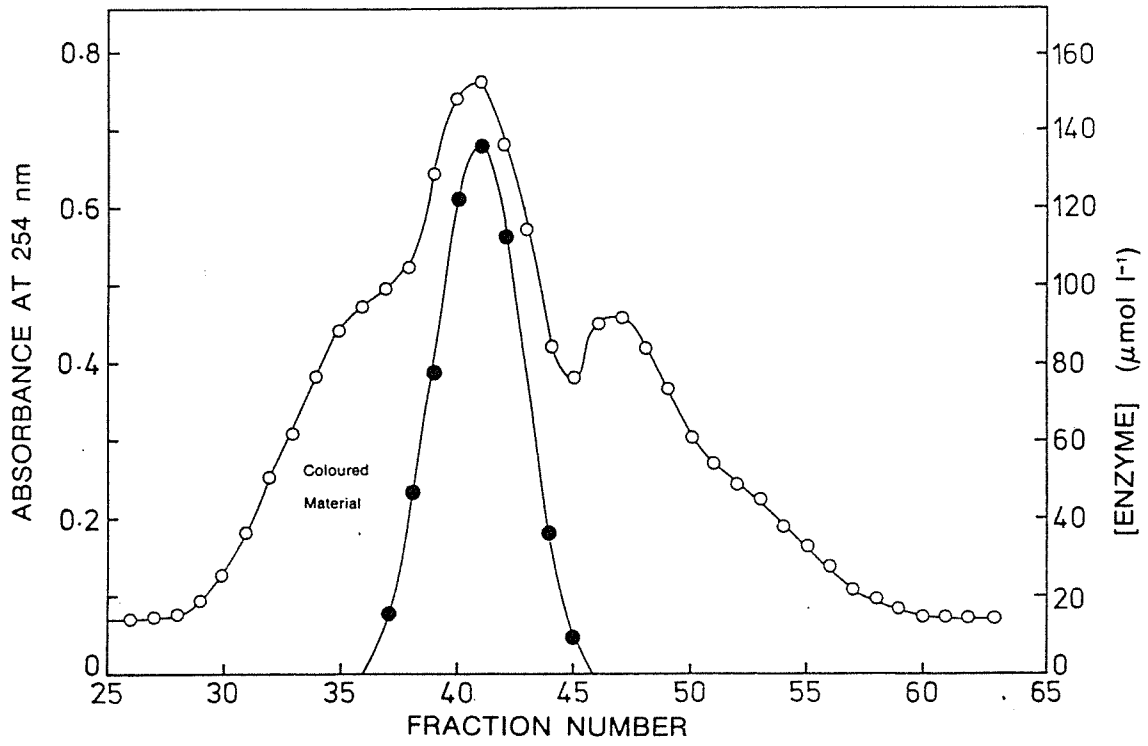


Figure 1:3 ELUTION PROFILE OF ALDEHYDE DEHYDROGENASE ON SEPHACRYL S-200 GEL FILTRATION COLUMN

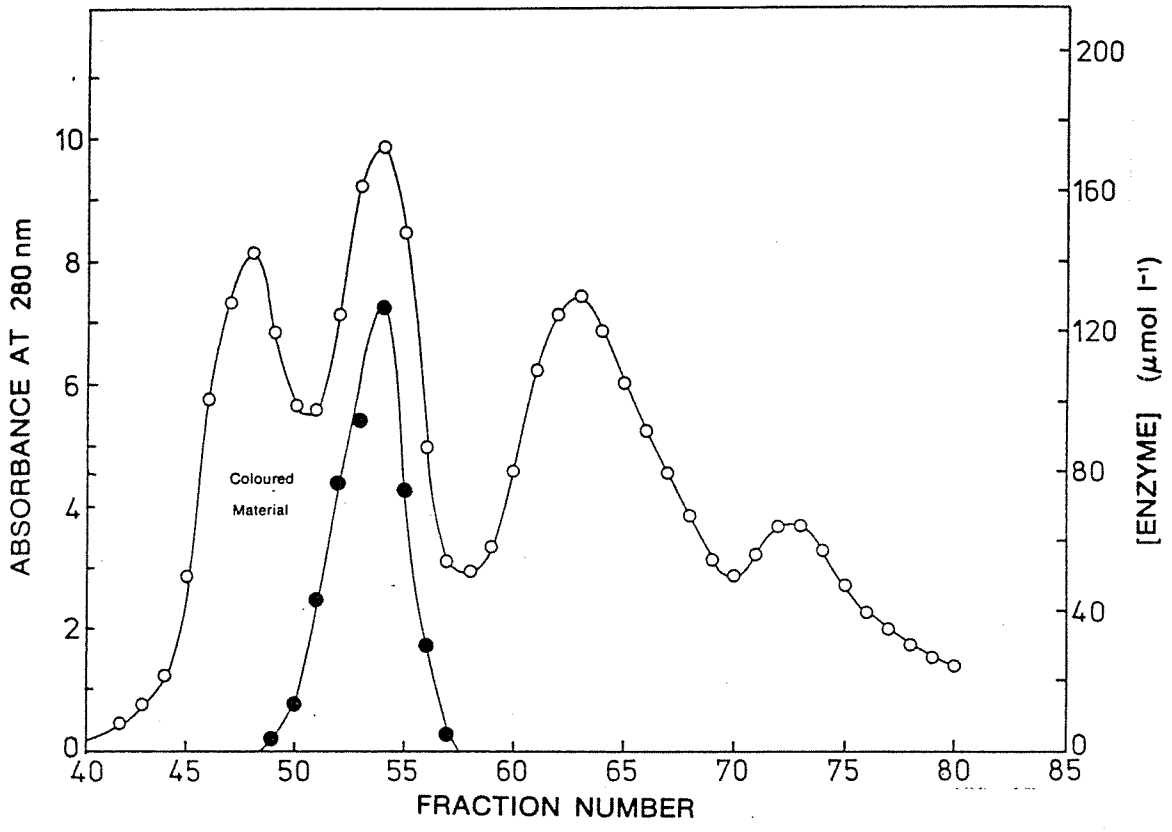


Figure 1.3 Enzyme Samples were centrifuged following concentration by ultrafiltration and applied to the column, which was eluted with 22 mM pH 7.3 phosphate buffer. A 12 cm^3 sample was loaded and approximately 8 cm^3 fractions collected.

(o) Absorbance at 280 nm (absorbance units).

(●) Concentration of aldehyde dehydrogenase (μoles active sites/l).

Figure 1.4 Enzyme samples were concentrated by ultrafiltration, centrifuged and applied to the column, which was eluted with 22 mM pH 7.3 phosphate buffer at a rate of 10-12 cm^3/hour.

(o) Absorbance at 280 nm (absorbance units).

(●) Concentration of aldehyde dehydrogenase (μmoles active sites/l)

1.4(a) A 6 cm^3 sample was loaded and fractions of approximately 7 cm^3 were collected.

1.4(b) A 10 cm^3 sample was loaded and fractions of approximately 7 cm^3 were collected.

Contaminant Enzymes

(□) Lactate Dehydrogenase

(◆) Alcohol Dehydrogenase

Figure 1:4 ELUTION PROFILE OF ALDEHYDE DEHYDROGENASE ON SEPHACRYL S-300 GEL FILTRATION COLUMN

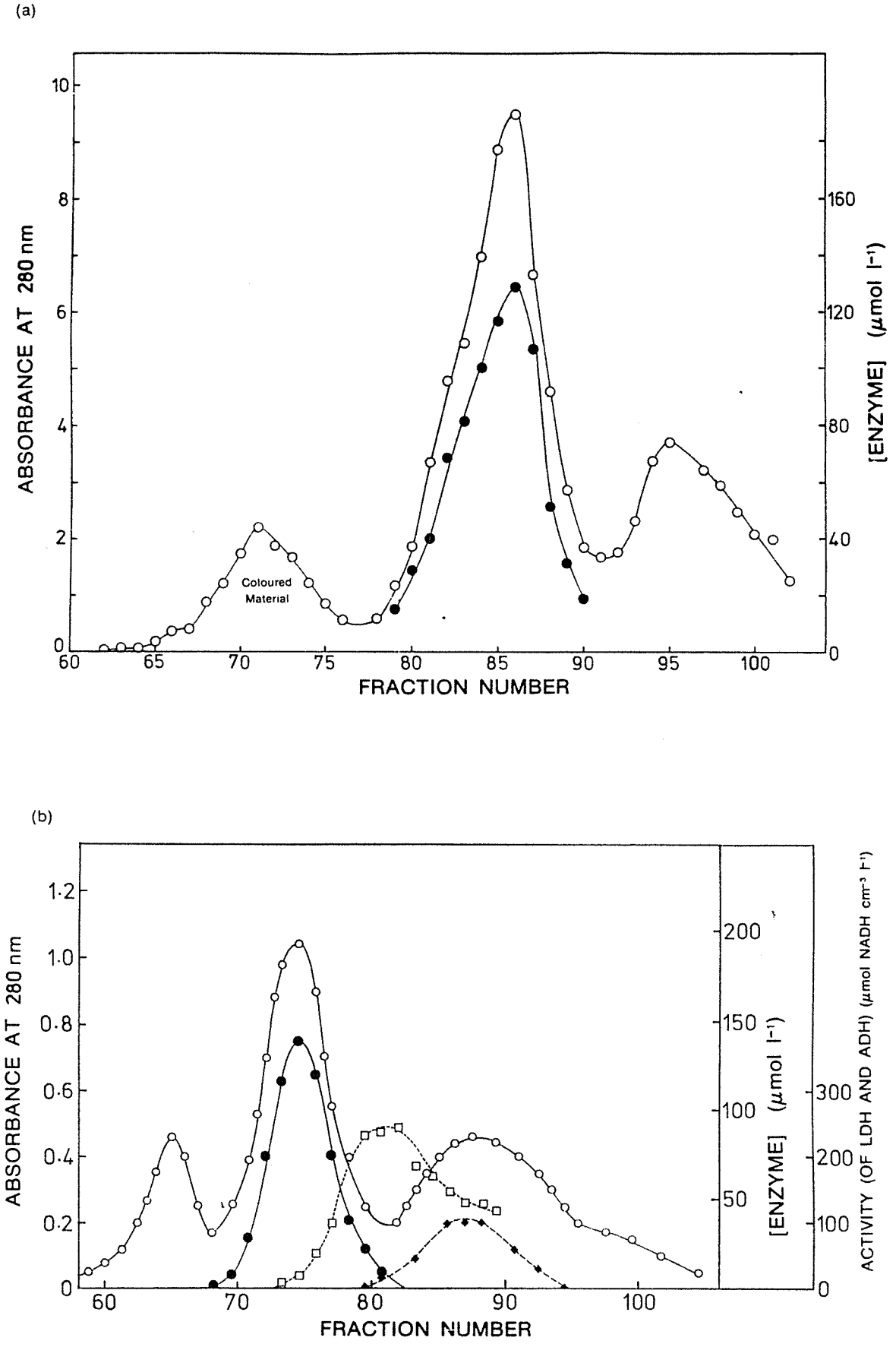


Figure 1.4 a. Slab Gel Electrophoresis of Fractions from the Sephacryl S-300 Column.

20 µl Enzyme (85 µM) from the fraction with highest activity eluted from the Sephacryl S-300 column was loaded onto a single-layer 15% acrylamide gel containing 1% SDS. For details see Methods.

1.3.5 DE Sephacel Column

Figure 1.5 shows the elution profile for enzyme using the DE Sephacel column. In this particular case, the enzyme had not been previously purified by gel filtration. Although two activity peaks were observed, the first (presumably mitochondrial aldehyde dehydrogenase) was so small as to be negligible. The column did produce some separation of lactate and aldehyde dehydrogenases (Blackwell, unpublished results), but the brown-coloured material described in the previous section was not removed from the fractions with aldehyde dehydrogenase activity.

Enzyme purified using this column was relatively unstable, almost all activity being lost over a two-week period.

1.3.6 AMP Sepharose Column

Aldehyde dehydrogenase was observed to bind readily to the AMP Sepharose column. All coloured material was removed during washing and the enzyme was eluted in a sharp peak, 2-3 10 cm^3 fractions of high activity generally being obtained. Negligible amounts of contaminating lactate dehydrogenase were observed and most of the NAD^+ could be removed by dialysis.

1.3.7 Purity of Enzyme Preparation

1.3.7.1 Contamination by other Proteins

Both precipitation methods were shown to produce enzyme essentially free of contaminating proteins and SDS gel electrophoresis showed only one major band. At very high loadings, other very faint bands were sometimes observed, but these did not represent significant contamination. The brown protein material observed in PEG purifications produced a readily identifiable sharp band on SDS gels, but this band was only present in fractions in the lower leading edge of the elution profile and these were not used for further work. Lactate dehydrogenase was the major enzyme contaminant, as described previously, and was readily detected by assay. Contaminated samples were not used for kinetic studies.

Whichever of the two precipitation methods was used, elution of

Figure 1.5 The Column was equilibrated with 10 mM pH 6.5 bistris buffer. The enzyme sample was titrated to the same pH and dilted to the same conductivity as the equilibration buffer before loading. A pH gradient was formed by eluting with 400 cm^3 10 mM pH 6.5 bistris buffer and 400 cm^3 10 mM pH 4.6 acetate buffer in a gradient mixer.

(●) Concentration of aldehyde dehydrogenase (μmoles active sites/l).

(o) pH.

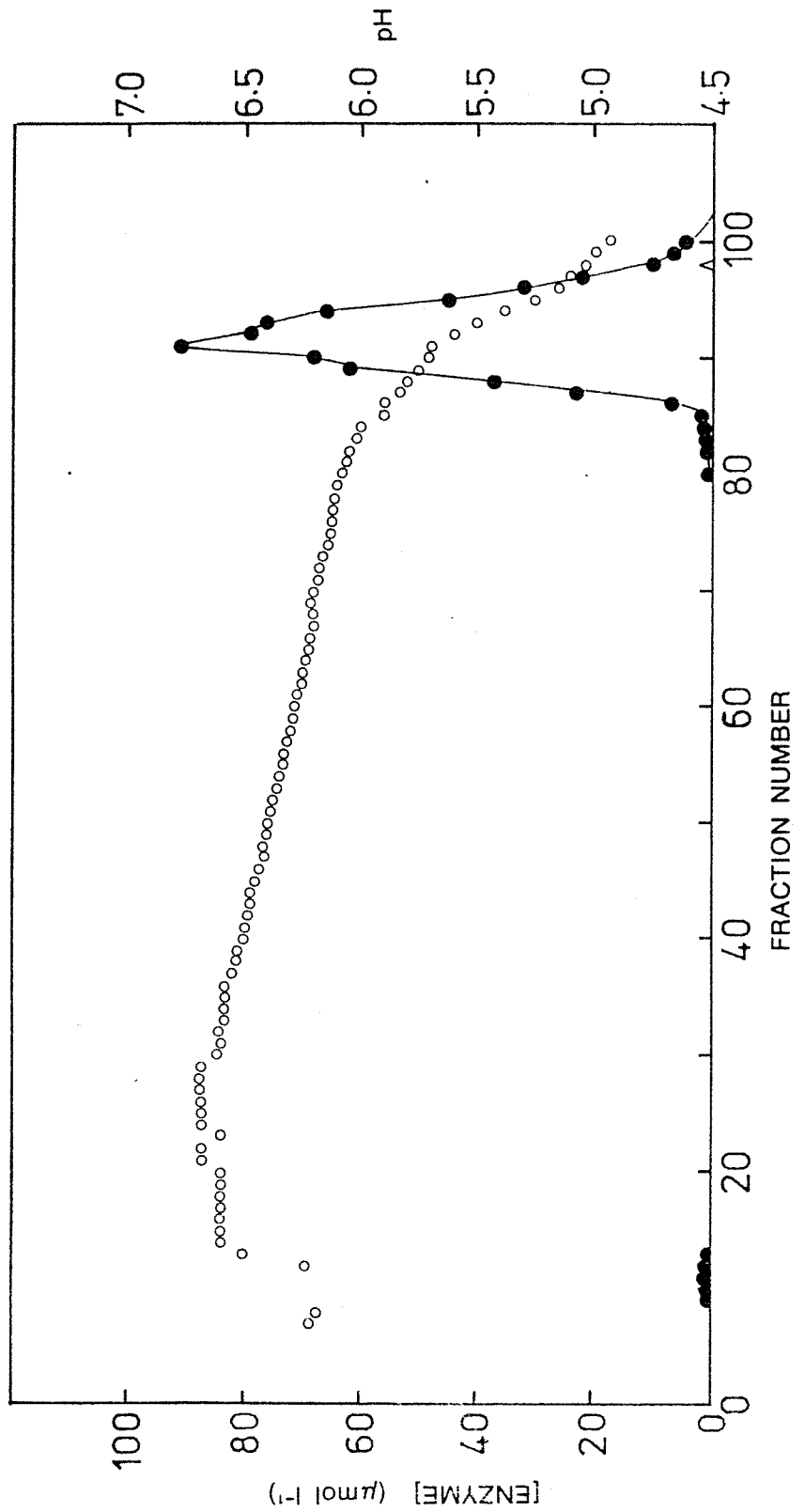


Figure 1:5 ELUTION PROFILE OF ALDEHYDE DEHYDROGENASE ON DE SEPHACEL COLUMN

the enzyme from the gel filtration column was followed by elution of other protein material (Figure 1.2). SDS gel electrophoresis of samples of this protein band from each method showed the same bands, indicating that despite different precipitation patterns for catalase and one or two coloured proteins, the methods were very similar.

1.3.7.2 Contamination by Mitochondrial Aldehyde Dehydrogenase

Using glutamate dehydrogenase as a marker enzyme, Agnew et al. (1981) found that consistently no more than 2% of the mitochondria were ruptured during the homogenisation and subsequent separation procedures using either method. This value was the same as that reported by Crow et al. (1974). Similar results were obtained for glutamate dehydrogenase assays in the present work.

Additional estimates of the contamination by mitochondrial enzyme could also be obtained by isoelectric focusing. Agnew et al. (1981) have shown that the mitochondrial enzyme produces seven bands on isoelectric focusing plates, two of which correspond with those observed for the cytoplasmic enzyme while the other five have higher isoelectric points (between 5.48 and 5.76). Mitochondrial enzyme was readily detected using this method but there was an almost complete absence of these bands in samples of cytoplasmic enzyme, except under extreme loading conditions. Activity staining of the two enzymes showed that the mitochondrial enzyme produces a much more intense stain than the cytoplasmic enzyme and this appears to be causing confusion as to the amount of contamination, hence it is important that both protein and activity stains are used with this method.

1.3.8 Enzyme Yields

Enzyme concentrations were expressed in terms of $\mu moles.l^{-1}$ of NADH binding sites as determined by assay (2.4.1).

Similar yields were obtained for both purification methods, an average preparation producing 5-6 µmoles of active sites in a total volume of about 100 cm^3. The concentration of active sites in the peak fraction varied from 80 to 200 µM.

Although it was possible to obtain higher yields using the second preparation method, presumably because of the reduction in the time required for the preparation, yields of enzyme were, on average, about the same because as stated above, significant loss of enzyme sometimes

occurred during the precipitation stage with PEG or enzyme was discarded after the DEAE cellulose column because of lactate dehydrogenase contamination.

1.4 DISCUSSION

Both enzyme purification procedures described above have been shown to produce cytoplasmic aldehyde dehydrogenase essentially free of contamination by the mitochondrial isoenzyme and by other protein material. The enzyme thus produced was of sufficiently high concentration and quality for use in steady state or stopped flow kinetic studies without further concentration, and was normally stable for at least four months if stored at 4°C in buffer containing 0.1% 2-mercaptoethanol.

The alterations to the method of MacGibbon *et al*. (1979) had several advantages, and significantly reduced the overall time required for the purification, particularly in the early steps.

Use of the Ultra-Turrax was found to be extremely fast and convenient, and to have no detrimental effect on the resulting product, provided care was taken to avoid frothing of the mixture. Shorter centrifugation times also reduced the time required for removal of the enzyme from the cellular material without jeopardising the purity of the final product.

Polyethylene glycol has been shown in the present work to be a satisfactory precipitant. There were two significant advantages to the use of PEG. Catalase was completely removed from the aldehyde dehydrogenase samples by precipitation, and the crude enzyme extract obtained following PEG precipitation did not require the exhaustive dialysis necessary to lower the conductivity of the solution when ammonium sulphate precipitation was used.

There were some slight disadvantages to PEG precipitation, which were not observed for ammonium sulphate precipitations. There was broadening of the DEAE cellulose elution profile, presumed to be caused by the high viscosity of the redissolved precipitate, which slowed the elution and ultrafiltration stages of the purification, and a coloured protein contaminant was eluted together with aldehyde dehydrogenase from the DEAE cellulose column. Neither of these factors was a serious problem however, as the total purification time was reduced by 24-36 hours and the coloured protein was removed from enzyme samples

during gel filtration. There was some evidence that longer washing of the DEAE cellulose column prior to elution reduced the broadening effect.

The use of Sephacryl S-300 for gel filtration provided some improvement in separation of the various proteins and this could most likely be improved further if a longer column was used.

Affinity chromatography using AMP Sepharose was very efficient for removal of the coloured protein material and contamination by lactate dehydrogenase, when it occurred, could be reduced to extremely low levels. However, the resulting enzyme solution contained large amounts of NAD^+ which was normally removed by dialysis. This NAD^+ contamination could be disadvantageous for some experiments and more efficient methods for removal would be necessary in such cases. The storage lifetime of enzyme samples eluted from this column is not known, as all such samples were used within 2-3 days. This column provided a convenient means of removing contaminants from enzyme samples whenever required and also frequently produced enzyme of a higher concentration than the sample originally applied to the column.

The DE Sephacel column described by Dickinson et al. (1981) was less useful, but appeared to be a good method for the removal of mitochondrial enzyme. However, as practically no contamination by mitochondrial enzyme was observed in enzyme preparations, this column procedure was not really necessary. Also, there was a detrimental effect on the stability of the enzyme. It is noted that, in the method published by Dickinson et al. (1981), the enzyme was reprecipitated with ammonium sulphate and dialysed following this column step, thus removing the bistris buffer. The stability of the enzyme after the ammonium sulphate procedure is not known, but it may be improved considerably. Studies of the enzyme activity in various buffers (Chapter 4) have shown that the enzyme has very low activity in tris buffers, so it is likely that the deterioration in stability is due to use of the bistris buffer.

There have been claims (Dickinson & Berrieman, 1979 and Dickinson et al., 1981) that there is more significant contamination of the enzyme by mitochondrial aldehyde dehydrogenase, than the 2% reported by Crow et al. (1974) and Agnew et al. (1981). Claims of 5-10% contamination, and of up to 17% in extreme cases, have been made for the method described by Dickinson et al. (1981) prior to use of the pH-gradient ion exchange chromatographic technique, and this method is

supposedly based on that of MacGibbon et al. (1979). However, the method described is actually different in several important respects, specifically the use of a Blender for homogenisation, and of shorter and slower centrifugation to remove mitochondria. Both of these changes could be expected to cause greater contamination by the mitochondrial enzyme.

There was no evidence of significant mitochondrial contamination in the preparations carried out as described here.

Enzyme prepared by the methods described above was therefore judged to be of sufficient purity for kinetic and stuctural studies. Most of the enzyme used in this study was prepared by the second method, unless clearly stated otherwise. Where Method 1 or additional columns were used exclusively, this is specified.

CHAPTER 2
GENERAL KINETIC METHODS AND STRUCTURAL STUDIES OF ALDEHYDE DEHYDROGENASE

2.1 INTRODUCTION

There has been a considerable amount of study of the kinetics of sheep liver aldehyde dehydrogenase, but much less study of the structure has been carried out to date. A number of structural and related investigations are therefore presented in this chapter. In addition the established work and the methods that have been developed for study of the kinetics are summarised in this section.

2.1.1 Enzyme Kinetics

Enzyme-catalysed reactions proceed via an intermediate, the enzyme-substrate complex.

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

where k_1, k_{-1} and k_2 are rate constants for each of the processes shown. Michaelis & Menten (1913) formulated a rate equation based on the above mechanism.

$$v = \frac{k_2[E][S]}{k_{-1}/k_1 + [S]}$$

where [E] = total enzyme
[S] = total substrate concentration
v = reaction rate

A more general solution was provided by Briggs & Haldane (1925) using the steady state approximation that the rate of change of the concentration of enzyme-substrate complex, [ES], is zero. The Michaelis-Menten equation has the general form

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

where V_{max} is the maximum reaction rate, obtained at high substrate concentrations and K_m is known as the Michaelis constant.
If $[S] = K_m$ then

$$v = V_{max}/2$$

and hence K_m may be found by determining the substrate concentration required to give half the maximum velocity. This maximum reaction rate, V_{max}, is dependent on enzyme concentration as follows:

$$V_{max} = k_{cat}\,[E]$$

where the constant k_{cat} is variously known as the catalytic constant, turnover number or catalytic centre activity.

Providing all the substrate binding sites on the enzyme for a particular substrate are identical, with no interaction between these sites, then values may be found for k_{cat} and K_m for any reaction, including those which involve more than one enzyme intermediate. This is the major disadvantage of steady state kinetics, since the same general form of the equation is obtained, irrespective of the mechanism, but the physical significance of the kinetic constants obtained depends upon the mechanism involved. The relationship between the observed values of K_m and k_{cat} and the actual rate constants for an assumed mechanism may be determined by the method of King & Altmann (1956). Although the relationships are complex, containing terms for each step in the mechanism, some of the individual rate constants may often be determined from the steady state data using these equations.

V_{max} and K_m are usually determined graphically. As a plot of V against [S] results in a rectangular hyperbola, the data is plotted in accordance with one of several possible transformations of the general equation, the most widely used form being the Lineweaver-Burk plot:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]}$$

A plot of $1/v$ against $1/[S]$ is linear with a slope K_m/V_{max} and an intercept of $1/V_{max}$ on the ordinate axis and hence both V_{max} and K_m may be determined.

2.1.2 Two Substrate Reactions

Many enzymes catalyse reactions with two or more substrates, and a general rate equation for two substrate reactions has been described by Cleland (1963a).

$$v = \frac{V_{max}[A][B]}{K_{ia}K_b + K_aK_b + K_b[A] + K_a[B]}$$

In this equation, A and B are substrates, and there are four constants V_{max}, K_a, K_b, and K_{ia}, compared with only two required for the single substrate case.
At very high concentrations of substrate B, the rate equation reduces to

$$v = V_{max}[A]/(K_a + [A])$$

and similarly at high concentrations of A it reduces to

$$v = V_{max}[B]/(K_b + [B])$$

These equations are of the same form as the original Michaelis equation and therefore the constants K_a and K_b are the Michaelis constants for substrates A and B respectively, at high concentrations of the other substrate. Hence V_{max}, K_a and K_b may be determined by varying one substrate at high concentrations of the other. Reaction mechanisms involving two substrates are generally divided into two categories, sequential or ping-pong. Sequential mechanisms are those in which all substrates must bind before any product is released. There are two groups of these; ordered mechanisms, in which one particular substrate must bind before the other, and random mechanisms, in which substrates may bind in any order. Ping Pong mechanisms are those in which a product may be released before both substrates have bound. Reactions mechanisms involving two substrates and two products are known as bi bi mechanisms.

Lineweaver-Burk plots with one substrate concentration fixed and the other varied result in a series of straight lines. Parallel lines are obtained in the case of a ping-pong mechanism, and for sequential

mechanisms, lines which intersect to the left of the ordinate axis. Thus the type of mechanism may be determined, and values for the Michaelis constants may be obtained by initial velocity studies. Product inhibition studies (Cleland (1963b) may be used to distinguish between ordered and random mechanisms and to give information about the order of substrate addition. (MacGibbon et al., 1977a)

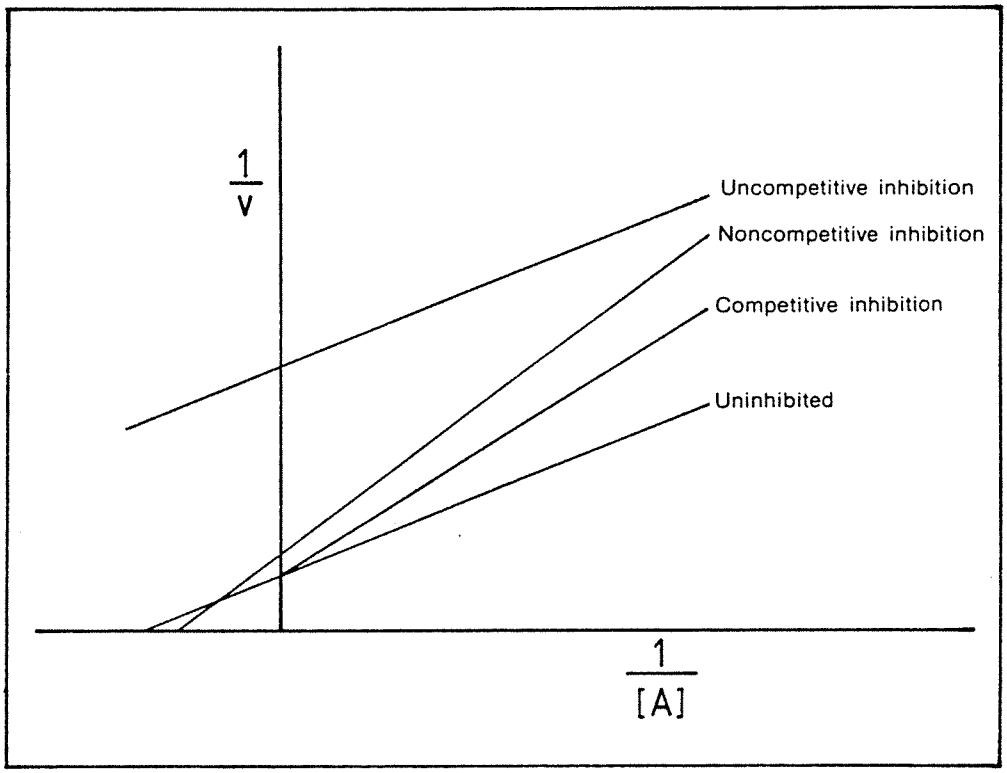
Inhibitors are substances which produce a reduction in the rate of the enzyme catalysed reaction. Reaction products may inhibit a reaction by shifting the equilibrium in favour of the reverse reaction, or in the case of an irreversible reaction may result in formation of an unreactive enzyme complex (dead end complex). Some inhibitors are substrate analogues, which bind to the enzyme active site but do not react further, thus resulting in a lowering of the active enzyme concentration. However many inhibitors belong to neither of these groups and usually cause inhibition by binding to the enzyme or an enzyme intermediate complex. Reversible inhibition is usually classed as one of three types; competitive, noncompetitive or uncompetitive, depending on how the inhibitor affects the slopes and intercepts of a Lineweaver-Burk plot. Different types of inhibition may be observed with the same inhibitor for the two different substrates of a two substrate reaction. A very brief description of these three types of inhibition follows.

Competitive Inhibition occurs when the inhibitor reacts with the same enzyme form as the substrate with which it is competitive. High concentrations of the substrate therefore removes the inhibition by saturating that form of the enzyme and high concentrations of the inhibitor will reduce the reaction rate to zero. Only the slope of the Lineweaver-Burk plot is affected in such cases.

Uncompetitive inhibition arises when the inhibitor binds to an enzyme form other than that to which the substrate in question binds, yielding an inactive E.S.I complex. This lowers the amount of enzyme available for distribution among the normal forms and cannot be overcome by saturation with substrate. The ordinate intercept, but not the slope of the Lineweaver-Burk plot is affected with this type of inhibition (Figure 2.1).

In the third type of inhibition, noncompetitive inhibition, the inhibitor can bind to the free enzyme or enzyme substrate complex, without affecting the binding or dissociation of substrate and hence the K_m is unaltered. The E.S.I complex is inactive, so the inhibition

Figure 2:1 EFFECT OF INHIBITION ON THE DOUBLE RECIPROCAL PLOT



cannot be removed by saturation with substrate with the result that both the slope and intercept of the Lineweaver-Burk plot are affected (Figure 2.1) More complicated types of inhibition are possible but these result in non-linear inhibition plots.

In steady state kinetic experiments, the overall rate of a reaction is studied, but information about the reaction intermediates can usually only be gained indirectly. For an enzyme catalysed reaction, the concentration of enzyme is kept sufficiently low (10^{-10} 10^{-6} M) so that the reaction rate is slow enough to be measurable, hence enzyme intermediates are normally not directly detectable. Measurements are made of the Michaelis constants (K_m) for each substrate and of the turnover number (k_{cat}). As previously stated, for reactions involving a number of sequential steps, these parameters are complex functions of the individual rate constants for each step and the determination of most of these rate constants is not possible from the steady state kinetic experiments alone, particularly if the reaction is irreversible, as in the case of aldehyde dehydrogenase.

However, some information may be gained from steady state kinetics, and the upper and lower limits of some individual rate constants, or combinations of rate constants, may often be determined.

In presteady state experiments, the reaction is followed during the first turnover of the enzyme-substrate reaction, before a steady state has been reached. More information about the reaction mechanism may therefore be obtained, as reaction steps other than the rate determining step may be studied. Higher concentrations of enzyme are required, and thus if an intermediate is formed which results in a change in a detectable physical property such as absorbance or fluorescence, then the rate of formation and decay of the intermediate may be observed directly. Generally the enzyme concentration is sufficiently high to allow observation of intermediates and to monitor the approach to the steady state, but if enzyme concentrations higher than the substrate concentration are used, the reaction may be observed during a single turnover, allowing observation of both formation and decomposition of intermediates.

For reactions involving several intermediate forms of the enzyme, the analysis of presteady state data may be potentially very complex. However, in practice the experimental data is usually more simple to interpret. There is no contribution from any steps occurring after the steady state rate limiting step and in addition, no change will be

observed until an intermediate exhibiting different observable physical properties to those of the reactants is formed. Even if more than one exponential formation or decay process occurs, some may not be observed because of their very small amplitudes (see below)

In some cases, changes in more than one physical property are observed during the presteady state phase of enzyme reactions. Some changes may occur as different intermediates form, and thus observation of the same reaction using different methods (e.g. absorbance and fluorescence at different wavelengths and proton release or uptake) can provide additional or complementary information.

The instrument that has been most widely used for presteady state kinetic studies is the stopped flow spectrometer, first described by Gibson & Milnes (1964). In this apparatus, two solutions are mixed very rapidly (within 1-4 ms), and the reaction is followed by monitoring subsequent physical changes in the resulting solution. The techniques and instrumentation have been reviewed by Hiromi (1979).

2.1.3 Kinetics of Sheep Liver Aldehyde Dehydrogenase

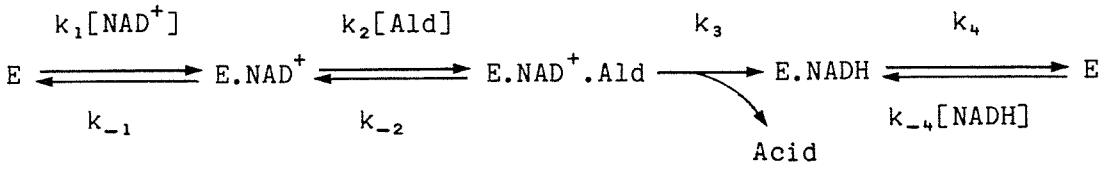
There have been a number of steady state and presteady state kinetic studies of the enzyme (MacGibbon et al., 1977a,b,c, Bennett et al., 1982, 1983, Blackwell et al., 1983a,b, Buckley & Dunn, 1982, 1985, Deady et al., 1985, Hart & Dickinson, 1978a, 1982, 1983, Dickinson, 1985) A brief summary of the major features of the kinetics follows. Figures quoted refer to experiments carried out at 25°C, in 0.025 M pH 7.6 phosphate buffer, using propionaldehyde as the aldehyde substrate, as these were the conditions used in the majority of experiments in this study. Esterase reactions were carried out using p-nitrophenylacetate (PNPA) as the substrate.

2.1.3.1 Steady State Kinetics

MacGibbon et al. (1977a) have shown that, in common with aldehyde dehydrogenases from other sources, cytoplasmic aldehyde dehydrogenase from sheep liver is NAD^+ dependent, is capable of oxidising a very wide range of aldehydes, and exhibits esterase activity, catalysing the hydrolysis of p-nitrophenylacetate (PNPA). Low Km values are obtained for both NAD^+ and aldehydes, indicating that the enzyme can operate at

very low substrate concentrations. Km values of 2.3 μM for NAD^+ and 1.1 μM for propionaldehyde are obtained at pH 7.6. The kinetic results are consistent with an ordered bi bi mechanism, with NAD^+ bound prior to aldehyde, and NADH dissociation as the last step. The simplest reaction scheme consistent with the steady state kinetics is shown below.

Scheme 2.1



King Altmann expressions were derived for this mechanism (MacGibbon et al. (1977a).

$$k_{cat} = k_3k_4/(k_3 + k_4)$$

$$K_a = k_3k_4/k_1(k_3 + k_4)$$

$$K_{ia} = k_{-1}/k_1$$

$$K_b = k_4(k_3 + k_{-2})/k_2(k_3 + k_4)$$

$$K_i = k_4/k_{-4}$$

Since $k_{cat}/K_a = k_1$

then by rearrangement $k_{cat} = K_1/K_a$

Using these expressions, values of $2\times10^5\ M^{-1}.s^{-1}$ and $1.6\ s^{-1}$ were determined for k_1 and k_{-1} respectively. Although k_2 and k_{-2} could not be determined uniquely, they are related by the equation $(k_{-2} + k_3)/k_2 = 50\times10^6\ M^{-1}$ (MacGibbon et al., 1977a). A value for k_3 of $11\ s^{-1}$ was obtained by MacGibbon et al. (1977c) from presteady state experiments, and values of $0.25\ s^{-1}$ and $5\times10^5 M^{-1}.s^{-1}$ have been obtained for k_4 and k_{-4} from studies of NADH binding and displacement (MacGibbon et al., 1977b). A value for k_{-2} of $50\ s^{-1}$ has been estimated by Bennett et al. (1982), and thus a value of $10^6\ M^{-1}$ has

been estimated for k_2.

Non-Linear behaviour is observed at high aldehyde concentrations for propionaldehyde and acetaldehyde (MacGibbon et al.,1977a). Double reciprocal plots are linear up to 50 μM propionaldehyde, and a k_{cat} value of 0.082 s^{-1} per active site was obtained. At higher propionaldehyde concentrations, faster rates are observed. A limiting value of 0.25 s^{-1} was obtained for k_{cat} and second K_m value of 3.5 mM (MacGibbon et al., 1977a). Blackwell et al. (1983a) have proposed that the enzyme possesses two distinct binding sites for propionaldehyde, designated P_1 and P_2. Propionaldehyde can only bind at the P_1 (K_m=1.1 μM) site in the presence of NAD^+ and reaction proceeds via the P_1 site only, but propionaldehyde binding at the low affinity P_2 site (K_m=3.5 μM) causes activation. Ester hydrolysis is proposed to occur at the P_2 site, ester being able to bind at this site irrespective of the presence or absence of NAD^+.

MacGibbon et al. (1977a) observed a 5.6-fold increase in the fluorescence of NADH upon binding to the enzyme. Titration of an enzyme sample with aliquots of NADH, when compared with a blank titration (NADH added to buffer), was used as a method for determination of the NADH binding site concentration of enzyme samples. Active site concentrations, were determined using the spectrophotometric assay described in section 1.2.2 , assuming a value for k_{cat} of 0.25 s^{-1} and were the same for a given enzyme solution as the NADH binding site concentrations determined by fluorescence titration.

2.1.3.2 Presteady State Kinetics

MacGibbon et al. (1977c) observed a presteady state phase (burst) in the production of NADH when enzyme was mixed with NAD^+ and propionaldehyde in the stopped flow spectrophotometer. This transient phase is first order, and is observed when either NADH fluorescence or absorbance is monitored, but is more readily observed in the fluorescence mode, because of the enhanced fluorescence of enzyme-bound NADH.

A linear dependence of the burst rate constant on the NAD^+ concentration was observed at low concentrations, but a maximum was attained at higher concentrations. The burst rate constant was also dependent upon propionaldehyde concentration (see below). At saturating concentrations of NAD^+ and propionaldehyde, the rate

constant for the burst is $11 \pm 2\ s^{-1}$ and the amplitude of the burst process is equal to 90% of the formal enzyme concentration, determined as described in section 2.1.3.1. The normal steady state production of NADH is observed following the burst. Rate constants of $2 \times 10^{-5}\ l.mol^{-1}.s^{-1}$ for NAD^+ binding and of $1.2 \times 10^{-6}\ l.mol^{-1}.s^{-1}$ for the aldehyde addition were obtained. Essentially the same burst rate constant and amplitude are observed at propionaldehyde concentrations of 100-200 μM as at 20 mM. Thus binding of propionaldehyde at the postulated P_2 site has no major effect on the presteady state phase of the reaction.

The same results are obtained whether the enzyme is premixed with saturating concentrations of NAD^+ or not, except that when enzyme and NAD^+ are not premixed, there is a slight lag phase observed in the first 100 ms of the reaction as NAD^+ binds to the enzyme.

The observation of a burst in the production of NADH indicates that formation of an enzyme-NADH intermediate occurs before the rate limiting step in the reaction. Using [1-^{2}H]propionaldehyde, no isotope effect on the burst rate constant is observed and therefore the rate limiting step in the presteady state phase must precede hydride transfer.

Proton Burst Studies

Using acid-base indicator dyes and very low buffer concentrations, Bennett et al. (1981) observed a presteady state release of protons (proton burst) when the enzyme was mixed with NAD^+ and propionaldehyde in the stopped flow spectrophotometer. The same dependence of the burst rate constant on substrate concentration was observed as for the burst in NADH production (NADH burst) and the same burst rate constants were obtained at saturating concentrations of substrates for both processes. Similar results were obtained for other aldehyde substrates, with the exception of 4-nitrobenzaldehyde, in which case there is no burst in NADH production, but a proton burst was observed, demonstrating that proton release precedes hydride transfer.

A conformational rearrangement resulting in proton release due to a perturbation of the pK of a functional group on the enzyme is proposed as the rate-limiting step in the presteady state phase. The amplitude of the transient proton release corresponds to release of one proton per active site although it is known from the stoichiometry that two protons are released during the reaction. It was proposed (Bennett et al., 1982) that the second proton release occurs in a slow step,

probably hydrolysis of the acyl enzyme intermediate, prior to NADH dissociation.

2.1.3.3 NADH Dissociation

MacGibbon et al. (1977b) have shown that, when NADH is displaced from the enzyme using NAD^+ or other displacing agents, a biphasic decrease in fluorescence is observed. When the binding of NADH to enzyme is studied, biphasic stopped flow traces are also obtained. Two apparent first order rate constants (decay constants) may be obtained for each experiment. In the case of NADH displacement by NAD^+, values for the fast and slow decay constants under saturating conditions of NADH are 0.85 s^{-1} for λ_F and 0.22 s^{-1} for λ_S and a relative amplitude of A_F/A_S of 0.5±1 is obtained. For NADH binding, values for λ_F of 4.8 s and λ_S of 0.85 s are obtained, providing concentrations of NADH are sufficiently high so that the process is pseudo first order.

No detectable proton release or uptake accompanies the dissociation of NADH from the enzyme (Bennett et al., 1982). It is postulated that the displacement is a two step process involving a conformational change of the enzyme-NADH intermediate.

$$^*E.NADH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.NADH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E$$

Values obtained for the rate constants (derived from the decay constants obtained experimentally) are $k_1 = 0.2\ s^{-1}$, $k_{-1} = 0.05\ s^{-1}$, $k_2 = 0.8 s^{-1}$ and $k_{-2} = 5 \times 10^{-5}\ s^{-1}$. The slow phase of this displacement is similar to the value reported for the steady state turnover number (k_{cat}) of 0.25 s^{-1} for propionaldehyde at high concentrations and it was proposed that the loss of NADH from the enzyme was rate limiting in the steady state under these conditions. At lower propionaldehyde concentrations, some additional step with a rate constant of the order of 0.13 s^{-1} was presumed to be partly controlling the steady state turnover rate in order to give the reported value of k_{cat} of 0.082 s^{-1} (Bennett et al.). As different values of k_{cat} are obtained for different aldehydes (MacGibbon et al., 1977a), the rate of this additional step was presumed to be due to the structure of the aldehyde.

2.1.3.4 Protein Fluorescence

MacGibbon et al. (1977c) observed that the protein fluorescence of the enzyme is quenched on binding of NAD^+ to the enzyme. The decrease in protein fluorescence is apparently first order and the rate constant for the process showed a linear dependence on the concentration of NAD^+. The apparent rate constant for NAD^+ binding obtained is 1.8×10^5 $l.mol^{-1}.s^{-1}$, in good agreement with the value obtained from NADH burst experiments of 2.3×10^5 s^{-1}. Some quenching of protein fluorescence is also observed when NADH binds to the enzyme, but less than for NAD^+.

It is proposed that a conformational change of the enzyme.NAD^+ intermediate occurs following binding of NAD^+, and that this change is responsible for the observed quenching of the protein fluorescence (MacGibbon et al., 1977c).

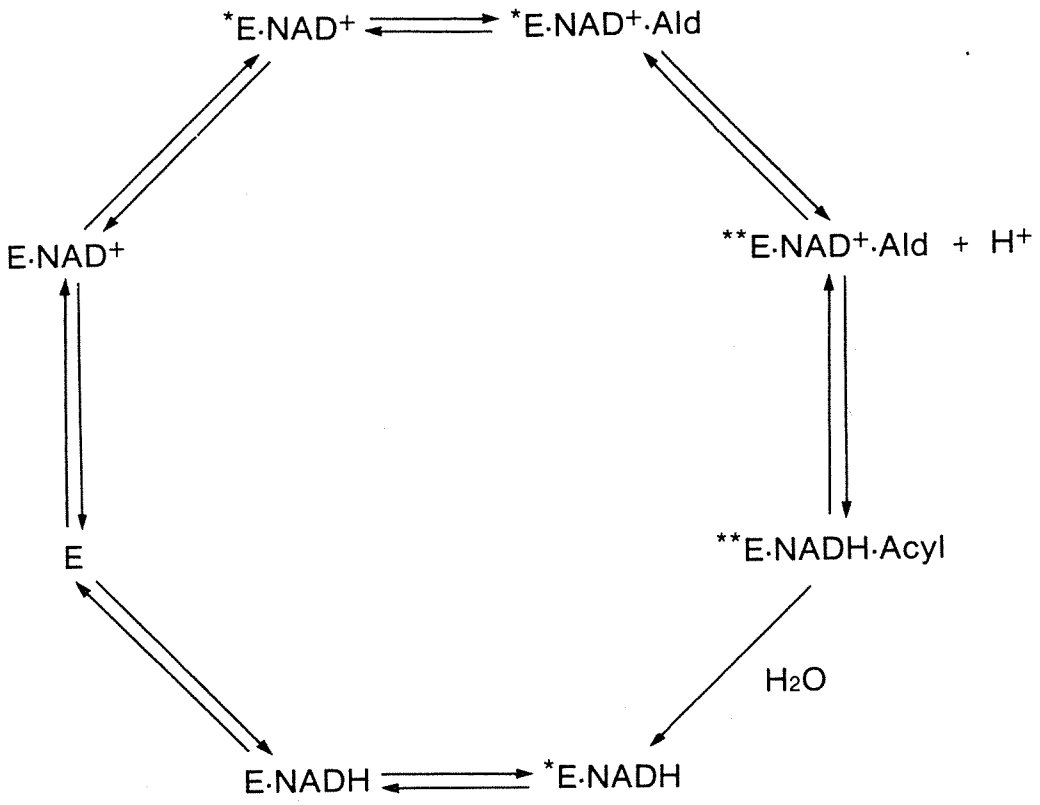
2.1.3.5 Reaction Mechanism

On the basis of the steady state and presteady state data, the mechanism shown in Scheme 2.2 has been proposed by Bennett et al. (1982).

NAD^+ binding to enzyme is followed by a conformational change in the enzyme-NAD^+ intermediate. A second conformational change occurs following aldehyde binding, accompanied by release of a proton. This process has a rate constant of 12 s^{-1} and is rate limiting in the presteady state phase of the reaction. Rapid hydride transfer follows and then irreversible hydrolysis of the proposed NADH-acyl-enzyme intermediate and dissociation of the acid product, combined with a reversal of the previous rate-limiting conformational change. Although there is uptake of a proton during this reversal, the net result of the combined hydrolysis and conformational rearrangement is the release of a second proton, thus satisfying the stoichiometric requirements (release of two protons) for the overall reaction. A slow conformational change which is considered to be the rate limiting step in the steady state phase follows, and finally dissociation of NADH yields free enzyme.

Computer simulations carried out recently by L.F. Blackwell and P.D. Buckley (personal communication) have shown that the rate constants for all of the steps between hydride transfer and the appearance of $^*E.NADH$ (summarised as a single acyl-enzyme hydrolysis step in Scheme 2.2) must be at least 5-10 s^{-1}. If this condition is

Scheme 2.2



not met marked lag phases follow the burst, which is clearly contrary to observation.

This step was previously considered to be slow, with a rate constant of about 0.15 s^{-1} (Bennett et al., 1982). This suggestion was based on a comparison between the k_{cat} value of 0.082 s^{-1} per active site at pH 7.6 reported by MacGibbon et al. (1977a) and the apparent first order rate constant (0.22 s^{-1}) for the isomerisation of the binary enzyme.NADH complexes as determined from the NADH displacement experiments at the same pH (MacGibbon et al., 1977b). Recent work (L.F. Blackwell, personal communication) has revealed a hitherto unknown time-dependent decrease in the enzymic activity on dilution. Hence the reported k_{cat} value of MacGibbon et al. (1977a) which was determined by monitoring nucleotide fluorescence on prediluted enzyme samples should be increased by a factor of almost two for comparison with the presteady state results. The apparent anomaly between the rate constant for the binding of NAD^+ obtained from protein fluorescence and burst experiments (MacGibbon et al., 1977b, c) and the k_{cat} value obtained from steady state studies (MacGibbon et al., 1977a) is eliminated if this higher k_{cat} value is used.

2.1.4 Consecutive Reactions

Frost & Pearson (1961) have described the solutions to a reaction scheme consisting of two consecutive first order reactions, as shown below.

$$E_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_3$$

Mechanism 2.1

Three decay constants, λ_1, λ_2, λ_3, describe the approach to equilibrium and the solutions obtained for these decay constants are

$$\lambda_1 = 0$$

$$\lambda_2 = (p + q)/2 \qquad 2.1$$

$$\lambda_3 = (p - q)/2 \qquad 2.2$$

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

$q = \sqrt{(p^2 - 4(k_1k_2 + k_{-1}k_{-2} + k_1k_{-2})}$

As the expressions for λ_2 and λ_3 are mathematical solutions of a quadratic equation, then also

$$\lambda_2 + \lambda_3 = k_1 + k_2 + k_{-1} + k_{-2} \qquad 2.3$$

$$\lambda_2.\lambda_3 = k_1k_2 + k_{-1}k_{-2} + k_1k_{-2} \qquad 2.4$$

It is possible to calculate the concentrations of the three species E_1, E_2, and E_3. If at time zero all enzyme is the the form E_1 so that:

$$[E_1] = [E_1]^\circ$$

$$[E_2] = [E_3] = 0$$

then

$$E_1 = E_1^\circ \left[\frac{k_{-1}k_{-1}}{\lambda_2\lambda_3} + \frac{k_1(\lambda_2 - k_2 - k_{-2})}{\lambda_2(\lambda_2 - \lambda_3)} e^{-\lambda_2 t} + \frac{k_1(k_2 + k_{-2} - \lambda_3)}{\lambda_3(\lambda_2 - \lambda_3)} e^{-\lambda_3 t} \right] \qquad 2.5$$

$$E_2 = E_1^\circ \left[\frac{k_1k_{-2}}{\lambda_2\lambda_3} + \frac{k_1(k_{-2} - \lambda_2)}{\lambda_2(\lambda_2 - \lambda_3)} e^{-\lambda_2 t} + \frac{k_1(\lambda_3 - k_{-2})}{\lambda_3(\lambda_2 - \lambda_3)} e^{-\lambda_3 t} \right] \qquad 2.6$$

$$E_3 = E_1^\circ \left[\frac{k_1k_2}{\lambda_2\lambda_3} + \frac{k_1k_2}{\lambda_2(\lambda_2 - \lambda_3)} e^{-\lambda_2 t} - \frac{k_1k_2}{\lambda_3(\lambda_2 - \lambda_3)} e^{-\lambda_3 t} \right] \qquad 2.7$$

These general equations can be applied to several of the experimental situations as follows:

(a)NADH Displacement Experiments

The following reaction scheme applies:

$$^{*}E.NADH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.NADH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + NADH$$

Mechanism 2.2

where E_1 is a conformationally altered form of the enzyme-NADH complex as proposed by MacGibbon et al. (1977b) and E_3 is the free enzyme. All three forms are present at time zero. However, under the experimental conditions, in which excess NAD^+ is present, the displacement is irreversible and thus k_{-2} is zero.

Hence

$$\lambda_2 + \lambda_3 = k_1 + k_{-1} + k_2 \qquad 2.8$$

$$\lambda_2\lambda_3 = k_1k_2 \qquad 2.9$$

(b)NADH Binding Experiments

Mechanism 2.2 applies in this situation also, but when the NADH concentration is much greater than the enzyme concentration k_{-2} can be regarded as a pseudo first order rate constant which depends on the NADH concentration. Equations 2.5, 2.6 and 2.7 can be expressed in terms of E_3, the initial concentration of free enzyme, because of the symmetry of the equations and the mechanism. Initially all enzyme is in the free enzyme form.

(c) Burst Experiments

With reference to the proposed reaction sequence (Scheme 2.2), the burst mechanism can be seen to be considerably more complex than the previous two cases and some simplification of the scheme is necessary for analysis.

Enzyme was usually preincubated with NAD^+, but since experiments in which NAD^+ was not premixed produced similar rate constants and amplitudes for the burst, the NAD^+ binding processes are assumed to be too fast to contribute significantly to the observed burst and are therefore disregarded in the mathematical treatment. When

propionaldehyde is the substrate, the rate determining step in the presteady state of the reaction is believed to be a conformational change and an associated release of a proton. No change in absorbance or fluorescence is observed until after the hydride transfer step, but this is believed to occur very rapidly. Therefore these steps have been combined in the step with rate constant k_2 of Mechanism 2.3. The slow steps which follow may be ignored in the presteady state phase (Shore & Gutfreund 1970).

Thus the mechanism reduces to the simpler form

$$E.NAD \underset{k_{-1}}{\overset{k_1[Ald]}{\rightleftharpoons}} E.NAD^+.Ald \xrightarrow{k_2} E.NADH$$

Mechanism 2.

Provided the aldehyde concentration is considerably larger than that of the enzyme, aldehyde addition can be treated as a pseudo first order process, and the mechanism is then of the same form as Mechanism 2.1, with k_{-2} equal to zero.
Hence

$$\lambda_2 + \lambda_3 = k_1[Ald] + k_{-1} + k_2 \qquad 2.10$$

$$\lambda_2\lambda_3 = k_1k_2[Ald] \qquad 2.11$$

As initially all enzyme is in the form $E.NAD^+$ equations 2.5, 2.6 and 2.7 may be used.

Experimentally the production of E.NADH (E_3) is monitored in the stopped flow apparatus, and only a single process is observed. From Equation 2.7 it can be seen that if only one process is seen, it will be the slower process with decay constant λ_3, as if the two rate constants are very different in magnitude, the amplitude of the fast process will be very much smaller, making it difficult to observe.

The dependence of the burst rate constant on aldehyde concentration may be derived using equations 2.10 and 2.11 above.
From equation 2.10

$$\frac{1}{\lambda_3} = \frac{1}{k_2} + \frac{(k_2 + k_{-1} - \lambda_3)}{k_1k_2[Ald]}$$

Since λ_2, the faster of the two processes is assumed to be of small amplitude, as it is not observed, substitution for λ_2 in equation 2.11 gives

$$\frac{1}{\lambda_3} = \frac{1}{k_2} + \frac{(k_2 + k_{-1})}{k_1k_2[Ald]}$$

Provided $(k_2 + k_{-1}) >> \lambda_3$ then a plot of $1/\lambda_3$ against 1/[Ald] produces a straight line (as is experimentally observed (MacGibbon et al., 1977c, Bennett et al., 1982)) with an intercept on the ordinate axis of $1/k_2$.

2.1.5 Structural Studies of the Enzyme

A few structural studies have been carried out on mammalian aldehyde dehydrogenases. The amino acid composition of several cytoplasmic aldehyde dehydrogenases from mammalian liver have been determined (Eckfeldt et al., 1976, Greenfield & Pietruszko, 1977, and Leicht et al., 1978).

Alcohol dehydrogenase (EC 1.1.1.1) has been shown to be a metalloenzyme containing zinc (Eichhorn, 1973), but studies of aldehyde dehydrogenases from human liver (Sidhu & Blair, 1975), rabbit liver (Duncan, 1977) and of the mitochondrial aldehyde dehydrogenase from sheep liver (Hart & Dickinson, 1977) revealed no metal ion content of these enzymes. MacGibbon (1976) has demonstrated that the cytoplasmic sheep liver enzyme did not contain zinc either.

In order to gain some further fundamental structural information about the sheep liver enzyme, the amino acid composition and metal ion content were therefore determined.

MacGibbon (1976) also reported an increase in absorbance at 340 nm when NADH was displaced from the enzyme with NAD^+, using the stopped flow spectrophotometer, but failed to observe any differences in the absorbance of enzyme-bound and free NADH at 340 nm by conventional spectroscopic methods. A further attempt to observe any spectral changes upon binding of NADH was therefore made in this work using a difference spectrum technique.

Although contamination of cytoplasmic aldehyde dehydrogenase prepared as described in the previous chapter by the mitochondrial enzyme has been shown to be consistently no greater than 2%, there have been claims (Dickinson & Berrieman, 1979, Dickinson et al., 1981a) of significant mitochondrial contamination, as discussed in the previous chapter. Chromatofocusing is a recently introduced chromatographic technique involving isoelectric focusing using ion exchange chromatography. Whereas in conventional ion exchange chromatography, the pH gradient is formed using a gradient mixer, in chromatofocusing the ion exchanger is initially equilibrated with buffer at the required

initial pH (start buffer) and the gradient forms automatically as a result of the titration of the anionic groups on the ion exchanger by the elution buffer, a multicomponent amphoteric buffer. As a result the pH varies down the length of the column and protein species migrate down the column in regions in which the pH is less than the isoelectric pH of the protein, but bind to the ion exchanger in regions of pH higher than the isoelectric point. Thus differential migration rates occur for proteins of different isoelectric points. An approximately linear pH gradient is produced over the chosen pH range, and proteins elute at a pH corresponding to their isoelectric point. The technique has a focusing effect, and therefore a small loading volume is not critical. An investigation of the possible advantages of this technique for obtaining very highly purified enzyme was undertaken.

Agnew et al. (1981) observed multiple bands with aldehyde dehydrogenase activity on isoelectric focusing gels for mitochondrial and cytoplasmic aldehyde dehydrogenases from sheep liver. For the cytoplasmic enzyme, two close bands with a mean isoelectric point of 5.22 ± 0.03 were observed, indicating the presence of two isoenzymes. This necessitated the reexamination of some of the results reported by MacGibbon et al. (1977a,b), in particular the biphasic NADH displacement and the non-linear dependence of the steady state rate on propionaldehyde concentration, to determine whether the observed effects are due to isoenzymes with different kinetic properties.

2.2 METHODS

All reagents were prepared in glass distilled deionised water using reagents of the highest quality available (refer Appendix III).

2.2.1 Spectrophotometric Assays

Assays were carried out using a Unicam SP 500 Series II spectrophotometer fitted with a Unicam SP 22 recorder or an American Instruments Corporation Aminco DW 2a uv-visible spectrophotometer. Both instruments were fitted to waterbaths thermostatted at 25°C. Reaction progress was followed by monitoring the appearance of NADH at 340 nm, assuming a molar extinction coefficient of 6220 $l.mol^{-1}.cm^{-1}$ (Horecker & Kornberg, 1948). The reaction was always followed for at least three minutes and as in some cases a slight lag was observed at

the start of the reaction, rate measurements were always made once a linear trace was obtained.

Assays at a propionaldehyde concentration of 100 μM, which is high enough to saturate the P_1 binding domain, but insufficient for significant binding at the low affinity P_2 site, will be referred to as "low propionaldehyde" assays throughout this work, and those at 20 mM propionaldehyde, sufficiently high to saturate both binding sites, as "high propionaldehyde" assays. Assay components are described below, with actual assay concentrations shown in parentheses. Reaction was initiated by the addition of propionaldehyde.

(a) High propionaldehyde Assay

2.20 cm^3 of 35 mM phosphate buffer	(25 mM)
0.45 cm^3 of 14 mM NAD^+ solution	(1.0 mM)
0.25 cm^3 of 240 mM propionaldehyde solution	(20 mM)
0.10 cm^3 of enzyme solution	

Propionaldehyde was distilled into glass vessels from which any traces of acid had been removed by heating in a large volume of boiling water after extensive cleaning. The refractive index of the distilled propionaldehyde was measured at 20°C and compared with the literature value of 1.3636 (Handbook of Chemistry and Physics, 1981).

(b) Low Propionaldehyde Assay

Assays at low propionaldehyde were exactly as above for the high propionaldehyde assay, but with the replacement of the 240 mM propionaldehyde solution by a 1.2 mM solution, giving a final propionaldehyde concentration in the assay of 100 μM. The 1.2 mM propionaldehyde solution was always prepared immediately before use by dilution from the 240 mM solution, using doubly distilled deionised water.

As the high propionaldehyde assay is a much more rapid and convenient means of determining the enzyme active site concentration than the NADH fluorescence titration, and both methods have been shown by MacGibbon (1976) to produce consistently the same results, the assay method was used throughout the present study. Unless otherwise stated, all enzyme concentrations given are active site concentrations determined by this method using the previously reported k_{cat} value of 0.25 s^{-1}, these being the results obtained from duplicate assays, with the requirement that there was agreement to within 2% of the two assays. Protein concentrations, determined as described in section 1.2.6, are also given where relevant.

Because the k_{cat} value which was used has very recently been shown to be incorrect (L.F. Blackwell, personal communication), all of the enzyme active site concentrations which are given in this work are incorrect as they are all about twice their true value. However, most of the concentrations have not been corrected using the higher revised k_{cat} value because further work is required before the precise nature of the loss of specific activity upon dilution of the enzyme is determined. Revised values for k_{cat} of 0.56 s^{-1} at high (20 mM) propionaldehyde concentrations and of 0.16 s^{-1} at low (100 µM) propionaldehyde levels based on preliminary investigations has been used for interpretation of the pH dependence of the enzyme in Chapter 4, and for certain calculations in which the exact enzyme concentration was important for the correct interpretation of the data, but in each case this has been specified.

2.2.2 NADH Titrations

NADH titrations were carried out as described by MacGibbon et al. (1979) using an Aminco SP 500 Ratio Spectrofluorimeter thermostatted at 25°C. The excitation wavelength was 340 nm (5 nm bandpass) and the emission wavelength was 435 nm (10 nm bandpass).

Aliquots of 0.01 cm^3 of NADH solution were added to the fluorimeter cuvette, by means of a glass syringe attached to a micrometer. A blank titration, in which NADH was added to a cuvette containing 3.0 cm^3 of 35 mM pH 7.6 phosphate buffer, was always carried out first, followed by titration of samples containing 3.0 cm^3 of enzyme buffer mixture with NADH. 20-30 aliquots of NADH were usually added, resulting in a final NADH concentration of 8-10 µM.

All solutions except the enzyme solution were filtered through sintered glass to remove dust before use.

2.2.3 Amino Acid Analysis

A sample of enzyme (prepared by method 1, 1.2.3) which had been shown to produce only one band when run on SDS gel electrophoresis, was dialysed for six hours against distilled water (2X4 l) and freeze dried in hydrolysis tubes which had been cleaned with AR nitric acid. Samples were weighed and then hydrolysed with 6 M HCl (containing 0.1% phenol) for 24, 48 and 72 hours at 100°C in sealed evacuated tubes.

The hydrolysates were analysed on a Beckman Model 120C Amino Acid Analyser.

The total cysteine and cystine content (half cystine) of the enzyme was determined as cysteic acid by the method of Hirs (1967), in which performic acid is produced from formic acid and hydrogen peroxide and used to oxidise the cysteine at -10°C. 2 mg of freeze dried protein was added to 0.2 mg pure formic acid and 0.1cm ^{3} methanol at -10°C. Performic acid (0.2cm ^{3}) was added and the solution maintained at -10°C for 2.5 hours, then frozen in liquid air and dried using a vacuum pump. The dried sample was hydrolysed as described above for 24 hours.

The tryptophan content was measured by the method of Matsubara & Sakasi (1969), which involved addition of 3% vol/vol thioglycolic acid to the 6 M HCl and phenol solution used for the hydrolysis. The sample was hydrolysed for 24 hours.

2.2.4 Metal Ion Analysis

2.2.4.1 Emission Spectrometry

Two samples of enzyme from different preparations (both method 1) which had been shown to produce only one band on SDS electrophoresis gels were dialysed for 24 hours against three 4-litre changes of 0.005 M pH 7.3 phosphate buffer, and then transferred to vials which had been washed with AR grade nitric acid (followed by deionised distilled water) and freeze dried. The dialysis tubing had previously been boiled in a solution containing 0.2 g/l EDTA and 0.2 g/l sodium bicarbonate. A sample of 1.0 M pH 7.3 phosphate stock buffer was also freeze dried. All samples were then dried at 110°C for several hours. 4-5 mg of enzyme was thoroughly mixed with spectroscopic grade graphite in an enzyme:graphite ratio of 1:2. 3 mg of the buffer sample was mixed in a buffer:graphite ratio of 1:5. Samples were loaded into a hollow graphite electrode (including a sample of pure graphite) and dried at 110°C for 24 hours.

The emission spectrum of the samples was recorded using a Hilger Watts Emission Spectrograph and the photographic plate was then compared with a spectral map and examined for spectral lines indicating the presence of metal ions.

2.2.4.2 Absorption Spectroscopy

A solution of enzyme (20 μM) and a blank of 0.022 M pH 7.4 phosphate buffer were directly analysed by aspiration on a Varian Techtron AA 5 Atomic Absorption Spectrometer at wavelengths corresponding to the metal ions of interest.

2.2.5 Spectral Changes on NADH Binding

Two pairs of matched cuvettes were prepared; one cuvette of each pair containing a 2.4 cm^3 solution of enzyme (39 μM) in 0.022 M phosphate pH 7.3 buffer and the other containing only buffer. Both pairs were placed in tandem in a Shimadzu MPS 500 double-beam spectrophotometer and a baseline absorption spectrum obtained.

NADH (0.10 cm^3 of a 635 μM solution, prepared in the same buffer) was added, by means of a micrometer regulated Hamilton syringe, to the enzyme solution in the sample beam and to the buffer solution in the reference beam to give a final NADH concentration of 25.4 μM. The same volume of water was added to the reference enzyme solution, and the difference spectrum was then recorded.

Separate absorption spectra for bound and free NADH were obtained by running the enzyme-NADH solution against the enzyme reference solution and the NADH solution against buffer.

2.2.6 Chromatofocusing

Chromatofocusing experiments were carried out using a 1 x 25 cm column of Polybuffer Exchanger PBE 94 (Pharmacia Fine Chemicals) according to the instructions supplied with the exchanger. All buffers contained 0.1% v/v 2-mercaptoethanol. The column was equilibrated with the starting buffer, 0.025 M pH 7.4 imidazole HCl, and the enzyme sample to be loaded was dialysed against the same buffer.

5 cm^3 of the eluant buffer, Polybuffer 74 (47 cm^3 diluted to 420 cm^3) was run on to the column, followed by the enzyme sample, and then the column was eluted with this buffer at a rate of 5-8 cm^3 per hour. About 50 hours were required for total elution. Fractions were collected and the pH, absorbance at 280 nm and the aldehyde dehydrogenase activity of each fraction measured.

2.2.7 Isoelectic Focusing

Isoelectric focusing was carried out using an LKB multiphor apparatus in conjunction with an LKB 2103 power supply. Narrow range ampholine polyacrylamide gel electrophoresis plates of pH range 4.0-6.5 were used, following instructions supplied with the polyacrylamide gel plates (LKB instruction leaflet 1804). Pieces of filter paper containing sample (approximately 10 µl of sample) were applied to the gel plate. After focusing for 30 minutes, the filter paper pieces were removed and focusing continued for a further 2-2.5 hours at 3-10°C, after which the gels were cut and stained separately for protein and enzyme activity. The pH gradient was determined by cutting 1cm^3 pieces from one edge of the gel plate, which had been kept free of sample. These pieces were placed in distilled water overnight and the pH of each resulting solution determined at the same temperature as the focusing run.

2.3 Stopped Flow Studies

2.3.1 Apparatus

Stopped-flow experiments were carried out using a Durrum-Gibson D 110 Stopped Flow spectrophotometer (Durrum Instruments Corporation, Palo Alto, California U.S.A.). In the stopped flow apparatus two reactant solutions in separate syringes are forced through a mixing jet into an observation chamber by a compressed-air driven plunger (Figure 2.2). Flow is halted by a stopping syringe which hits a trigger, activating the recording device and allowing changes in the observation chamber to be monitored. Mixing occurs within about 3 ms. The observation chamber is 1.7 cm long and 0.15 cm^3 of each solution is used in each run. Reaction progress is followed by monitoring radiation at an appropriate wavelength with a photomultiplier after it has passed through the observation chamber.

Figure 2:2 STOPPED FLOW APPARATUS

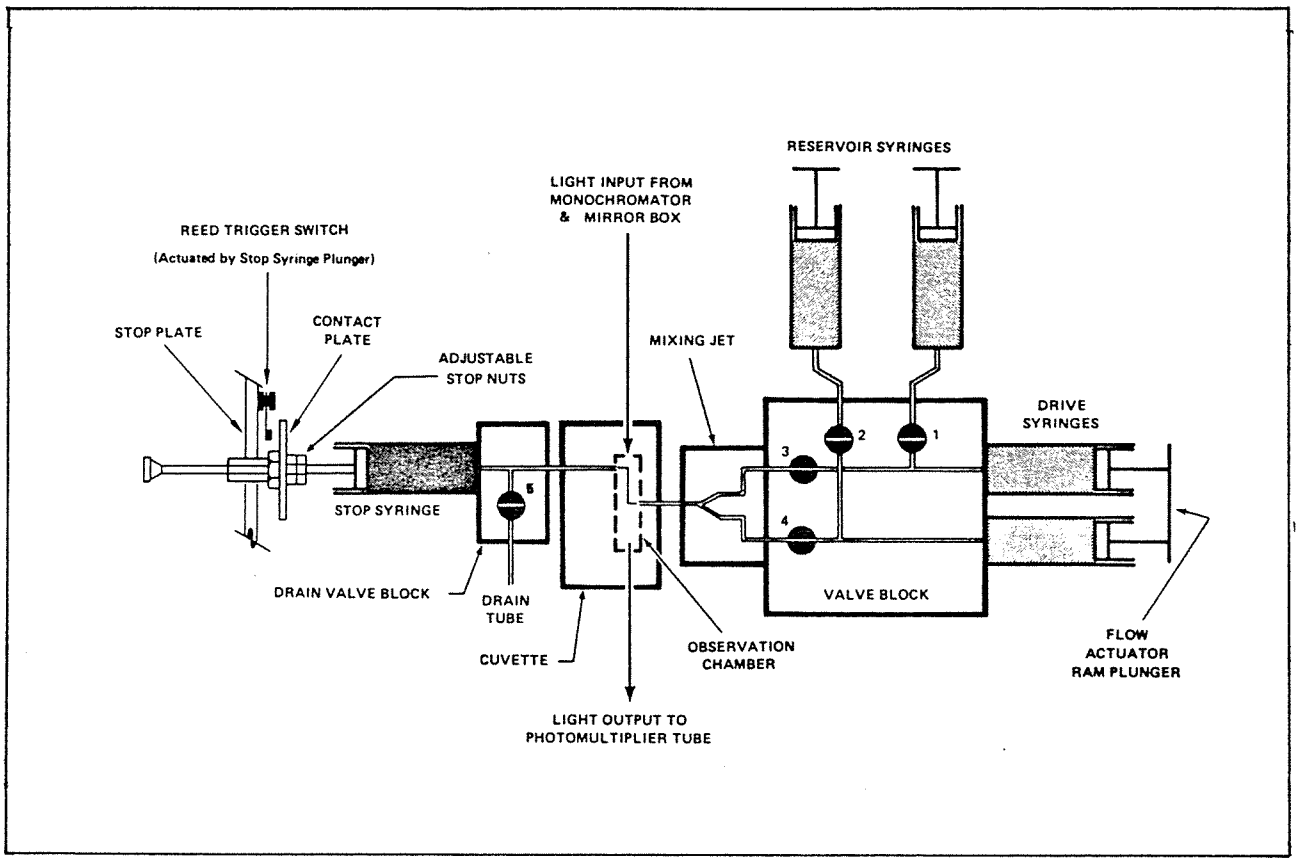


Figure 2.2 Schematic representation of the stopped flow apparatus (Durram Instruments Corporation Instruction Manual (D 110) 1974 p 3.14) In the fluorescence mode the light output to the photomultiplier tube is at right angles to the light source.

2.3.2 Observation and Recording of Data

For absorbance measurements, signals from the photomultiplier were passed via a log buffer amplifier to a Data Laboratories (Mitcham, Surrey, U.K) DL 905 or DL 901 transient recorder. The data were displayed on a Hewlett Packard 141B oscilloscope and, if the trace obtained was satisfactory, transferred to a Cromemco 2ZD minicomputer (Cromemco Inc., Mountain View, CA.) from the transient recorder via an interface. A permanent record of the reaction trace was printed using a Bryans 2900 A4 X-Y recorder (Figure 2.3). Data were either processed immediately or stored on disc for processing at a later date.

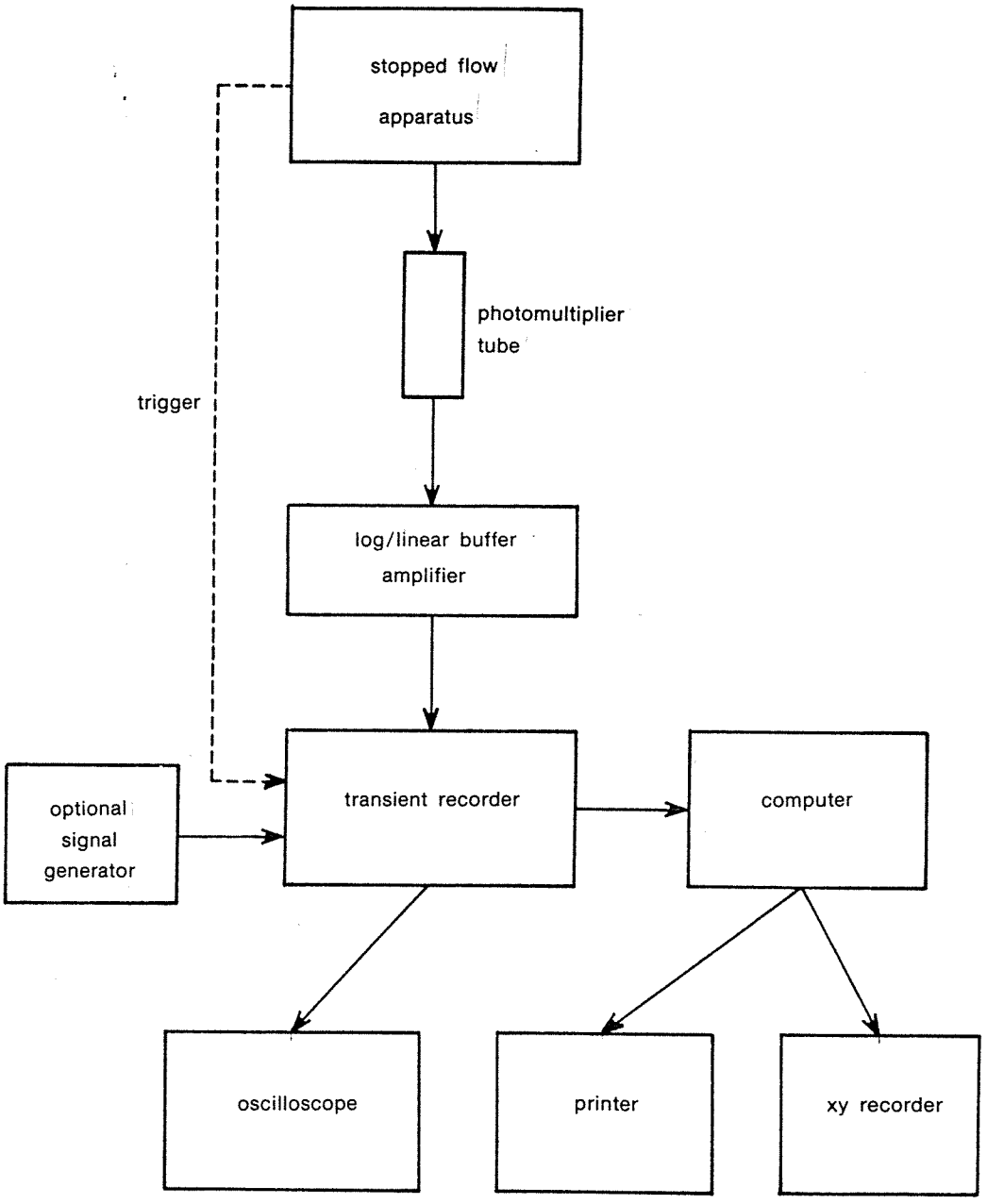
When traces with a low signal to noise ratio were obtained, it was possible to average several traces, thus improving the signal to noise ratio. Up to 8 traces could be stored by the computer, then each data point averaged to give a computer averaged transient or C.A.T. and calculations could then be performed on the averaged transient. This procedure could also be used for previously stored data.

The DL 905 transient recorder had a maximum time scale of 10 seconds, which was too short for some of the measurements required. For longer time scales, a Hewlett Packard 3311A function generator was connected to the transient recorder as an external timing device, as the DL 901 recorder which allowed time measurements of up to 200 s was not always available. For fluorescence measurements, the photomultiplier was mounted at 90° to the incident light.

Nucleotide fluorescence was excited with radiation at 340 nm and observed through Wratten 47B and 2B filters (sandwiched between glass discs) which together have maximum transmittance at 435 nm. Protein fluorescence was excited at 290 nm and observed through a Wratten 18A filter (maximum transmittance 335 nm).

For nucleotide fluorescence measurements, the fluorescence was standardised at the beginning of each experiment using a solution containing a known amount of NADH (usually 10 μM). There was, however, no reliable calibration of protein fluorescence. Use of enzyme solutions of known protein concentration was possible but the consistency of fluorescence of different enzyme samples is not known.

Figure 2:3 BLOCK DIAGRAM OF THE STOPPED FLOW APPARATUS AND ASSOCIATED EQUIPMENT



2.3.3 Preparation of Solutions

Solutions were prepared in 35 mM phosphate buffer which had been filtered through sintered glass to remove dust and degassed on a water pump for 10-15 minutes prior to use. Serious interference in the signal results from the presence of either dust or air bubbles in the reaction chamber, particularly in the case of fluorescence measurements. NAD^+ (Sigma Grade III) was prepared in water, but adjusted to pH 7.0 using 0.1 M NaOH. NADH concentrations were determined by absorbance measurements at 340 nm. Enzyme concentrations were determined by assay as described in section 2.2.1. Further degassing of the prepared solutions was possible by manually lowering the pressure in the loading syringes. However, solutions containing propionaldehyde or other volatile substances were not degassed in this manner.

2.3.4 NADH Burst Experiments

NADH burst experiments were usually carried out by mixing a solution containing enzyme, premixed with NAD^+ (2 mM) with a solution containing propionaldehyde (400 µM or 40 mM) in the stopped flow spectrophotometer, which was set up in the fluorescence mode, and NADH fluorescence was monitored.

2.3.5 NADH Displacement Experiments

Both NAD^+ and NADH bind at the same site on the enzyme. Thus if both species are present in a solution containing enzyme, there is competition between the two species for binding sites.

$$E + NADH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.NADH$$

$$E + NAD^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E.NAD^+$$

Thus, if a solution of NAD^+ is mixed with a solution containing enzyme and NADH, then some of the NADH will be displaced from the enzyme. At high NAD^+ concentrations, $k_2[NAD^+]$ will be much greater than both $k_1[NADH]$ and k_{-1} and the rate of dissociation of NADH from the enzyme will be irreversible and independent of NAD^+.

In displacement experiments, excess NAD^+ (2-8 mM) was mixed with a solution containing enzyme (2-5 μM) and NADH (10-40 μM) in the stopped flow spectrometer. The decrease in fluorescence at 435 nm caused by the conversion of the enzyme-NADH complex to the less fluorescent free NADH was monitored. This decrease in fluorescence upon NADH displacement has previously been shown to be biphasic (MacGibbon et al. (1977b) for all enzyme samples purified as described in Chapter 1.

2.4 TREATMENT OF DATA

2.4.1 Calculation of Active Site Concentration by Spectrophotometric Assay

The rate of oxidation of propionaldehyde, catalysed by aldehyde dehydrogenase, is equal to the rate of NADH production

$$v = \Delta[NADH]/\Delta t \qquad 2.12$$

The concentration of a solution of an absorbing species, such as NADH, which has an absorption maximum at 340 nm, can be related to absorbance using Beers Law

$$A = \varepsilon\, c\, l$$

where A = absorbance

ε = molar extinction coefficient of the absorbing species at the wavelength monitored.

c = concentration of species monitored

l = path length of the incident radiation through the solution

Hence, by rearrangement

$$c = A/\varepsilon.l$$

Therefore, if a reaction involves production or loss of the absorbing species

$$\Delta c = \Delta A/\varepsilon.l$$

Substituting for Δc in equation 2.12

$$v = \frac{\Delta A_{340}}{\Delta t \varepsilon_{NADH} l} \qquad 2.13$$

where ε for NADH at 340 nm is 6220 $l.mol^{-1}.cm^{-1}$ and A_{340} is the absorbance at 340 nm.

In the high propionaldehyde assay, both NAD^+ and propionaldehyde are present at saturating concentrations with respect to the enzyme concentration and thus the reaction rate is equal to V_{max}, the rate at infinite substrate concentrations. The value of V_{max} is dependent on the enzyme concentration.

$$V_{max} = k_{cat}[E]$$

Hence

$$[E] = V_{max}/k_{cat}$$

From 2.13

$$[E] = \frac{\Delta A_{340}}{\varepsilon_{NADH} l \Delta t k_{cat}} \qquad 2.14$$

$\Delta A_{340}/\Delta t$ can be measured by a spectrophotometer and at saturating concentrations of NAD^+ and propionaldehyde, k_{cat} was set equal to 0.25 s^{-1} per active site (MacGibbon et al., 1977a). Thus using l = 1cm and ε_{NADH} = 6220 $l.mol^{-1}.cm^{-1}$, the enzyme active site concentration can be calculated.

Active site concentrations can also be obtained from the low propionaldehyde assay, using the lower k_{cat} value of 0.082 s^{-1} (MacGibbon et al., 1977a) as the concentration of propionaldehyde used is sufficient to saturate the P_1 site alone. However, this method was not used to determine enzyme concentrations in the present study,

except for the purposes of comparison. As noted above, these k_{cat} values of 0.25 s^{-1} and 0.082 s^{-1} have very recently been revised, and thus all active site concentations reported are approximately twice their true values.

2.4.2 NADH Titrations

An enzyme molecule may possess a number of binding sites for a particular ligand. Provided all such sites are identical, they will act independently, and binding of ligand at any one site will have no effect on binding at any other site. In such a case the dissociation constant for the ligand binding is defined by the equation

$$K_D = \frac{[\text{Free Ligand}][\text{Free Binding Sites}]}{[\text{Bound Ligand}]}$$

In the case of NADH binding to the enzyme

$$K_D = [NADH][E]/[E.NADH] \qquad 2.15$$

where [NADH] is the concentration of free enzyme

[E] is the concentration of free binding sites

[E.NADH] is the concentration of occupied binding sites

As the total concentration of each species (NADH and enzyme binding sites) is the sum of the concentrations of the bound and free species, then

$$[E] = [E]_0 - [E.NADH]$$

and

$$[NADH] = [NADH]_0 - [E.NADH]$$

where $[E]_0$ and $[NADH]_0$ are the total concentrations of these species. Substituting in equation 2.15

$$K_D = \frac{([NADH]_0 - [E.NADH])([E]_0 - [E.NADH])}{[E.NADH]} \qquad 2.16$$

The fractional saturation of binding sites R is defined

$$R = [E.NADH]/[E]_o \qquad 2.17$$

Hence substituting for E.NADH in equation 2.16:

$$K_D = \frac{[E]_o(1 - R)([NADH]_o - R[E]_o)}{R[E]_o} \qquad 2.18$$

By rearrangement

$$\frac{1}{1 - R} = \frac{1}{K_D} \cdot \frac{[NADH]_o}{R} + \frac{[E]_o}{K_D} \qquad 2.19$$

Thus a plot of $1/(1 - R)$ against $[NADH]_o/R$ will have a slope of $1/K_D$ and an intercept on the abscissa axis of $[E]_o$, allowing determination of both K_D and $[E]_o$.

This form is useful because R is readily determined by experiment for the binding of NADH to the enzyme, since there is an enhancement of the nucleotide fluorescence observed upon binding, so that

$$R = \Delta F/\Delta F_{max}$$

where ΔF represents the difference in fluorescence between the blank titration containing NADH only and a titration containing enzyme and NADH. The fluorescence due to enzyme alone is also subtracted from the observed fluorescence. ΔF_{max} is the maximum change in fluorescence between the blank and enzyme titration, obtained when all the NADH sites are occupied. This value is determined graphically from the titration data, by plotting $1/\Delta F$ against $1/[NADH]$. ΔF_{max} is obtained from the intercept on the ordinate axis. Correction for the small amount of dilution was made by calculating a dilution-adjusted value for ΔF

$$\Delta F_{adj} = \Delta F(3 + 0.01n)/3 \quad \text{for each nth aliquot.}$$

If the concentration of enzyme molecules is known, then the number of active sites per molecule may be determined by the same method, using $n[E]_t$ to replace the $[E]_o$ term, where n is the number of binding sites per enzyme molecule and $[E]_t$ is the protein (tetramer) concentration of the enzyme sample.

Thus equation 2.18 has the alternative form

$$\frac{1}{1 - R} = \frac{1}{K_D} \cdot \frac{[NADH]_o}{R} + \frac{n[E]_t}{K_D} \qquad 2.20$$

An alternative graphical method, using the Scatchard Plot (Scatchard, 1949), in which a plot of

$$\frac{[NADH]_B}{[NADH]_F[E]_t} \quad \text{against} \quad \frac{[NADH]_B}{[E]_t} \qquad 2.21$$

i.e.

$$\frac{R}{[NADH] - R[E]_t} \quad \text{against} \quad R \qquad 2.22$$

where $[NADH]_B$ is the concentration of bound NADH

$[NADH]_F$ is the concentration of free NADH

and $[E]_t$ is the total protein concentration

results in a line with a slope of $-1/K_D$ and an intercept on the abscissa axis equal to n.

Both graphical methods produce straight lines only if all the binding sites are identical. The second method was found to be more convenient in cases in which the enzyme binding site concentration was already determined, and a comparison of the NADH binding in the presence and absence of a modifier was being made.

2.4.3 Amino Acid Analysis

The area under each peak of the amino acid analyser output trace was calculated and the actual amount of each amino acid determined by reference to the 'colour constant', the ninhydrin response of each amino acid determined from a set of standards run immediately prior to the enzyme hydrolysates. The mean amounts of each amino acid were determined from three samples for each hydrolysis time, and the results obtained for serine and threonine were extrapolated back to zero hydrolysis time, as these amino acids are slowly destroyed during hydrolysis.

Results are expressed in terms of moles of amino acid per 10,000 g of protein, but the number of moles of each amino acid per mole of protein can be calculated using the molecular weight of 212,000 reported by MacGibbon et al. (1979).

2.4.4 Processing of Burst Data

Photomultiplier signals were transferred by the transient recorder to the computer as 1028 8 bit data points. Burst experiments resulted in a single first order exponential and these were treated by a method described by Laidler & Bunting (1973), using a computer fitting programme developed by Hardman (1983b, 1985). The steady state slope was extrapolated back to zero time to obtain a baseline, then the $\log_{10}$ of the difference between the transient and the baseline plotted against time. A least squares analysis was used by the computer to fit a straight line to the $\log_{10}$ difference data. The rate constant was obtained from the slope of this line (k_b = slope x -2.303). After calculating the rate constant and amplitude of the burst process, the computer regenerated the burst curve, using the calculated values (Figure 2.4). The closeness in fit of the superimposed computer generated curve to the original reaction trace was taken as a measure of the accuracy of the calculated values.

2.4.5 NADH Displacement Experiments

NADH displacement experiments and other experiments, such as NADH binding experiments, which resulted in biphasic reaction traces were treated by the following methods, using computer programmes developed by Hardman (1983b).

The first method was based on that described by Gutfreund (1975 p126) and Hardman et al. (1978). The slower process was treated as a single exponential, with a baseline being drawn through the final baseline (or in the case of other biphasic processes which were followed by a steady state through the linear steady state part of the trace) to time zero. The $\log_{10}$ of the difference in fluorescence between the transient and the baseline was plotted as a function of time, and a least squares fit to this line calculated. The decay constant for the slower process was obtained from the slope of the straight part of this curve as described above. The decay constant for

the fast process was obtained by plotting the $\log_{10}$ of the difference between the line obtained from the first log difference plot (Figure 2.5) and the points on the first plot, and obtaining a least squares fit to this difference data. In this study, most of these calculations were performed by the computer interfaced to the stopped-flow apparatus

Figure 2:4 COMPUTER FITTING OF BURST DATA

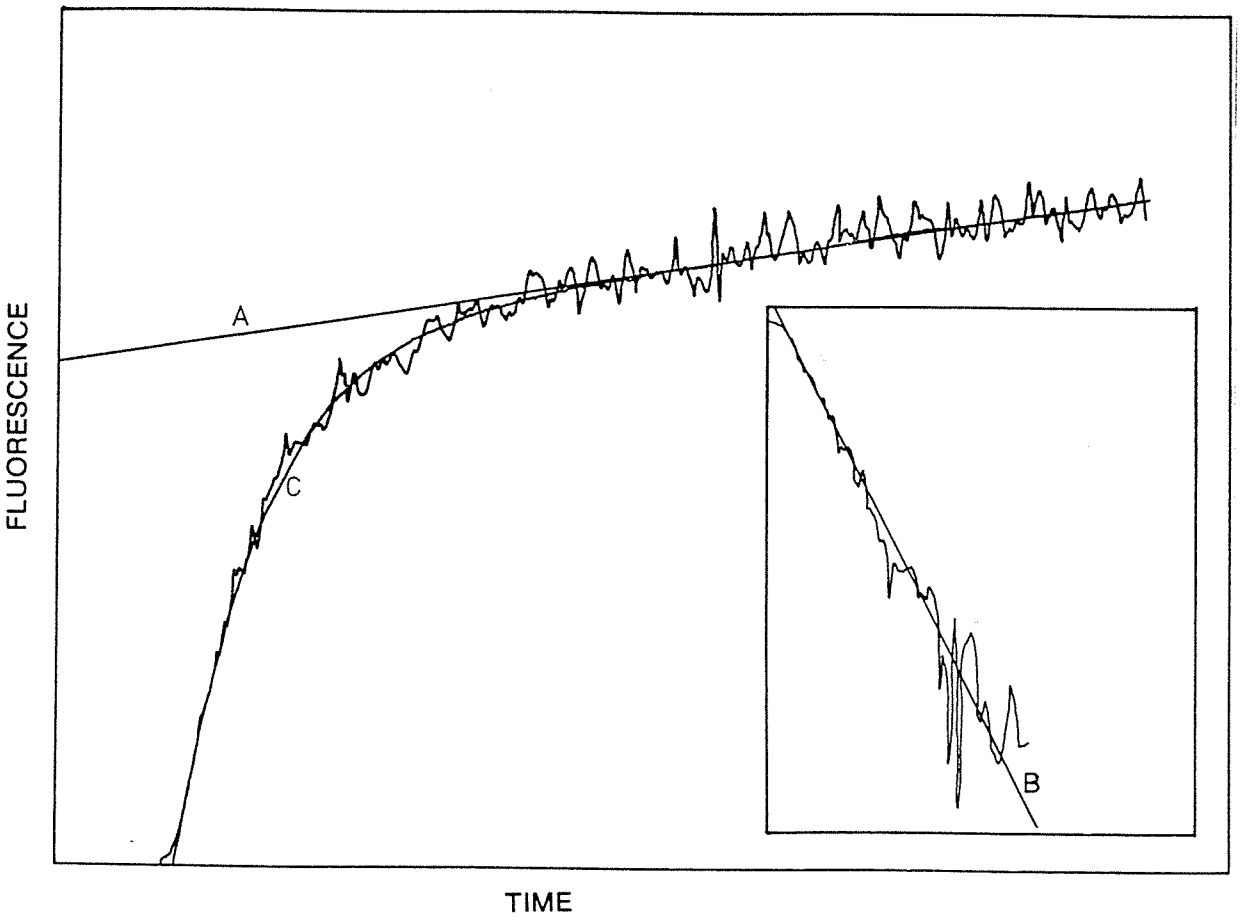
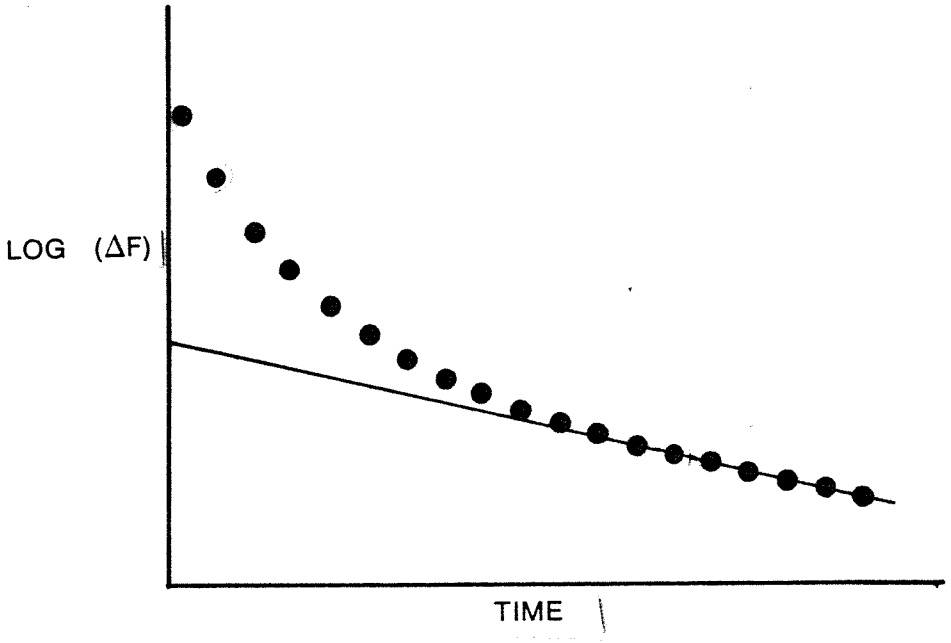


Figure 2.4 Line A is the extrapolated least squares line through the steady state production of NADH. Line B (inset) is the least squasres fit to the log of the difference in fluorescence between the transient and the extrapolated steady state line. Line C is the computer generated curve, obtained by using the calculated values for the burst rate constant and the steady state rate. The amplitude is obtained by measuring the difference between the fluorescence and the extrapolated steady state line at time zero.

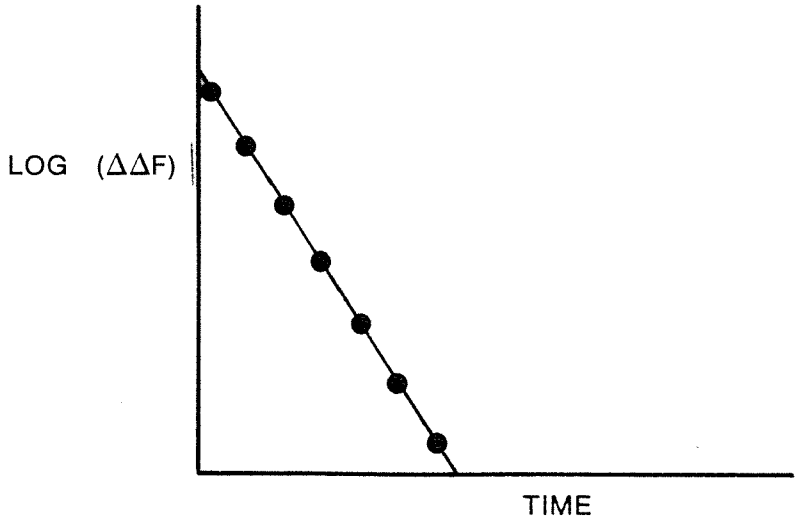
Figure 2:5 GRAPHICAL DERIVATION OF RATE CONSTANTS FOR A PROCESS INVOLVING TWO FIRST ORDER REACTIONS

(a) THE SLOWER PROCESS



The $\log_{10}$ of the difference between the transient and the baseline is plotted and the rate constant of the slow process is obtained from the slope of the extrapolated line (rate constant = -2.303 x slope)

(b) THE FASTER PROCESS



The $\log_{10}$ of the actual difference between the experimental ΔF values and the extrapolated values of ΔF (i.e. $\Delta\Delta F$) is plotted against time and the rate constant of the fast process is obtained from the slope of the line. The amplitudes of the fast and slow processes are the extrapolated intercepts.

(Figure 2.6), but in some cases the rate constants were obtained graphically.

In cases in which the baseline fluorescence had not been achieved at the end of the trace, a better fit was obtained by using the pretrigger level, but this was only possible if there was no machine drift.

The second method involved use of a non-linear least squares iterative curve fitting procedure (based on the Marquardt method (Marquardt, 1963)) using a FORTRAN programme (Daniel & Wood, 1971, Hardman, 1983a, 1985). Data were fitted to the equation for two exponentials:

$$Y = Y_f + A_1 e^{-\lambda_1 t} - A_2 e^{-\lambda_2 t}$$

where A_1 and A_2 are the amplitudes of the two phases and λ_1 and λ_2 represent the corresponding first order decay constants.

Initial estimates of the decay constants, amplitudes and the baseline intercept at time zero were required. These were obtained by use of the difference logarithmic method described above.

This method could be used for experiments in which two exponential processes followed by a steady state process were observed, or for simpler cases such as single exponential fits. The main requirement for the use of the method was that there must be sufficient data for each of the processes involved to allow a fit to be made (i.e. at least 5 half-lives for the slowest exponential and a good estimate of the steady state rate). A computer-generated curve was produced, using the rate constants and amplitudes obtained by iteration, and the closeness of the fit when superimposed on the original data was used as a measure of the accuracy.

Figure 2:6 ANALYSIS OF A BIPHASIC TRANSIENT BY COMPUTER

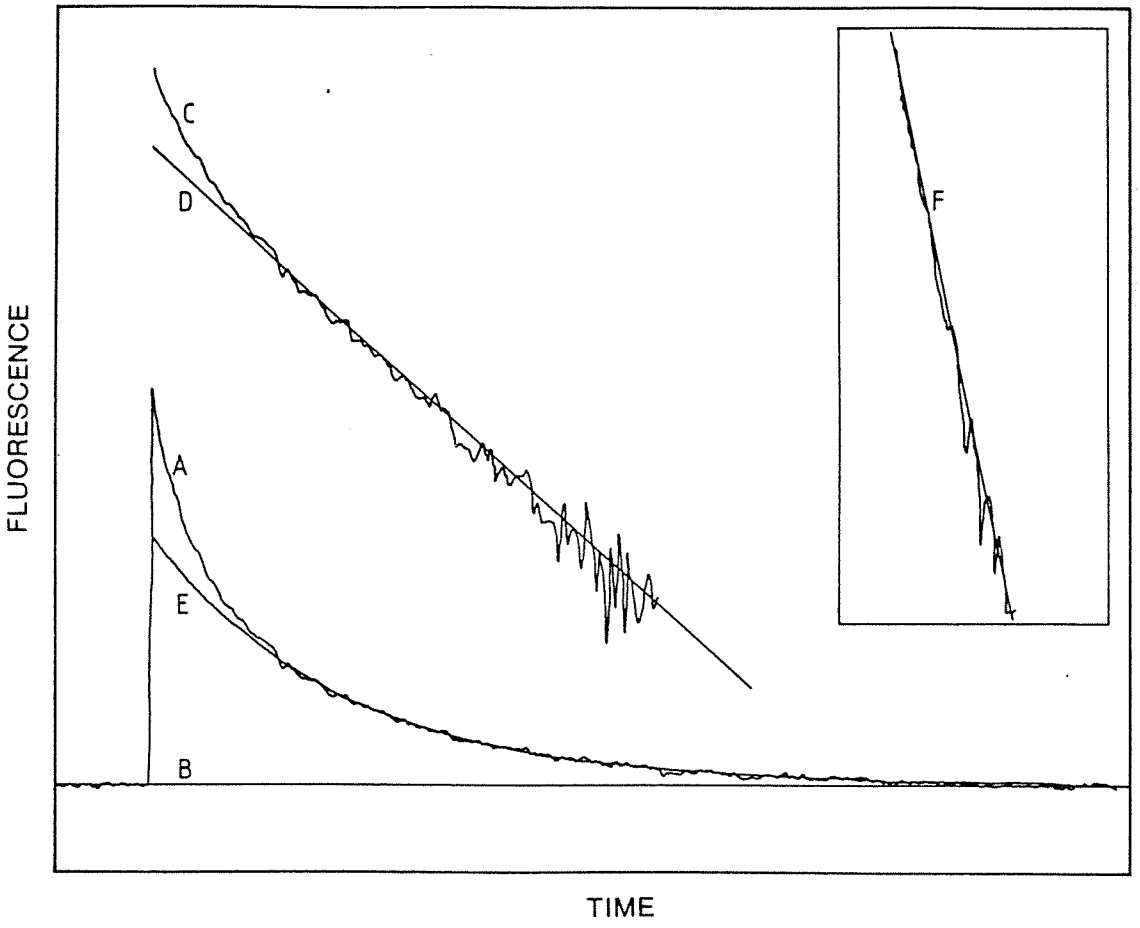


Figure 2.6 The trace shows the biphasic decrease in nucleotide fluorescence when NADH is displaced from the enzyme by NAD^+. A is the time dependent change in fluorescence. Line B is a horizontal line fitted through the last 10% of the trace. Line C is a log difference plot between the baseline (A) and the transient. Line D is a least squares line through the lower part of the log difference plot. E is a computer generated fit to the slower of the two exponentials. The inset (Line F) shows the least squares line through the log difference plot between line C and Line D.

2.5 RESULTS

2.5.1 Amino Acid Analysis

The results of the amino acid analysis are shown in Table 2.1. MacGibbon et al. (1979) reported a value of 36 thiol groups/enzyme molecule and the values obtained here of 34 thiol groups/212,000 g enzyme compares favourably with this value.

TABLE 2.1

AMINO ACID COMPOSITION OF THE ENZYME

Amino Acid	Yield after Hydrolysis mol/10^4 g Protein			Mean
	24 h	48 h	72 h	
Lysine	5.4	5.8	5.6	5.6
Histidine	1.1	1.2	1.1	1.1
Arginine	2.8	2.7	2.6	2.7
Cysteic Acid	1.6	-	-	1.6
Aspartic Acid	7.1	7.4	7.2	7.2
Threonine*	4.1	4.0	3.9	4.2*
Serine*	4.8	4.6	4.3	5.0*
Glutamic Acid	7.7	7.9	8.3	8.0
Proline	4.3	4.2	4.7	4.4
Glycine	6.9	7.1	7.0	7.0
Alanine	5.9	6.0	6.0	6.0
Valine	5.1	5.7	5.8	5.5
Methionine	1.5	1.5	1.5	1.5
Isoleucine	3.9	4.3	4.4	4.2
Leucine	5.5	5.6	5.6	5.6
Tyrosine	2.2	2.2	2.2	2.2
Phenylalanine	3.8	3.9	3.9	3.9
Tryptophan	2.1	-	-	2.1
Ammonia	-	-	-	-

* Determined by extrapolation to zero hydrolysis time

2.5.2 Metal Ion Analysis

Examination of the photographic plate obtained by emission spectroscopy revealed lines corresponding to sodium and phosphorus, which were both present in the buffer, and magnesium and copper.

Quantitative determinations of the amounts of magnesium and copper in two enzyme samples were made using atomic absorption spectroscopy. The levels of these elements present in the enzyme samples were considerably less than stoichiometric (Table 2.2), magnesium by a factor of 10-20, and copper by a factor of 200.

TABLE 2.2

METAL ION CONTENT OF ENZYME SOLUTION

Metal	Observed Amount of Metal ion µg/g	Amount Expected Stoichiometrically µg/g
Magnesium	0.025	0.590
Copper	0.008	1.65

2.5.3 Spectral Changes on NADH Binding

The difference spectrum between bound and free NADH is shown in Figure 2.7. The absorption maximum of NADH was shifted from 340 nm to 344 nm on binding to the enzyme. The greatest difference in absorbance occurred at 328 nm, when a value for the difference in absorption coefficient at 328 nm ($\Delta\varepsilon_{328}$) of 0.94 $mM^{-1}.cm^{-1}$ was obtained from this spectrum.

At 340 nm $\Delta\varepsilon_{340}$ was 0.73 $mM^{-1}.cm^{-1}$, in good agreement with the value of 0.66 $mM^{-1}.cm^{-1}$ obtained by MacGibbon et al. (1977b) for the absorbance change observed at 340 nm during displacement of NADH from the enzyme with NAD^+ on the stopped flow spectrophotometer.

2.5.4 Chromatofocusing

The results of two separate runs are shown as somewhat different results were produced. A third run produced results similar to the second of those shown here.

The elution profiles for the two runs are shown in Figure 2.8.

Figure 2:7 THE DIFFERENCE IN ABSORPTION SPECTRUM BETWEEN NADH BOUND TO ALDEHYDE DEHYDROGENASE AND FREE NADH

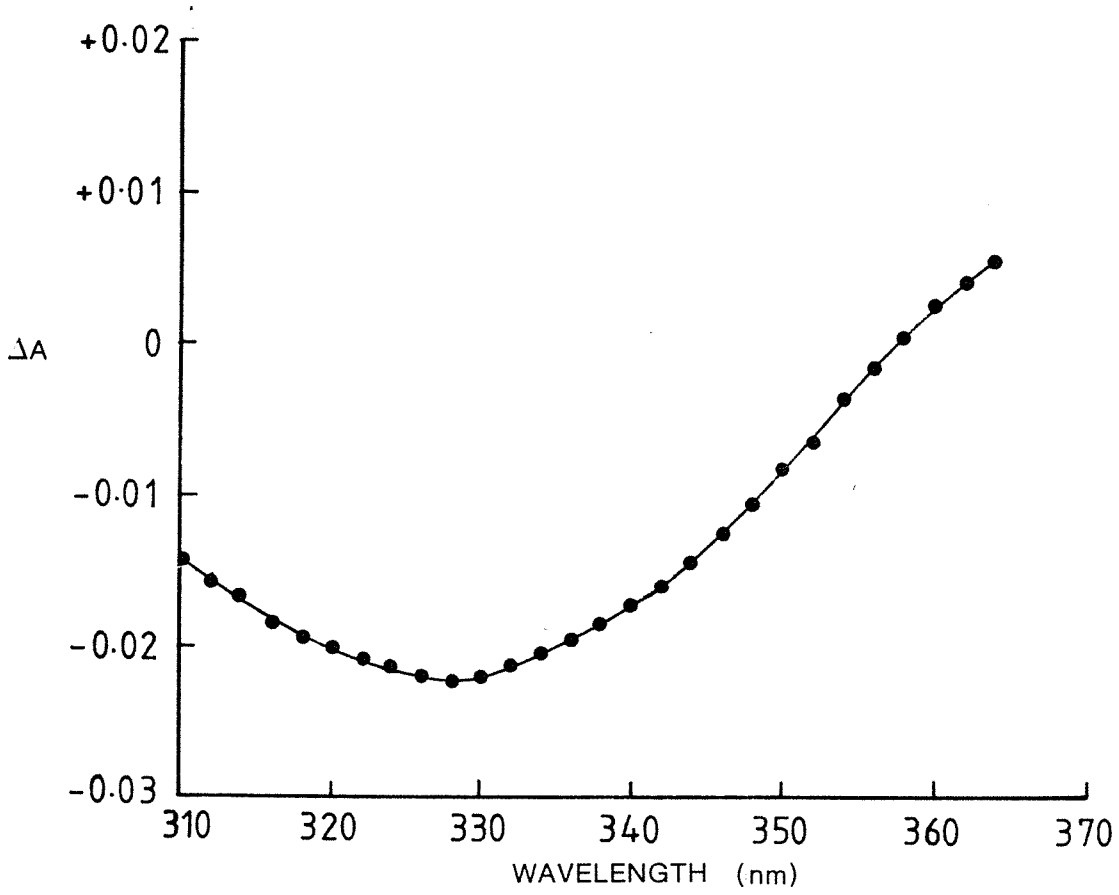


Figure 2.7 The difference spectrum was recorded with two pairs of 1 cm cuvettes mounted in tandem. The same amount of enzyme (2.4 cm^3 at 39 μM) was added to one of the cuvettes of each pair, while the two remaining cuvettes contained 2.4 cm^3 of 22 mM pH 7.3 phosphate buffer. Then 0.1 cm^3 of 635 μM NADH solution in 50 mM pH 7.6 phosphate buffer was added, by means of a micrometer-regulated Hamilton syringe, to the enzyme containing cuvette in the sample compartment and to the buffer-containing cuvette in the reference compartment. An equal volume of buffer was added to the enzyme-containing cuvette in the reference compartment and the difference spectrum was run several times over the wavelength range 300-360 nm. The base line was obtained in the absence of NADH and subtracted from the measured difference spectrum.

Isoelectric focusing on polyacrylamide gels plates was carried out on samples from the numbered marked fractions. In both these cases the enzyme was known to be contaminated with other protein.

(a)Run 1. A 5 cm^3 sample of 80 μM enzyme (unorthodox preparation method, D. Poll), heavily contaminated with mitochondrial aldehyde dehydrogenase and other protein, was applied to the chromatofocusing column and fractions of approximately 4 cm^3 were collected. Results are shown in Figure 2.8a. Enzyme activity was present in a number of partially resolved peaks over the pH range 5.2-5.6, highest activity being observed at 5.3. Minor peaks corresponded to pH values of 5.20, 5.25, 5.40 and 5.6. A large protein peak without aldehyde dehydrogenase activity, presumably the major contaminant protein lactate dehydrogenase, was also observed.

Agnew et al. (1981) observed multiple bands for mitochondrial and cytoplasmic aldehyde dehydrogenases on isoelectric focusing gels. Cytoplasmic enzyme produced two close bands with a mean isoelectric point of 5.22, and the mitochondrial enzyme produced the same two bands as the cytoplasic enzyme and also five other bands at pH values of 5.48, 5.56, 5.65, 5.70, and 5.76.

The results of isoelectric focusing of the original sample and the fractions marked are shown in Figure 2.9a. Dramatic reduction in the number of protein bands was achieved by the chromatofocusing method, and all remaining bands show aldehyde dehydrogenase activity. Different intensity ratios of the remaining bands were observed in fractions from the different activity peaks.

Comparison of the protein and activity stained samples reveals that, as reported previously by Agnew et al. (1981) and Dickinson & Berrieman (1979), the activity stain for the mitochondrial enzyme is more intense than for the cytoplasmic enzyme.

(b) Run 2. A 10 cm^3 sample of enzyme (95 μM), which appeared to contain significant amounts of mitochondrial aldehyde dehydrogenase, was loaded and 7 cm^3 fractions collected. A slightly steeper pH profile was observed (Figure 2.8b) and this factor and the larger fractions are probably the cause of the poorer resolution of the separate activity peaks compared to the previous run. Isoelectric focusing of marked fractions and of the original enzyme sample was then carried out. Duplicate or triplicate samples were focused and the plate stained for activity of aldehyde dehydrogenase (Figure 2.9b).

Total separation of the mitochondrial and cytoplasmic enzymes was

Figure 2.8 The column was equilibrated with 25 mM pH 7.4 imidazole HCL buffer. The enzyme sample was dialysed against this buffer, then applied to the column following a 5 cm^3 cample of Polybuffer 74 (diluted 1:9). The column was then eluted with Polybuffer 74 at a rate of 5-8 cm^3 per hour as described in methods. All buffers contained 2-mercaptoethanol.

2.8(a) A 5 cm^3 sample of 80 μM enzyme was applied to the column and 4 cm^3 fractions were collected.

2.8(b) A 10 cm^3 sample of 95 μM enzyme was applied to the column and 7 cm^3 fractions were collected.

Isoelectric focusing of samples from the marked fractions was carried out (see Figure 2.9).

(□) absorbance at 280 nm

(●) activity of aldehyde dehydrogenase

(○) pH

Figure 2:8 ELUTION PROFILES OF ALDEHYDE DEHYDROGENASE ON THE CHROMATOFOCUSING COLUMN

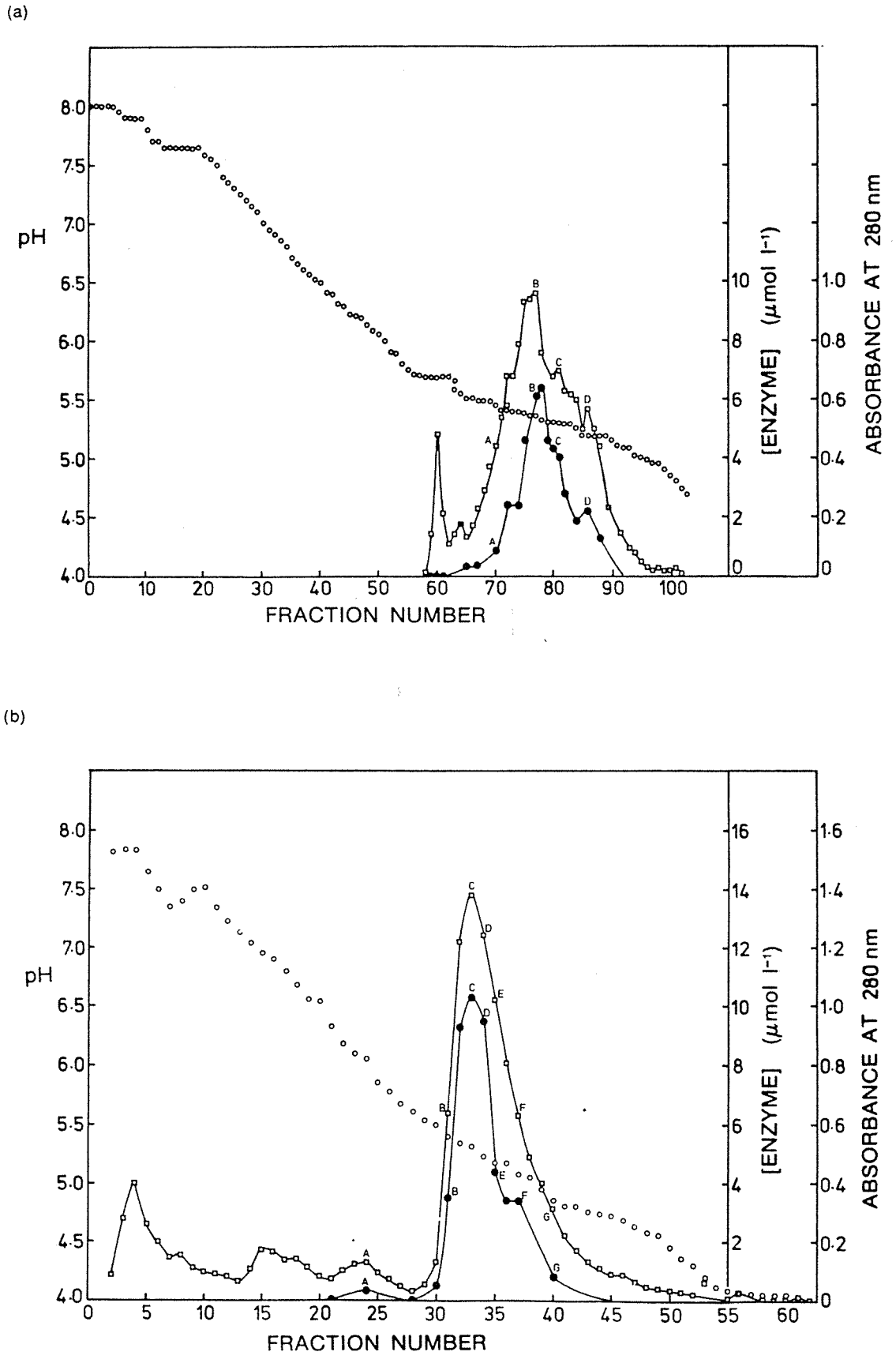


Figure 2.9 Isoelectric Focusing of Aldehyde Dehydrogenase Samples from the Chromatofocusing Column.
Isoelectric focusing was carried out as described in Methods.
2.9 (a) Samples from chromatofocusing column run 1. (refer Figure 2.8a). A, B, C, D, represent samples from the marked fractions; S is the sample originally applied to the column.
Photograph A. Stained for Activity of Aldehyde Dehydrogenase
Samples from left:

A (30 µl), S (15 µl), D (15 µl), C (15 µl), B (15 µl), A (15 µl)

Photograph B. Stained for Protein
Samples from left
S (15 µl), D (15 µl), C (15 µl), B (15 µl), A (15 µl)

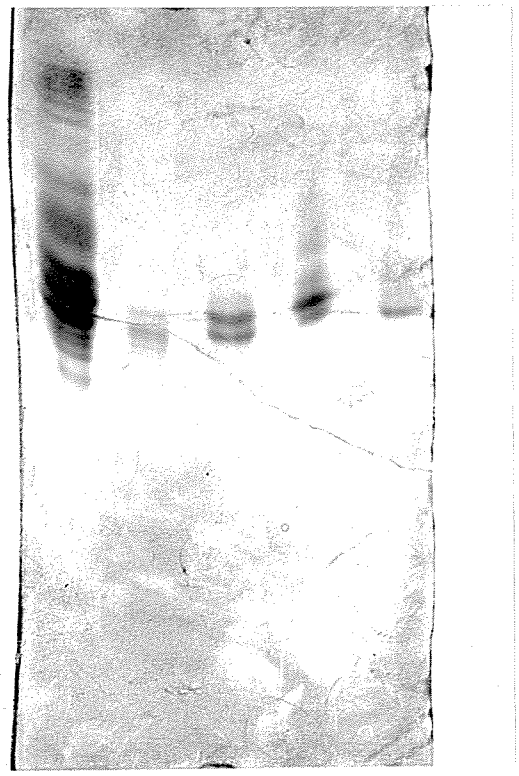
2.9 (b) Samples from chromatofocusing column run 2. (refer Figure 2.8b). A, B, C, D, E, F, G, represent samples from the marked fractions; S is the original sample applied to the column.
Photograph C. Stained for Activity

Samples from left (numbered)

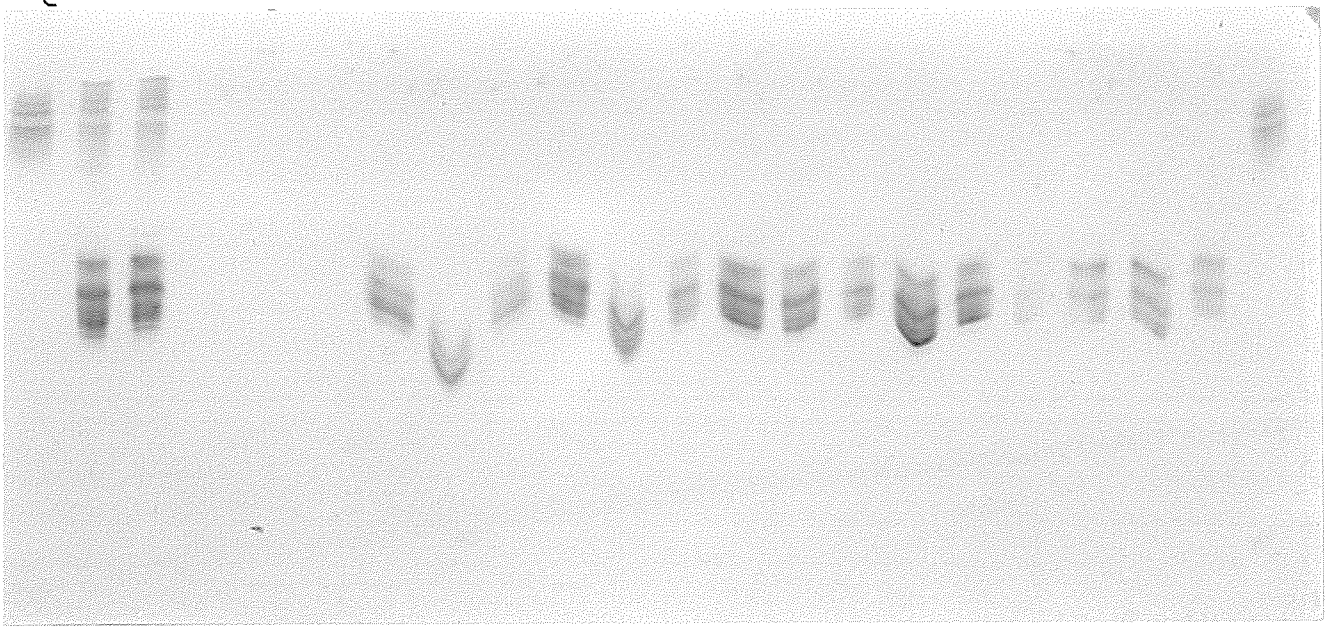
1.	A (30 µl)
2. 3.	S (5 µl)
4. 5. 6.	G (30, 20, 10 µl)
7. 8. 9.	F (20, 15, 10 µl)
10. 11. 12.	E (15, 10, 5 µl)
13. 14. 15.	D (15, 10, 5 µl)
16. 17. 18.	C (15, 10, 5 µl)
19. 20. 21.	B (20, 15, 10 µl)
22.	A (20 µl)

A

B



C



obtained using the chromatofocusing column. Although mitochondrial contamination of the original sample appears considerable from the activity staining, this is misleading, being due to more intense staining by the mitochondrial enzyme, as described above. As calculated from absorbance measurements at 340 nm and the activity assay (Figure 2.8b), the actual amount of mitochondrial enzyme was very small, representing only 3-5% of the total (determined by measurement of the area under the activity and A_{280} peaks).

In contrast with previous enzyme samples, 3 activity bands were observed for the cytoplasmic enzyme, corresponding to pH values of approximately 5.20. 5.25, and 5.50. However, narrower pH range plates were used than those used by Agnew et al. (1981), and this may be a result of greater resolution. Poorer separation of the 3 bands was achieved during this column run than in the previous one, but there was some variation in the relative intensities of the bands in the different fractions.

2.5.5 Enzyme Active Site Determinations

The concentration of active sites in an enzyme solution was determined by NADH titration and spectrophotometric assay at a high propionaldehyde concentration (20 mM). In addition, the spectrophotometric assays at the two different propionaldehyde concentrations were compared for a number of enzyme samples from all preparation methods, including a sample purified by chromatofocusing. In all cases there was good agreement between the various methods. The NADH titration indicated an NADH binding site concentration of 51 µM, compared with an active site concentration of 47 µM determined by the high propionaldehyde assay. For the large number of low propionaldehyde assays carried out during the course of experiments in the present work, the k_{cat} value determined from the low propionaldehyde assay was observed to vary from 0.05-0.15 s^{-1} when determined by comparison with the value of 0.25 s^{-1} assumed for the standard high propionaldehyde assay of the same enzyme sample. However, the majority of assays gave k_{cat} values of 0.08±0.01 s^{-1} at a propionaldehyde concentration of 100 µM, with good agreement between duplicate assays from the same enzyme sample. For the chromatofocused enzyme sample there was more variability in the rates of duplicate assays than was normal for these assays and the absorbance traces

showed considerable initial upward curvature. Both these effects were attributed to an effect of the polybuffer 74 which had not been removed from the sample. Average reaction rates of 4.4±0.4 μmoles/minute for the low propionaldehyde assay and of 14±1 mmoles/minute for the high propionaldehyde assay were obtained from six duplicate assays at each propionaldehyde level, demonstrating that despite the buffer effects the steady state rate at the higher propionaldehyde concentration was approximately three times the rate at the lower concentration as observed for other samples.

2.5.6 NADH Burst Experiments

The burst rate constant was determined at both low (200 μM) and high (20 mM) propionaldehyde levels for a sample of enzyme which had been purified using the chromatofocusing column (Peak tube, Run 2). These rate constants were compared with those obtained for enzyme from the same initial purification without chromatofocusing.

Similar results were obtained in all cases, burst rate constants of 7.3 s^{-1} and 7.4 s^{-1} being obtained at the lower propionaldehyde concentration and of 8.1±0.8 s^{-1} and 9±2s $^{-1}$ at the high propionaldehyde level for the purified and control enzyme respectively.

2.5.7 NADH Displacement Experiments

To determine whether the biphasic displacement of NADH could be a result, not of a two step process as concluded by MacGibbon et al. (1977b), but of two isoenzymes with different affinities for NADH, two displacement experiments were conducted. NAD^+ solution (4 mM) was used to displace enzyme-bound NADH from solutions of enzyme and NADH in both cases. In the first experiment an enzyme concentration of 7.0 μM and NADH concentration of 2.61μM were used, producing an [E.NADH]/[E] ratio of 0.3, assuming a K_D of 1.2 μM^{-1} (MacGibbon et al., 1977a, refer Appendix I). In the second experiment, NADH (45 μM) and a sample of enzyme (7.0 μM) purified by the chromatofocusing technique were used, and the results compared with the results of a duplicate experiment using a sample of enzyme from the same initial preparation, without the additional purification step.

In all cases biphasic traces were observed. The decay constants obtained were all very similar, as were the relative amplitudes of the

two processes, relative amplitudes of 0.5 ± 0.1 being observed for all three experiments.

2.6 DISCUSSION

In common with aldehyde dehydrogenases from other mammalian sources, sheep liver aldehyde dehydrogenase is not a metalloenzyme, as demonstrated by the absence of stoichiometric amounts of any metal ions in freeze dried enzyme samples or in solutions of active enzyme.

TABLE 2.3

AMINO ACID COMPOSITION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASES

Amino Acid	Sheep liver	Horse liver	Bovine liver	Human liver
	mol/10^4 g Protein			
Lysine	5.6	5.3	6.3	6.9
Histidine	1.1	1.0	1.1	1.5
Arginine	2.7	2.5	3.3	3.1
Cysteic Acid	1.6	1.4	1.4	2.0
Aspartic Acid	7.2	6.5	7.4	8.4
Threonine	4.2	4.1	3.4	5.1
Serine	5.0	4.1	4.7	5.3
Glutamic Acid	8.0	7.8	7.9	9.1
Proline	4.4	4.9	4.2	4.4
Glycine	7.0	6.7	7.5	8.8
Alanine	6.0	5.6	6.6	6.9
Valine	5.5	5.7	5.8	6.9
Methionine	1.5	1.5	1.6	1.5
Isoleucine	4.2	3.6	3.8	6.4
Leucine	5.6	5.7	5.8	6.7
Tyrosine	2.2	2.1	2.4	2.9
Phenylalanine	3.9	3.6	3.6	4.0
Tryptophan	2.1	0.9	1.3	1.3
Ammonia	-	3.4	5.3	-

A comparison of the amino acid compositions of cytoplasmic aldehyde dehydrogenases from sheep, human, bovine and horse liver is shown in Table 2.3. The primary structure of human liver aldehyde dehydrogenase has been published recently (Hempel et al., 1984) and the composition presented in the table for the human liver enzyme is

derived from that sequence, not from the earlier results of Greenfield & Pietruszko (1977).

The enzymes all have very similar amino acid compositions, apart from some variation in the amounts of tryptophan.

All of these enzymes have been shown to be tetramers of similar molecular weight and apparently identical subunits, thus it is likely that all are structurally and mechanistically very similar and a study of any one of the mammalian cytoplasmic aldehyde dehydrogenases may provide information of some relevance to human aldehyde metabolism.

The chromatofocusing column produced a dramatic improvement in the purity of the enzyme samples, with complete removal of mitochondrial enzyme and other proteins being observed. The different activity peaks of cytoplasmic aldehyde dehydrogenase were partially resolved, providing a possible means of separation of the isoenzymes observed by Agnew et al. (1981) and in the present study. If separation could be achieved by this method studies of single isoenzymes could then be undertaken to determine whether they have different kinetic properties. Some kinetic studies of the peak fraction from the second chromatofocusing run were made as described above.

Despite the fact that it is an exciting and efficient method for purifying enzyme samples, chromatofocusing is too expensive a technique to be included as a routine preparative procedure.

Both the NADH titration and the high propionaldehyde assay have been shown to produce the same results for determination of the active site concentrations of enzyme samples. The assay method was preferable as a routine method, because of its speed, reproducibility and ease of calculation, compared to the titration method and was used for all subsequent active site determinations.

Although the presence of isoenzymes of aldehyde dehydrogenase has been demonstrated using isoelectric focusing, there was no evidence that these isoenzymes had different substrate affinities or any other different kinetic properties. Regardless of the purity and method of preparation of the enzyme sample, a three-fold activation of the steady state rate was always observed using 20 mM propionaldehyde, compared with the rate when a propionaldehyde concentration of 100 µM was used.

In burst experiments, biphasic or multiphasic bursts would be expected for kinetically different isoenzymes, but there was no difference in the rate constants or amplitudes of the NADH burst when highly purified enzyme samples were used, compared with the samples

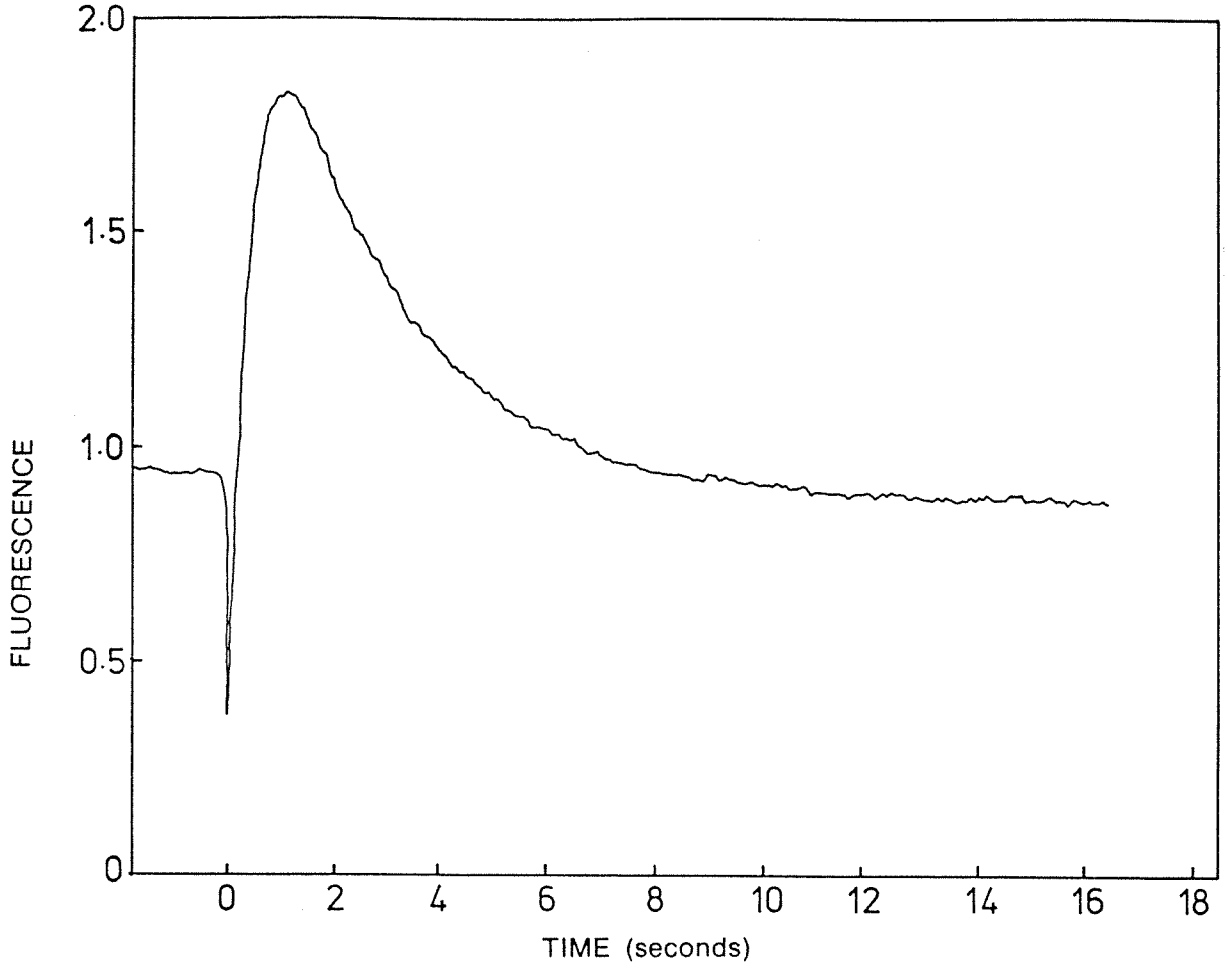
from the usual preparative procedure.

In addition, the displacement experiment in which sufficient NADH to bind to only 30% of the total enzyme was added would be expected to result in either a single exponential process, or if biphasic, then one process should be of much smaller amplitude than the other, which was not observed. Highly purified enzyme samples produced the same results as other samples. Even though total resolution of the different species was not achieved by isoelectric focusing using the chromatofocusing technique, the similarity of all three displacement experiments lends very strong support to the proposal of MacGibbon et al. (1977b) that the displacement of NADH from the enzyme is fundamentally a two step process, as a result of a slow isomerization step (conformational change) which must occur before NADH can dissociate from the enzyme.

Further clear evidence that such an isomerisation occurs during the normal dehydrogenase reaction in vitro is provided by single turnover experiments at a very low propionaldehyde concentration (Blackwell et al. 1986). As shown in Figure 2.10 (diagram courtesy of L.F. Blackwell and P.D. Buckley) a decrease in fluorescence was observed following the burst with a rate constant similar to that observed in the slow process during the NADH displacement experiment. Such a decrease is consistent with the mechanism proposed in Scheme 2.2 as under such conditions, all the aldehyde should react, rapidly converting an equivalent amount of enzyme to a mixture of the *E.NADH.acyl and *E.NADH complexes and producing a rapid increase in nucleotide fluorescence, due to the enhanced fluorescence of NADH when bound to the enzyme. After this, displacement of the NADH by NAD^+ (present in excess) should occur, limited by the rate of isomerisation of the binary E.NADH complexes, producing a decrease in fluorescence, as is observed. The fluorescence decay would not be expected to be biphasic, because under these conditions, at the end of the burst very little of the enzyme would have been converted to the E.NADH form, dissociation of which gives rise to the faster exponential observed in the NADH displacement experiments. Only a single exponential decrease in fluorescence was observed in the single turnover experiments and the decay constant for this process was 0.28 s^{-1}, very similar to the decay constant for the slower, isomerisation, process observed during NADH displacement experiments.

Therefore the biphasic nature of the NADH displacement is clearly

Figure 2:10 CHANGES IN NUCLEOTIDE FLUORESCENCE UNDER SINGLE TURNOVER CONDITIONS



Enzyme (5.3 μM) and NAD^+ (2.71 mM) in one syringe was pushed against propionaldehyde (16.4 μM) in the other. Both solutions contained 0.025 M pH 7.6 phosphate buffer. A rate constant of $0.28\ s^{-1}$ was obtained for the decrease in fluorescence following the burst.

a feature of the reaction mechanism, as previously proposed by MacGibbon et al. (1977b) and does not arise from the presence of two distinct types of NADH binding sites. Furthermore there is no evidence at present to suggest that any of the isoenzymes observed by isoelectric focusing have different kinetic properties, so separation of these enzyme forms is not necessary for valid kinetic studies of the enzyme. From the results presented here, there would not be expected to be any major differences between the isoenzymes of cytoplasmic aldehyde dehydrogenase.

In NADH displacement and binding experiments a horizontal baseline is obtained, following the biphasic displacement, as the final (constant) fluorescence level is achieved (Figure 2.6). Thus under conditions in which the electronic equipment was clearly stable and free of drift, so that the final fluorescence level was the same between separate runs, the pretrigger fluorescence could be used to construct an infinity line if the final fluorescence level had not been achieved at the end of the recording time frame. In test examples in which the fluorescence was still decaying at the end of the trace and the computer was instructed to construct a false infinity line through the last 100 points of the trace, higher incorrect values for the decay constants were obtained (approximately twice the real values). However, when an infinity line was constructed using the pre-trigger fluorescence level, then lower values were obtained, which were the same as those obtained using the iterative curve fitting procedure.

Thus it is essential that biphasic data be analysed carefully to avoid misleading results. The iterative least squares fitting procedure was the more reliable method of the two as it did not require an accurate infinity value, but in cases in which the infinity value was known accurately, the same results could be gained using the other method. In most cases, in this work, the difference logarithmic method was used to obtain estimates of the decay constants, and these were used to provide trial parameters for the iterative least squares programme. Normally very similar or identical results were obtained by the two methods. It must be noted here, however, that the trial parameters used for the iterative fit did not have to be particularly accurate as the same final results were obtained even when estimates incorrect by up to a factor of 10 were used inadvertently.

Recently it has been claimed by Dickinson (1985) that the decay constants for the NADH displacement at pH 7.0 are significantly greater

than those reported previously at pH 7.6 by MacGibbon et al. (1977b). However these faster decay constants can be shown to be the result of incorrect fitting of the data using a false infinity line as described above (Blackwell et al., 1986). The dependence of the decay constants for NADH displacement on pH is investigated further in Chapter 4.

CHAPTER 3
THE EFFECT OF p-MERCURIBENZOATE ON ALDEHYDE DEHYDROGENASE

3.1 INTRODUCTION

Aldehyde dehydrogenases that have been studied all possess a large number of thiol groups (2.6). The sheep liver cytoplasmic enzyme contains 34-36 thiol groups per tetramer, as determined previously by amino acid analysis (2.5.1) and by reaction of the denatured enzyme with Ellman's reagent (MacGibbon et al., 1979).

It has been suggested (Jakoby et al., 1963) that one of these groups is the site of acylation during the enzyme catalysed oxidation of aldehydes and it has sometimes been assumed that this is the case. However, the existence of a catalytically essential thiol group has not yet been established conclusively. Kitson (1978, 1982a, b) has shown that although the enzyme is very sensitive to inhibition by the thiol reagent disulphiram (tetraethylthiuram disulphide), it is not possible to reduce the enzyme activity to zero, even for highly purified samples and at very high disulphiram concentrations. This suggests that disulphiram is not reacting with the active site group but instead forms a modified enzyme with greatly reduced activity.

Activation of the dehydrogenase activity has been observed recently with another thiol reagent, 2,2'-dithiodipyridine (Kitson, 1978, 1982b, 1984) providing further evidence that disulphiram and related compounds can cause modification of the enzyme, but do not necessarily react at the site of aldehyde acylation.

The effect of the thiol reagent p-(chloromercuri)benzoate (PCMB) was studied in order to gain further information about the role of thiol groups in the enzyme activity. PCMB was chosen because of the reported high specificity of this reagent for thiol groups (Means & Feeney, 1971, pp198-205) and because it is possible to directly monitor mercaptide formation as the reagent reacts with thiol groups. PCMB has an absorption maximum at 233 nm with an extinction coefficient of $1.7 \times 10^4\ M^{-1}.cm^{-1}$, which increases to $2.2 \times 10^4\ M^{-1}cm^{-1}$ upon mercaptide formation (Riordan & Vallee, 1967). However the difference in absorption is greatest at 250 nm, the difference in extinction coefficients, $\Delta\varepsilon$, at this wavelength being $7.6 \times 10^3\ M^{-1}\ cm^{-1}$ (Boyer, 1954). This provides a means of studying the rate and extent of reaction of PCMB with proteins, independent of activity studies.

Preliminary studies indicated that high concentrations of PCMB reacted very rapidly with the enzyme, causing inhibition of the steady state rate, and that reaction of the enzyme with other thiol reagents, such as iodoacetamide and N-dansylaziridine was much slower. Thus PCMB was chosen as the preferred thiol reagent for study in the present work.

3.2 METHODS

As the enzyme was always prepared and stored in solutions containing 0.1% 2-mercaptoethanol, it was necessary to remove this reagent before any studies involving thiol reagents. Each enzyme sample was dialysed for at least four hours (usually overnight) against two 4-litre changes of 25 mM pH 7.3 phosphate buffer.

PCMB stock solutions were prepared as filtered saturated solutions in 0.2 M glycylglycine buffer and the concentration determined by absorbance measurements at 232 nm, using an extinction coefficient of 1.69×10^4 $M^{-1}cm^{-1}$ (Boyer, 1954).

3.2.1 Enzyme Assays

Enzyme active site concentrations were determined using the high propionaldehyde assay described in section 2.2.1.

The low propionaldehyde assay was used for most of the kinetic experiments in the presence of PCMB, so that the concentration of propionaldehyde was always just high enough to ensure that only the P_1 binding domain (2.1.3.) was occupied. Some experiments were,however, also carried out using high propionaldehyde concentrations.

A stock solution of PCMB in 0.2 M pH 7.6 glycylglycine buffer was prepared, and appropriate volumes added to assay solutions in place of phosphate buffer. The glycylglycine buffer used had no effect on aldehyde dehydrogenase activity.

The protein concentration was determined by absorbance measurements at 280 nm as described previously (1.2.6)

3.2.2 Titration of Thiol Groups with PCMB

Aliquots of PCMB (1.48 μM per aliquot) were added to a solution of enzyme (1.6 μM active sites, 1.6 μM protein) and the initial fast increase in absorbance at 250 nm was measured.

3.2.3 Determination of Total Thiol Content

Sodium dodecyl sulphate (SDS) is a detergent which acts as a denaturing agent, exposing previously inaccessible groups. Enzyme samples (0.3 μM) in 25 mM pH 7.6 sodium phosphate buffer were denatured with SDS (0.3% weight/volume). An excess of PCMB (196 μM) was added and the increase in A_{250} measured. Results were corrected for the absorbances of PCMB and enzyme at 250 nm.

3.2.4 PCMB Binding to Enzyme

An excess of PCMB (30-200 μM) was added to various enzyme samples (0.3-3.0 μM) in 25 mM pH 7.6 phosphate buffer. The increase in absorbance at 250 nm was monitored using a Unicam SP 500 Spectrophotometer until no further change in absorbance was observed. The reference solution contained an equivalent amount of PCMB in buffer, and the absorbance at 250 nm due to the enzyme was measured and this value subtracted from all absorbance measurements. Studies of PCMB binding to enzyme were also carried out using the stopped flow spectrometer, in which a solution containing enzyme (5 μM) was mixed with a solution of PCMB (30-500 μM) and the reaction followed by monitoring changes in absorbance at 250 nm. In a similar set of experiments, protein fluorescence was monitored at 335 nm (2.3.2).

3.2.5 NADH Titration

NADH titrations in the presence and absence of activating levels of PCMB were carried out using nucleotide fluorescence as described previously (2.2.2). 0.01 cm^3 aliquots (124 μM stock solution, 1.24 nmoles per aliquot) were added to 3.00 cm^3 solutions containing buffer only (the blank titration), enzyme solution (0.79 μM), and an enzyme, PCMB mixture (0.79 μM enzyme, 3.8 μM PCMB).

3.2.6 Esterase Assays

The effect of PCMB on the esterase activity of aldehyde dehydrogenase was studied by adding PCMB to solutions containing enzyme (1-3 μM) and p-nitrophenylacetate (PNPA, 100 μM) in 25 mM pH 7.6 phosphate buffer. Varying volumes of PCMB stock solutions were added in place of buffer to give a final total volume of 3.00 cm^3 in each assay. The reaction was followed by monitoring the production of the p-nitrophenoxide ion at 400 nm, the absorption maximum for this species, and the reaction rate compared with the control rate in the absence of PCMB. PNPA stock solutions were prepared in ethanol, but the final ethanol concentration in assays did not exceed 3%, a level which has been shown to have no effect on the enzyme activity (MacGibbon et al., 1978b).

3.2.7 Stopped Flow Experiments

3.2.7.1 NADH Burst Experiments

NADH burst experiments at pH 7.6 were carried out as described in section 2.3.4 using solutions containing 2.0-5.0 μM enzyme, 2 mM NAD^+, and 400 μM propionaldehyde. Various concentrations of PCMB were added, employing several mixing conditions in which NAD^+, PCMB or both were premixed with the enzyme. Control experiments in the absence of PCMB were conducted in each case. A second set of burst experiments were carried out using 40 mM propionaldehyde in place of the 400 μM solution.

3.2.7.2 Proton Burst Experiments

Proton burst experiments were carried out using the method of Bennett et al. (1982).

An enzyme solution which had been dialysed overnight to remove 2-mercaptoethanol as described above was dialysed for three hours against 2 x 3 litres of 0.5 mM pH 7.6 phosphate buffer to lower the buffer concentration of the solution. All solutions contained 20 μM phenol red, 0.1 M Na_2SO_4 and 0.1 M $NaNO_3$ and were adjusted to pH 7.6 with 0.01 M HCl or NaOH immediately before use.

Solutions containing 15 μM enzyme premixed with 2 mM NAD^+ and 60 μM PCMB were mixed with propionaldehyde (400 μM or 40 mM) in the

stopped flow spectrometer. Control experiments were conducted in the absence of PCMB. The reactions were followed by monitoring the decrease in absorbance of phenol red at 560 nm.

3.2.7.3 NADH Displacement Experiments

NADH displacement experiments were carried out at pH 7.6 as described in section 2.3.5 by mixing a solution containing 3 μM enzyme and 33 μM NADH with a solution containing excess NAD^+ (4 mM). PCMB was premixed with enzyme and NADH.

3.2.7.4 NADH Binding Experiments

NADH binding experiments were conducted by mixing a solution containing enzyme (5.8 μM) in 25 mM pH 7.6 phosphate buffer with a solution of NADH (84 μM) in buffer in the stopped flow spectrometer. The increase in NADH fluorescence was monitored at 435 nm following excitation at 340 nm. PCMB (24 μM) was premixed with enzyme.

3.3 Treatment of Data

3.3.1 Spectrophotometric Assays

The reaction rate for each assay was calculated as described previously (2.4.1). Results were calculated in terms of v/[E] and in the case of assays containing PCMB, rates were expressed relative to the control rate in the absence of PCMB.

3.3.2 NADH Titrations

Fluorescence titration data were analyzed as described previously (2.4.2) by means of Scatchard plots of

$$(R/([NADH] - [E]_t)) \quad \text{against} \quad R, \quad \text{where } R = \Delta F/\Delta F_{max}$$

and $[E]_t$ is the total enzyme concentration in moles per litre of protein. Dilution corrections for increasing volume were also made as described previously (2.4.2). A least squares fit of the data was calculated in each case.

3.3.3 Binding Studies

NADH displacement and binding data, which were biphasic, were analysed as described in section 2.4.5.

3.3.4 Burst Experiments

Burst data (for both the NADH and proton bursts) were analysed as described previously (2.4.4). Bennett et al. (1982) observed that the amplitude of the proton burst is equal to the enzyme active site concentration and therefore corresponds to release of a single proton. In the present work the amplitudes of the proton bursts in the presence of PCMB were compared with the amplitude for the control experiment, and are expressed as relative amplitudes.

3.4 RESULTS

Unless otherwise stated, all enzyme concentrations given are active site concentrations. Protein concentrations are specified as tetramer or protein concentrations.

3.4.1 Titration of Thiol Groups

The value for the increase in extinction coefficient of PCMB at 250 nm upon reaction with the enzyme was determined from the slope of plots of the change in absorbance, ΔA_{250}, against the total PCMB concentration. An average value of 7940 $M^{-1}.cm^{-1}$ was obtained, which was not significantly different from the value of 7600 $M^{-1}.cm^{-1}$ reported by Boyer (1954) for cysteine, hence the literature value was used for all subsequent calculations.

A plot of moles of mercaptide produced per mole of protein against moles of PCMB added per mole of protein is shown in Figure 3.1. An average of 12 thiol groups reacted with PCMB within the time of mixing. At PCMB/enzyme ratios higher than 12, there were significant departures from linearity.

Figure 3:1 TITRATION OF THIOL GROUPS ON NATIVE ALDEHYDE DEHYDROGENASE WITH PCMB

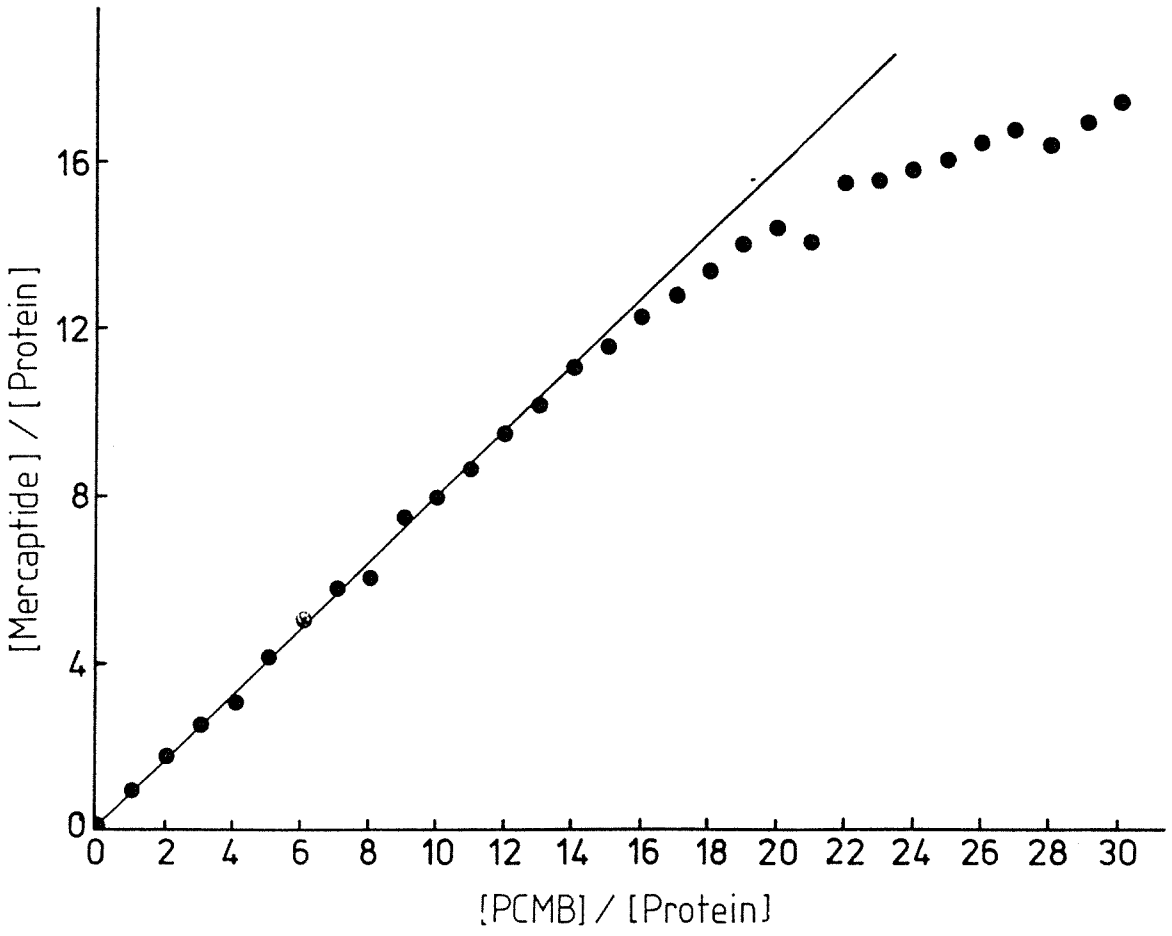


Figure 3.1 Titration of Thiol Groups on Native Aldehyde Dehydrogenase with PCMB at pH 7.6.

Enzyme (1.6 µM) was titrated with PCMB (1.48 µM per aliquot) by monitoring the increase in absorbance at 250 nm. The plot became non-linear when about 12-14 SH groups had been titrated.

3.4.2 Determination of Total Thiol Content

In the presence of SDS the increase in A_{250} due to mercaptide formation when excess PCMB was added to enzyme corresponded to an average of 34±2 thiol groups per mole of protein, using a value of $\Delta\varepsilon$ at 250 nm of 7600 $M^{-1}.cm^{-1}$. This was in good agreement with the results obtained from the amino acid analysis (2.5.1) and for the reaction of Ellmans reagent with the enzyme (MacGibbon et al., 1979).

3.4.3 PCMB Binding Studies

When an excess of PCMB (30-200 μM) was added to enzyme (0.3-3 μM) in a spectrophotometer cuvette, there was an initial increase in A_{250} which occurred within the time of mixing. This was followed by a slow increase in absorbance over about 180 minutes (Figure 3.2). Logarithmic plots of the increase in A_{250} against time showed that there was more than one process involved. Three rate constants were obtained by constructing logarithmic difference plots of the data using a graphical method similar to that shown in Figure 2.5, assuming that the observed time-dependent increase in A_{250} was the sum of three independent first order processes. i.e.

$$A_t = A_0 + A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) + A_3(1 - e^{-k_3 t})$$

where A_t is the absorbance at time t and A_1, A_2 and A_3 are the amplitudes, and k_1, k_2 and k_3 the rate constants of the three processes.

At the end of the reaction (t^∞),

$$A^\infty = A_0 + A_1 + A_2 + A_3$$

The average values obtained from several experiments for the rate constants and amplitudes of these processes, and the amplitude of the rapid process are shown in Table 3.1. There was considerable variation in the amplitudes of the two most rapid processes, most probably due to the difficulty of obtaining accurate data for the first few seconds of the reaction using a conventional spectrophotometer. The total amplitude of these rapid processes was however consistently about 12-16 moles of mercaptide produced per mole of protein. The PCMB concentration had no obvious effect on the rate constants or amplitudes

Figure 3:2 PROGRESS CURVE FROM REACTION OF PCMB WITH ALDH

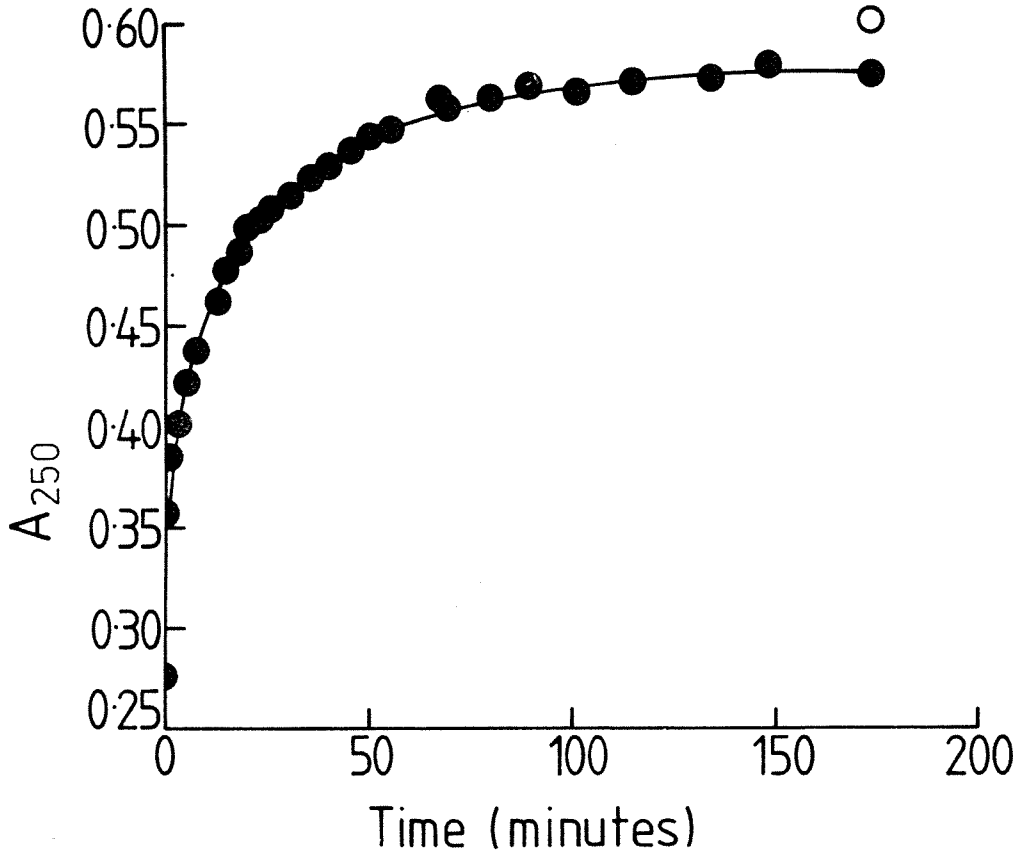


Figure 3.2 Progress Curve for Reaction of PCMB with Aldehyde Dehydrogenase.

The time dependence of mercaptide formation at pH 7.6 is shown. Enzyme (1 μM) was reacted with PCMB (54 μM) in a spectrophotometer cuvette and the increase in A_{250} was monitored as a function of time. The initial rapid increase in absorbance corresponded to 11 SH groups per tetramer and the total change was equivalent to 36 SH groups per tetramer.

obtained, within a PCMB/enzyme ratio range of 20-300.

TABLE 3.1

REACTION OF PCMB WITH ALDEHYDE DEHYDROGENASE

	Rate Constant s^{-1}	Moles Mercaptide/ Mole Protein	
Rapid process	fast*	2-8	
Slow process 1	0.05±0-03	8-14	total 14±3
Slow process 2	$(1.9\pm0.6) \times 10^{-3}$	14±2	
Slow process 3	$(2.5\pm0.9) \times 10^{-4}$	30±10	
		58 Total	

* within time of mixing

It is apparent from the Table that the total amplitude was considerably greater than that which would be expected on the basis of the number of thiol groups on the enzyme as determined above.

Further study of the initial rapid increase in A_{250} was conducted using the stopped flow spectrometer. As for the steady state changes, multiple processes were observed. It was not possible to resolve these into distinct first order processes, even by using the iterative nonlinear curve-fitting programme described in chapter 2 (2.4.5). Although apparently very close fits of the data for each individual run were obtained using this programme (Figure 3.3), different rate constants were obtained for duplicate runs carried out on different time scales, and it was possible to fit the data for runs on any time scale from 0.2-50 seconds assuming two first order exponential processes followed by a steady state, to get rate constants ranging from 20-30 s^{-1} to 0.02 s^{-1}. However, the amplitudes of some of these processes corresponded to mercaptide to enzyme ratios of less than one. The total amplitude of the absorbance change at 250 nm as determined over 50 seconds was 2-3 moles mercaptide/mole active sites. This was somewhat lower than that expected on the basis of the steady state results above, but if it is assumed that there are four active sites per subunit this gives a value of 8-12 moles mercaptide per mole of protein formed within 50 seconds of mixing. There was no detectable effect of PCMB concentration on these rate constants over the range 15-250 μM.

For similar experiments, when protein fluorescence was monitored using the stopped-flow spectophotometer, instead of PCMB

Figure 3.3 Stopped Flow Trace showing Rapid Reaction of PCMB with Aldehyde Dehydrogenase.

A solution of enzyme (14 μM) in 0.025 M pH 7.6 phosphate buffer was pushed against a solution of PCMB (250 μM) in the same buffer. The increase in absorbance at 250 nm was monitored over several time intervals. In each case, it was possible to obtain a close computer generated fit to the data (also shown) using the iterative curve-fitting procedure, but the rate constants obtained depended upon the time frame chosen for the experiment and in many cases the amplitudes obtained corresponded to mercaptide to enzyme ratios of less than one (refer results).

Figure 3.4 Protein Fluorescence Changes on Reaction of PCMB with Aldehyde Dehydrogenase.

Enzyme (5.0 μM) was rapidly mixed with PCMB (15 μM) and the resulting decrease in fluorescence was monitored at340 nm following excitation of the protein fluorescence at 280 nm. Although apparent rate constants of $0.31s^{-1}$ and $2.0s^{-1}$ were obtained using the iterative curve fitting programme described in Chapter 2, the process was clearly multiphasic, and representative individual rate constants could not be obtained.

Figure 3:3

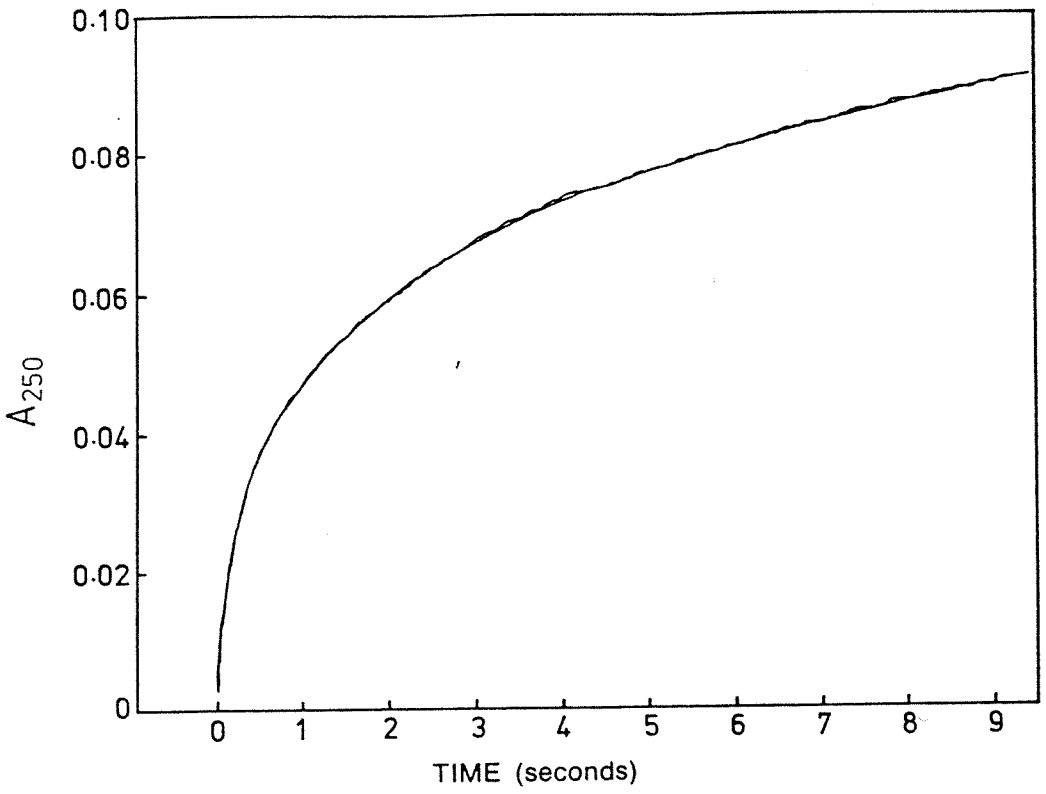
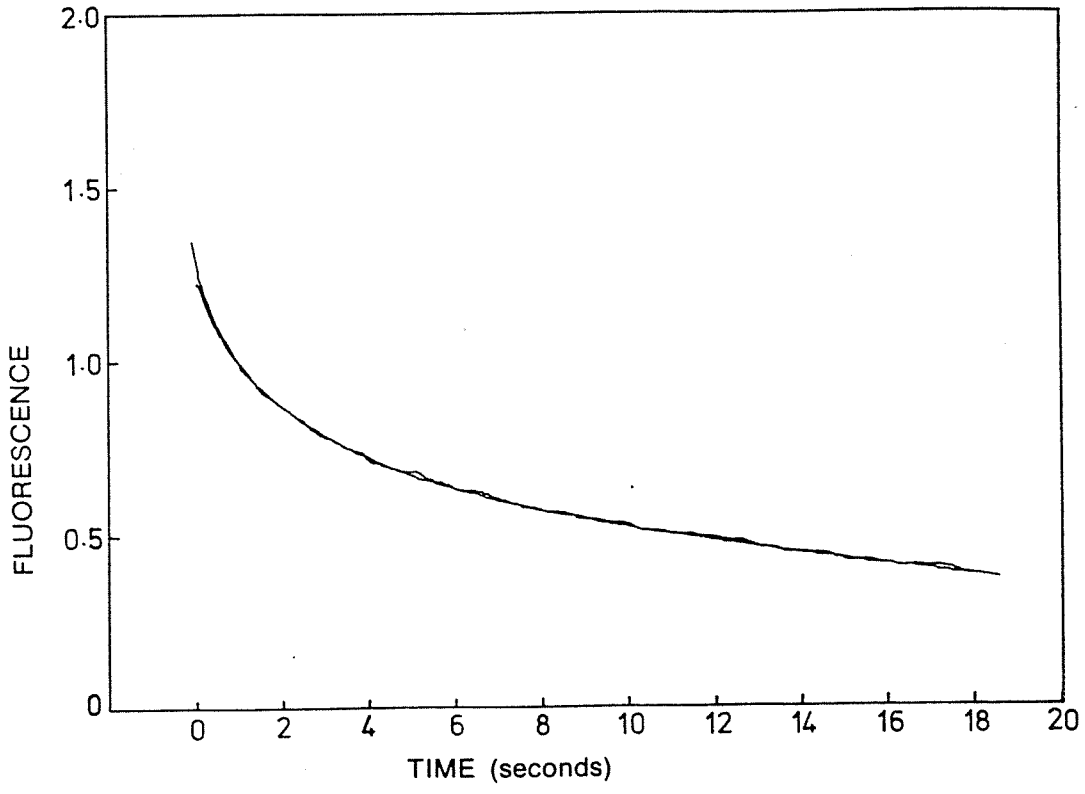


Figure 3:4



absorbance, a multiphasic decrease in protein fluorescence was observed (Figure 3.4). Rate constants obtained were similar to those observed for PCMB absorbance measurements at 250 nm.

It was not possible to study the binding of PCMB to enzyme in the presence of cofactors, because of the strong absorbance of NAD^+ and NADH at 250 nm.

3.4.4 The Effect of PCMB on Dehydrogenase Activity at Low Propionaldehyde Levels

The effect of PCMB on the enzyme activity at low concentrations of propionaldehyde was determined by adding different amounts of PCMB to separate low propionaldehyde assays at pH 7.6. The results of a typical experiment are shown in Figure 3.5a for an enzyme concentration of 2.32 μM.

At low concentrations of PCMB (1.08-4.31 μM) there was an increase in the steady state rate (v) as compared with the control rate (v^o). The amount of activation increased with increasing PCMB concentration to reach a maximum stimulation of 3.0 at a PCMB/enzyme ratio of 1.9. At higher concentrations of PCMB a time dependent decrease in reaction rate was observed (Figure 3.5b). Although the initial rate following the addition of PCMB (measured as a tangent to the absorbance versus time trace) remained approximately constant at the maximum activation of 3, the final steady state rates (measured from the linear part of the absorbance curve, Figure 3.5b) decreased with increasing PCMB concentrations until all activation was lost at a PCMB/enzyme ratio of 4.2. At still higher PCMB levels there was inhibition of the final steady state rate, until at a ratio of 9.0, the rate was almost zero.

A similar experiment was carried out at a lower enzyme concentration (0.23 μM), but keeping the concentrations of NAD^+ and propionaldehyde constant (Figure 3.6). At this enzyme concentration, hysteretic effects were observed for all assays after the addition of PCMB. At low PCMB concentrations the initial rate increased to a final activated rate, whereas at higher levels of PCMB, the initial activated rate decreased to a final slower rate (as observed above for the higher enzyme concentration).

Because of these hysteretic effects, although a maximum activation of 3 was again observed in each case, the PCMB/enzyme ratio at which this maximum occurred differed for the initial rate and final rate

Figure 3.5 The Effect of PCMB on Dehydrogenase Activity at Low Concentrations of Propionaldehyde.

3.5(a) Titration of steady state activity with PCMB at pH 7.6. PCMB was added to assays which contained enzyme (2.32 μM), NAD^+ (1 mM) and propionaldehyde (100 μM). The steady state rate immediately following the addition of PCMB (●) and the final steady state rate (O) are plotted against the [PCMB]/[enzyme active site] ratio.

3.5(b) Hysteretic Approach to the Final Steady State Rate at pH 7.6. The time dependent decrease in the steady state rate after the addition of more than activating levels of PCMB (16.2 μM) to an assay containing enzyme (2.32 μM), NAD^+ (1 mM) and propionaldehyde (100 μM) at pH 7.6 is shown. A value of 0.048 s^{-1} was obtained for k_{app}.

Figure 3:5 THE EFFECT OF PCMB ON ACTIVITY OF ALDEHYDE DEHYDROGENASE AT LOW CONCENTRATIONS OF PROPIONALDEHYDE

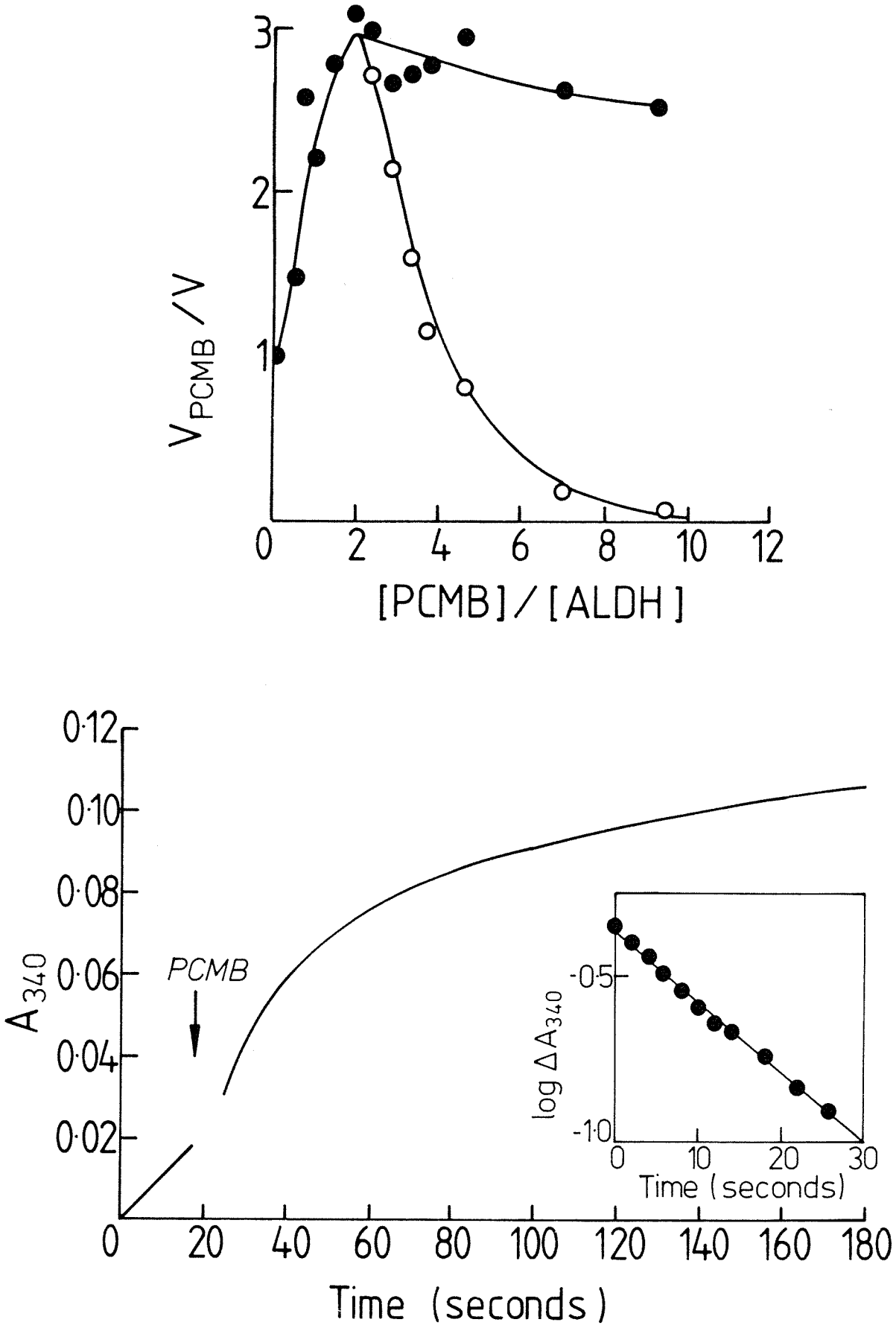


Figure 3:6

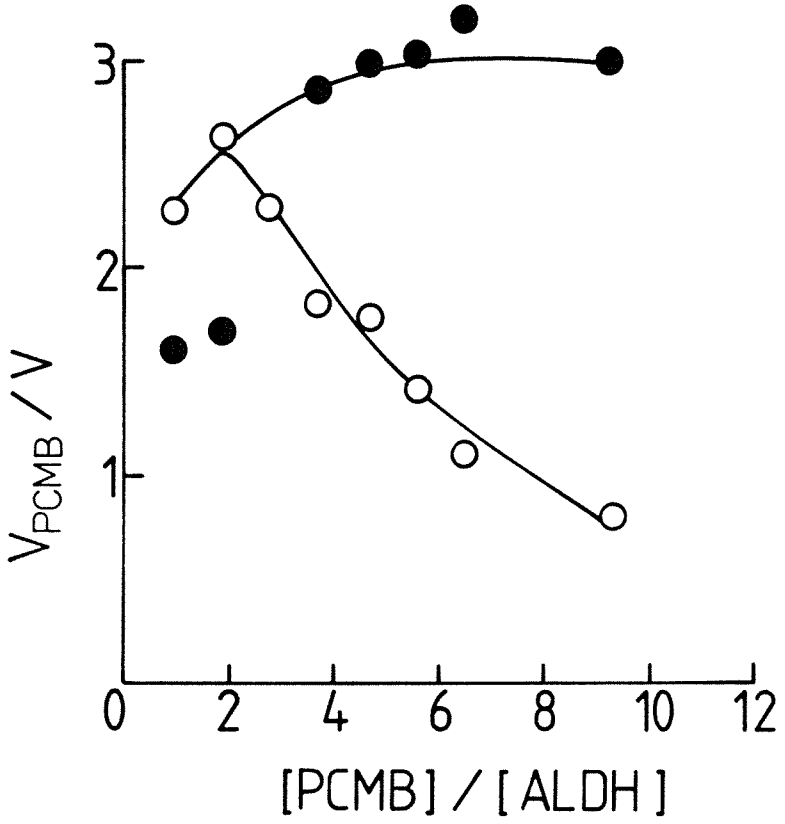


Figure 3.6 Titration of Steady State Activity with PCMB at Low Enzyme Concentrations. PCMB was added to assays which contained enzyme (0.23 μM), NAD^+ (1 mM) and propionaldehyde (100 μM) at pH 7.6. Initial rates (●) immediately following PCMB addition and final rates (○) are shown.

measurements. Compared to the assays with 2.32 μM enzyme there was much less activation of the steady state rate at comparable low PCMB/enzyme ratios, and maximum activation was observed at the higher ratio of 4.0. However, the final rate measurements showed a maximum activation of 2.64 at a PCMB/enzyme ratio of 1.9, as for assays at the higher enzyme concentration.

At high PCMB/enzyme ratios, for the lower enzyme concentrations, net activation of the final linear part of the assay was observed until a PCMB/enzyme ratio of 7.8 was reached and the activity was not reduced to almost zero until the ratio reached 23.

For all experiments at activating levels of PCMB, the PCMB/enzyme ratio required to produce the maximum activation at pH 7.6 for a particular enzyme sample was determined by a titration similar to those described above. PCMB was added last to assays containing 1 mM NAD^+ and 100 μM propionaldehyde (low propionaldehyde assays). With enzyme samples from different preparations the maximum activation did not always occur at PCMB/enzyme ratios of 1.9 as above, but varied from 1.0-4.0 depending on the sample and its age. As observed previously (Bennett et al., 1983), the number of active sites per tetramer varied from sample to sample, even though the enzyme samples did not contain extraneous bands on isoelectric focusing (2.2.7) or polyacrylamide gel electrophoresis (1.2.5). Maximum activation at a PCMB/enzyme ratio of 4 was obtained for enzyme samples with only one active site per tetramer, and a ratio of 1.0 obtained for the few enzyme samples which had four active sites per tetramer (as determined by NADH titration and activity assay). Thus the number of thiol groups which were modified to cause maximum activation was clearly one per subunit, irrespective of the number of active sites per tetramer.

3.4.4.1 Order of Mixing Effects

The extent of activation of the steady state rate showed some dependence on the order of mixing of the substrates and PCMB to the enzyme. Generally, the greatest activation was observed if PCMB was added last and this mixing condition was used for all activation experiments unless otherwise stated. Slightly less activation was observed at a given PCMB concentration if PCMB was added before, or simultaneously with the propionaldehyde, but if PCMB was mixed with the enzyme prior to the addition of NAD^+, considerably less activation was observed. (activation by a factor of 1.4 compared with 2.3 for the

control experiment in which PCMB was added last).

3.4.4.2 The Effect of 2-Mercaptoethanol on Activation

The reversibility of the activation process was investigated by using 2-mercaptoethanol to displace PCMB. PCMB (2.9 μM) was added last to a low propionaldehyde assay solution (containing 2.0 μM enzyme) and activation of the steady state rate by a factor of 2.2 was observed. Subsequent addition of excess 2-mercaptoethanol (100 μl) completely removed the activating effect. When enzyme, NAD^+ and PCMB were premixed for 10 minutes prior to the addition of propionaldehyde, an activated rate was still obtained, but the activation was still completely removed when excess 2-mercaptoethanol was added. The inhibitory effect of high concentrations of PCMB (PCMB/enzyme ratios of 20-40) could be reversed by the addition of 2-mercaptoethanol within 2-5 minutes. After 45 minutes, only partial reactivation of the enzyme activity by 2-mercaptoethanol occurred.

3.4.4.3 Time Dependent Changes in Steady State Activity

At PCMB concentrations greater than those causing maximum activation, a time dependent decrease in the activated steady state rate was observed (Figure 3.5). An apparent first order rate constant (k_{app}) could be determined in each case for the approach to the final steady state rate. The final linear portion of the curve was extrapolated back to time zero and the differences between the observed absorbances and the extraplolated values measured. k_{app} was obtained from the slope of a logarithmic plot of these differences as a function of time.

Figure 3.7 shows the effect of the PCMB concentration on k_{app} for three different enzyme concentrations (0.23, 1.61 and 2.32 μM). The plot was linear, even though the concentration of PCMB was well below the 8-10 fold excess required for pseudo first order conditions to apply. There was no effect of enzyme concentration as within experimental error, all data for the three enzyme concentrations lay on this same line.

The pH dependence of this process was investigated over the pH range in which activation is observed (6.5-9.0). PCMB (8.19 μM) was added last to assays in a PCMB/enzyme ratio of 4.1 using enzyme for which maximum activation was observed at a ratio of 1.45 at pH 7.6. Hysteresis was observed at all pH values from 6.5 to 9.0, but the

Figure 3:7

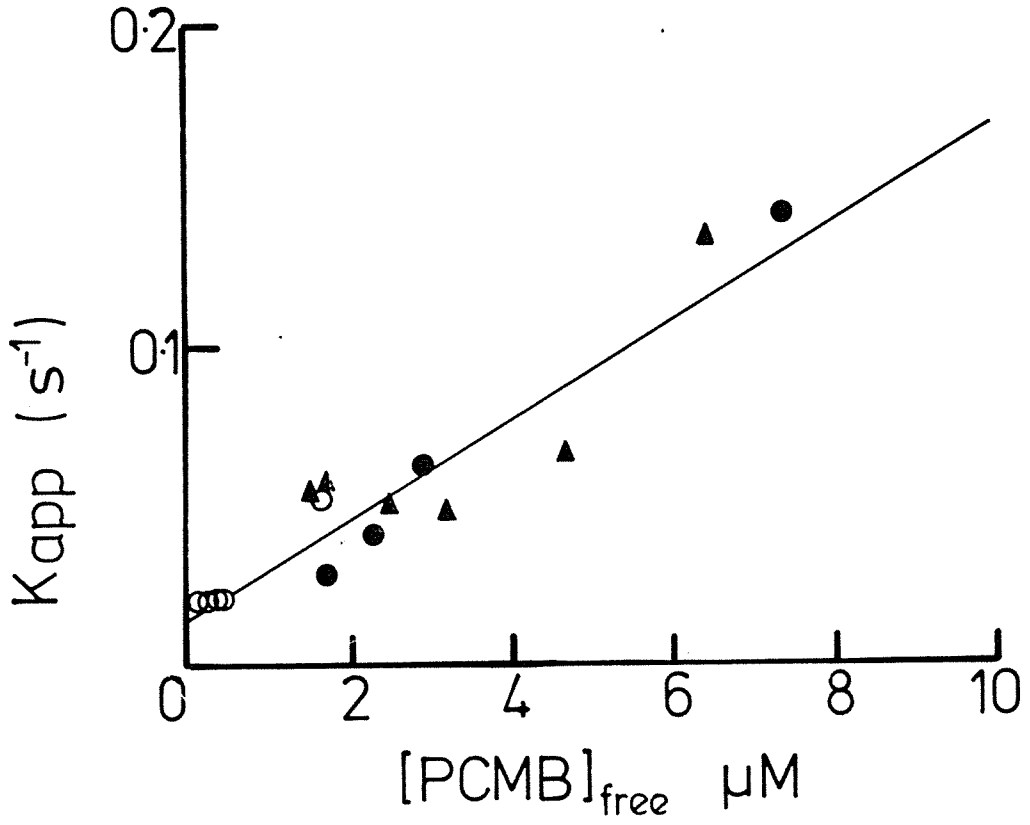


Figure 3.7 Plot of k_{app} against the free PCMB concentration at pH 7.6. k_{app} was plotted against the free PCMB concentration estimated from the PCMB titration curve for enzyme concentrations of 0.23 (○), 1.6 (●) and 2.32 µM (▲).

values determined for k_{app} did not vary as a function of pH, remaining approximately constant at 0.03 ± 0-06 s^{-1} (Table 3.2).

TABLE 3.2

EFFECT OF pH ON HYSTERESIS

pH	k_{app} s^{-1}	Amplitude (abs units)	Buffer
6.5	0.033	0.039	phosphate
7.0	0.027	0.040	phosphate
7.6	0.030	0.055	phosphate
8.0	0.032	0.027	phosphate
9.0	0.018	0.026	carbonate

A time dependent increase in the steady state rate until the activated rate was obtained was observed for the lowest PCMB concentrations (0.216 and 0.432 µM) used in the activity titration with the lowest concentration of enzyme (0.23 µM, refer Figure 3.6). For a PCMB concentration of 0.216 µM the half life of the lag was determined to be about 14 s, and the process became faster as the PCMB concentration increased. Thus although there appears to be some activation of the initial rate for the two lowest concentrations of PCMB, rates could not be determined until 5-10 seconds after the addition of the PCMB, and could have been nearly 50% activated before measurements were made.

3.4.4.4 The Effect of Disulphiram on Activation by PCMB

PCMB (4.68 µM) was added to an assay containing enzyme (1.2 µM), NAD^+ (1 mM) and propionaldehyde (100 µM) and the k_{cat} value, determined as described in section 2.2.1 ,increased from 0.0732s^{-1} to 0.219 s^{-1} (an increase of 302%). Disulphiram (5.2 µM) was added to this solution and an immediate decrease in the k_{cat} value to 0.027 s^{-1} was observed. This value decreased with time to a final value of 0.010 s^{-1}, which was similar to the value of 0.014 s^{-1} obtained for the control experiment in the absence of PCMB.

When disulphiram was added to the enzyme before PCMB, no activation of the steady state rate of the disulphiram-modified enzyme was observed. An immediate decrease in k_{cat} to 0.002 s^{-1} occurred, followed by a time dependent increase to a final value of 0.014 s^{-1} , which was the same as that obtained for the control experiment.

However, if PCMB was added only 10 seconds after the addition of disulphiram, linear traces were obtained with a k_{cat} value of 0.013 s^{-1}.

3.4.4.5 The Effect of PCMB on the Steady State Rate at 35°C

Assays were carried out at 25°C and at 35°C using activating levels of PCMB (5.2 µM PCMB, 1.5 µM enzyme) and also at a concentration of PCMB double that required for maximum activation. Results are shown in Table 3.3.

TABLE 3.3

THE EFFECT OF TEMPERATURE ON ACTIVATION BY PCMB

Temperature	[PCMB]	k_{cat} (s^{-1})		v/v^0	
°C	µM	initial	final	initial	final
25	7.55	0.211	0.211	2.59	2.59
25	15.1	0.146	0.041	1.89	0.53
35	7.55	1.18	1.18	2.08	2.08
35	15.1	0.91	-	1.59	-

Activation was still observed at 35°C, in contrast with the results obtained by Kitson (1982d, 1983) for activation of the enzyme by diethylstilboestrol in which activation was lost at the higher temperature. The relative activation observed at 35°C for both PCMB concentrations was only slightly less than that observed at 25°C and the differences may not be significant.

3.4.4.6 Acetaldehyde as Substrate

When acetaldehyde was used as a substrate in place of propionaldehyde, activation was again observed. A two-fold increase in the steady state rate was observed when PCMB (3.1 µM) was added last to assays containing 0.83 µM enzyme (PCMB/enzyme = 3.7) NAD^+ (1 mM) and acetaldehyde (100 µM-2 mM) in 25 mM pH 7.6 phosphate buffer.

3.4.5 The Effect of PCMB at High Propionaldehyde Concentrations

Inhibition of the steady state rate was observed when PCMB was added to assay mixtures at high concentrations of propionaldehyde. In Figure 3.8 the results of an experiment in which varying amounts of PCMB were added to separate assay mixtures containing enzyme (2.22 μM) in buffer, followed immediately (within 10 seconds) by NAD^+ (1 mM) and propionaldehyde (20 mM) are shown. A non-linear decrease in reaction rate with increasing PCMB concentration was observed. The rates shown are all final rates, as there was some slight hysteresis (from an initial slower rate) observed in the first minute for these assays.

As an attempt to obtain a dissociation constant for the reaction

$$E + PCMB \rightleftharpoons E.PCMB$$

the data was replotted (Figure 3.8b) in the form of a Scatchard plot of

$$(R/[PCMB] - R[E]_t) \quad \text{against} \quad R$$

where $R = 1 - v/v^0$

and $[E]_t$ is the enzyme tetramer concentration

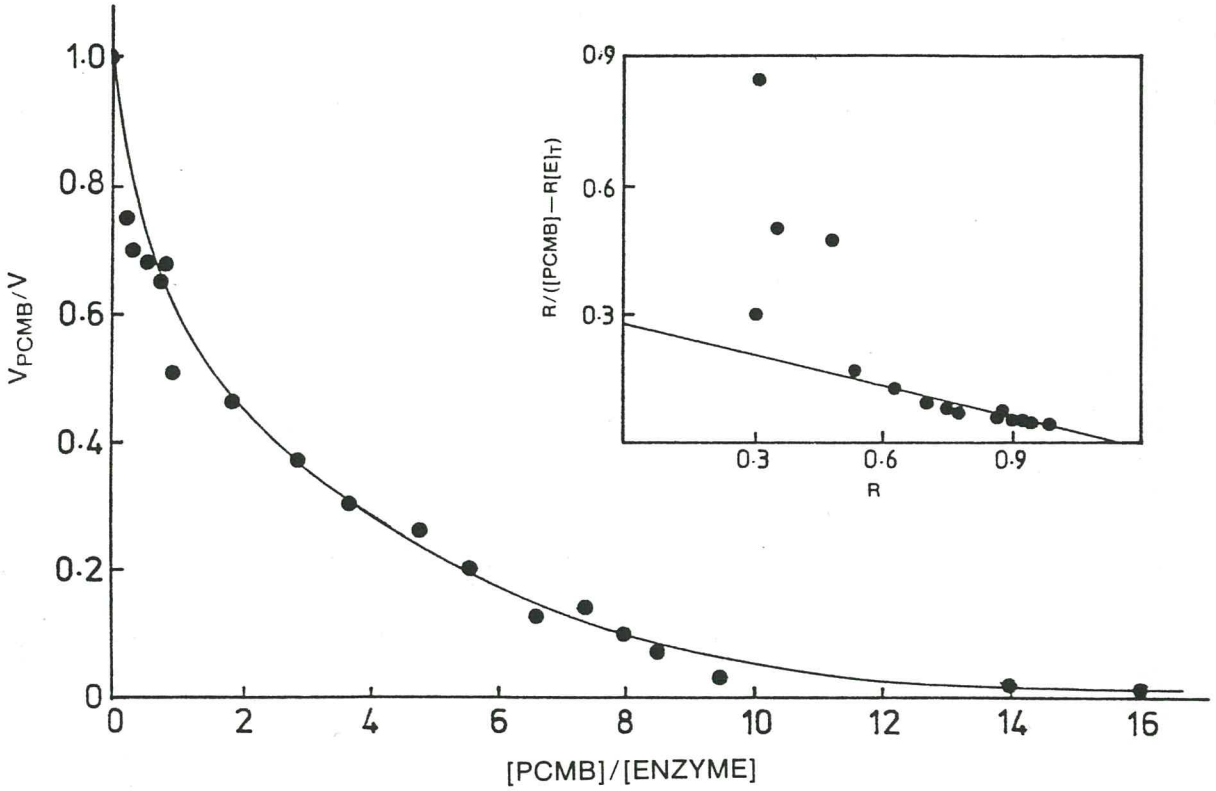
This plot has validity only if E.PCMB has no activity so that R represents the fraction of occupied PCMB binding sites (refer section 2.4.2).

Order of mixing effects were less marked than at low propionaldehyde concentrations, but in general about 5% greater inhibition was observed if PCMB was added to enzyme before both NAD^+ and propionaldehyde. The reaction of PCMB with the enzyme was very rapid however, as when PCMB was added last to assays, inhibition occurred within the time of mixing and there was no significant hysteresis.

The reversibility of the inhibition was investigated. PCMB (54 μM) was added to assay solutions containing enzyme (2-7 μM), and NAD^+ (1 mM). Propionaldehyde was added after various incubation times, followed by 100 μl of pure 2-mercaptoethanol. In the presence of PCMB, the reaction rate was reduced to 12% of its control value. If 2-mercaptoethanol was added within 5 minutes of the addition of PCMB, the original activity was completely restored, but after this time an increasing percentage of the original activity remained inhibited.

In several experiments, a high concentration of propionaldehyde

Figure 3:8 THE EFFECT OF PCMB ON DEHYDROGENASE ACTIVITY AT HIGH CONCENTRATIONS OF PROPIONALDEHYDE



Varying amounts of a solution of PCMB were added to solutions containing enzyme (2.22 μM) in a cuvette. NAD^+ (1 mM) and propionaldehyde (20 mM) were added immediately afterward. The reaction rate is expressed relative to the control rate in the absence of PCMB. A Scatchard plot of the data is also shown (inset). From this plot values of 1.2 for n (the number of binding sites per tetramer) and of 4.95 μM for K_D (for binding of PCMB to enzyme) were obtained.

(20 mM) was added to activated low propionaldehyde assays containing PCMB. An immediate reduction in the reaction rate to 25-40% of the activated rate was observed. At pH 7.6, the final rate was very close to the original pre-activation rate at the low propionaldehyde level.

3.4.6 The Effect of PCMB on the Esterase Activity of the Enzyme

The effect of adding different amounts of PCMB to separate esterase assay solutions containing enzyme (1.29 μM) and PNPA (116 μM) at pH 7.6 is shown in Figure 3.9. PNPA was added last to initiate the reaction, immediately after the addition of PCMB. All results are given relative to the control rate in the absence of PCMB. A Scatchard plot of

$$(R/([PCMB] - R[E]_t)) \quad \text{against} \quad R,$$

where $R = v/v^o$

and $[E]_t$ is the enzyme protein (tetramer) concentration

is shown (inset Figure 3.9). A value of 4.9 μM was obtained for K_D and of 1.23 for n. These results are very similar to those obtained above for the effect of PCMB on the dehydrogenase activity of the enzyme. The Scatchard plot is of the same form as for the high propionaldehyde assay and the same assumptions have been made.

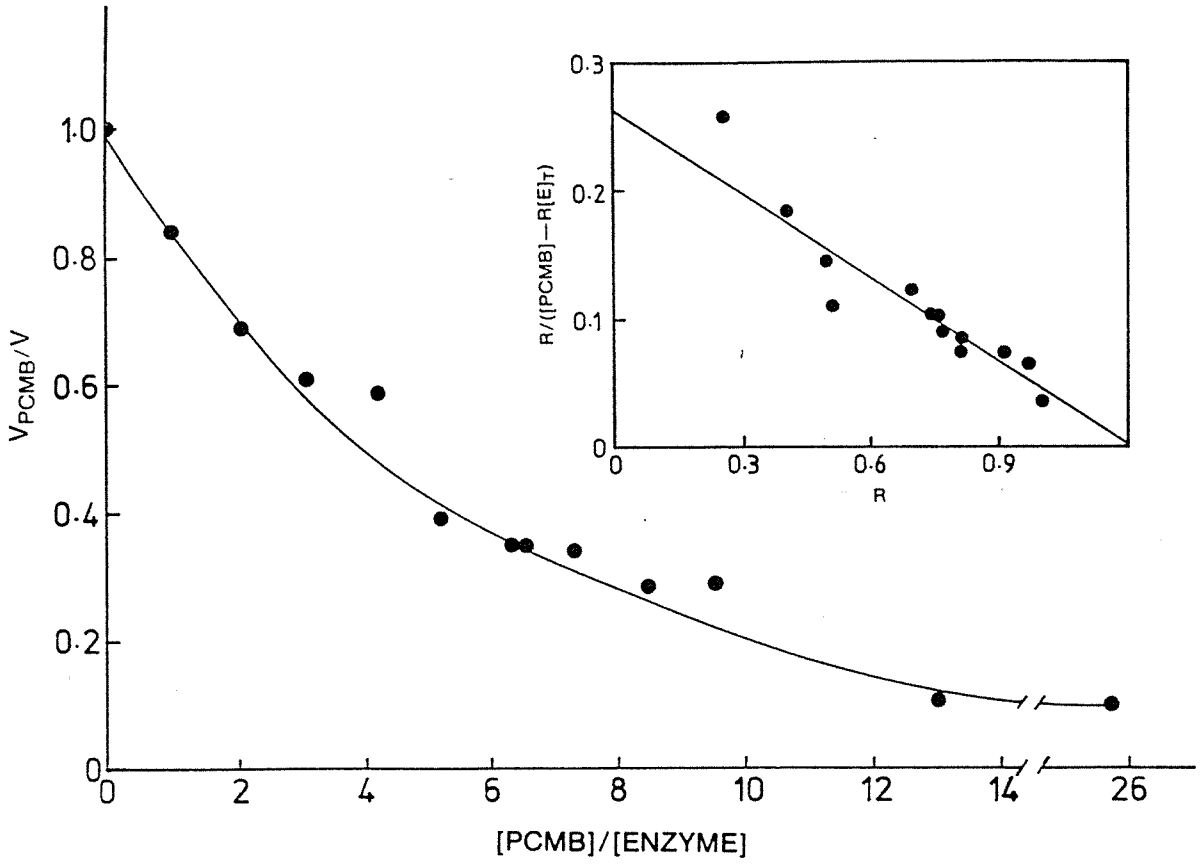
3.4.7 Effect of Activating Levels of PCMB on the NADH Titration

There was no significant difference in the fluorescence of enzyme bound NADH in the presence of an activating level of PCMB, as is apparent from Figure 3.10a. The Scatchard replot of the enzyme only data (Figure 3.10b) resulted in a K_D value of 2.0 μM and a value of 1.24 for the number of NADH binding sites per tetramer. Similar values of 2.7 μM for K_D and 1.46 for the number of NADH binding sites were obtained from the Scatchard replot of the data for enzyme (0.79 μM) to which PCMB (3.8 μM, the maximum activating level) had been added (Figure 3.10c).

3.4.8 The Effect of PCMB on the NADH and Proton Bursts.

The results obtained for burst experiments in the presence of activating levels of PCMB, using various mixing conditions and low propionaldehyde concentrations, are summarised in Table 3.4. All results are expressed relative to the control values in the absence of PCMB.

Figure 3:9 THE EFFECT OF PCMB ON ESTERASE ACTIVITY



Varying amounts of PCMB were added to solutions containing enzyme (1.29 μM) at pH 7.6 and p-nitrophenylacetate (116 μM) was added immediately afterwards. The rate of the resulting esterase reaction was measured and is expressed relative to the control rate obtained in the absence of PCMB. The inset shows a Scatchard plot of the data, from which a value of 4.9 μM was obtained for K_D and a value of 1.23 for n.

Figure 3.10 NADH Titration in the Presence of PCMB.
Enzyme (0.79 μM) was titrated with NADH in the presence (●) and absence (○) of activating amounts of PCMB. A control titration in which aliquots of NADH were added to buffer solution is also shown (■).
3.10 (b) and (c)The Scatchard replots of the data are also shown. For the enzyme only data (b) a K_D value of 2.0 μM and a value of 1.24 for n (the number of binding sites per tetramer) were obtained. For enzyme mixed with PCMB similar values of 2.7 μM for K_D and 1.46 for n were obtained.

Figure 3:8

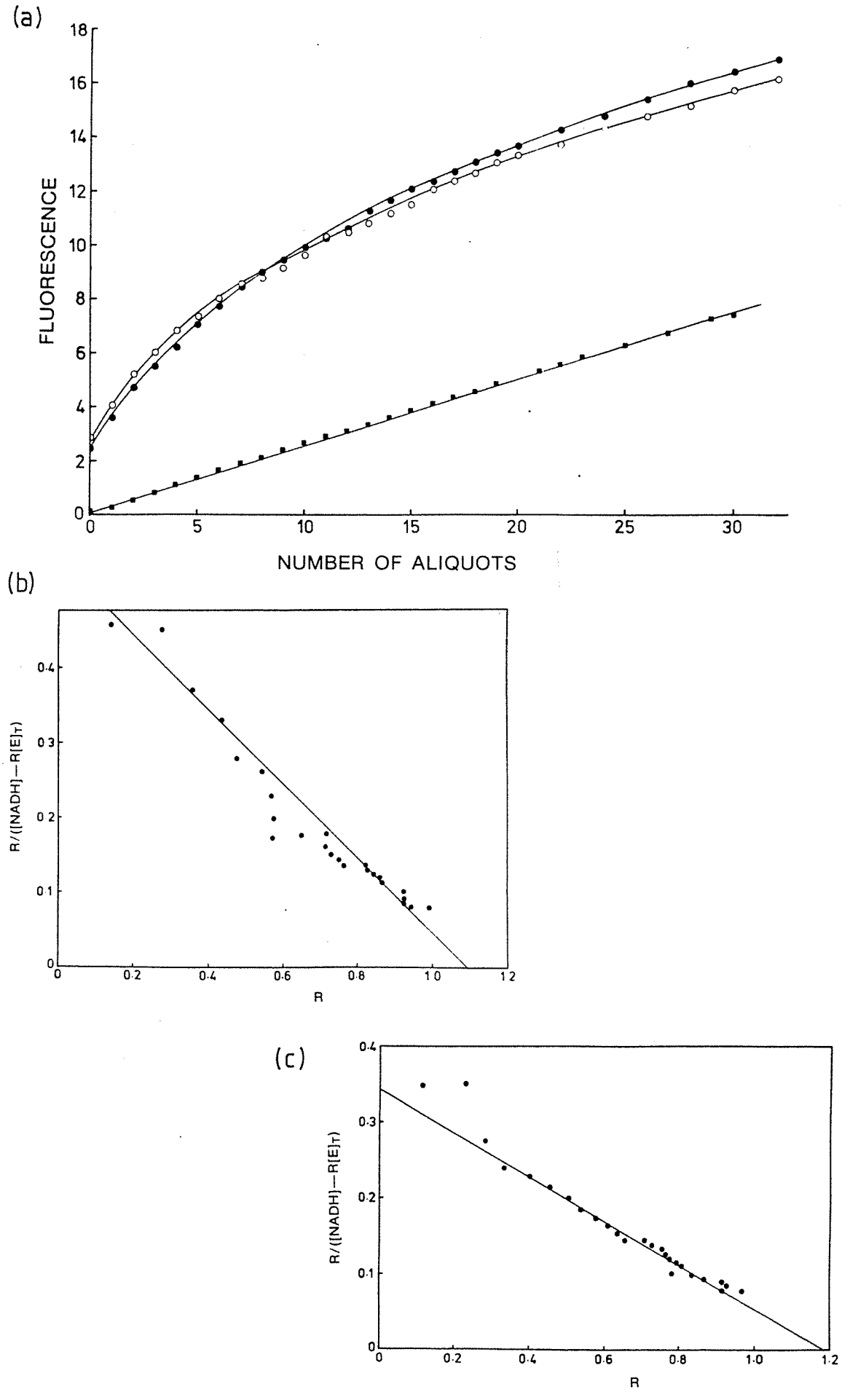


TABLE 3.4

THE EFFECT OF PCMB ON THE BURST RATE CONSTANTS AND AMPLITUDES AT LOW PROPIONALDEHYDE CONCENTRATIONS

[PCMB]/[ENZ]	Mixing Conditions	k_b/k_b^o	A_b/A_b^o	Burst Type
4.13[a]	E,PCMB/NAD^+,Prop[c]	0.62	0.23	NADH
1.91[a]	E,NAD^+/PCMB,Prop	1.1	0.94	NADH
1.91[a]	E,NAD^+,PCMB/Prop	0.92	0.63	NADH
4.0 [a]	E,NAD^+,PCMB/Prop	0.9	0.66	Proton
6.0 [b]	E,NAD^+/PCMB,Prop	1.0	0.77	NADH
20.0	E,NAD^+/PCMB,Prop	0.67[d]	1.1	NADH
20.0	E,NAD^+,PCMB/Prop	e	e	NADH
20.0	E,PCMB/NAD^+,Prop	e	e	NADH

a The maximum activating level of PCMB, as determined at the beginning of each experiment

b Using a [PCMB]/[Enz] ratio which in the steady state turnover of propionaldehyde gave initial activation followed by hysteresis to a final slower linear steady state rate.

c Prop is propionaldehyde

d The burst was followed by a decrease in fluorescence

e No burst or steady state was observed

In general, for enzyme premixed with NAD^+, there was very little effect of PCMB on either the burst rate constant or amplitude, although the amplitude of both the proton and NADH bursts was reduced by about 40% for PCMB premixed with enzyme and NAD^+. For PCMB premixed with enzyme alone a significant reduction of the burst amplitude and some reduction of the rate constant was observed. The steady state rates immediately following the burst were activated except for the first mixing condition in Table 3.4 for which no activation was observed.

3.4.9 NADH Burst Experiments at High Concentrations of PCMB

The effect of PCMB concentrations significantly greater than the activating level on the NADH burst were investigated and the results are included in Table 3.4. When PCMB was not premixed with the enzyme there was very little effect on the burst, even at a PCMB/enzyme ratio

of 20:1. However for enzyme premixed with enzyme, with or without NAD^+, no burst or steady state was seen. At a ratio of 6:1, the steady state following the burst showed initial activation followed by hysteresis to a slower linear steady state rate, as observed in steady state experiments.

At a PCMB/enzyme ratio of 20:1, a decrease in fluorescence was observed following the burst (Figure 3.11a,b). This process had a decay constant of 0.23 s^{-1} and was followed by a slow increase in NADH fluorescence with an apparent k_{cat} of 0.002 s^{-1} (Figure 3.11b).

3.4.10 Burst Experiments at High Propionaldehyde Concentrations

The effect of PCMB on the burst was also investigated using high concentrations of propionaldehyde. PCMB concentrations that produced activation of the steady state rate at low propionaldehyde (activating levels) and also higher concentrations of PCMB were used. Different mixing conditions were used as above and control burst experiments using high propionaldehyde concentrations were conducted in each case. These results are summarised in Table 3.5. Rate constants and amplitudes are expressed relative to the control values as above for the low propionaldehyde bursts.

TABLE 3.5

THE EFFECT OF PCMB ON THE BURST RATE CONSTANTS AND AMPLITUDES AT HIGH PROPIONALDEHYDE CONCENTRATIONS

[PCMB]/ [ENZ]	Mixing Conditions	k_b/k_b^o	A_b/A_b^o	Burst Type
3.67[a]	E,PCMB/NAD^+,Prop[b]	0.71	0.34	NADH
1.91[a]	E,NAD^+/PCMB,Prop	0.96	1.44[c]	NADH
1.91[a]	E,NAD^+,PCMB/Prop	0.81	0.62[c]	NADH
4.0 [a]	E,NAD^+,PCMB/Prop	0.70	1.85	Proton
20.0	E,NAD^+/PCMB,Prop	1.71	1.0[c]	NADH
20.0	E,NAD^+,PCMB/Prop	d	d	NADH
20.0	E,PCMB/NAD^+,Prop	d	d	NADH

a The maximum activating level of PCMB as determined at the beginning of each experiment

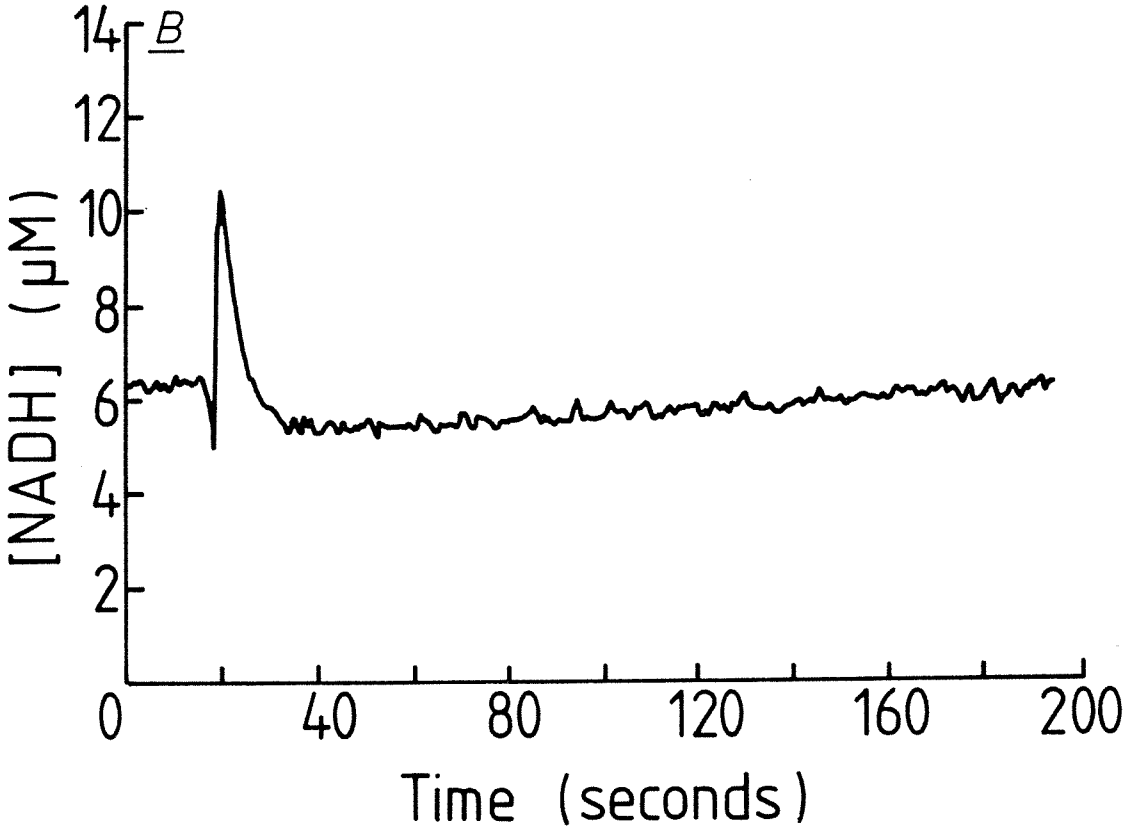
b Prop is propionaldehyde

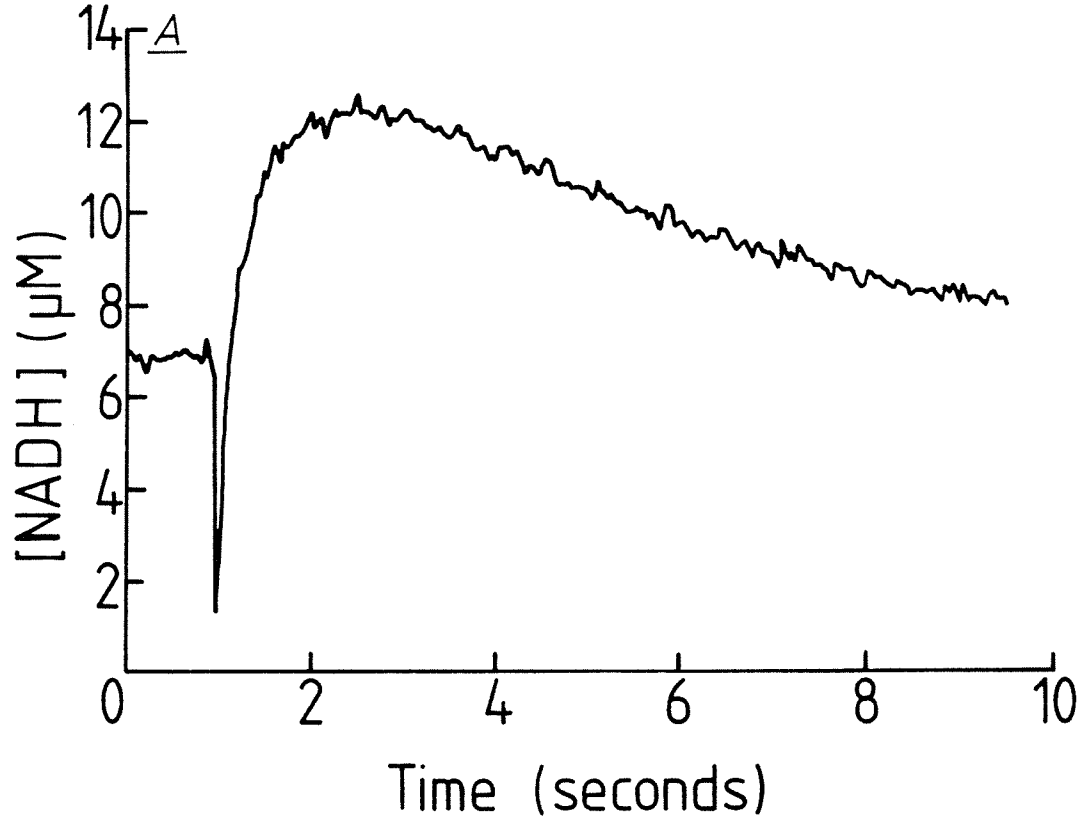
c The burst was followed by a decrease in fluorescence

d No burst or steady state was observed

Figure 3.11 The Effect of Excess PCMB on the NADH Burst at pH 7.6. Enzyme (5.6 μM) and NAD^+ (2 mM) from one syringe were rapidly mixed with propionaldehyde (400 μM) and PCMB (104 μM) from the second syringe. (A) The burst rate constant was 5.3 ± 0.7 s^{-1} and a decrease in fluorescence followed the burst. (B) On a longer time scale the decrease in fluorescence was more obvious. The decay constant was 0.23 s^{-1}.

Figure 3:11 THE EFFECT OF EXCESS PCMB ON THE NADH BURST





Both activating and higher concentrations of PCMB showed similar effects on the NADH burst at high propionaldehyde concentrations as observed at low propionaldehyde levels. However, following the burst, different effects were noted. In cases in which enzyme was premixed with NAD^+, a decrease in fluorescence was observed following the burst, similar to that observed previously for the low propionaldehyde burst, but considerably faster (Figure 3.12a,b). The data for this process is summarised in Table 3.6.

TABLE 3.6

SPONTANEOUS FLUORESCENCE DECREASE FOLLOWING THE BURST

[PCMB]/ [ENZ]	[Prop[a]] (mM)	Mixing Conditions	Rate Constant	Amplitude[c]	k_{cat}[d]
1.91[a]	20	E.NAD^+,PCMB/Prop	3.5	0.40	0.135
1.91[a]	20	E,NAD^+/PCMB,Prop	1.0	0.35	0.100
20	20	E,NAD^+/PCMB,Prop	1.4	0.66	0.062
20	0.2	E,NAD^+/PCMB,Prop	0.23	0.50	0.002

a Prop is propionaldehyde

b Maximum activating level

c Relative to the burst amplitude

d For the steady state following the burst

Whereas no fluorescence decrease following the burst was observed at activating levels of PCMB for the low propionaldehyde burst experiments, at high propionaldehyde concentrations such a decrease in fluorescence was observed in all cases except that in which PCMB was premixed with enzyme in the absence of NAD^+. The rate constants determined for this process were much faster than that obtained at low propionaldehyde concentrations, by a factor of about 15 for enzyme premixed with PCMB and NAD^+ and by a factor of 4 for enzyme not premixed with PCMB. Excess PCMB had little effect on the displacement rate for this latter mixing condition, but for enzyme premixed with PCMB, no burst or steady state was observed at high PCMB concentrations, in agreement with the results obtained using low propionaldehyde concentrations.

A marked increase in the amplitude of the proton burst at high

Figure 3.12 The effect of PCMB on the NADH burst at high concentrations of propionaldehyde.

3.12 (a) Enzyme (5.6 μM) and NAD^+ (2 mM) from one syringe were rapidly mixed with propionaldehyde (20 mM) and PCMB (104 μM) from the second syringe. (A) The burst rate constant was $13\pm1s^{-1}$ compared with the control value of 8±1, but this may not be significant, as a decrease in fluorescence was observed following the burst (B) which affected the appearance and fitting of the burst process. A decay constant of $1.4s^{-1}$ was obtained for the decrease in fluorescence. These results may be compared with those at the same concentrations of enzyme and PCMB, but at low propionaldehyde concentrations (Figure 3.11).

For the amplitude of this process, 1 V corresponds to an enzyme concentration of 5·1 μM.

3.12 (b)Enzyme (6.8 μM) was premixed with NAD^+ (2 mM) and PCMB (13.0 μM, the maximum activating level of 1.91 for this sample of enzyme) in one syringe and was rapidly mixed with propionaldehyde from the other syringe. (A) The burst rate constant was 13±1 compared with the control value of 16±1 (also obtained using 20 mM propionaldehyde). A rapid decrease in fluorescence followed the burst (B), and the decay constant obtained for this process was $3.5s^{-1}$.

For the amplitude of this process, 1 V corresponds to an enzyme concentration of 10·8 μM.

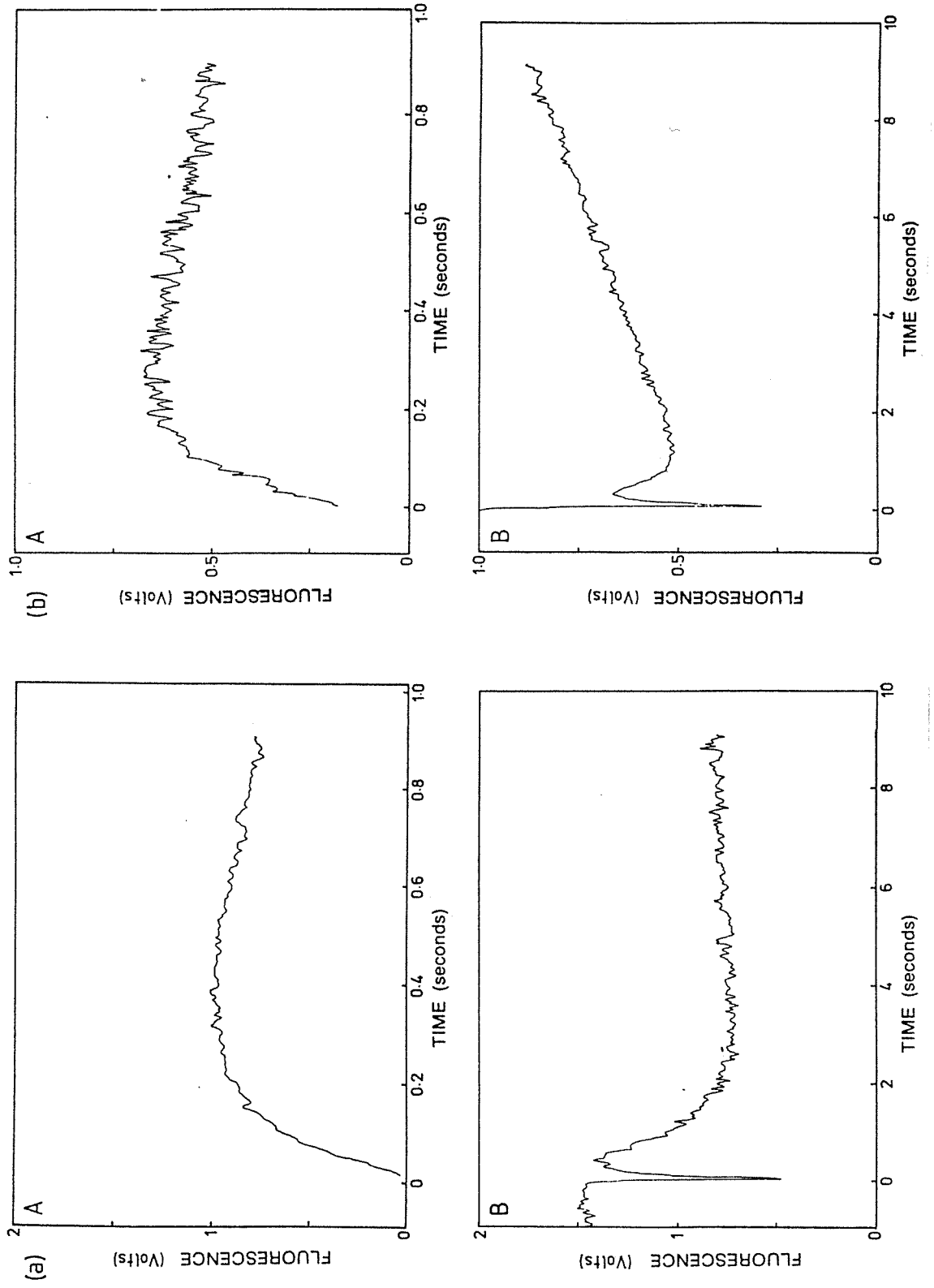

(a)
A
FLUORESCENCE (Volts)
TIME (seconds)
B
(b)
A
FLUORESCENCE (Volts)
TIME (seconds)
B

Figure 3:12

propionaldehyde concentrations was observed when activating levels of PCMB were premixed with enzyme and NAD. The amplitude was almost double that observed for the control proton burst at the same propionaldehyde concentration, suggesting that two protons were released in the presence of PCMB. The same amplitude was obtained for the control bursts at both propionaldehyde concentrations.

3.4.11 Effect of PCMB on NADH Displacement and Binding

The effect of activating levels of PCMB on the binding of NADH to the enzyme and on the displacement of NADH from the binary E.NADH complexes by excess NAD^+ were studied. In both experiments PCMB was premixed with the enzyme. The results are shown in Table 3.7, from which it is apparent that both binding and displacement of NADH are practically unaffected by activating levels of PCMB.

TABLE 3.7

EFFECT OF ACTIVATING LEVELS OF PCMB ON NADH DISPLACEMENT AND BINDING AT pH 7.6

Experiment	$[Enz]^a$ (μM)	$[PCMB]^a$/ $[Enz]$	$[NADH]^a$ (μM)	λ_f (s^{-1})	λ_s (s^{-1})	$A_b{}^b/A_b^o$
displacement	3.0	0	33	0.93±0.02	0.23±0.06	1.0
displacement	3.0	3.0	33	1.4±0.03	0.18±0.01	1.0
binding	5.8	0	84	7.9±0.1	1.05±0.01	1.0
binding	5.8	4.14	84	8.9	1.08	0.84[c]

a All concentrations are those prior to rapid mixing

b Amplitudes are expressed relative to the value in the absence of PCMB as 1.0. A_b^o was always 85-90% of the active site concentration.

c The decrease in amplitude resulted almost entirely from a decrease in the amplitude of the slow process (0.4 μM in the presence of PCMB compared with 1.0 μM in its absence).

The effect of high concentrations of PCMB on the NADH displacement was also studied, using a PCMB/enzyme ratio of 20:1 and the results are shown in Table 3.8. As these experiments were carried out at a temperature of 29°C, the decay constants obtained are all faster than

at 25°C. In the presence of high levels of PCMB, the rate of displacement of NADH was significantly increased. Both λ_s and λ_f were increased by a factor of about 2.5. Some increase in the amplitude of the slow process at the expense of the faster process was observed.

TABLE 3.8

THE EFFECT OF HIGH PCMB CONCENTRATIONS ON NADH DISPLACEMENT

[PCMB]/ [Enz]	λ_f (s^{-1})	λ_s (s^{-1})	λ_f/λ_f^o	λ_s/λ_s^o	A_f/A_f^o	A_s/A_s^o	A_t/A_t^o *
0	3.3±0.5	0.47±0.03	1.0	1.0	1.0	1.0	1.0
20	7±3	1.36±0.08	2.1	2.89	0.78	1.64	1.1

* A_t is total amplitude

3.5 DISCUSSION

PCMB is known to react rapidly with thiol groups of proteins (Means & Feeney, 1971, pp 198-205, Boyer, 1954) producing thiol mercurial complexes with low dissociation constants. Reaction with multiple thiol groups of the same protein and at differing rates, depending on their environment, is also well documented (Webb,1966). The value obtained for the change in extinction coefficient at 250 nm for reaction of PCMB with aldehyde dehydrogenase was very similar to that obtained by Boyer (1954) for reaction with cysteine, providing good evidence that PCMB is reacting with thiol groups of the enzyme. When the enzyme was denatured with SDS, a total of 34 -SH groups were rapidly modified per mole of protein by PCMB, in good agreement with previous determinations of the total thiol content of the enzyme (MacGibbon et al., 1979, section 2.5.1)

For native enzyme in the presence of a large excess of PCMB, about 12-14 thiol groups per mole of protein were observed to react rapidly, with further reaction with the remaining thiol groups over a three hour period. The time dependence of the reaction was complex, with a number of processes being observed in this time with different apparent rate constants. The total number of groups modified, calculated from the total absorbance change at 250 nm, was quite variable, and greater than that expected on the basis of the total thiol determinations,

indicating that groups other than -SH groups alone were reacting under these conditions. However, if the process (or processes) with the slowest rate constant was ignored, the total number of thiol groups modified by the faster processes was of the order of 30 per mole of protein. Such reaction of PCMB with other protein groups has been observed previously under similar conditions, but it has been clearly established that in the presence of available thiol groups, no other functional group can compete successfully for reaction with PCMB (Webb, 1966).

After a very long period in the presence of a large excess of PCMB, or more quickly if the enzyme concentration was also high, precipitation of the enzyme occurred, suggesting that extensive modification and denaturation of the enzyme finally occurred in the presence of excess PCMB.

On the basis of the PCMB binding experiments it is proposed that, in the absence of any added cofactors or substrates, PCMB reacts rapidly and possibly reversibly with all accessible thiol groups on the enzyme. Thus, it seems likely that 12-14 -SH groups per mole of protein are situated either on the enzyme surface or in shallow crevices. Further reaction with the remaining -SH groups occurs more slowly as the enzyme molecule unfolds, producing irreversible changes in the enzyme structure as less accessible thiol groups and then finally other groups are modified by the relatively bulky PCMB molecule.

The decrease in protein fluorescence observed upon reaction of PCMB with native enzyme was also clearly multiphasic and probably arises from significant disruption of the protein structure. Although the number of individual observable processes may be expected to be fewer than for absorbance measurements at 250 nm in which each molecule of PCMB reacting produces an increase in absorbance, the results were still too complex for meaningful data analysis.

Thiol reagents, such as disulphiram, normally cause inhibition of the enzyme and it was expected that reaction with PCMB would also produce inhibition. However, at low ($\leq$100 μM) concentrations of propionaldehyde, activation was in fact observed. As discussed above in results, the amount of PCMB required for maximum activation was one molecule per subunit, irrespective of the number of active sites per tetramer. Thus the mere fact that a single thiol group is rapidly modified by a thiol reagent must not be taken as evidence that it is

essential for catalytic activity.

At PCMB concentrations higher than the maximum activating level, further thiol groups were modified, but there was no immediate effect on the enzyme catalytic activity and an initial activation of the steady state rate was still observed. As the enzyme has a large number of thiol groups which react very rapidly with PCMB, it is to be expected that some of these groups will be relatively exposed so that reaction with PCMB will cause little disruption of the protein structure and hence will have very little immediate effect on the enzyme activity.

In the absence of premixed NAD^+ very little activation of the steady state rate was observed (none at all in the case of stopped flow experiments for the steady state immediately following the burst), indicating that PCMB cannot react with the activating group in its absence. This observation strongly suggests that the activating group is only exposed to PCMB after NAD^+ binding indicating that a conformational change occurs in the $E.NAD^+$ binary complex as suggested by MacGibbon et al. (1977c) and Buckley et al. (1982).

i.e.

$$E.NAD^+ \rightleftharpoons {}^*E.NAD^+ \rightleftharpoons {}^*E.NAD^+.PMB$$

where PMB is the p-mercuribenzoate group

In burst experiments, when PCMB was added to enzyme which was premixed with NAD^+ there was no effect on the rate constants or amplitudes of either the NADH or proton bursts, but the steady state rate following was activated.

These results demonstrate that the activation produced by PCMB does not affect substrate binding or hydride transfer. Activation such as that reported by Takahashi et al. (1981) for horse liver aldehyde dehydrogenase which involves dissociation into dimeric units is also excluded for PCMB, as this would produce an increase in the amplitude of the NADH burst, which is contrary to experiment.

At activating levels, PCMB had no effect on the rate constants for NADH binding or dissociation, and both the K_D for dissociation of E.NADH and the fluorescence enhancement of bound NADH were unaltered. Hence the conformational change controlling the rate of release of NADH from the $^*E.NADH$ complex (MacGibbon et al., 1977b) does not appear to be affected by this level of PCMB. However, it must be noted that NAD^+

was not present with enzyme in any of these experiments. In view of the conclusion above that the activating thiol group is only exposed in the presence of NAD^+, the possibility of changes in fluorescence or the NADH binding and displacement processes when PCMB was bound at the activating site cannot be entirely excluded. The same thiol group may not be exposed when NADH is bound. At higher PCMB concentrations the rate of NADH dissociation was increased significantly, both rate constants being increased by a factor of about 2.5. Again it is unlikely that PCMB was reacting with a thiol group in the activating site, but the possibility cannot be entirely excluded.

Using the model shown in Scheme 2.2 (2.1.3), the observed activation could result from an increase in the rate constant for hydrolysis of the acyl enzyme intermediate, **E.NADH.acyl. However, as discussed in Chapter 2, L.F. Blackwell (personal communication) has presented evidence that for propionaldehyde the hydrolysis of the acyl-enzyme intermediate is not rate limiting in the steady state phase of the reaction, and that the steady state turnover is limited by the slow release of NADH from the enzyme. If this is so, the rate of isomerisation of the enzyme-NADH complexes must have been increased by PCMB during the catalytic turnover when NAD^+ was also present, even if no effect is observed in the isolated displacement studies described above.

The time dependence of the activation observed at low concentrations of PCMB may be due to either slow binding of PCMB to the enzyme, or alternatively, slow isomerisation to produce an activated **E.NAD^+.PMB complex. For the latter proposal, the following scheme may be considered:

$$^{*}\text{E.NAD}^+ + \text{PCMB} \underset{k_{-1}}{\overset{k_1[\text{PCMB}]}{\rightleftharpoons}} {}^{*}\text{E.NAD}^+\text{.PMB} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} {}^{**}\text{E.NAD}^+\text{.PMB}$$

fast slow

Reaction of the E.NAD^+ complex with PCMB was followed by slow isomerisation to form an activated complex. Decay constants for this process, in which it is assumed that $k_1 >> k_2$ so that only the second process is observed are

$$k_f = k_{-1} + k_1[\text{PCMB}] \qquad 3.1$$

$$k_S = k_{-2} + k_2[PCMB]/([PCMB] + K_S) \qquad 3.2$$

where $K_S = k_{-1}/k_1$ (Fersht, 1977)

If k_1 is of the order of $10^7\ M^{-1}.s^{-1}$ then even at PCMB concentrations as low as 0.23 μM, k_f will be too fast to be observable using steady state techniques, and therefore the increase in steady state rate will be described by the slow decay constant k_S. Provided [PCMB] < K_S an increase in k_S would result from an increasing PCMB concentration as is experimentally observed. On the basis of the experimental data, a value for K_S of 1-10 μM is required, and this is within the normal range for the binding of PCMB to proteins (Webb, 1966)

The alternative proposal of slow binding of PCMB may be excluded as an inverse relationship between PCMB concentration and the slow decay constant k_S would be expected (Fersht, 1977) which is clearly contrary to experiment.

Thus the activation can be explained by a model in which PCMB reacts rapidly and reversibly with a thiol group exposed after the conformational change which follows NAD^+ binding. This is followed by an isomerization (conformational change) to produce an activated enzyme-NAD^+-PMB complex which, on the catalytic pathway, leads to an E.NADH.PMB complex from which the rate of release of NADH is about three times greater than that observed for the slower process of NADH release from the unmodified enzyme.NADH complex.

Studies of the effect of PCMB in the presence of disulphiram provide information about the location of the activating group. When disulphiram was added to enzyme activated by PCMB there was an immediate reduction in the activity to 10% of its former value. However, there was still activation of the rate in comparison with control samples inhibited by disulphiram but without PCMB. This suggests that PCMB and disulphiram produce their respective effects by reaction with different thiol groups of the enzyme. There is some interaction between the two, however, as the activating effect is slowly lost after reaction with disulphiram, and if the disulphiram is added first, no activation is observed. There is evidence (Vallari & Pietruzko, 1982, Kitson, 1983) that reaction of the enzyme with disulphiram results in slow formation of an enzyme-disulphide bridge and thus a second -SH group apart from that which normally reacts with

disulphiram must be present. Further evidence that there is a pair of reactive thiol groups has been provided recently by Kitson & Loomes (1985) who observed that one of these groups was modified by disulphiram, resulting in inhibition, and the other was modified by 2,2'-dithiodipyridine, resulting in activation of the enzyme. These authors also observed that disulphiram slowly removed the activating effect of 2,2'-dithiodipyridine in a similar way as was observed in this work for PCMB.

Therefore, it seems likely that both PCMB and disulphiram bind in the same region of the protein, at least at low concentrations of PCMB, but with different thiol groups. Hempel et al. (1982), by sequencing peptides isolated after reaction of the human liver enzyme with radioactively-labelled iodoacetamide, another thiol reagent, have shown that iodoacetamide reacts with one specific thiol group of a pair of adjacent cysteine residues in a single peptide fragment. This modification causes inibition of the enzyme activity, but like disulphiram, does not reduce the enzyme activity to zero, even at high iodoacetamide concentrations (Hempel & Pietruszko, 1981). Since disulphiram has been shown to block the selective alkylation by iodoacetamide (Hempel et al. 1982), it is highly likely that either PCMB or disulphiram reacts with this same group and it is possible that the other may react with the adjacent -SH group. In addition, it has been suggested that there may be another thiol group in the vicinity of the NAD^+, NADH cofactor binding site (von Bahr Lindstrom et al., 1981). Thus there are probably sufficient thiol groups in demonstrably influential regions of the protein to account for the various effects observed with reagents such as PCMB, disulphiram, 2,2'-dithiodipyridine, and iodoacetamide.

Blackwell et al. (1983a,b) have demonstrated that the ester PNPA binds in or near the low affinity (P_2) propionaldehyde binding site, and PNPA has been shown to protect the enzyme against inhibition by disulphiram (Kitson 1982b). Hence disulphiram must also bind in or near the P_2 site, suggesting that PCMB also binds in this vicinity. The three-fold activation of the steady state activity observed for PCMB is also observed at high concentrations of propionaldehyde (MacGibbon et al., 1977a, Bennett et al., 1982, Blackwell et al., 1983a,b) and it is possible that both substances act in the same manner and at the same site.

The two-fold activation produced by PCMB when acetaldehyde was

used as the aldehyde substrate was similar to the activation observed with this substrate in the presence of the steroidal compound diethylstilboestrol (Kitson, 1982b,c,d), suggesting that the two substances act in the same manner. Since activation has also been observed for the thiol reagent 2,2'-dithiodipyridine (Kitson, 1978, 1982b, 1984) it may be that when a substance either binds in the P_2 site or reacts with one of a pair of thiol groups in the vicinity, activation is observed.

For experiments at low levels of propionaldehyde, at PCMB concentrations higher than the maximum activating level, a first order decrease in the reaction rate to a final linear rate was observed. Large amounts of PCMB were required to reduce the final enzyme activity to zero, with higher PCMB ratios required at lower enzyme concentrations (Figure 3.6), suggesting that the inhibition may be due to reversible PCMB binding in which PCMB reacts rapidly and reversibly with the enzyme, but with a different thiol group than that causing activation, followed by a slow conformational change which now results in an inactivated or severely inhibited $E.NAD^+.PMB$ complex. Two decay constants would again be expected for such a scheme, but if as in the previous case, PCMB binding is very rapid, then only the isomerization will be seen. If $[PCMB] << K_s$ for this scheme, then the approach to the inhibited rate should be first order with a linear dependence on PCMB concentration (see equation 3.2), as was observed experimentally.

At high propionaldehyde concentrations, there was no activation of the steady state rate in the presence of PCMB. Instead inhibition was observed at all PCMB concentrations, but the activity was not reduced to zero, even at high PCMB concentrations, except upon precipitation of the enzyme. In steady state experiments in which high concentrations of propionaldehyde were added to enzyme activated by PCMB the reduction in the steady state rate to a rate very similar to that prior to activation clearly indicates that both substances must bind to the enzyme, as if one merely displaced the other, then the activation should remain. Similarly, activating levels of PCMB removed the activating effect of high propionaldehyde concentrations, as shown by inhibition studies of the high propionaldehyde activity (Figure 3.8).

The esterase activity of the enzyme was also inhibited in the presence of PCMB. Since NAD^+ was not present in the esterase experiments PCMB cannot be reacting with the activating thiol group. This requires that a second thiol group be involved in inhibition of

the esterase activity.

Very similar results were obtained for the inhibition of the dehydrogenase activity at high propionaldehyde concentrations and the esterase reaction. The results shown in Figures 3.8 and 3.9 suggest that either PCMB produces inactive enzyme upon binding to a low affinity thiol group, or alternatively, that a modified enzyme with reduced activity is formed. In the former case, the dissociation constants for the PCMB binding at the site causing inhibition could be obtained from the Scatchard replots of the data shown as insets in Figures 3.8 and 3.9. Very similar K_D values were obtained for the inhibition of esterase and high propionaldehyde dehydrogenase activity (provided that the first few points for the high propionaldehyde case, those at low [PCMB]/enzyme ratios, were disregarded), suggesting that the same thiol group is involved in each case. A fit to the low PCMB concentration points, assuming that inhibition at high propionaldehyde concentrations may involve a second higher affinity thiol group, gave a value for K_D of 1.0±0.5 μM.

The results above are explicable on the basis of the suggestion from the activation data that high concentrations of propionaldehyde and PCMB compete for the same (activating) -SH group when NAD^+ is present. A second lower affinity thiol group is also present, which on modification leads to inactivation. Thus when PCMB cannot react with the activating -SH group either because it is not exposed in the absence of NAD^+ as in the esterase experiments, or because the site is already occupied by propionaldehyde as in the high propionaldehyde assays, the second (inhibiting) thiol group is modified leading to inhibition of the steady state activity. Similarly, if PCMB reacts before propionaldehyde, then propionaldehyde binds at the lower affinity site producing inhibition.

Further evidence to support this proposal was obtained from the presteady state experiments in the presence of PCMB. In burst experiments at activating levels of PCMB, there was a substantial decrease in the amplitude of the NADH burst when PCMB was premixed with enzyme in the absence of NAD^+. Such a reduction was not observed for other mixing conditions in which enzyme was preincubated with NAD^+. As the evidence from steady state experiments suggests that reaction of PCMB with the thiol group which causes activation can only occur in the presence of NAD^+, the reduction in burst amplitude is consistent with modification of a second thiol group leading to an inactive form of the

enzyme or to formation of an enzyme-NADH-PMB complex with reduced fluorescence at 435nm. However, since the amplitude of the fluorescence decrease in the NADH displacement experiments was unaffected by the same concentrations of PCMB there is no evidence to support the latter proposal. If PCMB was not premixed with enzyme, then even when a 20-fold excess of PCMB over enzyme was used there was essentially no effect on the burst, which is consistent with previous observations that the inhibition of the steady state rate observed at this level of PCMB occurs relatively slowly.

In burst experiments in which propionaldehyde and excess PCMB were pushed against enzyme premixed with NAD^+, a decrease in fluorescence was observed following the burst (Table 3.7). At low propionaldehyde concentrations, the rate constant for this process of 0.23 s^{-1} was very similar to the slower decay constant obtained from NADH displacement experiments, suggesting that the fluorescence decrease was a result of dissociation of NADH from either an inactive enzyme form which was no longer able to bind NADH, or from a modified enzyme form with a higher NADH dissociation constant.

In NADH displacement experiments, enzyme is initially distributed between the *E.NADH and E.NADH forms, and biphasic fluorescence traces are observed arising from the two consecutive processes of isomerisation and NADH dissociation. However, in the above experiments in which the fluorescence decrease occurs immediately after the burst and therefore before a complete turnover of the enzyme, there would be no buildup of the E.NADH form and the rate of NADH dissociation would be controlled solely by the isomerisation process. A similar single exponential decrease in fluorescence following a burst was observed by A.K.H. MacGibbon (unpublished results) and by L.F. Blackwell and P.B. Buckley (personal communication, see Figure 2.10) in single turnover experiments as discussed previously (2.6).

Alternatively, the fluorescence decrease could possibly result from formation of an enzyme.NAD.PMB form with reduced fluorescence. In such a case, the rate constant observed for the fluorescence decrease would reflect the rate of PCMB binding or consequent rearrangements resulting in fluorescence quenching, and any similarity to the NADH dissociation rate would be coincidental. However, as noted previously, there was no evidence for such quenching, as the same total amplitude was observed for NADH displacement experiments in the presence and

absence of excess PCMB.

The fluorescence decrease following the burst was not observed using activating levels of PCMB with low propionaldehyde concentrations, the conditions which resulted in activation of the enzyme steady state activity, but only under conditions in which excess PCMB and/or high propionaldehyde concentrations were used. In the presence of high concentrations of propionaldehyde, the fluorescence decrease was observed even at activating levels of PCMB. This again suggests that the activation by PCMB and high concentrations of propionaldehyde may be the result of reaction at the same site, the postulated P_2 site, so that in the presence of high concentrations of propionaldehyde, PCMB is unable to bind at this site, and therefore binds at a second site, producing the observed fluorescence decrease. If the decrease in fluorescence represents inactivation of the enzyme or an alteration in the ability of the enzyme to bind NADH and NAD^+, this would account for the inhibition observed.

At high concentrations of propionaldehyde, the rate constant for the fluorescence decrease was increased to at least 1.0 s^{-1}, a value which is similar to the decay constant for the faster of the two processes observed in NADH displacement experiments. An even higher rate constant of 3.5 s^{-1} (Figure 3.12) was obtained for for the fluorescence decrease following the burst at high propionaldehyde concentrations when activating amounts of PCMB were premixed with enzyme and NAD^+. This suggests that PCMB and propionaldehyde both react with the enzyme at two non-catalytic sites, but that there is competition for the higher affinity site. It is proposed that, when propionaldehyde and PCMB are added together, propionaldehyde binds at the activating (P2) site, and the NADH dissociation process after the burst (from inactive or modified enzyme) is considered to be a result of PCMB binding at a second and possibly adjacent thiol group, as it is not observed in the absence of PCMB. However when PCMB is premixed with the enzyme, then it is able to bind at the activating site, and propionaldehyde binds at the alternative site, producing conditions in which dissociation of NADH occurs even more rapidly than in the previous case.

There are, therefore, four ways in which these two proposed sites may be occupied by the two substances. It seems reasonable to assume that if the bulkier PCMB can bind at the second site when the P_2 site is occupied by propionaldehyde, then propionaldehyde should also be

able to bind at both sites under the conditions used in high propionaldehyde assays. The results above, however, indicate that PCMB has a higher affinity for this second site than propionaldehyde, when both are present.

When both sites are occupied by PCMB, as in the excess PCMB, low propionaldehyde experiment, there is still a dissociation of some of the enzyme-bound NADH, but the rate is apparently controlled by the same slow isomerisation as is observed in the normal NADH displacement experiments.

Although this conclusion is not entirely consistent with the observation of an apparent increase in the decay constants for NADH displacement experiments in which excess PCMB was premixed with the enzyme, it is considered that these increases probably arise from even more extensive modification of the enzyme by PCMB, which occurs more slowly than the 10-20 second time scale over which the fluorescence decrease was observed (Figure 3.11a,b). Since no burst was observed under conditions in which excess PCMB was premixed with the enzyme (with or without premixed NAD^+) and regardless of the propionaldehyde concentration, it is clear that the slow modification of many thiol groups produces inactive enzyme. Therefore, it is reasonable to assume that the binding and hence the rate of dissociation of NADH will also be affected.

When propionaldehyde is present at either site, with PCMB at the other, then the NADH dissociation rate is considerably faster than normal, the extent of the increase depending on the position of each substance on the enzyme molecule.

As discussed above, activation by PCMB and high concentrations of propionaldehyde clearly results from an increase in the rate of release of NADH from the enzyme. This must occur, either by an increase in the rate of the isomerisation of the enzyme.NADH complexes or by causing a conformational change in the enzyme, eliminating the slow isomerisation step. There is some evidence that the isomerisation process may be bypassed when high concentrations of propionaldehyde are present. In NADH displacement experiments carried out recently by P.D. Buckley (personal communication) in which 1.10-phenanthroline was used instead of NAD^+ as the displacing agent, conditions which MacGibbon et al., (1977b) have previously shown to produce biphasic traces with similar rate constants as for displacement by NAD^+, addition of 20 mM propionaldehyde resulted in a displacement that was still biphasic, but

the amplitude of the slow phase was decreased to about 10% from its former value of 40-50% of the total amplitude. (NAD^+ cannot be used in these experiments as in the presence of propionaldehyde, reaction will occur.) Hence it appears likely that most of the reaction now occurs via a faster pathway, largely bypassing the slow isomerisation step and accounting for the activation. Since PCMB appears to produce activation by modifying the same site as $^{high}_{propH}$ (the P_2 site), it is probable that the mechanism of activation is the same in each case.

3.6 CONCLUSION

As a large number of thiol groups are present in aldehyde dehydrogenase it was expected that several of these would be modified by PCMB. Certainly the complexity of the kinetics of the reaction of excess PCMB with enzyme was not unexpected. However it was hoped that there would be one sulphydryl group per active site which would be particularly active and reaction of this group only with PCMB would lead to complete inactivation of the enzyme, a result which would have been consistent with such a group being involved in the chemical binding of aldehyde to enzyme, or at least to the group immediately adjacent to such a site.

It was a surprise therefore to find that reaction of the most reactive sulphydryl group led not to inactivation but to activation of the enzyme. The enzyme was only inhibited when there was sufficient PCMB present to react with other thiol groups. Detailed order of mixing studies, particularly those involving the burst kinetics in the presence of PCMB, revealed two sites that specifically affected the activity of the enzyme. One of these sites appeared to be the low affinity propionaldehyde site which when occupied at high concentrations of propionaldehyde also leads to activation of the enzyme.

These results support the proposals made by Bennett *et al*. (1982) that conformational changes are important on the catalytic pathway for aldehyde dehydrogenase. The subtle effects that occur as PCMB reacts at different sites on the enzyme can be understood in terms of this model. Direct evidence for the idea that a conformational change follows NAD^+ binding (MacGibbon *et al*., 1977 b, c, Buckley *et al*., 1982) is provided by the observation that reaction of PCMB with the enzyme leads to activation when NAD^+ is present. The complex

interactions between high concentrations of propionaldehyde (as propionaldehyde attempts to bind to the high affinity propionaldehyde site) and PCMB, which differ with the order of mixing, make sense only for an enzyme which must undergo a number of conformational shifts to carry out its catalytic role. No evidence was obtained for the existence of a thiol group essential for aldehyde binding, and the speculation, based mainly on the analogy made between this enzyme and glyceraldehyde 3-phosphate dehydrogenase, that the aldehyde binds through such a group, must remain just speculation.

In addition these results provide a warning that the presence of a highly reactive and accessible thiol group does not necessarily imply direct involvement in the catalytic mechanism.

CHAPTER 4
THE EFFECT OF pH ON ALDEHYDE DEHYDROGENASE

4.1 INTRODUCTION

There have been some previous studies of the pH dependence of aldehyde dehydrogenase. MacGibbon et al. (1977a) reported that there was little pH dependence of the enzyme activity over the pH range 6-10, for studies at low propionaldehyde concentrations. However differences were observed between different buffers, specifically an apparent anomaly between K_m values obtained using pyrophosphate buffers and those obtained using barbitone (veronal) buffers of the same pH (MacGibbon 1976).

Bennett (1981) reported two effects of ionic strength (I) on the enzyme activity. There was a linear dependence of k_{cat} on ionic strength up to about 80 mM and maximum activity was obtained at ionic strengths of 100 mM and above. In addition there was a shift in the pH at which this maximum occurred towards higher pH values as the ionic strength increased, from pH 5.2 at I = 3.3 mM to pH 7.7 at I = 250mM.

The pH profile of horse liver aldehyde dehydrogenase was determined by Takahashi et al. (1981) who observed a slow increase in reaction rate as the pH increased from 3.0 to 7.5, and a very large increase in rate between pH 8.0 and 10.0. Blair and Bodley (1969) in studies of human liver aldehyde dehydrogenase, using 5 mM actaldehyde as the substrate, obtained three different bell-shaped curves for three different buffers (sodium and potassium pyrophosphate and aminoethanol). The peak activity of each profile occurred between pH 9.0 and 10.0.

During the course of the PCMB experiments presented in the previous chapter, the effect of pH on the enzyme reaction in the presence of PCMB was studied. Control experiments in the absence of PCMB were carried out at each pH, and the results obtained were not in agreement with those of MacGibbon et al. (1977a). Some of the buffers used were however different for the two studies. In order to resolve the anomalies described above, the reasons for the apparent differences in pH dependence of the aldehyde dehydrogenases from other mammalian sources and to investigate the effects observed by Bennett (1981) further study of the buffer and pH dependence of aldehyde dehydrogenase was undertaken.

4.2 METHODS

4.2.1 Buffer Solutions

A variety of buffer solutions at a constant concentration were prepared according to Dawson et al. (1969) as shown in Table 4.1. Solutions were adjusted to the correct pH using a Radiometer 28 pH meter and diluted to give a final concentration of the major component species of 0.035 M. The pH of each solution was also determined after dilution. All solutions were filtered through sintered glass to remove particulate matter.

TABLE 4.1

BUFFER SOLUTIONS

Buffer	pH	Component Solutions
Acetate	4.0 - 5.5	0.2 M acetic acid/ 0.2 M sodium acetate
Citrate	4.1 - 5.0	0.1 M citric acid/ 0.1 M trisodium citrate
Succinate	4.0 -6.0	0.2 M succinic acid/ 0.1 M NaOH
Phthalate	4.5 - 5.5	0.1 M potassium hydrogen phthalate/ 0.1 M NaOH
Phosphate	6.0 - 8.0	0.1 M KH_2PO_4/0.1 M K_2HPO_4
Tris[a]	7.0 - 9.0	0.1 M Tris/0.1 M NaOH
Pyrophosphate	5.0 - 6.5	0.1 M tetrasodium pyrophosphate/
	7.5 - 10.0	0.1 M HCl
TEA[b]	8.0 - 9.0	0.1 M TEA/0.1 M NaOH
Borate	8.0 - 10.2	0.1 M Borate,0.1 M KCl/0.1 M NaOH
Carbonate	9.0 - 10.5	0.1 M $NaHCO_3$/0.1 M Na_2CO_3

a tris is tris(hydroxymethyl)aminomethane hydrochloride

b TEA is triethanolamine hydrochloride

Buffer solutions of constant ionic strength were prepared according to Dawson et al.,(1969) using buffers designed by Miller & Golder (1950), as shown in Table 4.2. Solutions were diluted to an ionic strength of 0.2 M.

TABLE 4.2

Buffer	pH Range	Buffer Component Solutions
Acetate	4.0 - 5.5	0.2 M sodium acetate/8.5 M acetic acid/ 5 M NaCl
Phosphate	6.0 - 7.5	0.5 M $NaH2PO_4$/4.0 M Na_2PO_4/ 5.0 M NaCl
Barbitone*	8.0 - 9.0	2.0 M HCl/0.5 M Na Barbitone*/5 M NaCl
Glycine	9.5 - 10.5	1.0 M Glycine, 1.0M NaCl/ 2.0 M NaOH/5 M NaCl

* sodium 5:5-diethylbarbiturate

For experiments involving pyrophosphate buffers solutions were maintained at an ionic strength not less than 0.1 M by the addition of sufficient 1.0 M KCl to give a final KCl concentration of 0.1 M. Pyrophosphate buffer solutions contained 0.050 M pyrophosphate.

4.2.2 NAD^+ Solutions

All NAD^+ solutions used were prepared using Sigma Grade III NAD^+ (unless otherwise specified) in deionized distilled water and were adjusted to pH 7.0 using 0.1 M NaOH.

4.2.3 Enzyme Assays

Enzyme activity was determined over a range of pH values at both low and high propionaldehyde concentrations by using the spectrophotometric assays described previously (2.2.1) replacing the pH 7.62 phosphate buffer by buffer solutions of the appropriate pH.

In initial experiments, the 0.035 M buffer solutions described above were used, the final buffer concentration in each assay being 0.025 M. For experiments at constant ionic strength, 1.5 cm^3 of buffer solution was used in each assay, giving a final ionic strength of 0.1 M. When pyrophosphate buffers were used, assay solutions contained 0.032 M pyrophosphate and 0.1 M KCl.

Duplicate standard assays at pH 7.6 using 20 mM propionaldehyde, were used to determine the enzyme active site concentration, using a value for k_{cat} of 0.56 s^{-1} per active site, This k_{cat} value (which is

used throughout this chapter) for the enzymic reaction at a propionaldehyde concentration of 20 mM was determined recently by L.F. Blackwell (L.F. Blackwell, personal communication, 2.2.1, 2.1.3.5). Using this figure, k_{cat} values at pH values other than 7.6 were obtained from the reaction rate data as described previously (2.4.1).

4.2.4 The Effect of pH on Activation and Inhibition by PCMB

The PCMB/enzyme active site ratio required to produce maximum activation in the low propionaldehyde assay (2.2.1) was determined at pH 7.6 as described in Chapter 3 (3.4.4). Once this ratio had been established, similar assays were carried out with the same ratio in a series of buffers which covered the pH range 4-10 (pH 4-6, acetate, pH 6-8, phosphate, pH 8-9, triethanolamine, and pH 9-10, carbonate). A control assay was conducted at each pH value in the absence of PCMB and the relative rate, v/v^{o} calculated. Final buffer concentrations were 25 mM in all cases.

The same PCMB/enzyme ratio was used in the high propionaldehyde assays (2.2.1) to determine the effect of pH on the inhibition by PCMB observed at these high concentrations of propionaldehyde.

4.2.5 NADH Burst Experiments

NADH burst experiments were carried out essentially as described previously (2.3.4). A solution containing enzyme (9.0 μM) and NAD^{+} (2.0 mM) in distilled water was placed in one syringe of the stopped flow apparatus and a solution containing propionaldehyde (40 mM) and buffer (I = 0.2 M) of the appropriate pH in the other. Enzyme solutions were used without prior dialysis to remove buffer, but the concentration of pH 7.3 phosphate buffer in the enzyme-NAD^{+} solution was only 0.005 M.

MacGibbon (1976) showed that there was very little variation (<10%) in the fluorescence of NADH over the pH range 6.9-9.6, allowing fluorescence amplitudes to be compared directly, at least over this pH range. The burst rate constant and amplitude at each pH was determined as described previously (2.4.4). Several (7-12) separate runs using the same initial solutions were carried out and the mean values of the rate constants obtained. The catting procedure described previously

(2.2.3 or 2.3.4) was also used to analyse data stored on a computer disc.

4.2.6 NADH Displacement

NADH displacement experiments were carried out as described previously (2.3.5). Solutions containing enzyme (9.0 μM) and NADH (40 μM) in 3.3 mM pH 7.3 phosphate buffer were pushed against solutions containing NAD^+ (2.5 mM) and buffer (I = 0.1 M) of the appropriate pH.

Biphasic displacement data were analysed as described previously (2.4.5). The values from at least 7 runs were averaged to give the reported values of the apparent rate constants, λ_s and λ_f, and the amplitudes of the two phases, A_s and A_f.

4.3 RESULTS

4.3.1 Steady State Studies

4.3.1.1 Buffer and Ionic Strength Effects

The pH dependence of the enzyme steady state reaction rate over the pH range 4.0 to 10.5 was determined, both at concentrations of propionaldehyde sufficient to saturate the high affinity propionaldehyde binding site (100 μM) and at concentrations sufficient to saturate the low affinity propionaldehyde binding site (20 μM). Buffers used were: pH 4.0 - 6.0 acetate, pH 6.0 - 8.0 phosphate, pH 8 - 9.0 triethanolamine hydrochloride, pH 9.0- 10.5 carbonate.

The reaction rate was calculated at each pH value and the results are shown in Figure 4.1. A marked pH dependence was observed at both propionaldehyde concentrations. Similarly shaped pH profiles were obtained in each case, although the reaction rates at high propionaldehyde were generally about 3 times those at 100 μM propionaldehyde. The maximum rate at low propionaldehyde concentrations was 34% of the maximum at high propionaldehyde concentrations. The pH profiles had several features in common. Maximum steady state reaction rates were obtained at pH 5.3, there was a plateau or perhaps a second peak in the pH region 6.0 - 7.6, and very low activity was observed at pH 9.0.

Figure 4:1

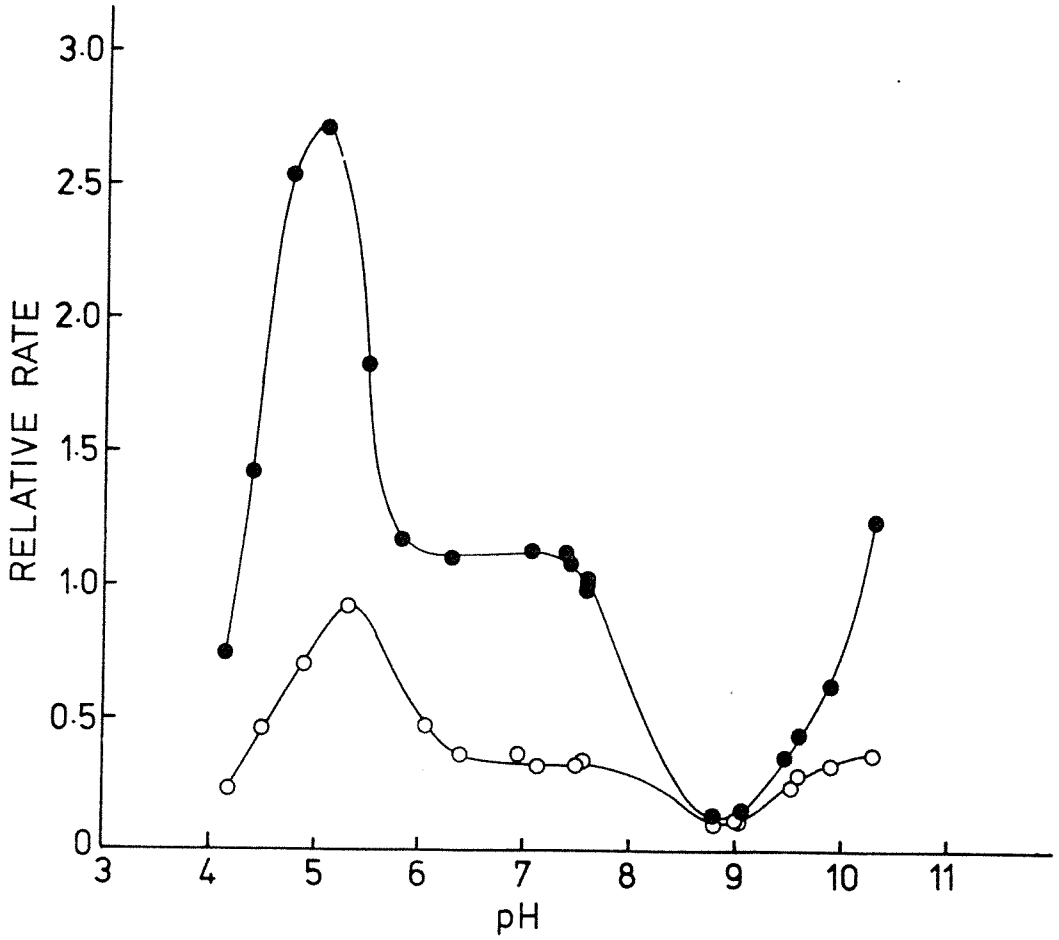


Figure 4.1 The pH Profile for Aldehyde Dehydrogenase at High and Low Concentrations of Propionaldehyde using Acetate, Phosphate, Triethanolamine and Carbonate buffers. The enzyme (2.8 μM) was assayed in buffers covering the pH range 4-9. From pH 4-6 acetate (25 mM) buffers were used; from pH 6-9 phosphate (25 mM) buffers were used; from pH 8-9 triethanolamine buffers (25 mM) were used; from 9-10 carbonate buffers were used. The NAD^+ concentration was 1mM and the propionaldehyde concentration was 20 mM (●) and 100 μM (○). The steady state rates are expressed relative to the rate at pH 7.6 as 1.0.

Values of k_{cat} were not calculated for these data, because the experiments were carried out using Sigma Grade AA1 instead of Sigma Grade III, and subsequent tests showed that lower reaction rates obtained when Grade AA1 NAD^+ was used. The manufacturers claim that this product is not a highly purified substance and may contain up to 4% methanol as an impurity, whereas the major contaminant in the more highly purified and characterised Grade III NAD^+ is acetone (up to 3%). Thus there appears to be some inhibition of the steady state activity by an as yet unidentified impurity in the poorer quality NAD^+. This inhibition was also pH or buffer dependent, less inhibition being observed at either higher or lower pH values than at pH 7.6. As a result k_{cat} values have only been calculated in cases in which Grade III NAD^+ was used, for comparison with the presteady state data.

Solutions were retained after rate measurements for a further pH measurement, and assay solutions containing excess propionaldehyde (initially 20 mM) at pH 9.5 or higher were observed to turn yellow upon standing. Subsequent studies showed that this yellow substance was formed independently of enzyme, in solutions at pH 9.0 or higher. The yellow product had an absorbance maximum at 345 nm and was highly fluorescent. Therefore, the apparent rapid increase in enzyme activity in the pH range 9.5 - 10.5 was an artefact. In subsequent experiments a control experiment in the absence of enzyme was carried out for all reaction mixtures at a pH higher than 9.0. The control rate was subtracted from the rate obtained when enzyme was present, and results corrected in this manner are given. The enzyme-independent increase in absorbance at 340 nm observed at pH 9.0 or higher was considerably slower when borate or pyrophosphate buffers were used than for carbonate buffers.

Figure 4.2 shows the results obtained using a variety of buffers over the pH range 4.0 - 10.5. Enzyme activity was measured at a propionaldehyde concentration of 20 mM. Although sigmoidal or complete bell-shaped curves were obtained in each case, the pH at which the maximum relative rate occurred varied between pH 5.0 and 8.0, depending on the buffer system used, and it was impossible to construct a single pH profile. Very low activity was observed using triethanolamine or tris buffers and use of these buffers with this enzyme was subsequently abandoned.

As the buffers used were all of constant concentration (25 mM) but not constant ionic strength, and since a number of polybasic buffer

Figure 4:2

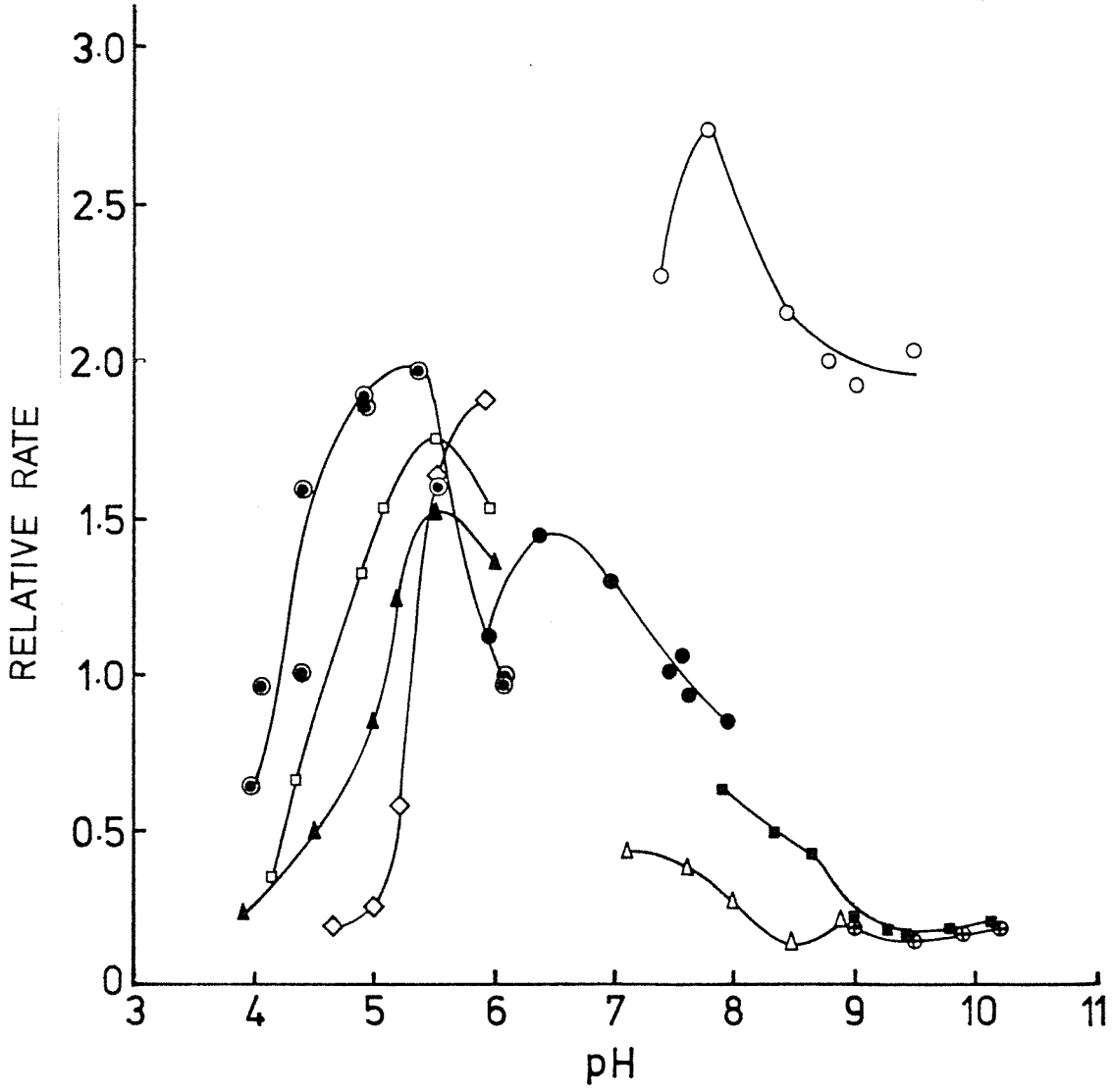


Figure 4.2 pH profiles for aldehyde dehydrogenase in constant concentration (25 mM) buffers. Aldehyde dehydrogenase (2.9 μM) was assayed in buffers of different pH prepared as described in Methods with NAD^+ (1 mM) and propionaldehyde (20 mM). The different buffer systems were as follows: (○) pyrophosphate; (◉) acetate; (●) phosphate; (■) borate; (Δ) tris; (⊕) carbonate; (□) citrate; (▲) succinate; (◇) phthalate.

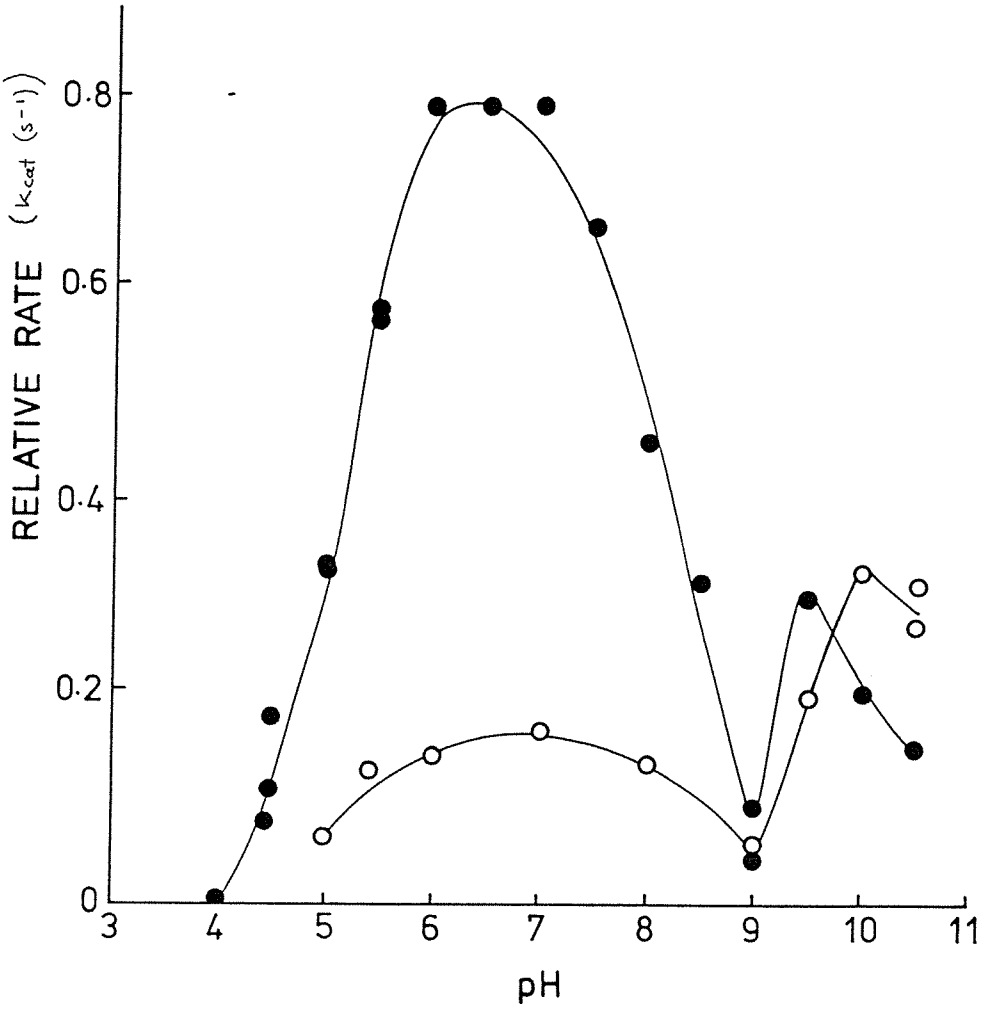
systems were used, the observed differences in the pH profiles could be partially attributable to ionic strength effects similar to those reported by Bennett (1981). In order to eliminate this additional effect, all subsequent experiments were carried out at a constant ionic strength of 100 mM. In addition the pH profile of activity obtained using pyrophosphate buffers appeared to be sufficiently different to those obtained using other buffers to warrant further study.

4.3.1.2 Constant Ionic Strength Buffers

Figure 4.3 shows the k_{cat} values obtained over the pH range 4.0 - 10.5 using buffers of constant ionic strength (I=100 mM) for both high (20mM) and low (100 µM) propionaldehyde concentrations. The pH profile differed from that obtained previously (Figure 4.1) in several respects. At the high propionaldehyde level, a single bell-shaped curve was obtained in the pH range 4.0 to 9.0 and maximum activity was observed at pH 6.0 to 7.0. An apparent pK value of 5.3 and a slope of +1 were obtained from a plot of log k_{cat} against pH for the ascending arm of the curve and a pK value of 8.3 and a slope of -1.05 for the descending arm. At low propionaldehyde concentrations a similar curve was obtained, with similar apparent pK values but with much lower activity. At pH 5.9, the low propionaldehyde reaction rate was only 18% of the rate at 20 mM propionaldehyde. Very low activity was again observed at pH 9.0 for both propionaldehyde concentrations. Above pH 9.0, k_{cat} values were obtained from the rate measurements as described in the previous section. A further small apparent increase in k_{cat} was observed in the pH region 9.5 to 10.0 at both propionaldehyde concentrations, although at pH 10 a higher k_{cat} value was obtained using 100 µM propionaldehyde than when 20 mM propionaldehyde was present. However, this may merely reflect an overcorrection, as the effect of enzyme on any reaction between NAD^+ and propionaldehyde, either by competition or catalysis, could not be determined.

The maximum k_{cat} values obtained were 0.80 s^{-1} for 20 mM propionaldehyde assays and 0.16 s^{-1} for 100 µM propionaldehyde assays.

Figure 4:3 pH PROFILE OF ALDEHYDE DEHYROGENASE ACTIVITY



Figures 4.3 pH Profile of Steady State Activity of Aldehyde Dehydrogenase. The enzyme (2.1 μM was assayed in buffers of constant ionic strength (I = 0.1 M, refer methods) covering the pH range 4-10 at both high (●, 20 mM) and low (○, 100 μM) levels of propionaldehyde. The NAD^+ concentration was 1.0 mM in all assays. Values of k_{cat} were determined from the reaction rate at each pH value, relative to the value of $0.56 s^{-1}$ obtained at pH 7.62 in 25 mM phosphate buffer.

4.3.1.3 Pyrophosphate Buffers

The steady state pH profile was determined using pyrophosphate buffers at a constant ionic strength of 100 mM and, as shown in Figure 4.4, the results differed significantly from those obtained in the previous buffer systems. At high propionaldehyde concentrations, the maximum value for k_{cat} of 0.76 s^{-1} was similar to that shown in Figure 4.3, but this maximum value was obtained at a higher pH (8.0) than previously. The curve was broader than for the other buffer systems in Figure 4.3 but an apparent pK value of 5.6 was determined from a plot of log k_{cat} against pH. Although there appeared to be a decrease in k_{cat} above pH 9 at this propionaldehyde concentration, there were insufficient data to determine the exact pH dependence in this region. Control samples lacking enzyme showed a negligible rate of increase in absorbance at 340 nm at pH 9 and 10 so that no correction of these results for non-enzymic reaction of propionaldehyde and NAD^+ was necessary.

At low propionaldehyde concentrations, the results obtained in the pH range 5-7 were similar to those shown in Figure 4.3 for the other buffer systems. Above pH 7, however, there was a marked increase in k_{cat} until at pH 9, the value of k_{cat} was equal within experimental error to that obtained at high propionaldehyde concentrations. There were insufficient data to determine an apparent pK for this increase, but an approximate value of 9.0 was assigned, assuming that only a single ionizable group was involved. Above pH 9.2, the k_{cat} value obtained at low propionaldehyde concentrations appeared to be higher than that obtained at high propionaldehyde levels, although this could not be established conclusively from these data.

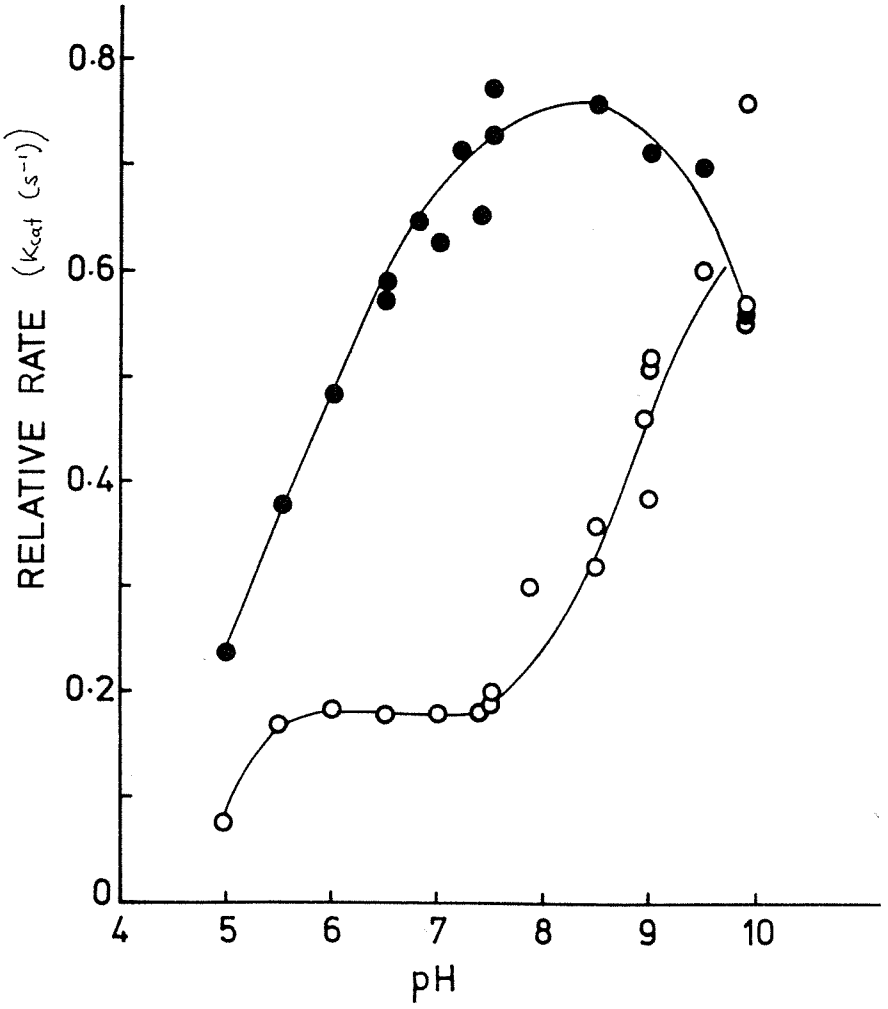
High k_{cat} values at pH 9.0 were obtained for both propionaldehyde concentrations, in contrast with the very low values obtained using other buffers.

4.3.1.4 The Effect of pH on Modification by PCMB

At a PCMB/enzyme ratio of three (the maximum activating level for this particular enzyme sample at pH 7.6) the pH profile obtained at low propionaldehyde concentrations (Figure 4.5) was markedly different to the control profile obtained in the absence of PCMB using 25 mM buffers, (not including pyrophosphate).

The pH profiles for PCMB-modified and control samples are shown in Figure 4.5a, and the relative values for v/v^0 shown in Figure 4.5b.

Figure 4:4



Figures 4.4 The Effect of Pyrophosphate Buffers on the Steady State Activity.

The k_{cat} values were determined at both low (O , 100 µM) and high (● , 20 mM) concentrations of propionaldehyde. Assays contained enzyme (2.3 µM) and NAD^+ (1 mM) in a range of sodium pyrophosphate buffers.

Figure 4.5 The pH Dependence of the Steady State Activity at Activating Levels of PCMB.

PCMB (3.0 μM) was added last to assays containing enzyme (1.2 μM), NAD^+ (1 mM) and propionaldehyde (100 μM) over the pH range 5-10.

4.5 (a) The rates of both the control assays and those in which PCMB was present are both shown.

4.5 (b) The rate of the PCMB-modified reaction rate is shown relative to the control rate at the same pH. Apparent pK_a values of 6.25 and 8.8 were obtained for the ascending and descending arms of this curve respectively, in separate experiments (Insets A, B).

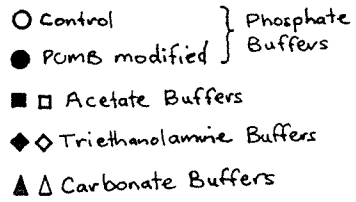
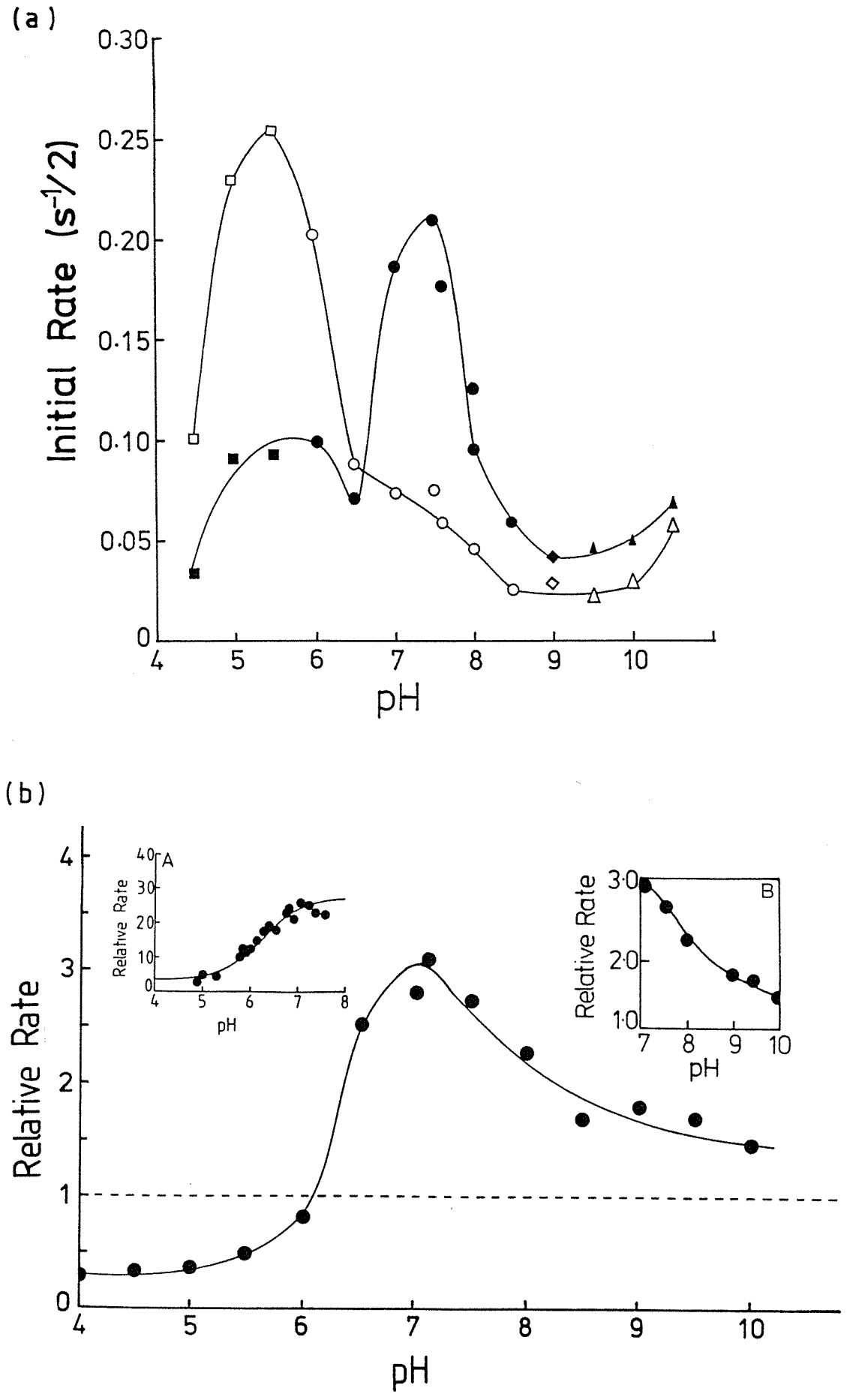


Figure 4:5 THE EFFECT OF pH ON ACTIVATION BY PCMB



Although a net inhibition was observed at pH values of 6.5 and below and net activation at higher pH values, it is clear from Figure 4.5a that the true pH dependence of the activation is more complex than is immediately apparent from Figure 4.5b. Maximum activation was observed at pH 7.2 (Figure 4.5b), but the modified enzyme produced apparent activity peaks at pH 5.5 and 7.6 (Figure 4.5a), very similar to the peak pH values of the control samples in phosphate and acetate buffers respectively.

4.3.2 The Effect of pH on the NADH Burst

4.3.2.1 Constant Ionic Strength Buffers

The effect of pH on the burst rate constant (k_b) over the pH range 5.0 - 10.0 is shown in Figure 4.6. The burst rate constant was determined at high (20 mM) propionaldehyde concentrations. The buffers used were those used previously (4.3.1.2).

As shown in Figure 4.6a, the burst rate constant increased over the pH range 7.0 - 9.0. A maximum burst rate constant of $30\pm4\ s^{-1}$ was observed at pH 10.0, a three-fold increase over the rate constant at pH 5.0. An apparent pk value of 8.5 was obtained from a plot of log k_b against pH.

The variation in the amplitude of the burst as a function of pH was observed to increase with increasing pH (Figure 4.5b) to reach a maximum between pH 9 and 10. The amplitude at pH 5.0 was only 4% of the value at pH 9.0 and there was a ten-fold increase between pH 5.0 and 6.0. An apparent pK value of 6.5 was obtained fom the curve shown in Figure 4.6b. Experiments at pH 4 or below were not attempted because of the instability of NADH in acid solution and to avoid the possibility of enzyme precipitation in the reaction chamber of the stopped flow apparatus, which could have caused blockage or serious interference with the fluorescence signal.

Burst experiments using low propionaldehyde concentrations (100 μM after mixing) were conducted at pH 7.0 and pH 10.0 but the results were unsatisfactory because the propionaldehyde concentration was less than that required for saturation of the low propionaldehyde (P_1) binding site (MacGibbon et al., 1977c). Therefore because the rate of binding of propionaldehyde could still be partly rate limiting at these low concentrations, the results could not be compared directly with

Figure 4.6 pH Profile for the NADH Burst in Constant Ionic Strength Buffers.

The burst pH profile (●) was determined in constant ionic strength buffers (see Methods) by rapidly mixing solutions of aldehyde dehydrogenase (9.0 μM) and NAD^+ (2 mM) dissolved in distilled water with a second solution containing propionaldehyde (40 mM) dissolved in the appropriate constant ionic strength buffer (0.2 M).

4.6(a) pH Dependence of the Burst Rate Constant. The open circle shows the value for the burst rate constant at low concentrations of propionaldehyde (see Results).

4.6(b) pH dependence of the burst amplitude determined relative to the amplitude at pH 7.6.

Figure 4:6 THE pH PROFILE OF THE NADH FLUORESCENCE BURST

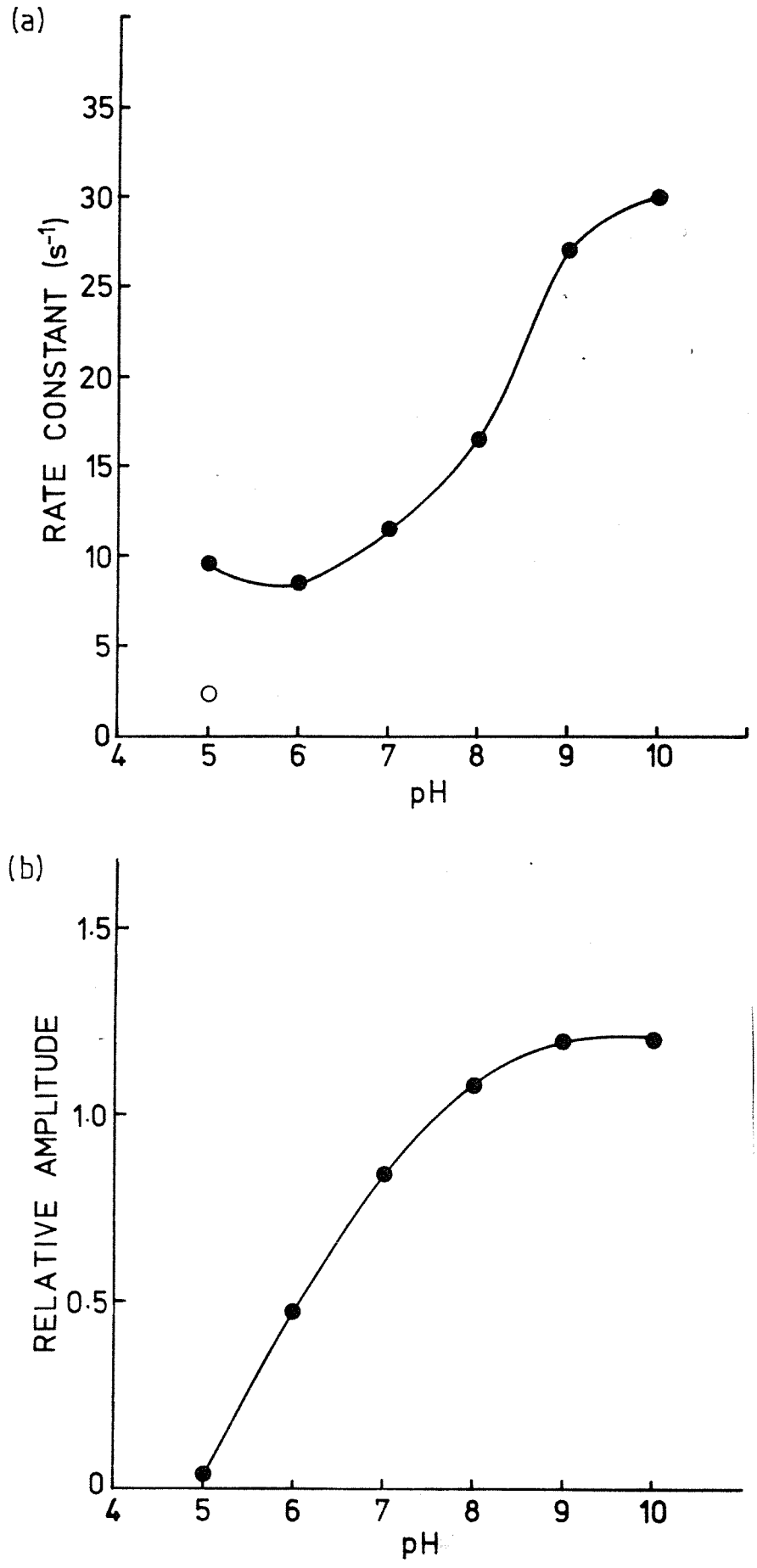


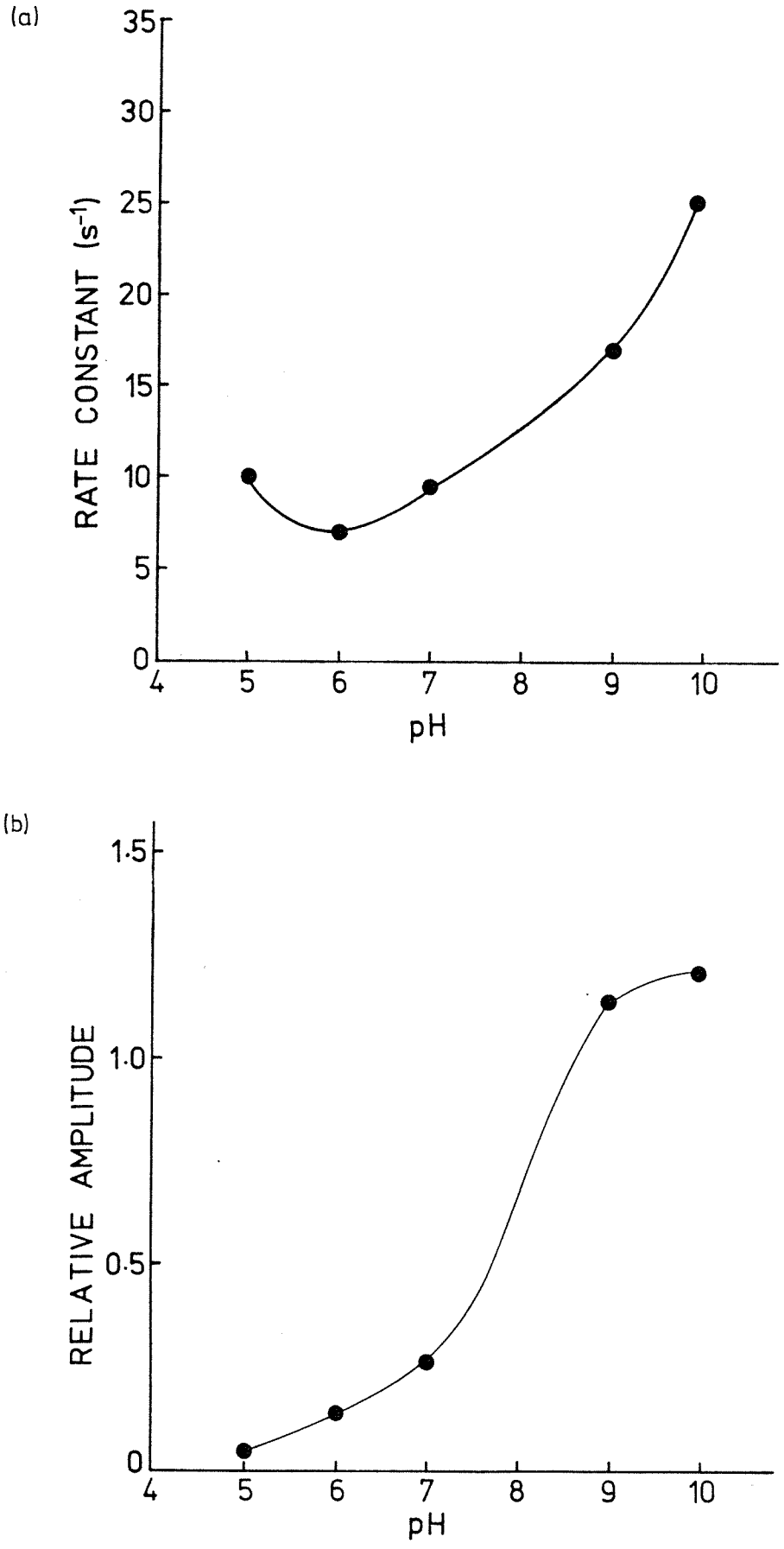
Figure 4.7 The Effect of pH on the NADH Burst at High Concentrations of Propionaldehyde and in Pyrophosphate Buffers.

The burst pH profile was determined in sodium pyrophosphate buffers (see Methods) by rapidly mixing solutions of aldehyde dehydrogenase (9.0 μM) and NAD^+ (2 mM) in water with a second solution containing propionaldehyde (40 mM) dissolved in the buffer of the appropriate pH (0.2 M).

4.7 (a) pH profile of the burst rate constant

4.7 (b) pH dependence of the burst amplitude determined relative to the amplitude at pH 7.6

Figure 4:7


(a)
RATE CONSTANT (s^{-1})
35
30
25
20
15
10
5
0
4
5
6
7
8
9
10
pH
(b)
RELATIVE AMPLITUDE
1·5
1·0
0·5
0
4
5
6
7
8
9
10
pH

the high propionaldehyde burst results reported under saturating conditions. Other studies (A.F. Bennett, unpublished results) showed that lower rate constants were observed at low propionaldehyde concentrations at pH values below pH 7.0, but no differences between the two burst profiles were observed at higher pH values. In either case, it was not possible to fit the low propionaldehyde burst data to a single pK value as the spread of the pH curve was too wide.

The amplitude of the burst at low propionaldehyde concentrations was also too complex to fit to a single pK value but a large increase in amplitude was observed between pH 5.0 and 6.0 similar to that observed at high propionaldehyde levels.

4.3.2.2 Pyrophosphate Buffers

Burst rate constants using high propionaldehyde concentrations were also determined over the pH range 5.0 - 9.9 using pyrophosphate buffers. Results are shown in Figure 4.7. The profile is basically similar to that obtained using other buffers although the curve was shifted towards higher pH. A pK value could not be obtained as there is insufficent information at high pH, but is expected to be 9.5 or greater if a single pK is involved. Rate constants were in the range 7.0 to 30.0 s^{-1} for the high propionaldehyde NADH burst, values which were very similar to those described above for the other buffers.

Burst amplitudes are shown in Figure 4.6b and an increase in amplitude as the pH increased was again observed. As for the rate constants, the curve was shifted towards higher pH, consistent with a higher apparent pK (8±1). The amplitude at pH 5.0 was again only 4% of the maximum observed at pH 9-10.

4.3.3 The Effect of pH on Displacement of NADH by NAD^+

4.3.3.1 Constant Ionic Strength Buffers

Biphasic NADH displacement traces were obtained over the pH range 5.0 to10.0. The two apparent rate constants λ_f and λ_s and the amplitudes A_f and A_s of the two phases were determined and are shown as a function of pH in Figure 4.8 Similar trends were observed for both λ_s and λ_f. At pH 5.0, values of 1.25±0.09 s^{-1} for λ_s and 6.4 ±0.4 s^{-1} for λ_f were obtained, both of which were six to seven times larger than the control values determined at pH 7.6. However, at pH 6.0 λ values had

decreased markedly to 0.31±0.01 s^{-1} for λ_s and 1.68±0.09 s^{-1} for λ_f. There was a further decrease in λ_s to 0.08±0.01 s^{-1} and in λ_f to 0.30±0.07 s^{-1} at pH 9.0, followed by an increase to values at pH 10 of 0.34±0.06 s^{-1} for λ_s and of 1.0±0.1 s^{-1} for λ_s.

Amplitudes are expressed relative to the total enzyme active site concentration and as observed in Figure 4.8c, there was an increase in the total amplitude of the displacement process over the pH range 5-10, the total amplitude at pH 5.0 being only 60% of that at pH 10.0. However, different trends were shown for the individual amplitudes of the two phases. A minimum relative amplitude of 0.28 and a maximum of 0.67 were obtained for A_s (Figure 4.8c). When the results were expressed in terms of the percentage of the total amplitude due to the fast phase (Figure 4.8d) a maximum value of 46% was obtained at pH 6.0, but it must be noted that the errors involved in the measurements were large, as indicated in the figures.

4.3.3.2 Pyrophosphate Buffers

NADH displacement experiments were carried out in pyrophosphate buffers over the same pH range as above and the traces were also biphasic. Results are shown in Figure 4.9. In contrast with the results in other buffer systems, different profiles were obtained for λ_s and λ_f. High values were again observed at pH 5.0, but λ_s decreased from 1.2±0.1 s^{-1} at pH 5.0 to a minimum value of 0.4±0.2 s^{-1} at pH 7.0 and then increased again to a second peak value of 0.97±0.09 s^{-1} at pH 9.0 (Figure 4.9a), whereas λ_f decreased from a value of 7±2 s^{-1} at pH 5.0 to a minimum value of 3.3±0.4 s^{-1} at pH 8.5 and then increased again at higher pH to a value of 4.2±0.5 s^{-1} at pH 10.0 (Figure 4.9b).

The dependence of amplitude on pH as shown in Figure 4.9c was also considerably different to that observed in other buffer systems, although a plot of the percentage fast phase (Figure 4.9d) was similar to that obtained previously. The total amplitude did not increase in a smooth fashion over the pH range as for previous buffer systems, although such a trend was observed for A_s.

Figure 4:8 THE EFFECT OF pH ON THE DISPLACEMENT OF NADH BY NAD^+

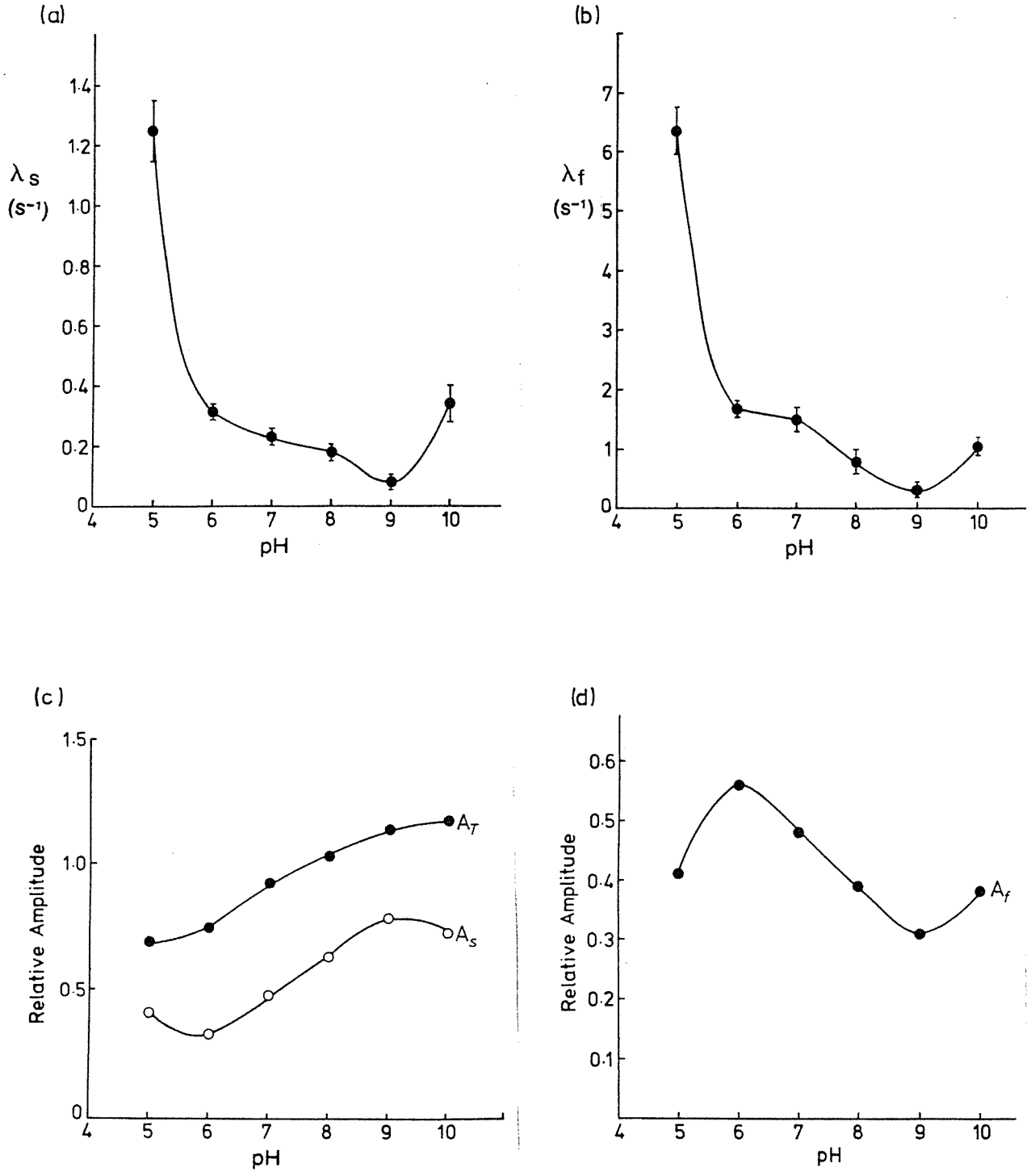


Figure 4.8 The Effect of pH on the Displacement of NADH

NADH displacement experiments were carried out over the pH range 5-10 using buffers of constant ionic strength (I = 0.1 M, refer Methods). Solutions containing enzyme (9.0 μM) and NADH (40 μM) in 3.3 mM pH 7.3 phosphate buffer were rapidly mixed with solutions containing NAD^+ (2.5 mM) in the buffer of the appropriate pH.

Figure 4:9 THE EFFECT OF pH ON NADH DISPLACEMENT IN PYROPHOSPHATE BUFFERS

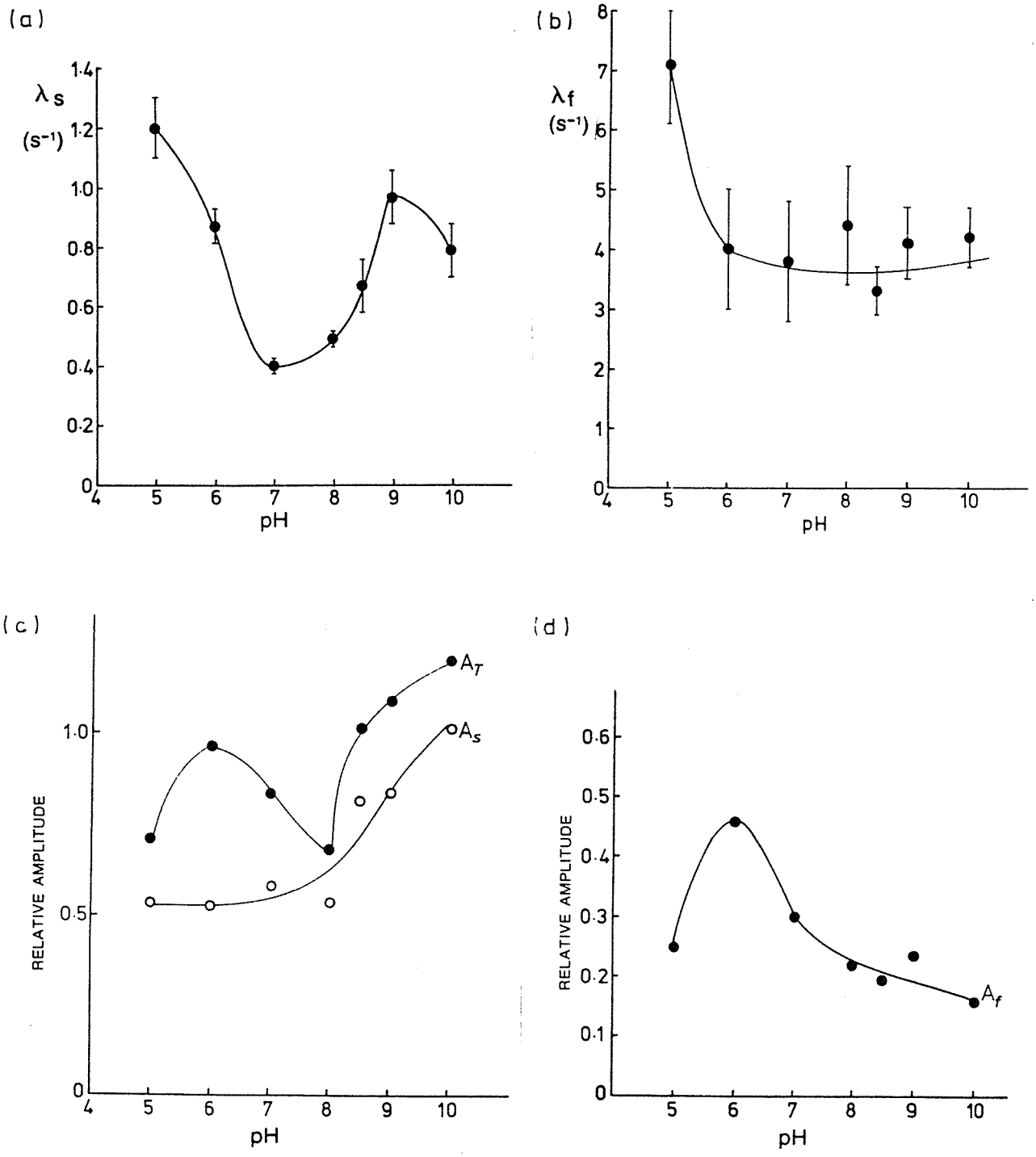


Figure 4.9 The Effect of pH on the Displacement of NADH in Pyrophosphate Buffers.

NADH displacement experiments were carried out over the pH range 5-10 using sodium pyrophosphate buffers. Solutions containing enzyme (9.0 μM) and NADH (40 μM) were rapidly mixed with solutions containing NAD^+ (2.5 mM) in the buffer of the appropriate pH.

4.4 DISCUSSION

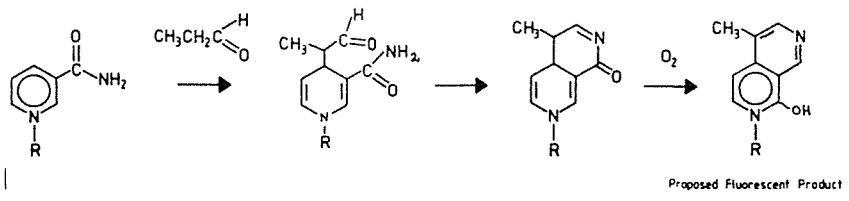
The effects of pH on aldehyde dehydrogenase appear to be complex, and to include numerous specific buffer effects. Interpretation is also complicated because of the dependence of enzyme activity on ionic strength as reported by Bennett (1981).

The results shown in Figure 4.2, in which slightly different pH profiles were obtained for each buffer system used demonstrate that the shape of the pH profiles shown in Figure 4. arises as a consequence of combining the data obtained in several different buffer systems when the buffers involved produce different profiles with different apparent pK values and different maximum rates.

Although the data presented in Figure 4.1 does not represent the pH profile of the enzyme, it does demonstrate that the reaction rates obtained at low propionaldehyde concentrations, although always lower than those at high propionaldehyde levels, followed the same general pattern.

The rapid increase in k_{cat} with increasing pH above pH 9.5 at high propionaldehyde concentrations has been demonstrated to be an artefact due to production of a highly fluorescent species with an absorption maximum at 345 nm. This substance was produced in the presence of NAD^+ and propionaldehyde and appeared to be catalysed by carbonate or bicarbonate anions. Addition reactions of NAD^+ with organic compounds, such as ketones, at high pH to produce fluorescent NAD^+ derivatives is well documented (Metzler, 1977) and it is likely that propionaldehyde reacts in a similar manner, forming the product shown below in Scheme 4.1.

Scheme 4.1



Addition of propionaldehyde at the 4 position of the pyridine group is followed by condensation and ring closure. The resulting product is slowly converted to a fluorescent compound in the presence of oxygen (Metzler, 1977). Such a slow conversion is consistent with the data, as the yellow fluorescent species was only detected when unstoppered cuvettes were allowed to stand at room temperature. As a result of this reaction, the pH profiles cannot be considered to be very accurate above pH 9.0.

The very poor activity observed when tris and TEA buffers were used can be attributed to reaction of propionaldehyde with the amino groups of tris and ethanolamine (an impurity (1-2% v/v) present in the triethanolamine) to form Schiff's base compounds.

Soluble proteins such as enzymes generally contain a large number of charged groups and are therefore polyelectrolytes. As the tertiary structure of enzymes is not rigidly constrained, but is subject to influence from the environment, the molecule adopts a conformation in which the distribution of charged groups is such as to provide maximum solubility in any particular medium, and to minimise unfavourable electrostatic interactions. Thus it is to be expected that the presence of other electrolytes, and the total concentration of ions in the environment of the enzyme will affect the structure, and consequently the catalytic activity of the enzyme.

At very low ionic strengths, it is possible that interactions between oppositely charged groups or regions on the protein molecule may cause a distortion from the optimum conformation for catalytic activity. At high ionic strengths, such intramolecular effects would be minimised because of the greater probability of alternative ionic interactions.

The dependence of the catalytic activity of aldehyde dehydrogenase on ionic strength as observed by Bennett (1981) and in the present work can be explained in terms of effects such as those discussed above. The loss of activity in low ionic strength would be expected to be reversible, as was experimentally observed.

Similar dependences of enzyme activity on ionic strength have been observed previously for other enzymes, such as lysozyme (Maurel & Douzou, 1976) and ribonuclease (Irie, 1965, and Kalnitsky et al., 1959)

As the ionization state of acidic and basic groups on the enzyme will change as the pH of the environment is altered, the electrostatic interactions and total charge on the molecule will change considerably,

and it is to be expected that this will also affect the catalytic activity.

Comparison of Figures 4.2 and 4.3 shows that when the ionic strength of acetate and phosphate buffers was increased, there was a shift in both the apparent pK values and the pH of maximum activity for each buffer system towards higher pH values, in agreement with the results reported previously by Bennett (1981)

Maurel and Douzou (1976) have shown that electrostatic fields, such as those produced by a polyelectrolyte molecule, influence the hydrogen ion concentration in the immediate vicinity. Thus the pH experienced by a particular ionizable group may be different from the pH measured in the bulk solution. Such effects are also true for concentrations of ions other than hydrogen, but it is the proton concentration concentration which is of interest in pH studies.

$$[H^+]_L = [H^+]_B \, e^{-Z/kT} \qquad 4.1$$

where the subscripts L and B refer to the local and bulk phases, and Z is a function of the electrostatic potential prevailing in the neighbourhood of the polyelectrolyte, and may be either positive or negative. As a result, the difference in pH (ΔpH) between the local and bulk phases is proportional to the local electrostatic potential:

$$\Delta pH = pH_L - pH_B = 0.43 \, Z/kT \qquad 4.2$$

The polyelectrolyte theory also states that Z decreases as the logarithm of the ionic strength inceases, and therefore at sufficiently high ionic strengths pH_L and pH_B become identical as the concentration of ions in solutions becomes too great for the hydrogen ion concentration to be subject to local influence (Maurel & Douzou, 1976).

Thus if an enzyme reaction occurs under the influence of an electrostatic potential field, the activity of the enzyme will depend on pH_L not pH_B. However, when pH_L is significantly different from pH_B, as at low ionic strength, the activity profile, which can only be plotted in terms of pH_B, will be displaced by an amount ΔpH which depends on Z as above (equation 4.2). In the presence of a positively charged electrostatic field, the pH dependence of the enzymic activity

will be displaced to lower pH values at low ionic strength, and will therefore be observed to shift towards higher pH values as the ionic strength of the solution increases. Thus observation of a pH dependency of activity for a particular enzyme which is itself a function of ionic strength will reveal the importance of electrostatic field effects for that particular enzyme (Maurel & Douzou, 1976).

For aldehyde dehydrogenase, the pH dependence of the activity was observed to shift towards higher pH values with increasing ionic strength, indicating that, according to the polyelectrolyte theory, the ionizable groups which give rise to the pH optimum are subject to the influence of a positively charged local environment, even though the enzyme has a net negative charge above pH 5.3 (Agnew et al., 1981).

When a number of different buffer systems were used, the maximum catalytic activity varied considerably, and the optimum pH varied from 5.0 in acetic acid to 8.0 in pyrophosphate (Figure 4.2). Part of this difference may have been attributable to the fact that polybasic buffer systems have higher ionic strengths than for corresponding monobasic buffer systems at low pH, and the exact ionic strength of each solution will depend on the pK values for that particular buffer. There was a general tendency for the pH optimum to occur at higher pH values for higher ionic strength buffers as expected from the unbuffered experiments. At all pH values between 4 and 6, citrate buffers (pK_1 = 3.13; pK_2 = 4.76) have a higher ionic strength than acetate buffers (pK = 4.76) and the pH profile is shifted to higher values in agreement with the polyelectrolyte theory.

However, this cannot be the only important factor since at pH 4.5, with acetate and succinate buffers (pK_1 = 4.21; pK_2 = 5.64) which have similar ionic strengths, there is a marked shift in the optimum pH towards higher pH values for the succinate buffer system. Also, the phthalate buffer system (pK_1 = 2.95; pK_2 = 5.41), which would have only a slightly higher ionic strength at pH 4.5 than the succinate buffer system, nevertheless has an activity maximum at an even higher pH. These results therefore indicate that the enzyme activity is also affected by the nature of the anions present in the solution. In addition, the pH profiles clearly demonstate how results gained at a single pH value may be subject to misinterpretation. For example, at pH 5.0, the relative enzyme activities in each buffer were 1.0 (acetate), 0.77 (citrate), 0.45 (succinate), and 0.14 (phthalate), values which, taken in isolation, would suggest that citrate, succinate

and phthalate ions were inhibitors of the enzymic activity relative to the acetate buffer system. However the maximum enzyme activities in each buffer were very similar for all these buffers (Figure 4.2). Thus any apparent inhibition or activation by non-specific reagents should be investigated over a wide pH range with respect to a control system.

Further evidence for specific anion effects has been provided by A.F. Bennett (unpublished results) who demonstrated that the enzyme has a specific requirement for anions such as carbonate, sulphate or phosphate. In experiments in which degassed bicarbonate and carbonate-free distilled water was used instead of buffer, the enzyme activity could not be restored by addition of KNO_3, but was immediately restored upon addition of sulphate anions. However, removal of dissolved carbon dioxide from solution containing phosphate buffers of low ionic strength had no detrimental effect (A.F. Bennett, unpublished results). These results, and those shown in Figure 4.2 indicate that the enzyme activity requires the presence of certain anions. The lack of specificity for such anions suggests that the anion plays a structural role, rather than being involved directly in a catalytic role. Such a requirement could arise if there is a region on the protein in which a number of basic residues (such as lysine or arginine) were in close proximity, as this would constitute an anion binding site. In the absence of a suitable anion to bind in this site, a situation which is probably unlikely to occur under physiological conditions, the electrostatic repulsions involved could result in disruption of a vital region of the tertiary structure even at high ionic strength. The nature of the effects of anions such as succinate, phthalate and citrate has not yet been established, but it is possible that they may interact at this same site or in a similar manner at a different site.

When the pH dependence of the enzyme activity was determined using buffers of a constant ionic strength of 100 mM, a smooth pH profile was obtained (Figure 4.3) at both low (100 µM) and high (20 mM) propionaldehyde concentrations with none of the apparent anomalies observed when the data from different buffer systems at low ionic strength were combined. The pH profile was clearly biphasic at both propionaldehyde concentrations, both profiles being apparently controlled by two pK values of about 5.3 and 8.3. The data obtained for the slow and fast decay constants for the NADH displacement experiments in Figure 4.6a,b appeared to be controlled by the same two

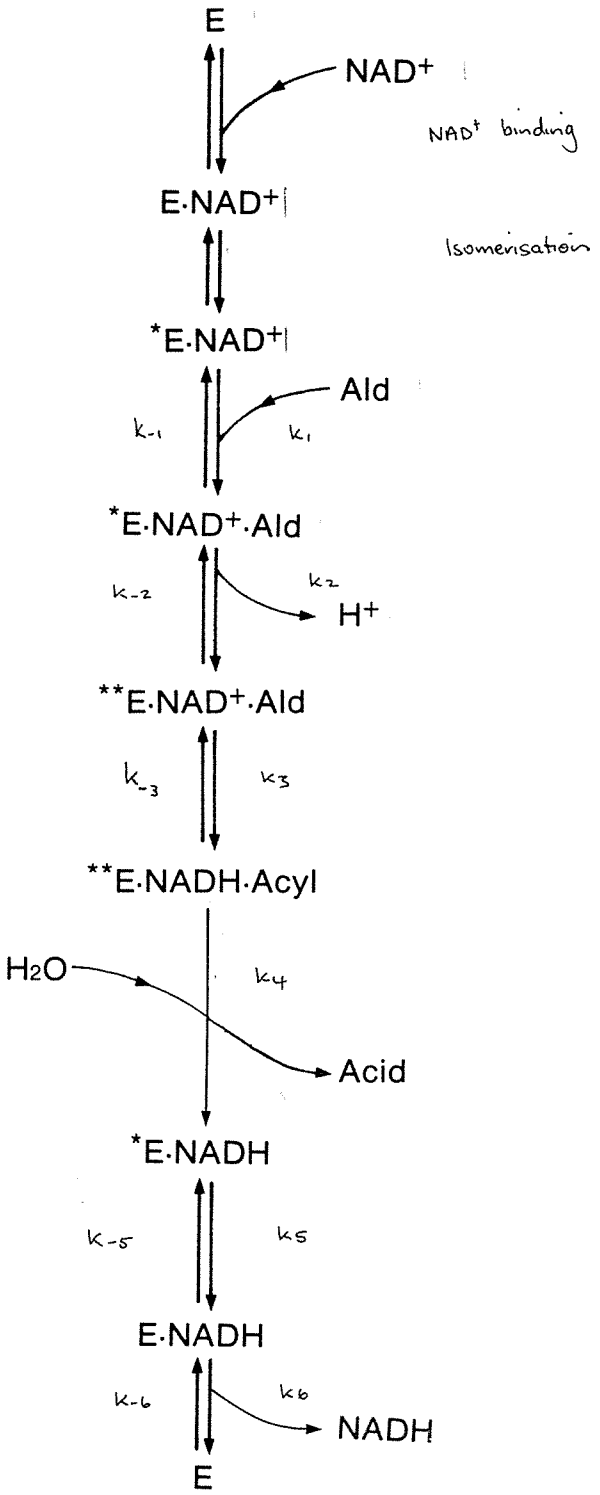
pK values.

The bell-shaped pH profile observed for the activity of aldehyde dehydrogenase is similar to that obtained for many other enzymes (Tipton and Dixon, 1983). However such profiles can arise under a variety of circumstances, from the simplest case in which only a single ionization state of an enzyme has catalytic activity to those in which different ionization states are important for different steps in the mechanism. Although ionization of many of the available groups of an enzyme must take place if the pH is altered over a wide pH range, only those ionizations which directly affect the slowest steps in the reaction mechanism will produce an effect on the enzyme activity. This is particularly true if one or more of the ionization states has negligible catalytic activity. Although two apparent pK values may often be obtained from a bell-shaped pH profile, these values may not necessarily be representative of individual ionizable groups on the enzyme. In some cases, including those in which there is a change in the rate determining step over the pH range, even if only a single ionizable group is involved two apparent pK values may be obtained (Tipton and Dixon, 1983).

As discussed previously (2.1.3), at low concentrations of propionaldehyde (<100 μM) and at pH 7.6, the oxidation reaction is thought to be controlled by means of two conformational changes (Scheme 4.2), one of which occurs after NAD^+ binding (MacGibbon et al., 1977c, Buckley et al., 1982, 3.5) and is slowly reversed during release of NADH from the enzyme. (MacGibbon et al., 1977b, Bennett et al., 1982, Buckley et al., 1982). This reversal (isomerisation of binary E.NADH complexes in Scheme 4.2) is considered to be the rate limiting step in the reaction (2.1.3.5,2.6). The second rapid conformational change, which occurs after aldehyde binding, controls the rate of the presteady state phase of the reaction (Bennett et al., 1982) and is presumably reversed upon deacylation of the acyl-enzyme intermediate as suggested by Bennett et al. (1982). The chemical steps in the reaction are therefore not rate limiting and no information relating to these steps has been obtained from kinetic studies.

A burst in NADH fluorescence is observed as an NADH containing enzyme intermediate is formed, indicating that hydride transfer occurs before any steps controlling the steady state rate. Since a burst was observed at all pH values over the range 5-10, the steps in the mechanism which control the steady state rate must follow hydride

Scheme 4.2


E
NAD+
NAD+ binding
E·NAD+
Isomerisation
*E·NAD+
Ald
k-1
k1
*E·NAD+·Ald
k-2
k2
H+
**E·NAD+·Ald
k-3
k3
**E·NADH·Acyl
H2O
k4
Acid
*E·NADH
k-5
k5
E·NADH
k-6
k6
NADH
E

transfer, irrespective of the pH.

The slow decay constant, λ_S, obtained from NADH displacement experiments, is a function of the rate constant for the isomerisation process (Scheme 4.2) which, as discussed above, controls the steady state rate at pH 7.6 and at low propionaldehyde concentrations. When the k_{cat} values obtained at a propionaldehyde concentration of 100 μM are compared with the values of λ_S at each pH (Figure 4.10) it is clear that these two parameters show a similar pH dependence at pH values of 7.0 and greater, but not at low pH.

The King-Altmann expression derived for the mechanism proposed in Scheme 4.2 leads to the relationship between k_{cat} and the rate constants of Scheme 4.2 as given below

$$k_{cat} = k_5 k_6/(k_5 + k_6 + k_{-5}) \qquad 4.3$$

Values for k_5, k_{-5} and k_6 have been determined previously by MacGibbon et al. (1977b) at pH 7.6 and substitution of these values in Equation 4.3 gives a k_{cat} value of 0.15 s^{-1} per active site which is in good agreement with the recently revised experimental k_{cat} value of 0.16 s^{-1} for low concentrations of propionaldehyde (L.F. Blackwell and P.D. Buckley personal communication). Thus, the rate of the isomerisation step (governed by k_5) is sufficient to completely account for the observed k_{cat} value at low concentrations of propionaldehyde within experimental error, and the second slow step proposed earlier by Bennett et al., 1982 has been shown to be unnecessary. Unless k_4 is 5 s^{-1} or greater at pH 7.6 a lag would follow the burst, a prediction that is inconsistent with the data (3.1.5.2).

It is clear that k_{cat} must always be less than λ_S (and less than k_5) because of the reversibility of the isomerisation (Scheme 4.2). However, it is not a simple matter to determine the magnitudes of k_5, k_{-5} and k_6 (MacGibbon et al., 1977b) over the entire pH range so that the contribution of the rate of isomerisation to k_{cat} may be determined. However, an estimate of the expected k_{cat} value can be made from the displacement data by using the expression

$$k_{cat} = \lambda_F \lambda_S/(\lambda_F + \lambda_S) \qquad 4.4$$

Figure 4:10 THE RELATIONSHIP BETWEEN λ_s AND k_{cat} AT LOW PROPIONALDEHYDE CONCENTRATIONS

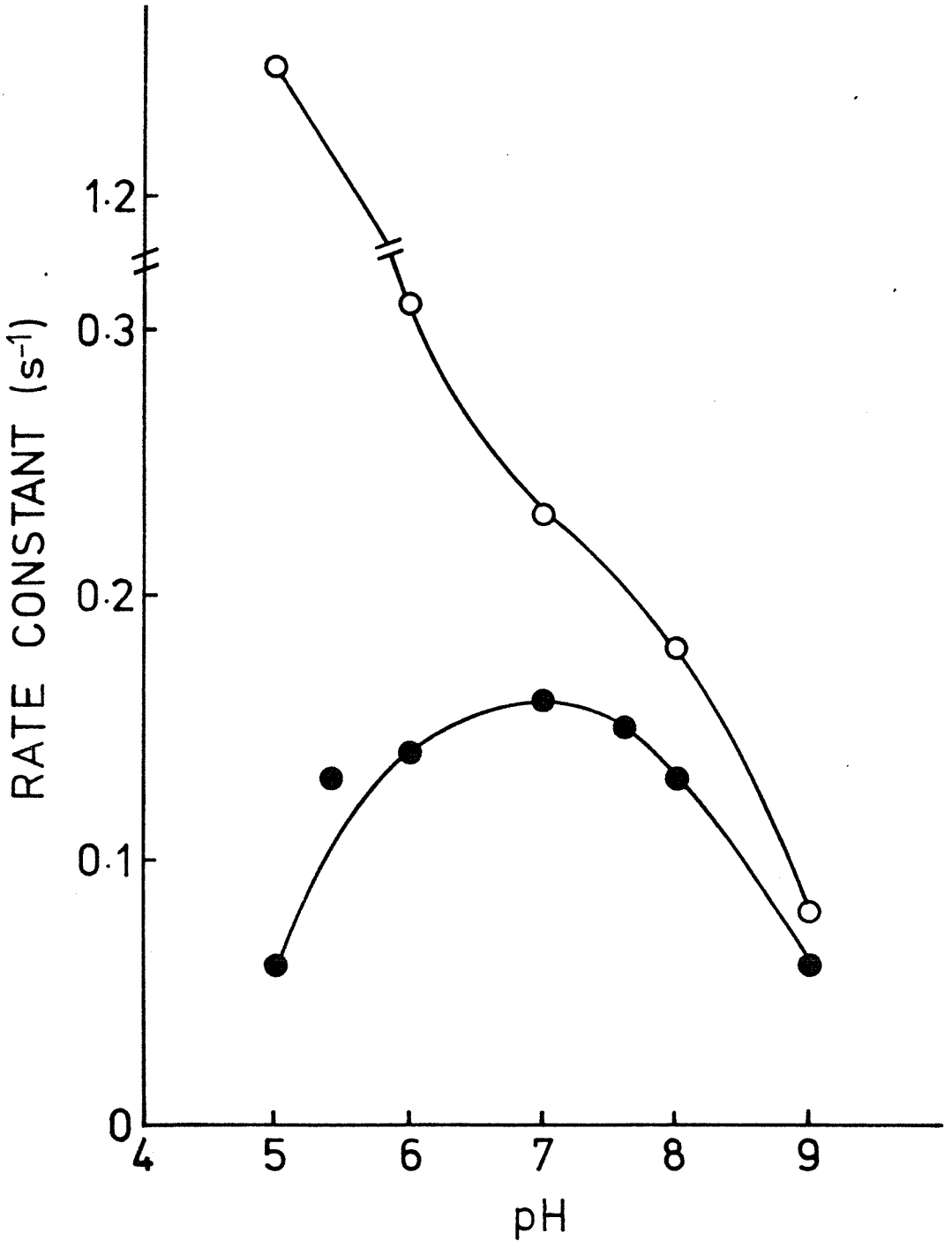


Figure 4.10 pH profile for k_{cat} and λ_s at low (100 µM) concentrations of propionaldehyde. The profile for k_{cat} (●) was determined for assays containing aldehyde dehydrogenase (2.1 µM) and NAD^+ (1 mM) and propionaldehyde; all components were dissolved in the appropriate constant ionic strength buffer (for details see Methods). The pH profile for λ_s was determined also in constant ionic strength buffers. One syringe contained aldehyde dehydrogenase (9.0 µM)and NADH (40 µM) in 3.3 mM pH 7.3 phosphaste buffer and the other contained NAD^+ dissolved in the appropriate constant ionic strength buffer (0.2 M).

Since $\lambda_F + \lambda_S = k_5 + k_{-5} + k_6$ when the concentration of NADH is small and $\lambda_F > k_6$ and $\lambda_S < k_5$, Equation 4.4 approximates to Equation 4.3. Estimates of k_{cat} calculated using equation 4.4 are in good agreement with the experimentally determined values over the pH range 7-9, confirming that in this region, isomerisation of the binary enzyme.NADH complexes constitutes the major rate limiting step. However, if the isomerisation step were rate limiting over the whole pH range (5-9) then the steady state pH profile should follow the pH profile of λ_S. This is not observed experimentally (Figure 4.10) thus some other step after hydride transfer in Scheme 4.2 must become rate limiting at pH values below 7, since the isomerisation clearly occurs much more rapidly than the steady state turnover rate.

At these low pH values it is therefore necessary that the rate of acyl-enzyme hydrolysis, an accompanying isomerisation, or dissociation of the acid product (all included in the k_4 step) now becomes rate limiting. Buckley and Dunn (1985) have shown recently that the overall rate of acyl-enzyme hydrolysis (k_4) is the limiting step in the oxidation of the substrate p-dimethylaminocinnamaldehyde (DACA) at all pH values between 4 and 11. For this substrate the rate of acyl-enzyme hydrolysis is so slow that it is less than the rate of isomerisation of the binary enzyme.NADH complexes over the entire pH range. It has also been demonstrated that the k_4 step becomes extremely slow at acid pH values, so much so that the acyl-enzyme intermediate becomes a quasi-stable species.

The apparent pK value for the k_4 step using DACA as the substrate is 8.95 (Buckley & Dunn, 1985) which is much higher than the value of 5.3 estimated from the ascending arm of the pH profile in the present work. It is clear however that the true pK for the increase in the steady state rate between pH 5 and 7 must be greater than 5.3 if the requirement demonstrated by computer simulations (L.F. Blackwell P.D. Buckley personal communication, refer section 2.1.3.5) that k_4 be greater than 5 s^{-1} above pH 7 is to be met. It seems unlikely that the pK value for acyl-enzyme hydrolysis using propionaldehyde as substrate will be different from the value determined with DACA. Hence the apparent pK value determined with propionaldehyde probably results from the fact that the maximum rate for the k_4 step (which must be much grea ter than 5-10 s^{-1} for the deprotonated form of the enzyme at pH values greater than the pK) is much greater than the maximum value of the rate constant for isomerisation (1.24 s^{-1}). Thus, very little of the pH

profile for acyl-enzyme hydrolysis (and the accompanying changes) will be observed before isomerisation becomes rate limiting.

The amplitude of the NADH burst in fluorescence decreases dramatically between pH 5 and 6 and thus the possibility that the enzyme is converted (rapidly) into an inactive form at acidic pH values giving rise to the observed decrease in activity must be considered. However, the burst in the production of the acyl-enzyme intermediate for DACA (which is followed in absorbance) shows no decrease in amplitude between pH 5 and 7. Since the appearance of the acyl-enzyme intermediate occurs simultaneously with, or immediately following the hydride transfer step (Buckley & Dunn 1982) this indicates that there can be no such loss of activity and hence the decrease in nucleotide fluorescence which is observed must be due to some other factor. At acid pH values the rate of acyl-enzyme hydrolysis is much slower than the rate of isomerisation of the binary enzyme.NADH complexes and the accompanying dissociation. Thus, it is possible that the release of NADH now occurs rapidly immediately after the burst so that acyl enzyme hydrolysis and acid release are the last steps in the reaction sequence. This would account for the low amplitude observed for the burst experiments and for the fact that the amplitude of the NADH displacement experiments was only slightly lowered at pH 5. If this is the case then it raises the possibility that the release of products from the enzyme during catalysis is not necessarily ordered but depends on the relative rates of the isomerisation step and the rate of acyl-enzyme hydrolysis. For DACA, at all pH values, the rate of acyl-enzyme hydrolysis is slower than the rate of isomerisation of the NADH complexes and thus it might be expected that NADH release preceeds the release of the acid product. Propionaldehyde at alkaline pH values and DACA thus represent two extremes and it is also possible there are intermediate situations in which both these processes proceed at comparable rates.

Therefore, the best explanation of the effect of pH on the mechanism at low concentrations of propionaldehyde is that it results from a change in the rate determining step which gives rise to the bell shaped pH profile. Increasing the pH increases the rate of acyl-enzyme hydrolysis and at the same time decreases the rate of the k_5 step so that at a pH of about 7 isomerisation becomes the slowest (and therefore the last) step on the pathway.

High concentrations of propionaldehyde (20 mM) activate the steady

state rate by a factor of 3 to 3.5 at pH 7.6 (MacGibbon et al., 1977a) and similar activation was observed over the entire pH range studied in the present work (Figures 4.1, 4.2, 4.3). When the magnitudes of λ_F and λ_S are compared with the corresponding k_{cat} values over the pH range 7-9 (Figures 4.10 and 4.11) it is clear that the mechanism shown in scheme 4.2 cannot alone account for the observations. If all of the reaction flux passed through the isomerisation step of Scheme 4.2 k_{cat} could not exceed the value defined by equation 4.4 (which is less than λ_S) at each pH value. However, if high concentrations of propionaldehyde modified the enzyme so that the k_s step was completely circumvented, k_{cat} should become equal to, or greater than, λ_F (since λ_F is determined under conditions in which NADH dissociation is irreversible) which is clearly also contrary to what is observed (Figure 4.11). There are therefore two possibilities to account for the fact that the experimental value for k_{cat} lies between λ_S and λ_F at each pH between 7 and 9 at 20 mM concentrations of propionaldehyde. Either some of the enzyme oxidises the aldehyde by a pathway which does not include the isomerisation step but the remainder still oxidises the aldehyde according to Scheme 4.2 or, alternatively none of the enzyme is limited by the isomerisation step but some other step after hydride transfer now becomes partly rate limiting as well.

There is some evidence to support the former of these proposals since as described previously (3.5) NADH displacement experiments carried out by P.D. Buckley (personal communication) in the presence of 20 and 40 mM propionaldehyde and using 1,10-phenanthroline as the displacing agent, showed that although displacement of NADH was still biphasic under these conditions, and λ_S was unchanged with respect to the control value, λ_F was approximately three times greater and the amplitude of the slow phase was reduced to only 10% of the total amplitude. This appears to indicate that, although a small proportion (probably less than 10%) of the enzyme continues to undergo the slow isomerisation from *E.NADH to form E.NADH, the majority of the enzyme somehow bypasses this process in the presence of high propionaldehyde concentrations.

Below pH 7 k_{cat} is much less than λ_F and at pH 5 it is also significantly less than λ_S, indicating that the decrease in k_{cat} which is observed (Figure 4.11) is not simply due to the isomerisation step becoming rate limiting. One of the steps associated with acyl-enzyme hydrolysis is clearly rate limiting at pH values below pH 7 at both

Figure 4:11 THE RELATIONSHIP BETWEEN λ_f AND k_{cat} AT HIGH PROPIONALDEHYDE CONCENTRATIONS

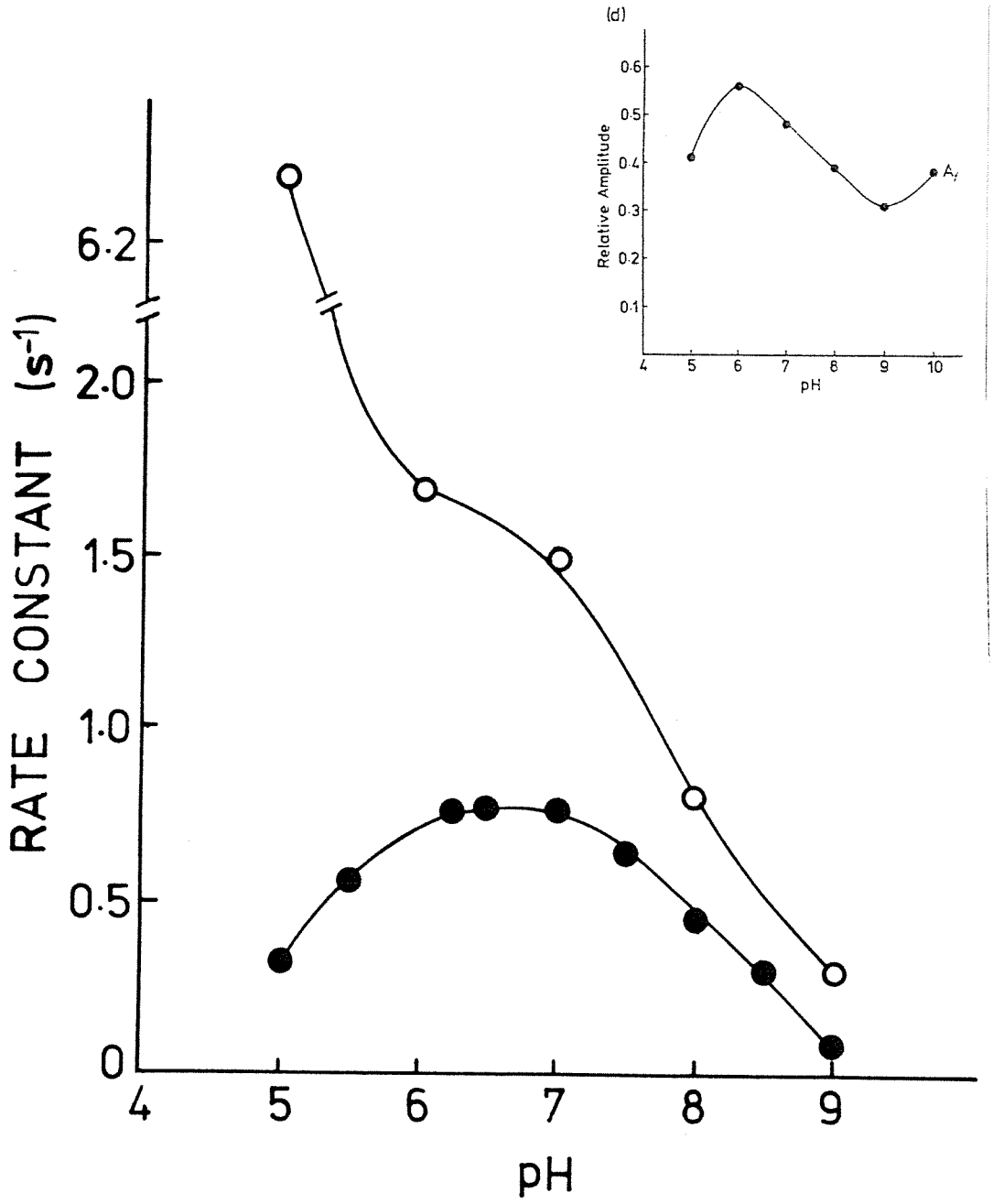


Figure 4.11 pH profile for k_{cat} and λ_F at high (20 mM) concentrations of propionaldehyde. The profile for k_{cat} (●) was determined for assays containing aldehyde dehydrogenase (2.1 μM) and NAD^+ (1 mM) and propionaldehyde; all components were dissolved in the appropriate constant ionic strength buffer (for details see Methods). The pH profile for λ_F was determined also in constant ionic strength buffers. One syringe contained aldehyde dehydrogenase (9.0 μM) and NADH (40 μM) in 3.3 mM pH 7.3 phosphate buffer and the other contained NAD^+ dissolved in the appropriate constant ionic strength buffer (0.2 M). The insert shows the relative amplitude of the fast phase as a function of pH.

high and low concentrations of propionaldehyde (compare Figures 4.10 and 4.11).

The fact that high concentrations of propionaldehyde affect the steady state rate at both acidic and basic pH values has implications for the two-site model as proposed by Blackwell et al., (1982). The mechanism by which propionaldehyde causes activation has been discussed essentially in terms of a second low affinity binding site (MacGibbon et al., 1977a, Blackwell et al., 1982, Dickinson, 1985, Duncan, 1985) but there has been a divergence of opinion as to the location of this second P2 binding domain. There is now clear evidence that high concentrations of propionaldehyde can bind to one, or both, of the enzyme.NADH complexes of Scheme 4.2 (Dickinson, 1985, Blackwell personal communication). Therefore, one possibility for the location of the P2 binding domain is at the catalytic functional group in the enzyme.NADH complexes. However, it is obvious that propionaldehyde cannot be binding at the catalytic group in the active site below pH 7 since acyl-enzyme hydrolysis, an accompanying conformational change, or acid dissociation is rate limiting. In all of these steps the active site group is either acylated or protected by the acid product yet activation still occurs at high propionaldehyde concentrations. A second binding domain for propionaldehyde is therefore required by the data at these pH values.

At pH 5 the rate constant for the burst in NADH fluorescence is greater (10 s^{-1}) at 20 mM concentrations of propionaldehyde than at 200 µM concentrations (ca 2 s^{-1}). Since the burst amplitudes were similar at all pH values the increase in k_b as the concentration of propionaldehyde is increased is not simply due to a decreased affinity of the enzyme for the propionaldehyde at acid pH values. Thus the observed increase in k_b probably reflects an increase in the rate of the conformational change which controls the burst (Bennett et al., 1982). High concentrations of propionaldehyde are therefore also causing changes in the protein prior to the hydride transfer step in the burst experiments and this means that an aldehyde binding site other than the active site must be present on the enzyme.

High propionaldehyde concentrations not only affect the isomerisation of binary enzyme.NADH complexes, but also produce changes in the enzyme prior to hydride transfer and give rise to higher k_{cat} values over the entire pH range. It is possible therefore that the binding of a second propionaldehyde molecule in a non-catalytic

(effector) site, may facilitate both the conformational changes (and their subsequent reversal) in the mechanism. Thus high propionaldehyde may increase the rate of all four isomerisation steps in the reaction sequence, although the effect on the conformational change of $E.NAD^+$ complexes would not be observed under the experimental conditions (NAD^+ premixed with enzyme) used in the experiments.

There were considerable differences between the pH profiles which were obtained using pyrophosphate buffers and those for other buffer systems. At low propionaldehyde concentrations, below pH 7, reaction rates in pyrophosphate were basically similar to those obtained in other buffer systems, suggesting that the catalytic activity of the low pH forms of the enzyme are relatively unaffected by pyrophosphate. However, there was an apparent activation of the steady state rate at higher pH values.

A comparison of λ_S and k_{cat} Figure 4.12) shows that the activation can be accounted for in terms of an increase in the rate of isomerisation of the binary enzyme.NADH complexes in pyrophosphate buffers. The isomerisation step (k_s in Scheme 4.2), although increasing as a function of pH, nevertheless remains the major rate limiting step at low concentrations of propionaldehyde above pH 7.

It is clear that pyrophosphate ions are causing fundamental changes in the enzyme at alkaline pH values either by binding to the protein (perhaps in the anion binding site) or chemically modifying it at some site. In either case the effect of the pyrophosphate is to allow the conformational change which is part of the NADH release process to occur more easily than is the case when other buffer anions are present.

The data obtained at high concentrations of propionaldehyde in pyrophosphate buffers are similar in the pH range 5-7 where λ_S is much greater than k_{cat} (Figure 4.11) but above pH 7 it is obvious that even λ_F is too large to be rate limiting (inset Figure 4.12). It might be expected therefore that the pH profile above pH 7 would follow the profile reported by Buckley and Dunn (1985) for DACA. However, there is very little pH dependence in pyrophosphate buffers above pH 7 and there may even be a small decrease in the steady state rate above pH 9. To explain this behaviour it is necessary to propose that a step between hydride transfer and NADH release is slowed down in the presence of high concentrations of propionaldehyde to such an extent that it takes over as the rate limiting step. This step must follow

Figure 4:12 COMPARISION OF THE VALUES OF k_{cat} AND THE DECAY CONSTANTS λ_s AND λ_f IN PYROPHOSPHATE BUFFERS

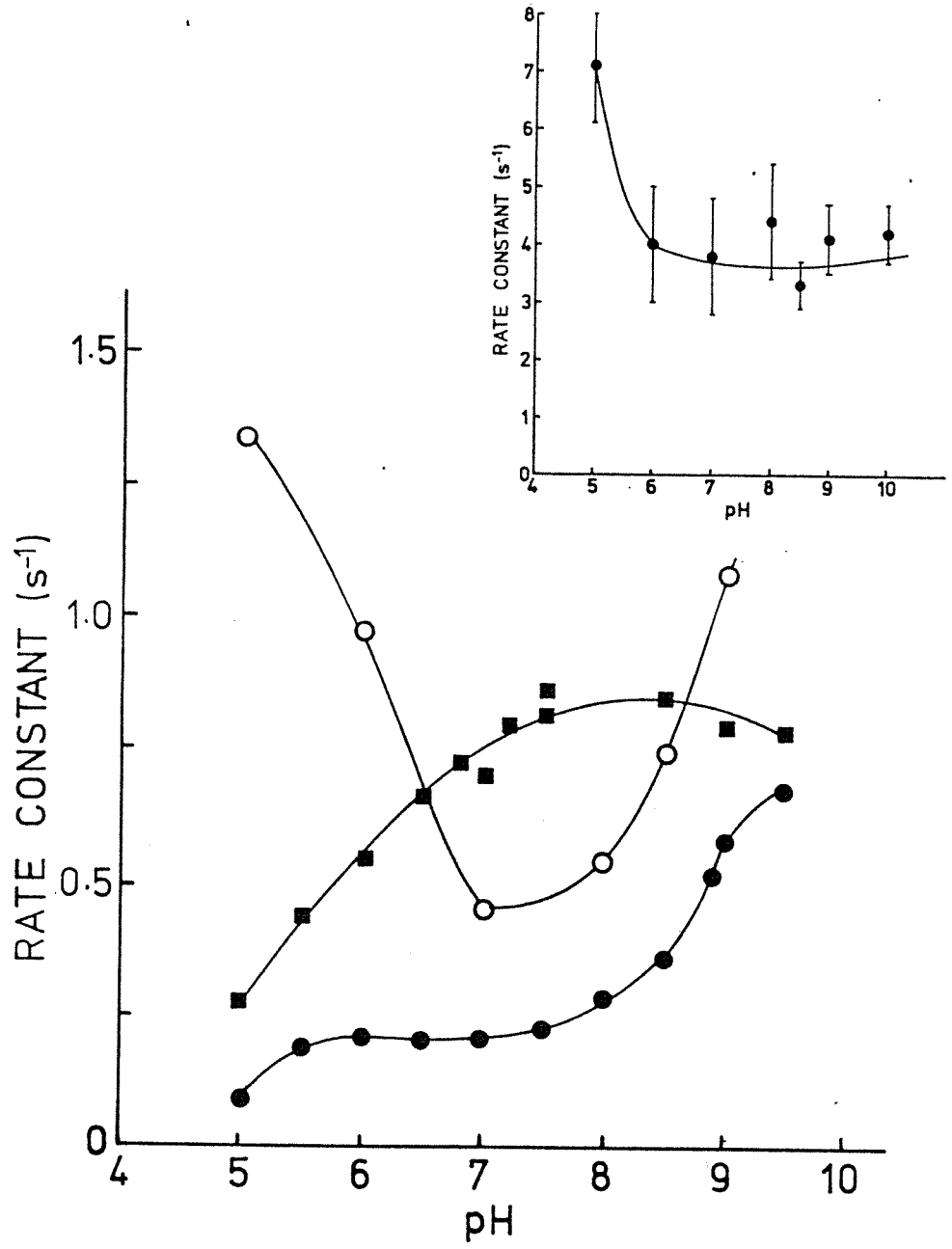


Figure 4.12 pH Profile in pyrophosphate buffers. The k_{cat} values were determined at both low (●; 100 μM) and high (■; 20 mM) concentrations of propionaldehyde. Assays contained aldehyde dehydrogenase (2.3 μM) and NAD^+ (1 mM) in a range of sodium pyrophosphate buffers. The pH profile for λ (○) and λ_F (●;inset) were determined as described in Methods. Syringe A contained aldehyde dehydrogenase (9.0 μM) and NADH (40 μM); syringe B contained NAD^+ (2.5 mM) in the appropriate pyrophosphate buffer.

soon after the hydride transfer step otherwise a lag would be seen in the burst experiments which is contrary to experiment. If a similar situation exists in the other buffer systems then the data at pH values above 7 could be accounted for if both NADH dissociation (as indicated by the λ_F values) and this second slow step control the steady state rate. However, in view of the markedly different behaviour observed in pyrophosphate buffers at low concentrations of propionaldehyde it may be that the two sets of data cannot be compared.

It is therefore not possible to directly compare results obtained in the presence of pyrophosphate with those obtained using other buffer systems as different enzyme forms may be involved. pH profiles which have been reported previously in which pyrophosphate buffers have been used cannot therefore necessarily be considered to be a correct representation of the pH dependence of the enzymic activity.

The shape of the pH profile of Takahashi et al. (1981) probably arises as a consequence of combining data obtained at low propionaldehyde concentrations (128 μM) from acetate, phosphate and pyrophosphate buffers. Similarly double peaks observed in the burst data of these authors may need to be reinterpreted with respect to the shift in the pH dependence of the burst in pyrophosphate buffers.

The pH profiles of Blair & Bodley (1969) for human liver aldehyde dehydrogenase in which maximum activity was observed at pH 9-10 at a high concentration of acetaldehyde (5 mM) in pyrophosphate buffers was similar to that obtained in this work for the enzyme activity at high concentrations of propionaldehyde in pyrophosphate. The anomalies reported by MacGibbon et al., (1976) between K_m values obtained in barbitone buffers and pyrophosphate buffer at 9.3 can also be explained in terms of modification of the enzyme by pyrophosphate.

The results obtained for the effect of pH on activation by PCMB, as shown in Figure 4.5, are difficult to interpret, particularly as the ionic strength was not constant over the pH range, but since the two activity peaks observed in both the control and modified pH profiles appear to coincide in each case, it appears unlikely that there is a shift in the pK values governing the steady state reaction in either buffer. As only inhibition was observed when acetate buffer was used and activation observed mainly when phosphate buffer was present this may indicate a requirement for phosphate ions in the activation process. Such a requirement would not have been apparent from the results in chapter 3 in which all activation studies were carried out

using phosphate buffer. However it is more likely that only the form of the enzyme which exists above pH 7.0 can be activated. Apparent pK values of 6.25 and 8.8 could be obtained from relative rate data (inset Figure 4.5b) values which are reasonably similar to the apparent pK values determined for native enzyme, but at higher ionic strength.

Meaningful interpretation of the inhibition data observed at low pH in the presence of PCMB is impossible considering the complicating factor of low ionic strength. Future experiments carried out at a constant ionic strength may provide more information about both the inhibition and the activation processes.

All of the steady state and presteady state kinetic effects described this work appear to be controlled by an apparent pK value of about 8.5. If the same ionizable group is involved in all of the processes reported in the present work a chemical role for it seems unlikely. The dependence of the rate of acyl-enzyme hydrolysis on pH could be explained in terms of a requirement for a deprotonated group which is perhaps required to function as a general base. This suggestion is supported by the fact that the DACA acyl-enzyme intermediate is stable at pH values up to pH 6.5 after denaturation of the enzyme (Buckley, personal communication) which shows that catalysis of acyl-enzyme hydrolysis is necessary. The identity of the functional group which is acylated has not been established from these pH studies but catalysis of acyl-enzyme hydrolysis by nitrogen bases would be expected on the basis of studies with model compounds (Bruice & Benkovic, 1966). However a similar role seems unlikely for the decrease in the rates of enzyme.NADH isomerisation and NADH dissociation together with the increase in the burst rate constants which also depend on the presence of a deprotonated group. It is more likely that the conformational changes which control the activity (MacGibbon et al., 1977b, Bennett et al., 1982) are dependent on the ionization state of the enzyme. Bennett et al. (1982) have demonstrated that an ionizable group with a pK_a value of about 8.5 is perturbed during the conformational change which controls the rate of the presteady state phase of the reaction.

The identity of the ionizable group responsible for the pH behaviour cannot be unambiguously determined from the kinetic data but the apparent pK_a value is consistent with a histidine, cysteine or ε-amino group.

The identification of possible active site groups was not the

primary aim of these pH studies, and for the reasons stated above, any such identification is likely to be difficult for this enzyme. However, the reasons for previous anomalies are now apparent, and the conditions of pH and ionic strength for maximum enzyme activity have been identified.

CHAPTER 5
A PRELIMINARY STUDY OF REVERSE REACTIONS OF ALDEHYDE DEHYDROGENASE.

5.1 INTRODUCTION.

The oxidation of aldehydes by aldehyde dehydrogenase is considered to be irreversible. There is no change in NADH absorbance or fluorescence when a carboxylic acid is added to a mixture of NADH and the enzyme (MacGibbon et al., 1977a), presumably because the **E.NADH.acyl ternary complex (Bennett et al., 1982, Figure 2.2) cannot form, due to the poor acylating ability of carboxylic acids. However, using the anhydrides of carboxylic acids, Hart and Dickinson (1978a) have observed a decrease in the absorbance of NADH when aldehyde dehydrogenase, NADH and anhydride were mixed together. The decrease was not simply due to the destruction of NADH, since when acetaldehyde was added to the mixture the absorbance at 340 nm increased to its original level. A high buffer strength was maintained in these experiments, to prevent significant lowering of the pH by the acid products of spontaneous anhydride hydrolysis. On the basis of these observations they concluded that there was a partial reversal of the reaction with the formation of NAD^+, the rate of the reverse reaction for mitochondrial aldehyde dehydrogenase from sheep liver at pH 7.0 in phosphate buffer being the same for acetic and butyric anhydrides.

It was decided to further investigate this reverse reaction to establish whether in fact any aldehyde had been formed and also to attempt to carry out systematic kinetic studies in order to determine to what extent the reaction pathway is the microscopic reverse of the normal pathway for oxidising aldehydes.

However it was realised from the outset that the spontaneous hydrolysis of anhydrides in water would create problems in the attempt to obtain precise and quantitative kinetic data for the reverse reaction. For example acetic anhydride has a first order rate constant for hydrolysis of $2.623 \times 10^{-3}\ s^{-1}$, so that the half-life in water is only 4.4 minutes (Rivett & Sidgwick, 1910a, Gold, 1948), while for propionic anhydride the half-life is 42.9 minutes and for butyric anhydride the half-life is 14.8 minutes (Wilsdon & Sidgwick, 1913). The effect of this hydrolysis on the reverse reaction also needed further investigation.

5.2 METHODS

5.2.1 Reverse Reactions.

Studies of the reversal of aldehyde dehydrogenase catalysed oxidation were initially carried out by mixing enzyme (0.2-8.0 µM), NADH (20-200 µM), and acetic anhydride (17 mM) in 0.2 M potassium phosphate buffer, pH 7.0. in a 3 cm^3 cuvette. The change in absorbance at 340 nm was monitored using a Unicam SP 500 Spectrophotometer. Acetic anhydride (5 µl) was added directly to the cuvette to initiate the reaction. However, this method was not suitable for the less soluble propionic anhydride or for lower levels of either anhydride. Thus, in subsequent experiments, solutions of anhydride dissolved in acetonitrile were used. At no time did the volume of acetonitrile in reaction solutions exceed 3% of the total assay volume since MacGibbon et al.. (1978b) have demonstrated that such an amount of acetonitrile has no modifying effect on the enzyme activity. The useful range of NADH concentrations was limited to between 20 and 160 µM (absorbances at 340 nm of 0.12 to 1.0) for absorbance measurements due to the relatively poor sensitivity of the SP 500 spectrophotometer which, at the time these preliminary studies were being carried out, was the only instrument available. For experiments involving lower concentrations of enzyme, NADH or anhydride, fluorescence measurements were made by following the radiation emitted at 455 nm after excitation of the NADH at 340 nm, using an Aminco SPF 500 Ratio Spectrofluorometer. Much greater sensitivity was achieved in this way and tnus enzyme concentrations could be reduced to 0.05-0.20 µM, NADH concentrations to 0.5-10 µM,and propionic anhydride concentrations to 50-500 µM.

All reactions were carried out at 25°C, and in 0.2 M pH 7.0 phosphate buffer unless otherwise stated.

5.2.2 Determination of Acetaldehyde Concentration

The following procedure was used to analyse samples taken from the NADH, acetic anhydride and enzyme containing reaction mixture for the presence of acetaldehyde. The samples were first treated with sufficient 60% perchloric acid to give a final concentration of 3% v/v

in order to precipitate the enzyme and halt the reaction. Following centrifugation, the sample was analysed by the Alcohol Research group in this Department using the automated acetaldehyde assay of Stowell (1978).

To monitor the acetaldehyde levels throughout a reaction, a solution was prepared at 25°C and 2.0 cm^3 samples were withdrawn from this solution at various time intervals for treatment and analysis as above. Simultaneous continuous monitoring of the absorbance at 340 nm was carried out in parallel on a sample removed from the same solution immediately after the solution was prepared.

Acetaldehyde determinations were also conducted in the same way on solutions containing p-nitrophenylacetate (PNPA), in the presence of enzyme and NADH. In these cases the enzyme was first dialyzed extensively to remove 2-mercaptoethanol as described previously (3.2). The production of the p-nitrophenoxide ion by the enzyme-catalysed hydrolysis of p-nitrophenyl acetate was monitored continuously at 400 nm for a duplicate sample and using an extinction coefficient of 18.3×10^3 $l.mol^{-1}.cm^3$ as reported by Kezdy & Bender (1962). Two sets of control samples were prepared for all acetaldehyde determinations, one set lacking enzyme and the other lacking NADH.

5.2.3 The Hydrolysis of Propionic Anhydride

The following pH Stat method was used to determine whether any of the assay components were catalysing the hydrolysis of either acetic or propionic anhydride. The method involved the automatic addition of 0.1 M NaOH to a solution of the anhydride in 0.5 M KCl to maintain a constant pH of 7.0. The rate at which hydroxide was added indicated the rate of production of carboxylic acid by hydrolysis. Experiments were conducted using a Radiometer Titrator TTT and Autoburette ABU 11 (Volume 2.5 cm^3), and the rate of addition of NaOH was recorded on a Radiometer Servograph REC 51 (adjusted for volume measurements). The effect of enzyme, NAD^+ and NADH on the rate of hydrolysis of propionic anhydride was studied by the addition of one or more of these substances to the 0.5 M KCl solutions and titration commenced immediately after addition of the anhydride. Control samples contained only propionic anhydride in 0.5 M KCl. Similar studies were carried out using acetic anhydride by L. Deady (personal communication).

5.3 RESULTS.

5.3.1 Reverse Reactions

When acetic anhydride (17 mM) was added to a solution containing enzyme (1-3 μM), and NADH (20-160 μM) at pH 7.0, a decrease in absorbance at 340 nm was observed. After 4.5 minutes, when the absorbance had reached a minimum, addition of excess aldehyde (2.0 mM acetaldehyde or 20 mM propionaldhyde) produced a rapid increase in absorbance back to the original level (Figure 5.1), as reported by Hart & Dickinson (1978a).

The initial rate of oxidation of NADH could be determined from these absorbance measurements and at saturating conditions of NADH and acetic anhydride was found to be dependent on the enzyme concentration (Figure 5.2a) in a linear manner. An apparent k_{cat} value of 0.087 s^{-1} per active site was derived from the slope of the plot.

No change in absorbance at 340 nm was observed in control solutions lacking enzyme, provided that the buffer concentration was sufficiently high (0.2-0.5 M), to prevent significant lowering of the pH of the solution by the acetic acid produced from spontaneous hydrolysis of the acetic anhydride. If the solution becomes acid NADH is destroyed and hence the absorbance will decrease. If reverse reactions were followed for longer than five minutes the absorbance was observed to level off at a low, but non zero level of NADH. Then, within 30 seconds, a spontaneous increase in absorbance occurred with the absorbance eventually returning to its original value. The higher the initial concentration of NADH, the greater the amount of NADH which remained after the absorbance decrease. A typical trace is shown in Figure 5.3a. Similar results were obtained with propionic anhydride (Figures 5.2b & 5.3b) but the initial reaction rate was considerably greater at saturating conditions of propionic anhydride and NADH than for acetic anhydride. Comparison of the slopes of the initial rate versus enzyme concentration plots in Figure 5.2 shows that the propionic anhydride rate was 3.6 times the acetic anhydride rate. Also, with propionic anhydride, the absorbance levelled off after only 2-3 minutes and the increase in absorbance which followed was much more rapid, reaching the initial level after only 14 minutes, compared with 150 minutes for acetic anhydride under the same conditions.

Figure 5:1 PROGRESS CURVE FOR THE REVERSE ALDEHYDE DEYDROGENASE REACTION

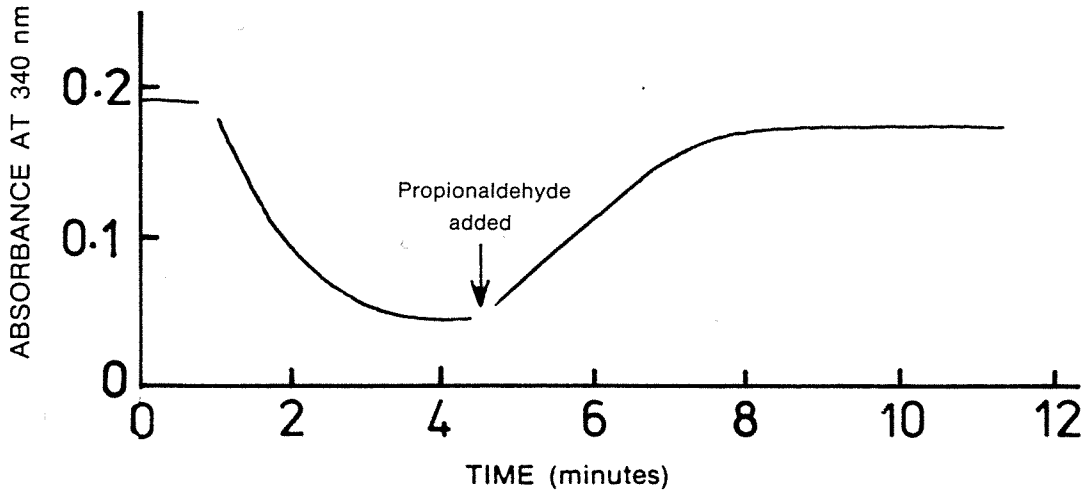


Figure 5.1. Progress Curve for the Reverse Aldehyde Dehydrogenase Reaction. Enzyme (3.0 µM) and NADH (27 µM) were mixed in 0.2 M pH 7.0 phosphate buffer and an absorbance reading of 0.19 at 340 nm was obtained. Acetic anhydride (5 µl of pure acetic anhydride, initial concentration in solution 17 mM) was added to initiate the reaction. Propionaldehyde (18 mM) was added after 3.5 minutes as indicated.

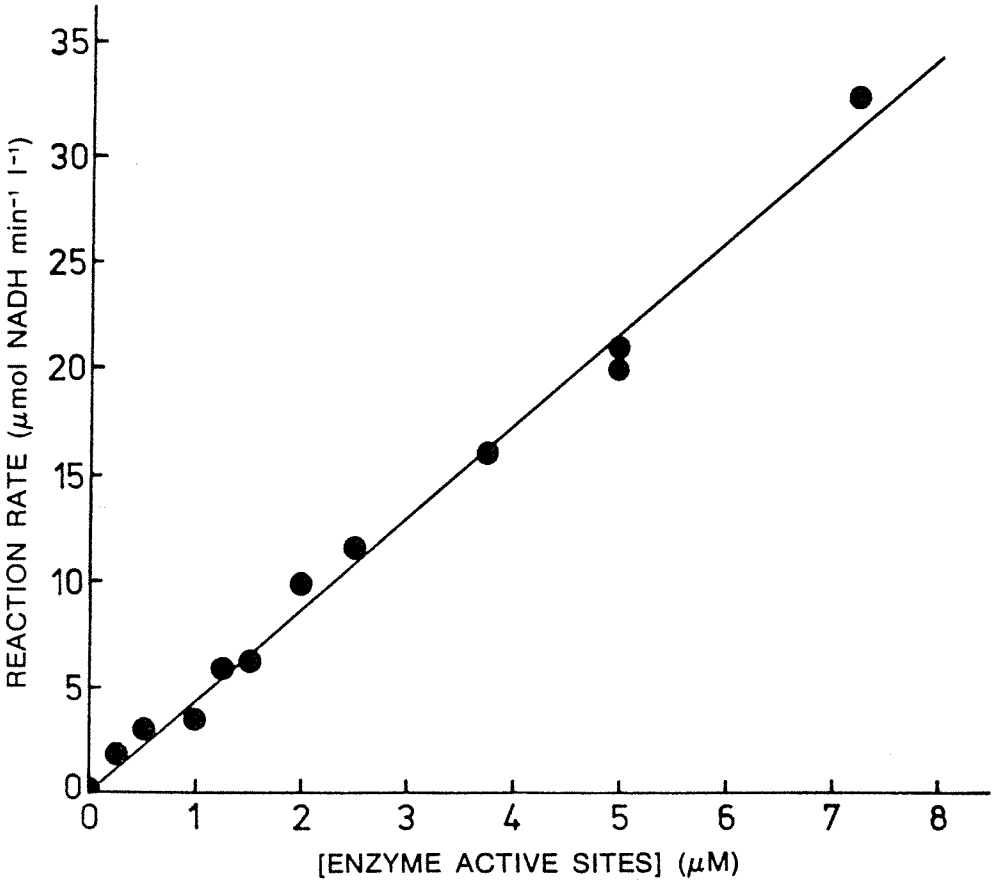
Figure 5.2 The Dependence of the Initial Rate of the Reverse Reaction on the Enzyme Concentration. The initial rate of the reverse reaction was determined in 0.2 M pH 7.0 phosphate buffer in each case.

5.2(a) The concentration of enzyme was varied at constant concentrations of NADH (60 μM) and acetic anhydride (17 mM). A value for V/[E] (kcat) of 0.087 s^{-1} was obtained from a least squares fit to these points.

5.2(b)The concentration of enzyme was varied at constant concentrations of NADH (● 60 μM or O 100 μM) and propionic anhydride (13 mM). A value for k_{cat} of 0.25 s^{-1} was obtained from a least squares fit to these points. However, it was noted (refer Chapter 6) that these data were better represented by a curve (...).

Figure 5:2 DEPENDENCE OF THE INITIAL RATE OF THE REVERSE REACTION ON ENZYME CONCENTRATION

(a) ACETIC ANHYDRIDE AS SUBSTRATE



(b) PROPIONIC ANHYDRIDE AS SUBSTRATE

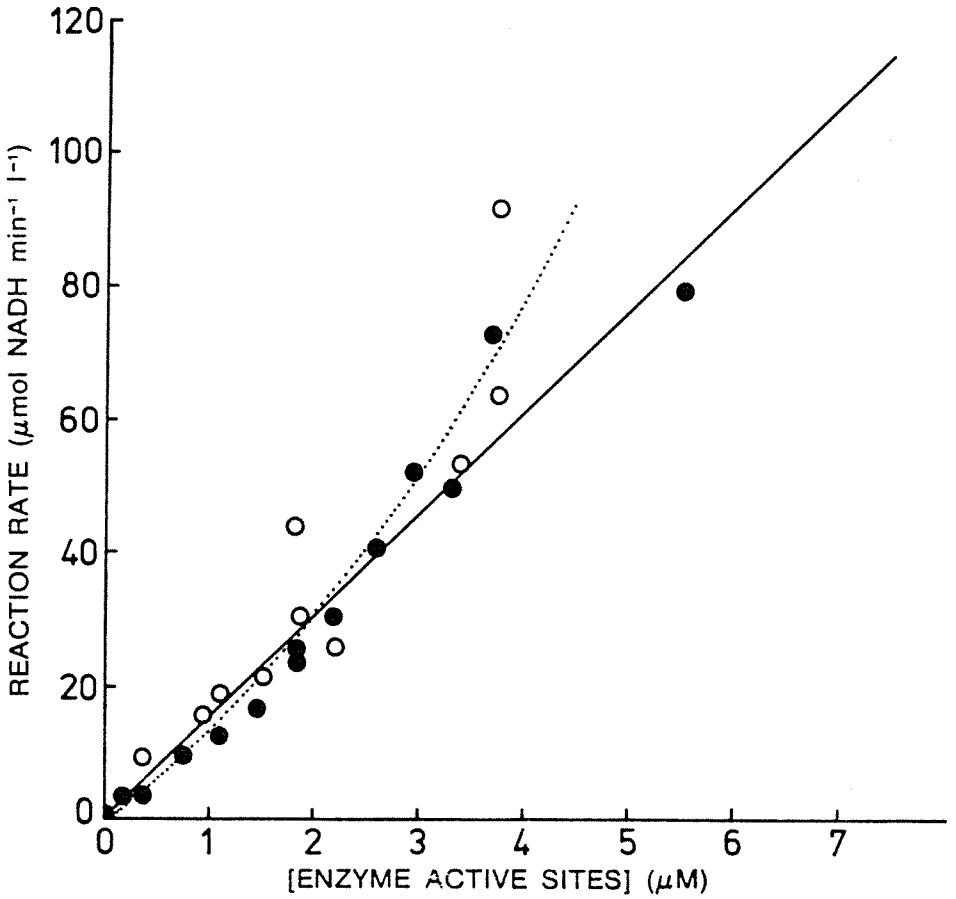


Figure 5:3 COMPLETE PROGRESS CURVES FOR THE REVERSE REACTION

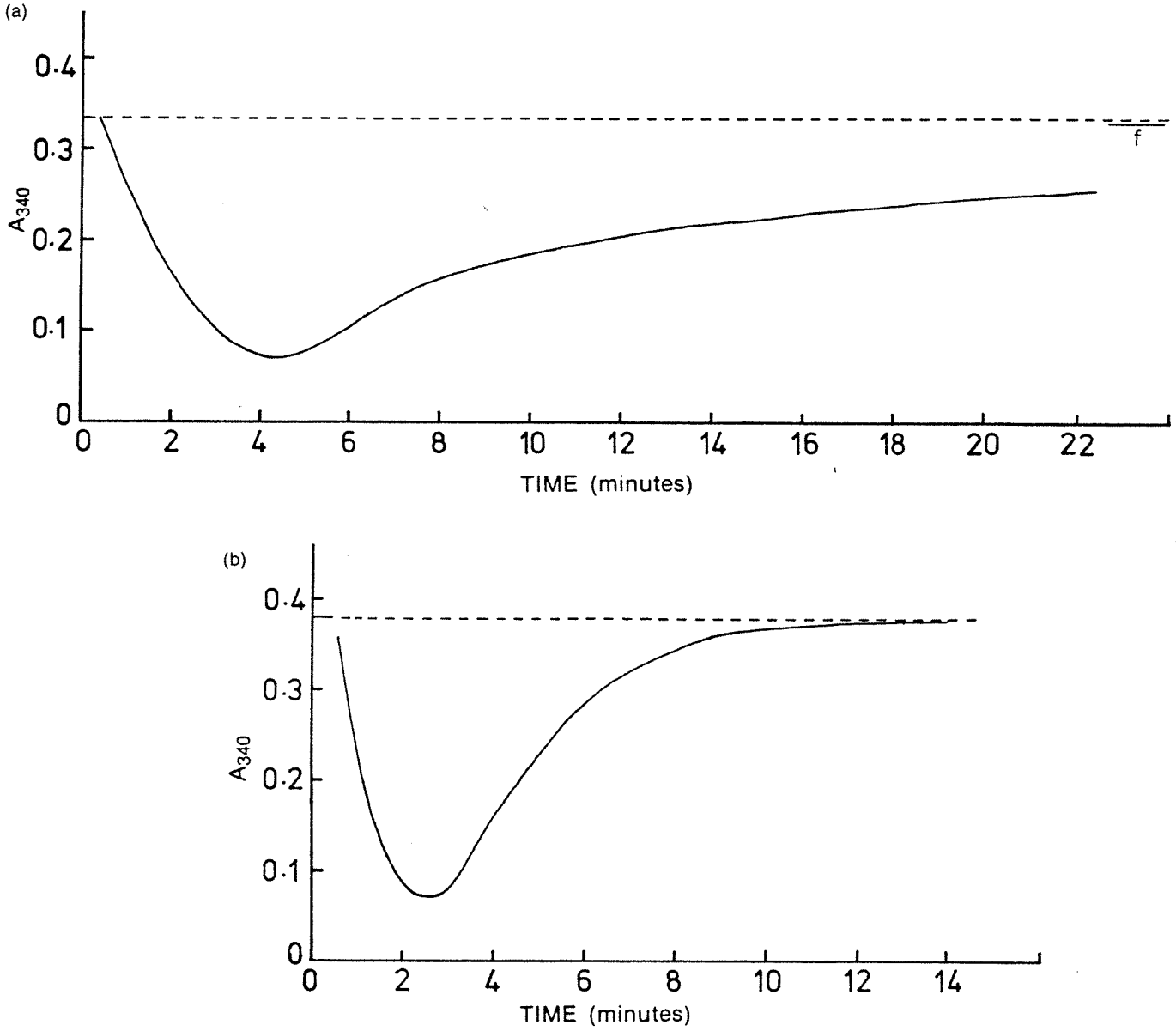


Figure 5.3 Complete progress curves for the reverse reaction are shown. Enzyme (5.0 μM) and NADH (60 μM) were mixed in 0.2 pH 7.0 phosphate buffer. The anhydride substrate was added last to initiate the reaction.

5.3(a) Acetic anhydride (17 mM) was used as the substrate. The final absorbance value after the reaction was 99% of the initial value, achieved after 110 minutes.

5.3(b) Propionic anhydride (13 mM) was used as the substrate. The final absorbance value after the reaction was 99% of the initial value, achieved after 14 minutes.

When a solution initially containing 1.7 μM enzyme, 42 μM NADH and 17 mM acetic anhydride and in which the reaction was terminated after 5 minutes by the precipitation of the enzyme was analysed for acetaldehyde by the method of Stowell (1978) an amount was found corresponding to a concentration of 38 μM in the original mixture. No acetaldehyde was produced in control experiments in which either enzyme or NADH was absent.

In order to study the acetaldehyde levels during the entire course of the absorbance changes at 340 nm, 2 cm^3 samples were withdrawn for analysis at various time intervals from a 40 cm^3 solution of enzyme (1.7 μM), NADH (51 μM) and acetic anhydride (17 mM). NADH levels during the reaction were determined by simultaneous continuous monitoring of the absorbance at 340 nm of another sample withdrawn from the same solution. The results obtained are shown in Figure 5.4. It can be clearly seen that the oxidation of NADH, measured by the decrease in absorbance at 340 nm, was accompanied by a corresponding production of acetaldehyde. The maximum amount of acetaldehyde produced (1.48 μmol) was equal (within experimental error) to the maximum amount of NADH oxidized (1.68 μmol), determined using an extinction coefficient of 6220 $l\ mol^{-1}\ cm^{-1}$ for NADH (Horecker & Kornberg, 1948). After reaching a maximum, the acetaldehyde level fell again and this drop was matched by an increase in absorbance attributable to reduction of NAD^+ back to NADH.

The use of semicarbazide hydrochloride to determine the aldehyde concentration by the method of Burbridge et al.(1950) was not possible as a large increase in absorbance at 233 nm was observed when semicarbazide hydrochloride and acetic anhydride were mixed, presumably because of a rapid reaction between the anhydride and the semicarbazide. Thus it was also impossible to use this reaction to remove the aldehyde produced which would have allowed study of the reaction without the added complication of accumulation of both of the substrates of the dehydrogenase reaction.

If additional acetic anhydride was added to a reaction solution after the absorbance had returned to its original level (56 minutes after mixing) only a very small decrease in absorbance was now observed (Figure 5.5a). This was not the case for propionic anhydride reactions, however. Addition of a second and even a third amount of anhydride still produced a sizable decrease in absorbance, although the

Figure 5:4 ACETALDEHYDE CONCENTRATIONS DURING THE REVERSE REACTION

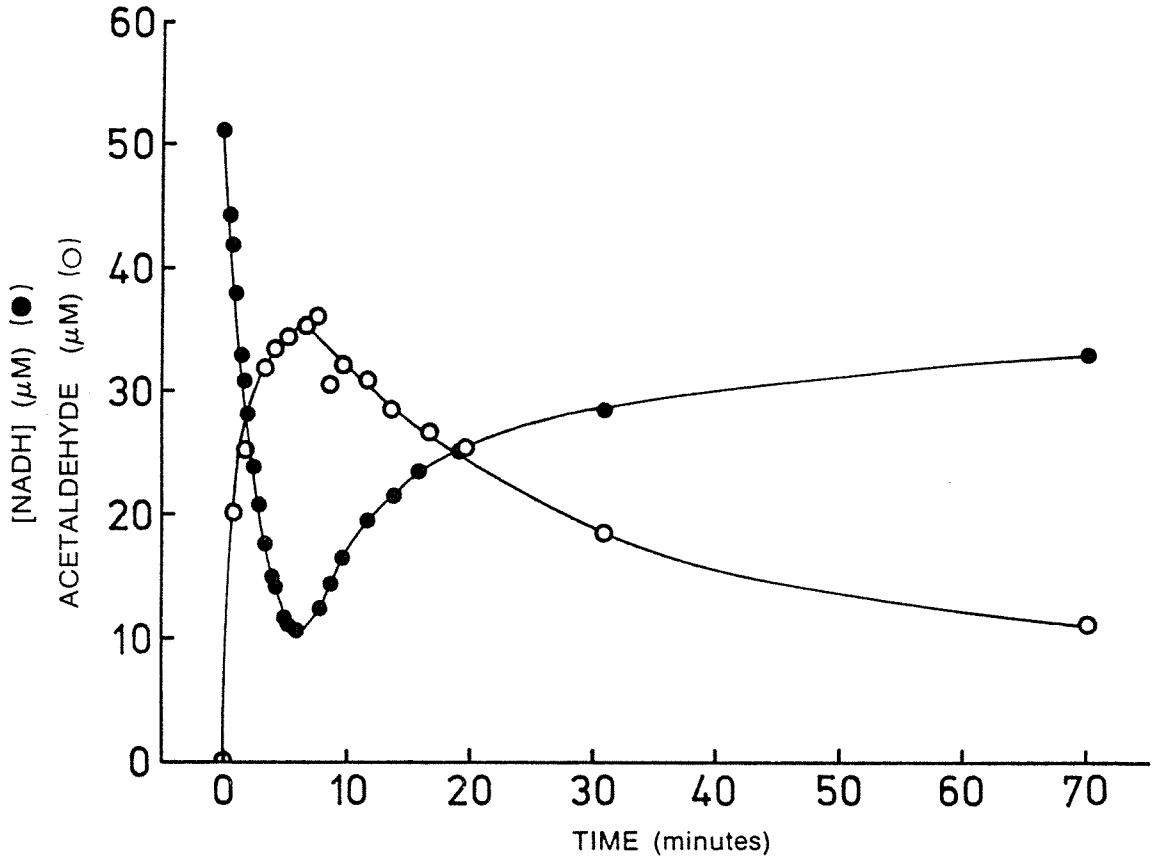


Figure 5.4. Acetaldehyde and NADH Concentrations during the Reverse Reaction.

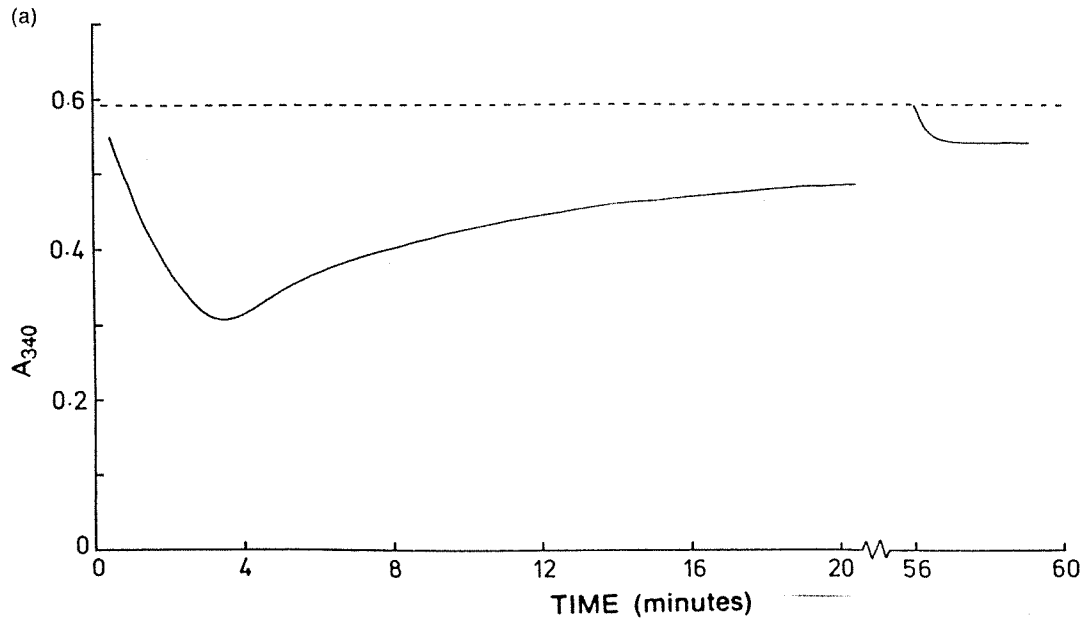
The solution initially contained enzyme (1.7 μM), NADH (51 μM) and acetic anhydride (17 mM) in 0.2 M pH 7.0 phosphate buffer. NADH levels were monitored by following changes in absorbance at 340 nm and acetaldehyde concentrations were determined by withdrawing samples from the solution, denaturing the enzyme with 3% perchloric acid, and then analysing for acetaldehyde using the method of Stowell et al (1978). The total volume of the solution was 40cm^3. A 3.0cm^3 sample was withdrawn immediately after mixing for the spectrophotmetric monitoring, and 2cm^3 samples were withdrawn at various time intervals for the acetaldehyde assays.

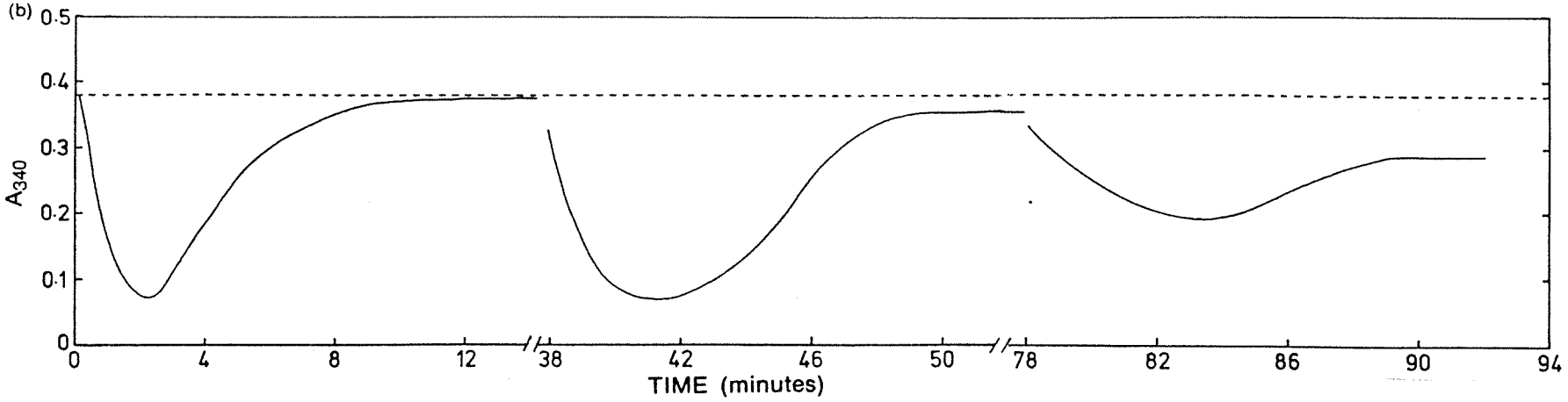
Figure 5.5. The Effect of Adding a Second Aliquot of Acetic or Propionic Anhydride to the Reaction Solution after the Absorbance returned to its Initial Level.

5.5(a). The solution contained enzyme (5 μM), NADH (93 μM) and acetic anhydride (17 mM) in 0.2 M pH 7.0 phosphate buffer. A second aliquot of acetic anhydride (5 μl pure anhydride) was added after 56 minutes.

5.5(b). The solution contained enzyme (5 μM), NADH (60 μM) and propionic anhydride (13 mM). Second and third additions of propionic anhydride (5 μl pure anhydride) were made at 37.7 and 78.4 minutes respectively.

Figure 5:5 REVERSIBILITY OF ACYLATION OF ALDEHYDE DEHYDROGENASE





reaction profile was somewhat altered on each subsequent addition (Figure 5.5b).

A second addition of acetic anhydride to the reaction mixture at the time at which the absorbance reached a minimum did however produce a large additional decrease in absorbance, provided that significant amounts of NADH remained. If there was only a small amount of NADH left, addition of further acetic anhydride produced only a slight decrease in absorbance but the additional acetic anhydride caused a marked flattening of the trace over a period of several minutes. Both of these cases are shown in Figure 5.6. The lack of sensitivity of the SP 500 spectrophotometer used in these studies severely limited the usable concentration range of NADH for kinetic studies of the reaction. Since the value of K_D for NADH is 1.2 μM (MacGibbon et al., 1977a,b) all NADH concentrations used in absorbance measurements were saturating and initial rates were therefore unaffected by the NADH concentration. However, fluorometric measurements allowed the use of much lower levels of all reagents and preliminary investigations indicated that the initial rate, v_i, was dependent on the NADH concentration at low levels of NADH as shown in Table 5.1a. This dependence is explored more fully in Chapter 6.

The levels of NADH used in the absorbance experiments appeared to have no effect on the total amplitude of the absorbance or fluorescence decrease or upon the time, t_m, taken for the NADH level to reach a minimum. Typical results are shown in Table 5.1b. Results for acetic anhydride were very similar.

Figure 5:6 UTILISATION OF NADH IN REVERSE REACTIONS

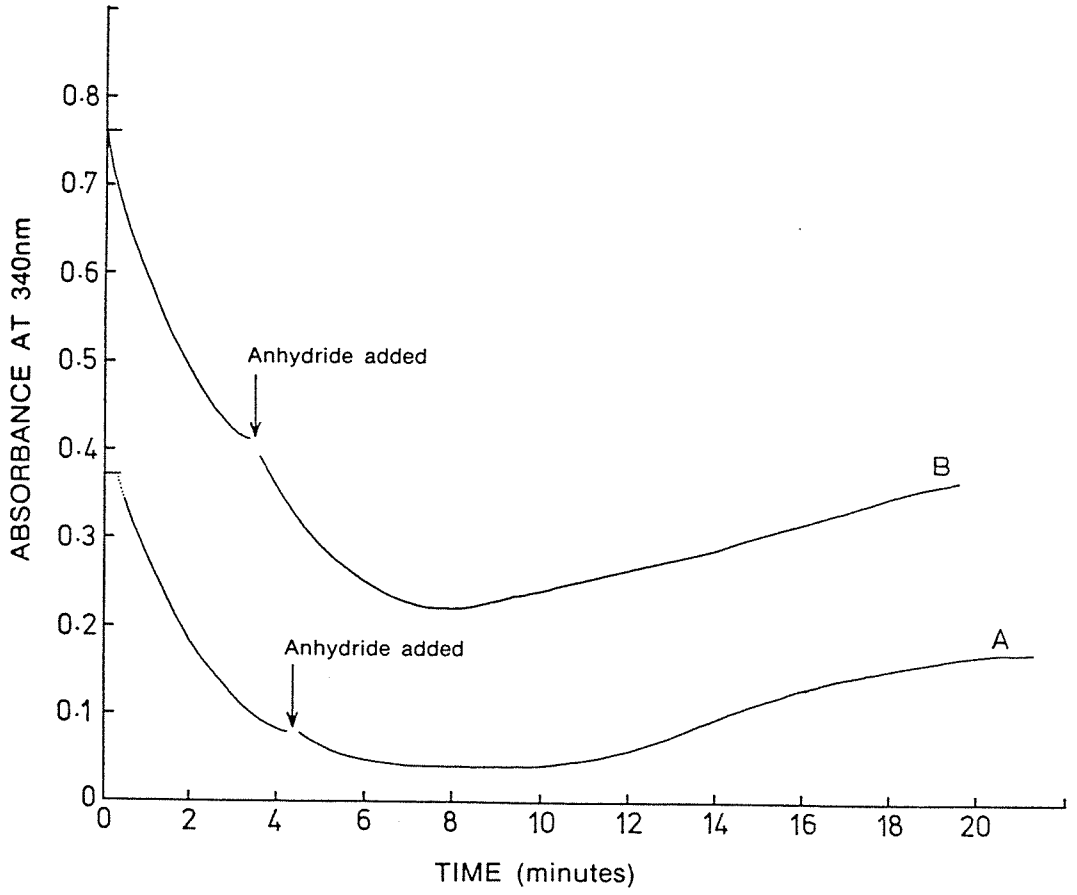


Figure 5.6 The effect of adding a second aliquot of acetic anhydride when the absorbance reached a minimum, at two different initial NADH concentrations is shown. The same concentrations of enzyme (5 μM) and acetic anhydride (17 mM) were used in each case. The NADH concentrations were 60 μM (Trace A) and 125 μM (Trace B). Both reactions were carried out in 0.2 M pH 7.0 phosphate buffer.

TABLE 5.1

The Effect of NADH Concentration on the Reverse Reaction

(a) Fluorescence Measurements

[NADH]	v_i	Δ[NADH][a]	t_m[b]
μM	μM.min^{-1}	μM	min
0.57	0.36	0.83	4.5
2.8	0.84	0.88	5.8
6.2	1.20	3.50	5.9
8.3	1.56	5.50	8.3

[E] = 0.18 μM, [Propionic Anhydride] = 6.5 mM

(b) Absorbance Measurements

[NADH]	v_i	Δ[NADH][a]	t_m[b]
μM	μM.min^{-1}	μM	min
30	24	28	3.1
60	20	36	4.0
90	31	37	4.3
120	24	40	4.4

[E] = 2.3 μM, [Propionic Anhydride] = 2.6 mM

a Δ[NADH] was calculated from the change in absorbance from the initial level to the minimum absorbance.

b t_m is the time to minimum absorbance from the start of the reaction.

No clear dependence of the initial rate on anhydride concentration (for either acetic or propionic anhydride) could be established, even using fluorimetric methods. However the total amplitude of the absorbance decrease was observed to decrease as the anhydride level decreased, the total amplitude becoming too small for accurate initial rate measurements even at concentrations of anhydride (260 μM) which represented a large molar excess of this species over enzyme in the reaction. The time taken to reach the minimum NADH point also decreased with lower acetic anhydride levels. Typical results from absorbance measurements are shown in Table 5.2.

TABLE 5.2

The Effect of Propionic Anhydride on the Reverse Reaction

[Anhydride]	v_i	Δ[NADH]	t
μM	$\mu M.min^{-1}$	μM	min
0.26	15	12	1.2
1.17	18	23	2.0
3.8	18	26	2.5
6.5	17	28	3.5
13	25	32	3.2
26	23	32	2.9

[E] = 1.6 μM, [NADH] = 43 μM

5.3.2 Anhydride Hydrolysis.

Using the same pH Stat method described above, L Deady (personal communication) established that the presence of enzyme, NAD^+, or NADH caused an increase in the rate of hydrolysis of acetic anhydride. In the presence of both enzyme and NAD^+, the catalysis of the hydrolysis was greater than the added effects of the individual reagents.

Preliminary studies of the hydrolysis of propionic anhydride by this method showed similar effects in the presence of enzyme, NAD^+ and NADH as shown in Table 5.3.

TABLE 5.3

Catalysis of the Hydrolysis of Propionic Anhydride

Solution	Relative Rates of Hydrolysis[a]	
	6.2 mM[b]	16 mM[b]
KCl	1.0	1.0
KCl,NAD^+	1.1	1.6
KCl,NADH	1.2	-
KCl,E	1.5	2.1
KCl,E,NAD^+	1.7	1.4

[E] = 1.0 μM, [NAD^+] = 1 mM, [NADH] = 0.3 mM

a Rate relative to hydrolysis in KCl solution

b Initial concentration of propionic anhydride

The method was not entirely suitable for study of this process, and large experimental errors were obtained for both anhydrides.

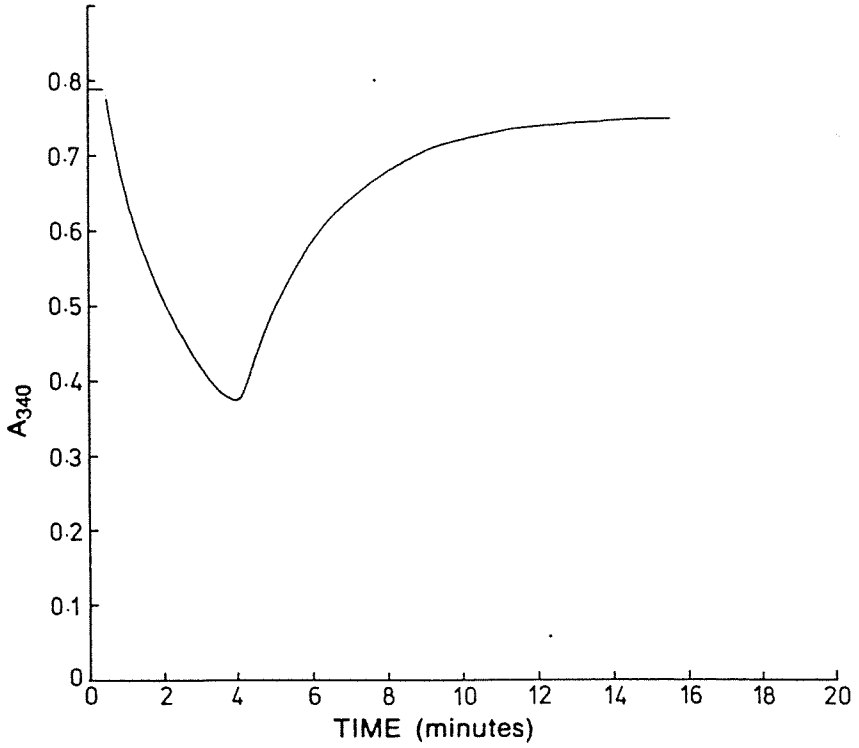
In the presence of buffer the spontaneous rate of hydrolysis of propionic anhydride was greatly increased. The exact rate of hydrolysis of propionic anhydride in buffer solutions could not be determined by the pH stat method which is dependent upon pH changes, but for reactions in 0.2 M buffer most of the anhydride appeared to have hydrolysed within 2-3 minutes. Reactions in which 0.5 M buffer was used instead of 0.2 M buffer reached the minimum point in a shorter time (2 mins) and the subsequent reduction of NAD^+ occurred more rapidly, the changeover between the two processes being very sharply defined. (Figure 5.7a)

To measure the rate of hydrolysis of propionic anhydride, a sample containing 0.5 M buffer, 108 μM NADH and 2.6 mM propionic anhydride was premixed for 3 minutes before addition of enzyme (2.3 μM) in 500 mM buffer. The total absorbance decrease at 340 nm observed for the reverse reaction upon adding enzyme was only 0.02 absorbance units, equivalent to production of 3 μmol l^{-1} compared to 35 μmol l^{-1} when propionic anhydride was added last.

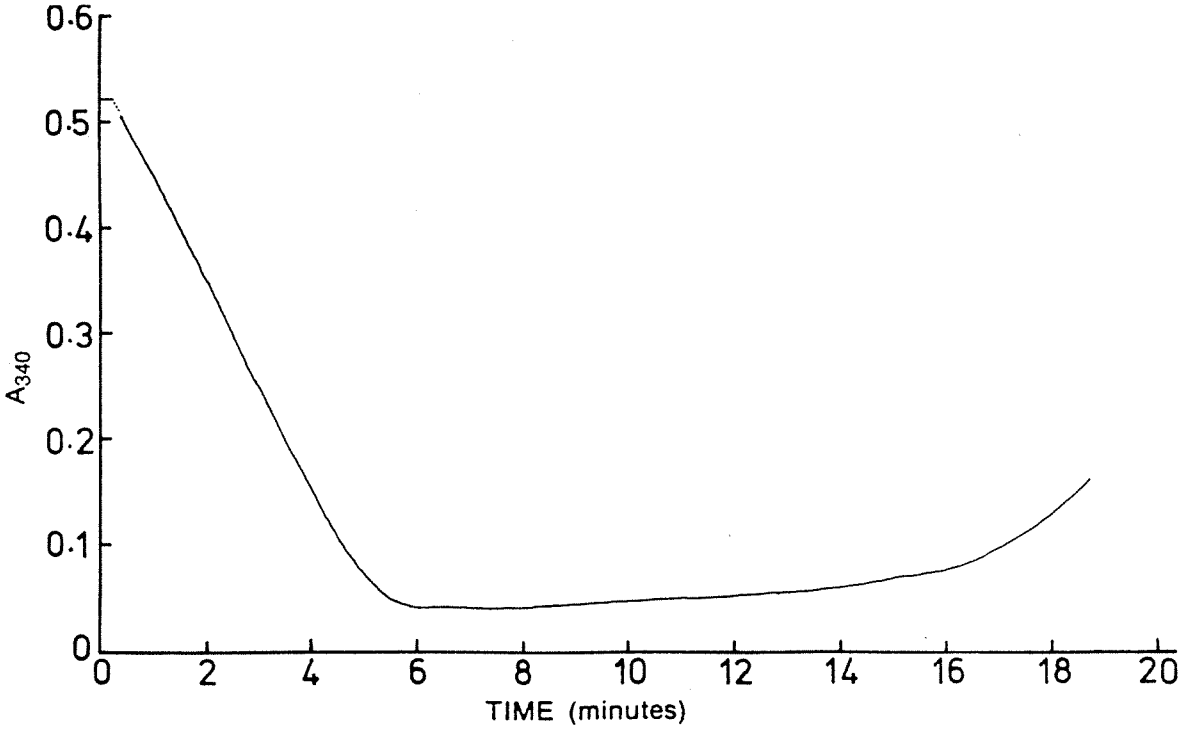
Figure 5.7b shows the results of a reverse reaction carried out with propionic anhydride in 0.5 M KCl solution. A linear decrease in A_{340} was observed for five minutes and almost all (about 90%) of the

Figure 5:7

(a) REVERSE REACTION IN 0.5M PHOSPHATE BUFFER



(b) REVERSE REACTION IN UNBUFFERED ELECTROLYTE SOLUTION



5.7 (a) The absorbance trace for a reverse reaction carried out in 0.5 M pH 7.0 phosphate buffer. The solution initially contained enzyme (3.1 μM), NADH (126 μM) and propionic anhydride (5.2 mM).

5.7 (b) The absorbance trace for a reverse reaction carried out in a solution of KCl (0.5 M). The solution initially contained enzyme (2.5 μM), NADH (84 μM) and propionic anhydride (2.6 mM).

NADH was oxidized. There was no significant increase in absorbance until about 17 minutes after the minimum had been reached. The final pH of the solution after 19 minutes was 5.3. These results may be compared with those in Figure 5.3b & 5.5b which were carried out in 0.2 M pH 7.0 phosphate buffers. The hydrolysis rate of acetic anhydride also appeared to be increased in buffer solutions but not to the same extent. It was not possible to carry out reactions using acetic anhydride in salt solutions as both the reaction rate and the amplitude of the decrease in A_{340} were reduced considerably in the absence of buffer. Low buffer concentrations such as 20-50 mM were found to be optimal in this case.

5.3.3 Production of Acetaldehyde from p-Nitrophenylacetate

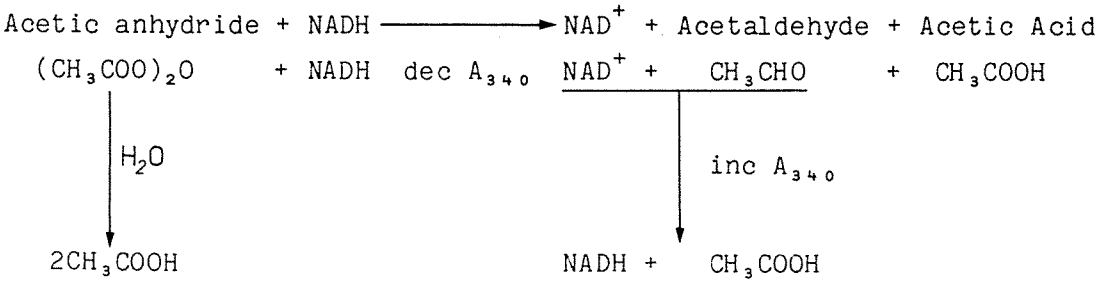
Only extremely small amounts of acetaldehyde were detected in solutions containing enzyme (2.4 μM), NADH (50 μM), and p-nitrophenylacetate (110 μM), even after 60 minutes. The rate of production of the p-nitrophenoxide ion as determined by monitoring the increase in absorbance at 400 nm was 5.7 $\mu mol.min^{-1}.l^{-1}$. In the absence of NADH the rate was 3.6 $\mu mol.min^{-1}.l^{-1}$ and the spontaneous rate of hydrolysis of PNPA was 0.1 $\mu mol.min^{-1}.l^{-1}$. In contrast, the amount of acetaldehyde detected in samples containing enzyme, PNPA and NADH was only 2 nmol (0.7 μM) after 2-5 minutes, and there was no increase in this amount after longer time intervals. An acetaldehyde concentration of 0.3 μM was detected in the control sample which contained PNPA and NADH and hence the corrected acetaldehyde concentration was 0.4 μM (1 nmol), which corresponds to oxidation of only 0.8% of the available NADH. A second experiment in which the PNPA, NADH and enzyme concentrations were 320 μM, 120 μM, and 1.0 μM respectively produced very similar results. A maximum acetaldehyde concentration of 1.2 μM was detected which corresponded to oxidation of 1% of the initial NADH concentration. However, in this case, an identical amount of acetaldehyde was detected in the control sample which contained only enzyme and NADH in buffer. Thus there is no conclusive evidence that any acetaldehyde was produced in the presence of PNPA and NADH.

5.4 DISCUSSION.

When enzyme, NADH and acetic anhydride were mixed, a decrease in absorbance at 340 nm was observed. As shown by Hart & Dickinson (1978a), addition of an aldehyde after several minutes produced an increase in absorbance back to the original level, thus suggesting that NADH is being oxidized to NAD^+, which in the presence of excess aldehyde, is reduced back again to NADH. This experiment also indicates that the enzyme retains its oxidative activity, at least for several minutes, despite a massive excess of a powerful acylating agent.

The results obtained by monitoring acetaldehyde and NADH concentrations simultaneously showed clearly that acetaldehyde was both produced and then utilized in a further reaction at the same rate as the accompanying changes in the NADH concentration. As NAD^+ and acetaldehyde are both substrates for the dehydrogenase reaction, the most likely explanation for these absorbance changes is given in scheme 5.1.

Scheme 5.1



Acetic anhydride is removed from the solution by reaction with the enzyme to form acetaldehyde and also by hydrolysis with water to form acetic acid. The rate of hydrolysis of acetic anhydride in pure water ($k = 2.263 \times 10^{-3}\ s^{-1}$) was sufficiently fast to cause removal of at least half of the anhydride by the time the NADH level reached a minimum (at 4-5 minutes). However, for those reactions in which 17 mM acetic anhydride was added to solutions of 2 μM enzyme and 30-100 μM NADH, even removal of half of the anhydride leaves a vast excess of the reagent. Reactions identical to this, but with half as much anhydride, still exhibited a decrease in absorbance at 340 nm upon mixing. Thus it would appear that a much greater proportion of the anhydride than expected had been hydrolysed within this time, indicating a much greater rate of hydrolysis in reaction solutions than

in pure water.

Experiments such as those shown in Figure 5.6 confirm this. Upon addition of a fresh sample of anhydride further reverse reaction occurred, provided sufficient NADH remained, as in the upper trace in this diagram. If most of the NADH had already been oxidised, as seen in the lower trace, a further decrease was therefore limited by the small remaining concentration of NADH. However, the presence of free anhydride was seen to inhibit the dehydrogenase reaction following, a flattening of the trace being observed before the absorbance increased. These results can best be explained in terms of rapid hydrolysis of the acetic anhydride so that the oxidation of aldehyde can subsequently take over.

Several species present in the solution have been shown to catalyse the hydrolysis of both acetic and propionic anhydrides. Enzyme, NAD^+ and NADH all independently increased the hydrolysis rate, and there were indications that it was also increased in the phosphate buffer used (5.3.1). In addition, enzyme, in the presence of NAD^+, was a considerably more powerful catalyst than either separately. This suggests that the conformationally-altered $*E.NAD^+$ species (2.1.3, 3.5) was more catalytically active.

It therefore seems likely that the absorbance reaches a minimum before all of the NADH is utilised, because the acetic anhydride is all removed by hydrolysis (and reaction to produce acetaldehyde) or at least reduced to such low levels that it can no longer inhibit the dehydrogenase reaction. The dehydrogenase reaction then proceeds to utilise irreversibly the available substrates NAD^+ and acetaldehyde via the normal dehydrogenase pathway. As these are produced in equimolar amounts, the reaction proceeds to give total utilization of these species and the original amount of NADH is regenerated, none being lost during this process. As shown in Figures 5.2(a) & (b), the reverse reaction was clearly enzyme catalysed since no reaction was observed in the absence of either enzyme or NADH.

Although the enzyme clearly retained some activity after the reverse reaction as it could utilise the NAD^+ and aldehyde products, there did appear to be a slow modification of the enzyme to a less active form. Thus a second addition of acetic anhydride after the original NADH was all regenerated produced only a very small reverse reaction. This modification appeared to be a slow process, only producing significant effects on the enzyme after 20-25 minutes. As

all of the acetic anhydride was proposed to have hydrolysed by this time, it is most likely that some acetylation of sidechain groups of the enzyme occurred while anhydride was still available, but that the modifying effect on the enzyme activity was only a consequence of a much slower rearrangement following this acetylation.

Duncan (1979) reported that very small amounts of acetaldehyde were produced when cytoplasmic aldehyde dehydrogenase from rabbit liver was mixed with NADH and PNPA, and concluded that the catalytic sites for the dehydrogenase and esterase reactions were the same. However, the amount of NAD^+ produced corresponded to only 1.2% of the amount of PNPA which was hydrolysed in the same length of time (135 minutes). The method used by Duncan (1979) for the detection of acetaldehyde, which involved addition of alcohol dehydrogenase to the reaction and hence forming NAD^+ by converting the acetaldehyde to ethanol, was similar in principle to the acetaldehyde assay used in the present work, in which acetaldehyde was removed from the solution by perfusion techniques to a separate solution containing alcohol dehydrogenase and NADH (Stowell *et al.*, 1978), but it is probably less accurate as the presence of any NAD^+ in the commercial alcohol dehydrogenase used would give a false result.

The failure to observe significant acetaldehyde formation during the hydrolysis of PNPA lends support to the proposal of Blackwell *et al.* (1983a) that the enzyme has distinct dehydrogenase and esterase catalytic sites. If the site of PNPA hydrolysis were the same as the site of oxidation of aldehydes, acetylation of aldehyde dehydrogenase by PNPA, with an accompanying loss of a p-nitrophenoxide ion, and acetylation by acetic anhydride, with loss of an acetate anion, would produce the same acetyl-enzyme intermediate. Thus any differences between these two acetylating agents in the rates of production of NAD^+ and acetaldehyde should only arise as a result of different rates of acetylation by these two substances. Although a burst in production of the p-nitrophenoxide ion has been observed by MacGibbon *et al.* (1978), indicating that the enzyme is acylated very rapidly, the rate of enzyme-catalysed production of acetaldehyde from PNPA was insignificant when compared with the rate of production of acetaldehyde from acetic anhydride. In addition, in the presence of acetic anhydride, up to 90% of the NADH available was oxidised, compared with only 1% of the NADH when PNPA was used. It is clear from these results that the increased rate of hydrolysis observed in the presence of certain levels of NADH

(MacGibbon et al. 1978b) is not a result of reaction of the NADH and acetate group to form acetaldehyde, but must involve some other process.

The suggestion by Duncan (1979, 1985) that the site of aldehyde oxidation and the so-called esterase site of the enzyme are one and the same may therefore be discounted. However it is quite possible that one of the sites of enzymic catalysis of the anhydride hydrolysis is the same as the site of hydrolysis of PNPA. Previous studies of the esterase reaction carried out by MacGibbon et al.. (1978), Blackwell et al.. (1983a,b), and Deady et al., (1985) also provide convincing evidence in favour of distinct sites.

The same general reaction scheme was proposed for propionic anhydride as for acetic anhydride with the products being in this case propionaldehyde and propionic acid. There are, however, two main differences. The reverse reaction with propionic anhydride was much faster, the initial rate being 3.6 times faster than the acetic anhydride rate. Also propionic anhydride did not appear to permanently modify the enzyme in the way observed for acetic anhydride. Second and even third additions of propionic anhydride produce a reverse reaction of sizeable, although slightly reduced, amplitude, as shown in Figure 5.5b, although the accompanying small changes in the reaction profiles may be due to chemical modification of the enzyme.

The apparent very high rate of hydrolysis of propionic anhydride in these reactions was most unexpected. The reported half life of propionic anhydride in pure water was 42.9 minutes and thus it was hoped that the experimental problems caused by hydrolysis of acetic anhydride would be avoided with propionic anhydride. However, almost all of the anhydride appeared to have been hydrolysed within 2-3 minutes. As was the case for acetic anhydride, enzyme, NAD^+ and NADH each separately catalysed this hydrolysis, with increased catalytic activity for the enzyme.NAD^+ binary complex.

Catalysis by these species does not account fully for the observed rapid hydrolysis however, and the the phosphate buffer used appears to be an even more important catalyst. Although this could not be demonstrated directly (by the pH stat method for example), there was much indirect evidence of buffer catalysis of propionic anhydride. Reactions in 0.5 M buffer instead of 0.2 M buffer reached a minimum absorbance more quickly, with an extremely sharp changeover between the decreasing and increasing absorbance processes (Figure 5.7a).

Premixing of NADH and anhydride in buffer for 3.0 minutes before addition of enzyme almost completely eliminated the reverse reaction, clearly indicating that hydrolysis of almost all the anhydride had occurred, since addition of fresh anhydride did produce the expected reverse reaction. Finally, reactions carried out in water or in salt solution in the absence of buffer were quite different to those in buffer. In both these later cases the decrease in NADH absorbance was linear until close to the minimum in the A_{340} versus time curve, despite pH changes from 7.0 to 5.3. The absorbance did not reach a minimum until 90% of the NADH was oxidised, and the dehydrogenase reaction, as shown by the increase in A_{340}, did not proceed to any great extent until 17 minutes after the reaction commenced.

Thus the high concentration of buffer used in order to maintain a pH of 7.0 during reactions was producing a very large increase in the hydrolysis rate of the propionic anhydride. This catalysis was much greater for propionic anhydride than for acetic anhydride and the combined effects of all the species in the solution increased the rate of propionic anhydride hydrolysis to a rate which was even faster than that for acetic anhydride, even though the rate of spontaneous hydrolysis of propionic anhydride in pure water is reported to be 9.8 times slower than acetic anhydride (Wilsdon & Sidgwick, 1913)

Gold (1948) showed that the hydrolysis of acetic anhydride was catalysed by both acids and bases, basic solutions being the most effective catalysts, and for this reason reactions were all conducted at pH 7.0. Pyridine has also been shown to be an effective catalyst of acetic anhydride hydrolysis (Bafna & Gold, 1953), so it is possible that reaction with the pyridine group of NAD^+ and NADH may account for the increased rate of hydrolysis of propionic anhydride and acetic anhydride in the presence of these substances.

Although it is possible that the basic components of this buffer were possibly responsible for the catalysis observed in the presence of phosphate buffer, it is also possible that reaction between propionic anhydride and the phosphate anion, to form propionyl dihydrogen phosphate (Bentley, 1949) may have occurred.

Kinetic information about the reverse reaction using acetic anhydride was strictly limited, due to the rapid hydrolysis of this anhydride. It was not experimentally feasible to produce low levels of anhydride and the absorbance versus time plots were frequently markedly curved, making initial rate measurements difficult and therefore

inaccurate.

In the absence of phosphate buffer, propionic anhydride was hydrolysed more slowly than acetic anhydride, so that the reaction could be studied using lower concentrations of anhydride. Linear plots were frequently obtained for several minutes and hence initial rates could be easily and more accurately measured. However, it was not possible to maintain the pH of reaction solutions at 7.0 since the high concentrations of buffer which would be required catalysed the hydrolysis of propionic anhydride and prevented meaningful data from being obtained. The linearity of the absorbance versus time plots in the absence of buffer for times sometimes in excess of 10 minutes and in spite of pH changes of up to 1.7 pH units (due to propionic acid production) suggested however that there was not a strong pH dependence of the reaction rate, at least in moderately acid solutions. This dependence is explored more fully in Chapter 7.

Although few conclusions can be drawn at this stage of the investigation, the products of the reverse reaction have been established, as has the clear demonstration of dependence of the rate of the reverse reaction on the enzyme concentration. Species catalysing the hydrolysis of acetic and propionic anhydrides have been identified and favourable conditions for further, more systematic, study determined. The rate of the reverse reaction of propionic anhydride has been shown to be several times greater than the rate for acetic anhydride. Also the failure to observe the production of acetaldehyde during the enzyme catalysed hydrolysis of PNPA in the presence of NADH argues for distinct dehydrogenase and esterase sites.

More detailed investigation of the reverse reaction is given in Chapter 6, and in Chapter 7, where the effects of various anhydrides as modifiers of the dehydrogenase reaction activity are investigated.

CHAPTER 6

FURTHER STUDIES OF THE REVERSE REACTIONS OF ALDEHYDE DEHYDROGENASE

6.1 INTRODUCTION

In the previous chapter, it was established that the products of the reverse reaction of aldehyde dehydrogenase in the presence of NADH and acetic anhydride are acetaldehyde and NAD^+. Propionaldehyde was proposed as the product for the corresponding reverse reaction with propionic anhydride, because of the similarities between the two reactions.

In both cases, as the anhydride concentration in the reaction solution was lowered by the accompanying hydrolysis the oxidation of NADH was followed by the usual enzyme catalysed oxidation of the aldehyde product (in the presence of NAD^+, the other product) thus regenerating the NADH. Under conditions in which the NADH was removed from the solution by the reverse reaction before complete hydrolysis of the propionic anhydride had taken place, the anhydride remaining was found to inhibit the dehydrogenase reaction.

According to the stoichiometry of equation 5.1, NADH and propionic anhydride react in equimolar amounts to produce NAD^+, propionaldehyde and propionic acid, also in equimolar amounts. The absorbance decrease ΔA_{340} measured for each reaction therefore gives not only a measure of the extent of NADH oxidation, but also of the amount of propionic anhydride utilized in the reverse reaction and hence the maximum amounts of NAD^+ and propionaldehyde formed. Similarly the absorbance trace allows the amounts of NADH, NAD^+ and propionaldehyde to be calculated at each point in the reaction. However, the rate of hydrolysis of propionic anhydride under reaction conditions was not known and therefore, propionic anhydride concentrations during the course of the reaction could not be assigned; only the concentration at the start of the reaction.

Of the many species in reaction solutions which have been shown to catalyse the hydrolysis of both acetic and propionic anhydrides, most are essential reaction components and therefore cannot be eliminated. By reducing the phosphate buffer concentration from 500 mM to 2-5 mM it was possible to extend the lifetime of the propionic anhydride species in reaction solutions sufficiently to allow quantitative kinetic

studies of the reverse reaction with propionic anhydride to be carried out. The spontaneous hydrolysis rate of acetic anhydride was however still too rapid to allow collection of precise data, as discussed in chapter 5. However the reaction with propionic anhydride is of more inherent interest, as a product of this reaction, propionaldehyde, is the enzyme substrate used elsewhere in the present work.

It was recognised at the outset that the removal of effective buffering capacity from the reactions was likely to create many experimental difficulties. However initial indications from reverse reactions carried out in very low buffer concentrations suggested that precise data could be obtained from initial rate determinations. The absence of marked pH effects on the reverse reaction was indicated by absorbance versus time plots which were linear for 5-6 minutes despite pH changes from 7.0 to 5.3 over this time.

No attempt was made to find a more suitable buffer, which did not catalyse the hydrolysis of the propionic anhydride, because any change of buffer system would have necessitated considerable study to redetermine the behaviour of the enzyme in that buffer. It was shown in Chapter 4 that the enzyme activity was not the same in all buffers. Also the nature of the catalysis of anhydride hydrolysis by phosphate buffer was not known, and without further study of the mechanism of catalysis, choice of a suitable buffer could only be by trial and error, not by an informed choice.

As apparently satisfactory data could be obtained in the absence of adequate buffer, it was decided to proceed to obtain as much quantitative kinetic data as was possible without the use of buffer, by both conventional steady state methods and also by the use of stopped flow rapid kinetic techniques.

6.2 METHODS

6.2.1 Reverse Reaction Studies using Propionic Anhydride

Reverse reactions were carried out essentially as described in chapter 5, except that all solutions contained 0.1 M Na_2SO_4 and 0.1 M $NaNO_3$ (which will be referred to subsequently as "electrolyte solution") instead of buffer. This was necessary to maintain a high ionic strength and to ensure that the enzyme has the optimal ionic

atmosphere. (Bennett, 1981, unpublished results, also refer to chapter 4)

In each experiment, a solution containing enzyme (0.10-8.00 μM) and NADH (0.20-400 μM) was prepared in a spectrophotometer cuvette. Reaction was initiated by the addition of a small volume (0.010-0.100 cm^3 added to a total volume of 3.0 cm^3) of a solution of propionic anhydride in acetonitrile to the cuvette.

The resulting changes in absorbance at 340 nm were monitored using an Aminco DW 2a u.v.- visible spectrophotometer. This instrument was very much more sensitive and adaptable than the spectrophotometer used for the early experiments outlined in chapter 5 and precise data could be obtained over a much greater NADH concentration range, eliminating the need for fluorescence studies of the steady state reaction. The reference cuvette always contained an amount of NADH (in 25 mM phosphate buffer pH 7.6) equivalent to the initial NADH concentration in the sample cuvette. Thus all measurements were made in terms of the change in absorbance, not as absolute absorbance readings, to enable the greater sensitivity of the spectrophotometer to be utilised.

Enzyme stock solutions contained 25 mM pH 7.3 phosphate buffer. NADH stock solutions were freshly prepared each day in 7 mM pH 7.6 phosphate buffer. The final buffer concentration in each experiment did not exceed 5 mM and in most experiments was about 2 mM. A low buffer concentration was necessary to reduce the rate of propionic anhydride hydrolysis, but since solutions of NADH in water are only stable for a short time it was therefore necessary to prepare stock solutions of NADH in buffer. Also, removal of buffer from enzyme samples by dialysis would have had a detrimental effect on the enzyme, reducing its activity (Bennett, 1981) and possibly its lifetime in storage. Therefore, no attempt was made to completely remove all the phosphate buffer.

Because the buffer concentration is so low the possibility of large pH changes caused by the production of propionic acid by hydrolysis had to be considered. The pH of reaction solutions was usually measured after the reaction as a check.

For reactions at low substrate concentrations in which the absorbance trace was curved, the initial rate was measured by constructing a tangent to the initial part of the curve. In addition to determination of the initial rate, v_i, other measurements were made for those reactions in which the reverse reaction was followed by a

dehydrogenase reaction when the reaction was monitored for a sufficiently long time. Measurement of the total absorbance change, ΔA_{340}, indicated how much NADH was oxidized. The maximum rate of the subsequent dehydrogenase reaction, v_f, was determined by constructing a tangent to the part of the absorbance curve at which the rate of increase in A_{340} was greatest (Figure 6.1). Two time measurements were also made: t_1, the time taken for the absorbance to reach a minimum, and t_2, the time at which the absorbance started to increase were both measured. Such measurements were made as shown in Figure 6.1 and results are given in terms of these parameters. In many cases t_1 and t_2 could not be determined separately (inset, Figure 6.1) and in such cases only a single time, t, was measured.

All experiments were carried out at 25°C and all absorbance measurements were at 340 nm. Unless otherwise stated, the anhydride substrate was propionic anhydride in all experiments.

6.2.1.1 The Effect of Modifier Reagents on the Reverse Reaction

To test the effect of the reaction products, propionaldehyde and NAD^+, various amounts of these reagents were added separately to reaction solutions, generally prior to the addition of propionic anhydride. The effects of several modifiers of this enzyme (PCMB, disulphiram, chloral hydrate, and glyoxylic acid) were also tested in this manner.

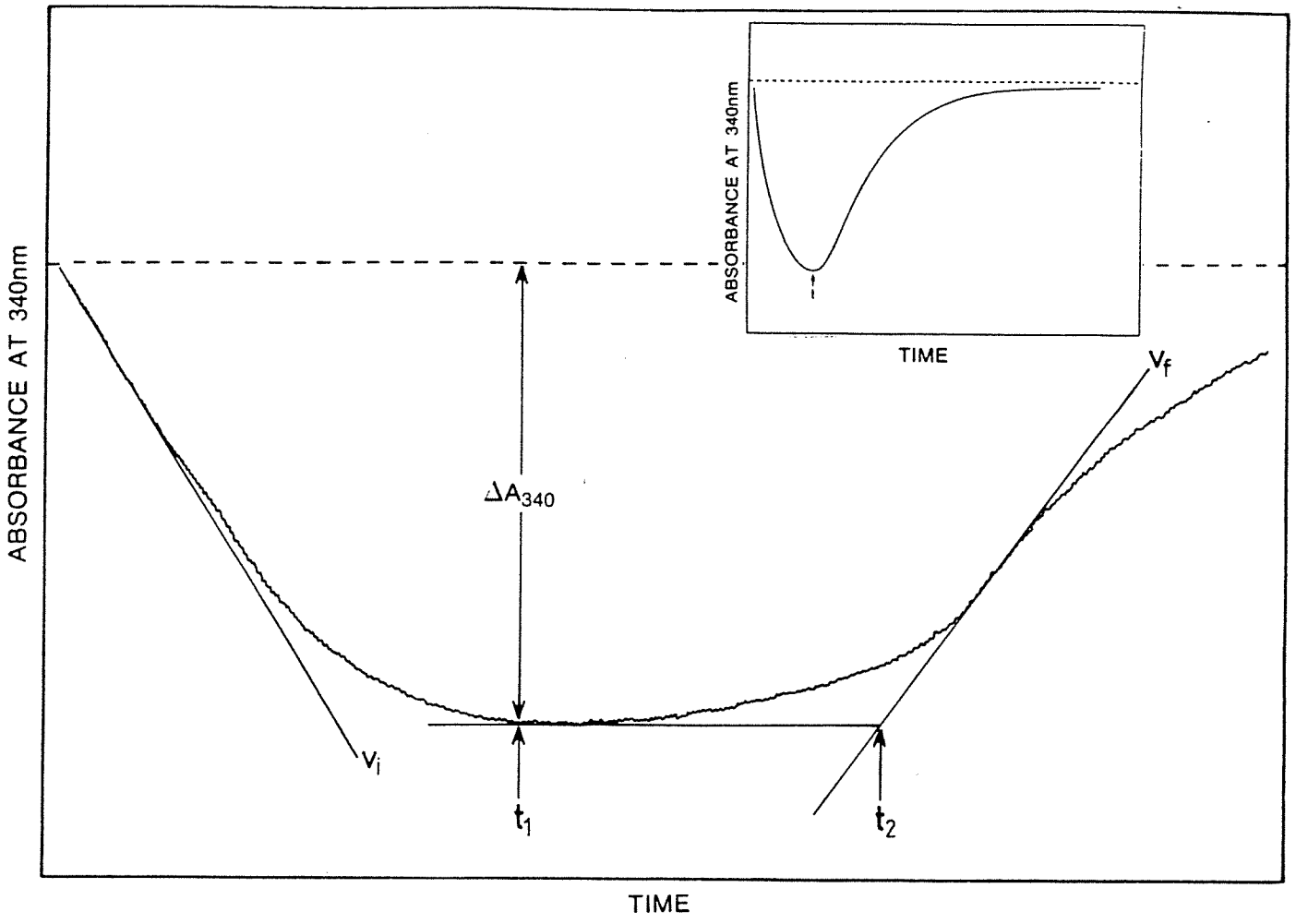
6.2.1.2 pH Profile of the Reverse Reaction

In order to keep the buffer concentration low, but to start at the desired pH, solutions containing enzyme and NADH were prepared in 25 mM buffer of the required pH. The true pH was then measured and adjusted if necessary with 0.1 M NaOH and HCl. Finally propionic anhydride was added to initiate the reaction.

6.2.2 Reverse Reactions involving Other Anhydrides

Attempts were made to produce a reverse reaction with a variety of anhydrides. A solution of each anhydride in acetonitrile was used in place of propionic anhydride in experiments as described above. In some cases, satisfactory data could not be obtained in the absence of buffer and in these cases 0.100 mM phosphate buffer, pH 7.0 was used.

Figure 6:1 INFORMATION OBTAINED FROM ABSORBANCE TRACES OF REVERSE REACTIONS



Information obtained from Absorbance Traces of Reverse Reactions

Figure 6.1 The dotted line shows the absorbance at 340 nm before addition of propionic anhydride.The initial rate of the reverse reaction, v_i and the maximum rate of the subsequent dehydrogenase reaction were determined from the slope of the tangents as shown. The maximum change in absorbance, ΔA_{340}, and the time at which the minimum absorbance was observed, t_1, were also measured. A second time measurement, t_2, was made as shown, but separate measurements of t_1 and t_2 could not always be made (Inset).

6.2.3 Presteady State and Stopped Flow Studies of the Reverse Reaction

Various methods for the specific study of this enzyme using the stopped flow spectrometer have been described previously (Chapters 2 & 3,) and most of these methods could also be used for studying the reverse reaction. Changes in nucleotide absorbance and fluorescence were produced by the reverse reaction, but were in the opposite direction to those normally observed for this enzyme. As for previous presteady state studies, nucleotide fluorescence was used for the most sensitive measurements. Measurements of absorbance at 340 nm were used to provide complementary information, and to distinguish between processes which involve only fluorescence enhancement or quenching and those which also involve spectral changes in absorbance. Protein fluorescence studies were also carried out, but were of limited use as discussed previously (2.1.3.4 and 3.2.4).

The proton burst method of Bennett et al. (1982) as described in chapter 3 (3.2.7.2) could not be used to study proton uptake in the reverse reaction, due to the spontaneous hydrolysis of anhydride in aqueous solution. Acid production from anhydride hydrolysis completely swamped any such small changes in pH produced by the reverse reaction and colour changes in the dye occurred before measurements could be made.

Stopped flow experiments involved rapid mixing of a solution containing enzyme premixed with NADH and a solution containing anhydride and some NADH. This was necessary to prevent dissociation of the enzyme-NADH species upon rapid mixing, as this involves a 50% dilution of each solution. The concentration of NADH premixed with anhydride was always chosen to be equal to the amount of NADH remaining unbound in the enzyme syringe, calculated on the basis of the known dissociation constant for the enzyme-NADH complex of 1.2 μM (MacGibbon et al., 1977a & b). No change in fluorescence was observed upon mixing of solutions containing enzyme and NADH with NADH solutions having concentrations determined in this way (see Appendix 1).

A solution of anhydride in acetonitrile was added directly to this NADH solution immediately prior to its introduction into the drive syringes of the stopped-flow spectrometer and used immediately. Fresh anhydride solutions were prepared frequently to minimise the effects of decreasing anhydride concentration as a result of hydrolysis. For lower concentrations of anhydride, when loss of reagent by hydrolysis

became a significant factor, additions of anhydride were made directly into the reservoir syringes by the use of a Hamilton syringe and then mixed, thus reducing handling time before the reaction. However, even this method was not suitable for anhydride concentrations lower than about 200 μM, so in order to make a qualitative study of very low anhydride levels it was necessary to utilise the hydrolysis of the anhydride itself as a means of lowering the concentration. Low concentration solutions containing NADH and anhydride were prepared and introduced into the drive syringes. Then for each successive run, the concentration of anhydride was lower than in the previous experiment. Although the exact amounts of anhydride in such experiments could not be determined, due to the complexity of the hydrolysis in reagent solutions, it was possible to gain some information by measuring the time of each experiment from the initial time of preparation of the bulk anhydride solution.

Unless otherwise stated, all solutions prepared for the stopped-flow experiments contained 0.1 M sodium sulphate and 0.1 M sodium nitrate to maintain a constant high ionic strength. Stock solutions of enzyme and NADH contained 25 mM and 7 mM buffer respectively, as described previously (6.2.1). As for steady state experiments, the final buffer concentration in each experiment did not exceed 5 mM.

6.2.3.1 Nucleotide Fluorescence

The stopped-flow spectrometer was set up for nucleotide fluorescence as described previously. (2.3) A solution containing enzyme (2-10 μM), premixed with NADH (5-100 μM), was pushed against a solution containing NADH (at a concentration equivalent to the free NADH in the previous solution) and anhydride (0.1-8.0 mM). Data were collected and analysed as described previously (2.4.4 & 2.4.5) by the use of the computer fitting programmes described in Chapter 2. Calibration of the fluorescence signal was carried out using a standard NADH solution (10 μM).

6.2.3.2 The pH Dependence of the PreSteady-State Process.

Experiments were carried out essentially as above, except that the anhydride and NADH solution contained 0.1 M buffer of the appropriate pH and the enzyme-NADH solution contained sodium sulphate and sodium nitrate (0.1 M in each). Buffer solutions were of constant ionic strength and prepared as described in Chapter 4. Buffer components at each pH were as follows: pH 5.0, acetate, pH 6.0 and 7.0, phosphate, pH 8.0 veronal/HCl, pH 9.0, Borate,KCl/NaOH, pH 10.0, Glycine/NaOH.

6.2.3.3 Protein Fluorescence.

Protein fluorescence measurements were carried out by monitoring the fluorescence emission at 340 nm, using a Wratten 18A filter (10 nm bandpass, maximum transmission at 335 nm). The wavelength of the incident (exciting) radiation was 280 nm. Experiments were conducted as for nucleotide fluorescence using solutions prepared in the same way. Calibration of the fluorescence signal was by the use of a known amount of enzyme (5 μM), but the different enzyme samples probably had slightly different protein fluorescence.

6.2.3.4 Absorbance.

Experiments similar to the nucleotide fluorescence experiments were carried out, using the stopped flow spectrometer in the absorbance mode, by monitoring the absorbance of NADH at 340 nm. As this method is less sensitive than nucleotide fluorescence higher concentrations of enzyme (10-30 μM) and NADH (0.1-0.3 mM) were used.

NADH was premixed with the enzyme and therefore changes in absorbance at 328 nm such as those indicated by the difference spectrum determined in chapter 2 (2.5.3) would not be expected to occur in the presteady state. Thus absorbance studies were restricted to 340 nm.

6.2.4 The Effect of Anhydrides on the Binding of NADH to the Enzyme

Presteady state studies of the reverse reaction were also carried out for enzyme not premixed with NADH. In this case the effect of the anhydride on the binding of NADH to the enzyme was also seen, by monitoring the increase in nucleotide fluorescence as NADH binds. For NADH binding experiments, solutions containing enzyme (10 μM) were pushed against solutions containing NADH (10-80 μM) and propionic anhydride (0.1-8 mM). All solutions contained 0.1 M Na_2SO_4 and $NaNO_3$.

As described previously (6.2.1 and 6.2.3), buffer concentrations did not exceed 5 mM.

Amplitudes and decay constants for the two binding processes were obtained by computer fitting of the data as described in chapter 2. As for steady state experiments, all measurements were carried out at 25°C.

6.3 RESULTS

6.3.1 The Effect of NADH Concentration on the Reverse Reaction

The effect of NADH concentration on the rate of the reverse reaction was determined by varying the amount of NADH at a fixed high concentration of propionic anhydride (1.3 mM). At this level of propionic anhydride, linear absorbance versus time curves were obtained for at least 5 minutes, providing that the NADH concentration was not limiting. Maximum reaction rates were achieved at this propionic anhydride concentration (refer below, 6.3.2).

The reaction rate increased until a maximum rate was reached at an NADH concentration of 30 μM (Figure 6.2a). At NADH levels higher than this, two effects were noted. Hysteresis became a feature of the reaction, a decrease in the reaction rate occurring over 20-60 seconds, after which a constant rate was generally obtained. This process appeared to be first order and rate constants obtained for the hysteresis were all of the order of $0.03\pm0.01\ s^{-1}$ over the NADH concentration range 50-300 μM. Measurements were made of the initial rate v_i and the reaction rate v_1 following this hysteresis and both are shown in Figure 6.2a. Secondly, as shown in Figure 6,2b, inhibition of the reaction rate was observed as the NADH concentration increased, this effect being more apparent for measurements of v_1 than for initial rate measurements.

A double reciprocal plot of the initial rate data for NADH concentrations up to 21 μM is shown in Figure 6.2c. From this plot values for K_m of 4.0 μM and for Vmax/[E] of $0.080\ s^{-1}$ ($0.17\ s^{-1}$ if a lower enzyme concentration actually applies (2.2.1)) were obtained.

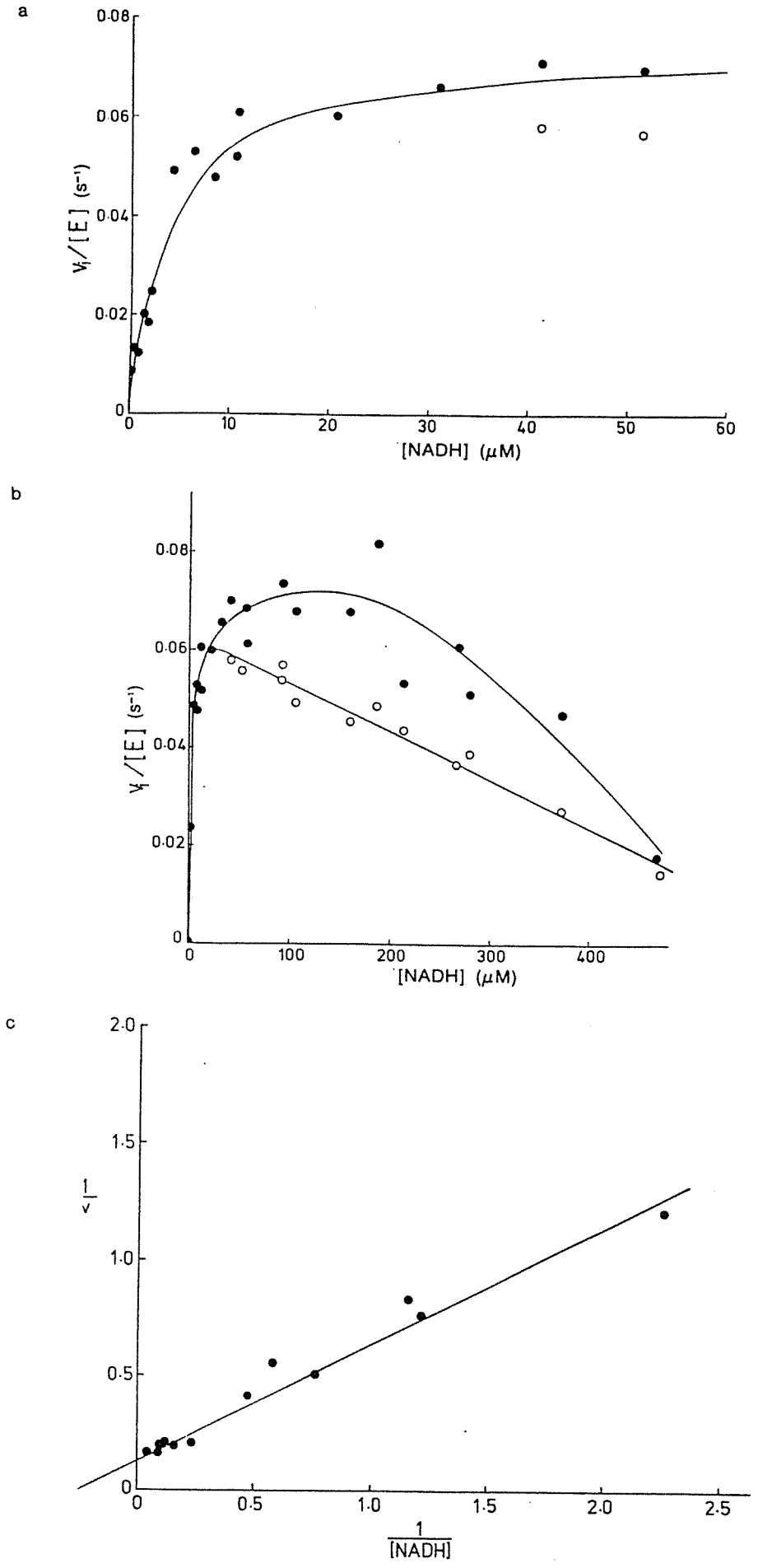
At NADH concentrations up to 75 μM, at a fixed propionic anhydride concentration of 1.3 mM, the change in absorbance at 340 nm represented almost total oxidation of the available NADH (calculated using an

Figure 6.2 The Effect of NADH Concentration on the Rate of the Reverse Reaction.

6.2 (a) and (b) The NADH concentration was varied in solutions containing enzyme (2.4 μM) and propionic anhydride (1.3 mM) in electrolyte solution. Both initial rates (●) and final rates (○) are shown.

6.2 (c) A plot of $1/v_i$ against 1/[NADH] for the lower NADH concentrations (up to 21 μM) is shown. From this plot values for K_m of 4.0 μM and for $V_{max}/[E]$ of 0.080 s^{-1} (or 0.017 s^{-1}) were obtained.

Figure 6:2 THE EFFECT OF NADH CONCENTRATION ON THE RATE OF THE REVERSE REACTION



extinction coefficient of 6220 $l.mol^{-1}.cm^{-1}$ (Horecker & Kornberg, 1948). For example in Figure 6.3a the A_{340} versus time traces for a series of reverse reactions at varying low concentrations of NADH are shown.

For enzyme samples from some preparations, at very low levels of NADH, the change in absorbance was greater than that which could be accounted for by the amount of NADH supposedly present, raising the possibility that a small amount (approximately 0.13 moles NADH/mole active sites) of NADH had remained bound to the enzyme throughout the preparative procedure. Other evidence (7.3.1) supported this proposal, and a similar conclusion has been reached by Hart & Dickinson (1983). Attempts to remove such NADH by the addition of activated charcoal to the enzyme solution and subsequent centrifugation were unsuccessful as this treatment had no effect on ΔA_{340}.

As shown in Figure 6.3b, curve A, except in those cases in which there was NADH already bound to the enzyme, the amount of NADH oxidized represented 91% of the initial concentration of NADH. At such high concentrations of propionic anhydride, it is not clear why all the available NADH was not oxidised. However, all results obtained, using many different NADH solutions in many separate experiments, were consistent with this figure of 91%. Since NAD^+ is produced by the reverse reaction it is possible that competition between NAD^+ and NADH for enzyme binding sites and inhibition of the reverse reacton by NAD^+ prevents the oxidation of all the NADH. Calculation of the relative amounts of Enzyme.NAD^+ and Enzyme.NADH based on the respective dissociation constants indicates that for an NAD^+:NADH ratio of 9:1 the ratio of the two enzyme species Enzyme.NAD^+:Enzyme.NADH should be approximately 57:43. As these two species are present in approximately equal amounts, it is also possible that there is competition between the reverse and dehydrogenase reactions, so that little or no change in absorbance is observed as a result.

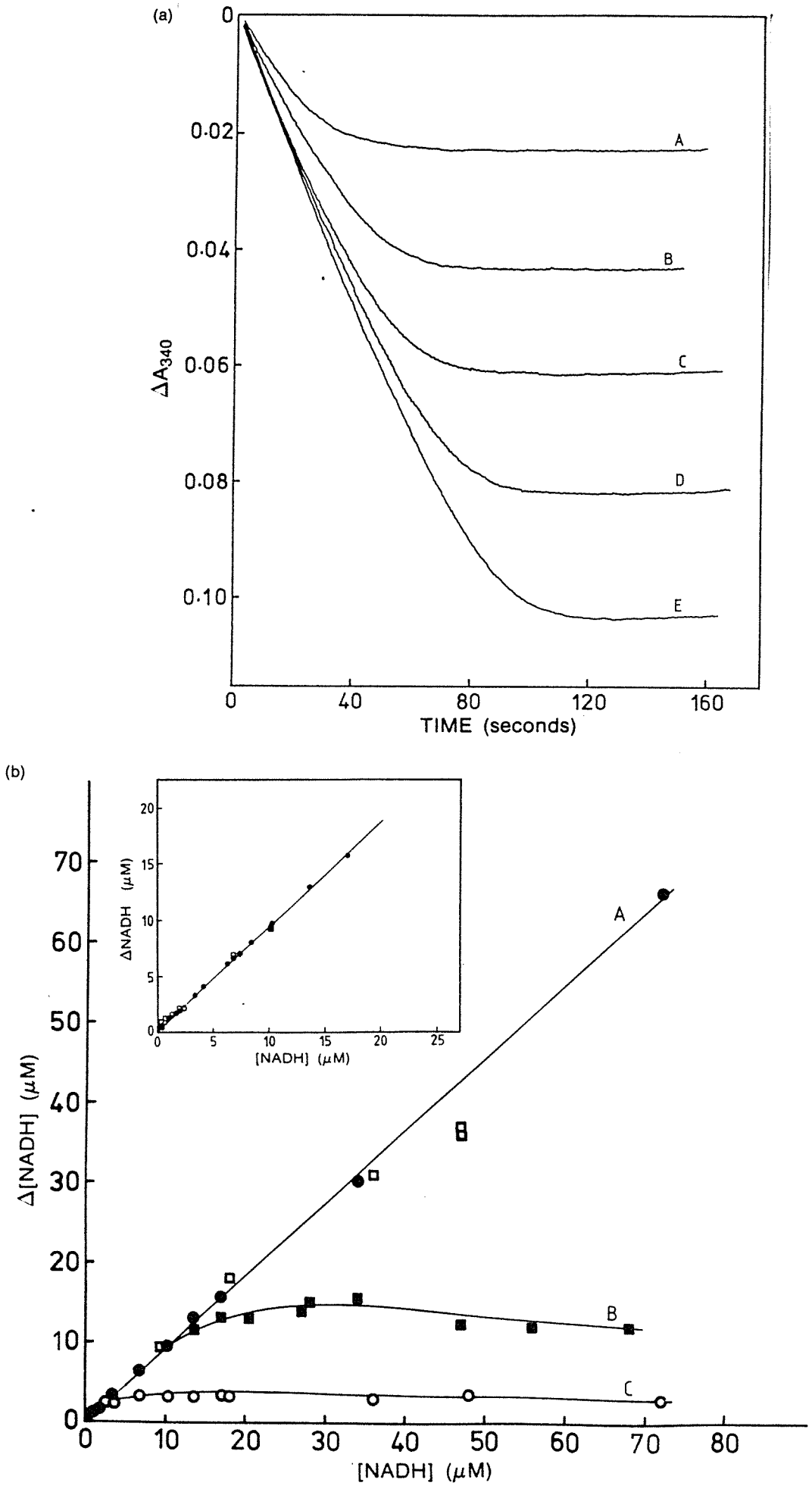
For reactions involving excess propionic anhydride, a very slow increase in A_{340} was frequently observed following oxidation of most of the NADH, but before enough of the propionic anhydride had hydrolysed to allow the dehydrogenase reaction to proceed at its maximum rate (Figures 5.8, 6.1, 6.3b, see also Figures 6.4a (A), 7.4a) suggesting that the dehydrogenase reaction is slightly more favoured under these conditions than the reverse reaction.

Figure 6.3 Utilisation of NADH in Reverse Reactions

6.3 (a) The NADH concentration was varied in solutions containing constant concentrations of enzyme (2.8 μM) and propionic anhydride(1.3 mM). Reaction traces (absorbance at 340 nm as a function of time) are shown. Concentrations of NADH in each reaction were 3.4 μM (A), 6.8 μM (B), 10.2 μM (C), 13.6 μM (D), and 17 μM (E).

6.3 (b) The amount of the available NADH used in the reverse reaction at four different initial propionic anhydride levels is shown. The enzyme concentration was 2.4 μM and the propionic anhydride concentrations were 78 μM (○), 260 μM (■), 520 μM (□), and 1.3 mM (●) The inset is an expansion of curve A at low NADH concentrations. A slope of 0.91 was obtained for this line.

Figure 6:3 UTILIZATION OF NADH DURING THE REVERSE REACTION



6.3.1.1 The Effect of NADH at Lower Propionic Anhydride Concentrations

At lower levels of propionic anhydride (≤520 µM), other effects were observed because, at these concentrations, the combined effects of reaction to form propionaldehyde and hydrolysis to propionic acid caused removal of propionic anhydride from the solution rapidly, usually within 4-5 minutes.

At these lower levels of propionic anhydride several effects of the NADH concentration were observed. Figure 6.4a shows a series of traces at differing NADH levels at an initial propionic anhydride concentration of 130 µM. At NADH concentrations of 7 µM or less, the absorbance reached a minimum value when most (90%) of the NADH had been oxidized. A significant subsequent increase in absorbance was not observed for several minutes, by which time sufficient propionic anhydride had hydrolysed so that the dehydrogenase reaction was no longer inhibited. Increasing the NADH concentration above 7 µM resulted in only a very small additional increase in ΔA_{340}, indicating that a much smaller proportion of the NADH was now being utilized. Also, after reaching an absorbance minimum, an immediate increase in A_{340} was observed.

The effects of NADH concentration on reaction rates were similar to those observed previously at higher propionic anhydride concentrations. At an initial propionic anhydride concentration of 130 µM the initial steady state rate reached a maximum at an NADH concentration of 7 µM (Trace A). Reference to Figure 6.4a shows that this rate was sustained for a longer time at higher NADH concentrations (14 µM, Trace B). The maximum rate obtained (9 µmoles NADH/min/l) was comparable to the rates obtained previously (7 µmoles NADH/min/l) at the higher propionic anhydride concentration of 1.3 mM (as for the data presented in Figure 6.2a). At much higher NADH concentrations (112 µM) a decrease in the initial rate (inhibition) was observed (Trace E), similar to that observed previously (Figure 6.2b)

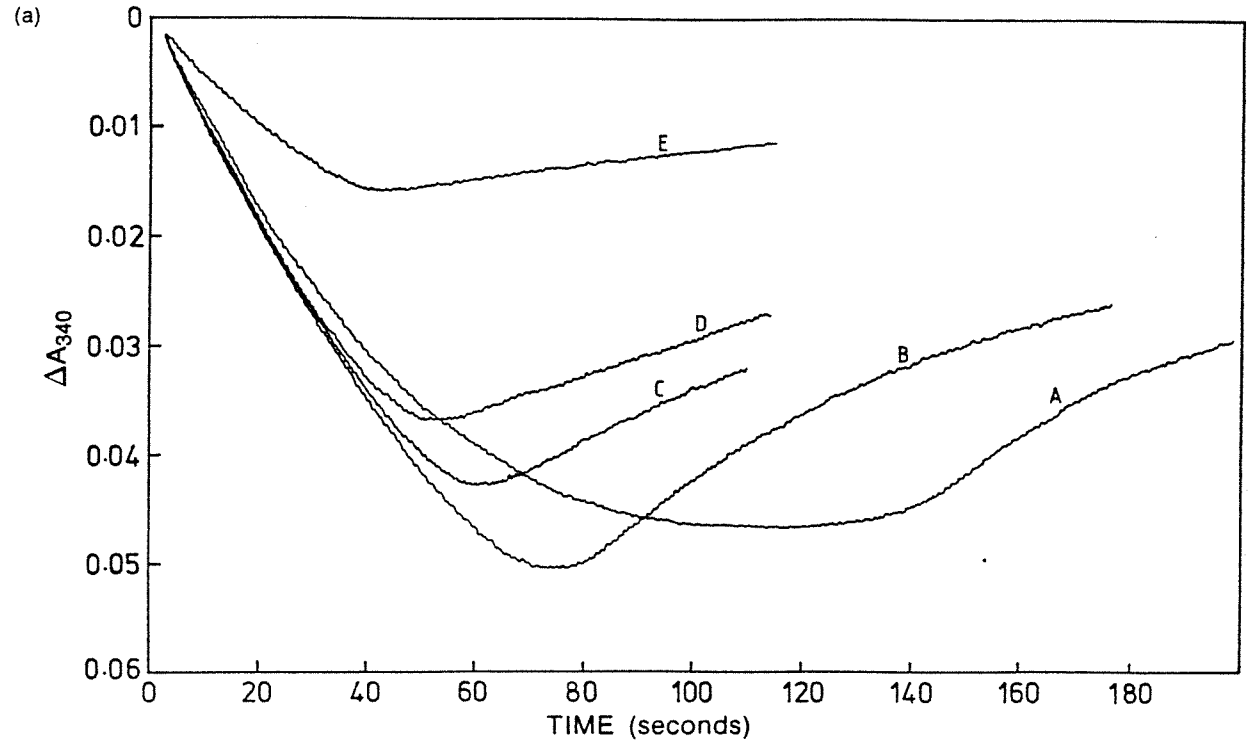
Figures 6.4b shows a similar set of traces at a higher propionic anhydride concentration. These show that at lower initial concentrations of propionic anhydride, the optimum utilisation of substrates occurs at correspondingly lower NADH concentrations. This trend is reflected in the two lower curves in Figure 6.3b in which a distinct maximum change in NADH concentration was observed at each initial concentration of propionic anhydride. The inset to Figure 6.3b is an expansion of this portion of the graph, showing results at

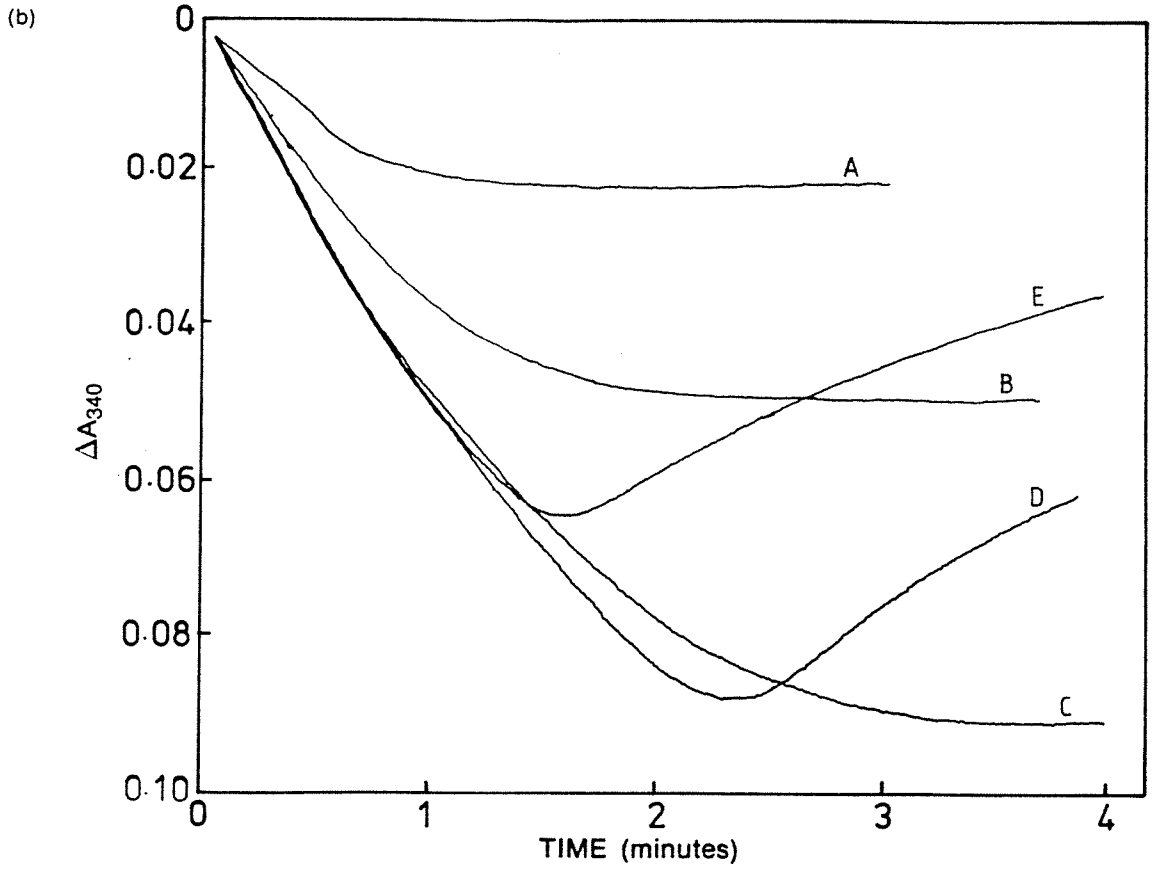
Figure 6.4 Reaction Traces for Reverse Reactions at Different NADH Concentrations.

7.4 (a) Reaction solutions contained enzyme (2.7 μM), propionic anhydride (130 μM) and NADH. NADH concentrations were 7 μM (A), 14 μM (B), 28 μM (C), 56 μM (D), and 112 μM (E).

7.4 (b) Reaction solutions contained enzyme (2.7 μM), propionic anhydride (260 μM) and NADH. NADH concentrations were 2.8 μM (A), 7 μM (B), 14 μM (C), 28 μM (D) and 56 μM (E).

Figure 6:4 PROGRESS CURVES FOR REVERSE REACTIONS AT VARYING NADH CONCENTRATIONS





several NADH concentrations. Thus, for each propionic anhydride concentration, there was an optimum NADH concentration. This was the highest concentration at which the maximum proportion (90%) of NADH was oxidised. (For example Trace A Figure 6.4a). The maximum initial reaction rate was also obtained at this optimum level. Doubling the amount of NADH produced only a slight increase in ΔA_{340} without affecting the initial rate. However an increase in the NADH concentration above this level caused a decrease in ΔA_{340} (Figure 6.5a). Also, as the amount of NADH increased above the optimum, there was a marked decrease in the time taken for the absorbance to reach a minimum (t_1 or t) and this was immediately followed by an increase in absorbance, suggesting that the propionic anhydride concentration becomes limiting in the reaction and oxidation of NADH does not proceed as a result. These results indicate that catalysis of anhydride hydrolysis by NADH becomes a very significant factor in controlling the reaction at higher NADH concentrations.

The results of an experiment designed to test this explanation are shown in Figure 6.5b. NADH (16.8 μM) and propionic anhydride (130 μM) in electrolyte solution were mixed for 15 seconds prior to addition of enzyme (3.13 μM) (Trace C). The results of two control experiments are also shown. In the first of these, (Trace B) propionic anhydride was mixed with electrolyte solution and then NADH and enzyme were added simultaneously 15 seconds later. In the other control experiment (Trace A) propionic anhydride was added last.

The change in absorbance ΔA_{340} and the time taken to reach the minimum absorbance, t, in traces B and C were measured and compared with those in trace A and used to provide estimates of the relative rate of hydrolysis, on the assumption that the reverse reaction ceases as the propionic anhydride concentration becomes limiting. For propionic anhydride premixed with NADH a reduction in ΔA_{340} of 40% and a decrease in t of 50% were observed, compared with reductions of only 14% and 3% respectively for propionic anhydride in electrolyte solution alone. This confirmed that the presence of NADH was causing an increase in the rate of hydrolysis of the propionic anhydride. Figure 6.6 shows that there was a linear relationship between the optimum amount of NADH used and the initial propionic anhydride concentration. At concentrations lower than or equal to this maximum, oxidation of NADH was consistent with the 90% observed at high levels of propionic anhydride, while at higher concentrations, a smaller

Figure 6.5 The Effect of NADH on the Hydrolysis of Propionic Anhydride.

6.5 (a) The Effect of NADH on ΔA_{340}.

Reaction solutions contained enzyme (2.7 μM) and propionic anhydride (130 μM (○) or 260 μM (●)).

6.5 (b) The Effect on the Reverse Reaction of Premixed NADH and Propionic Anhydride.

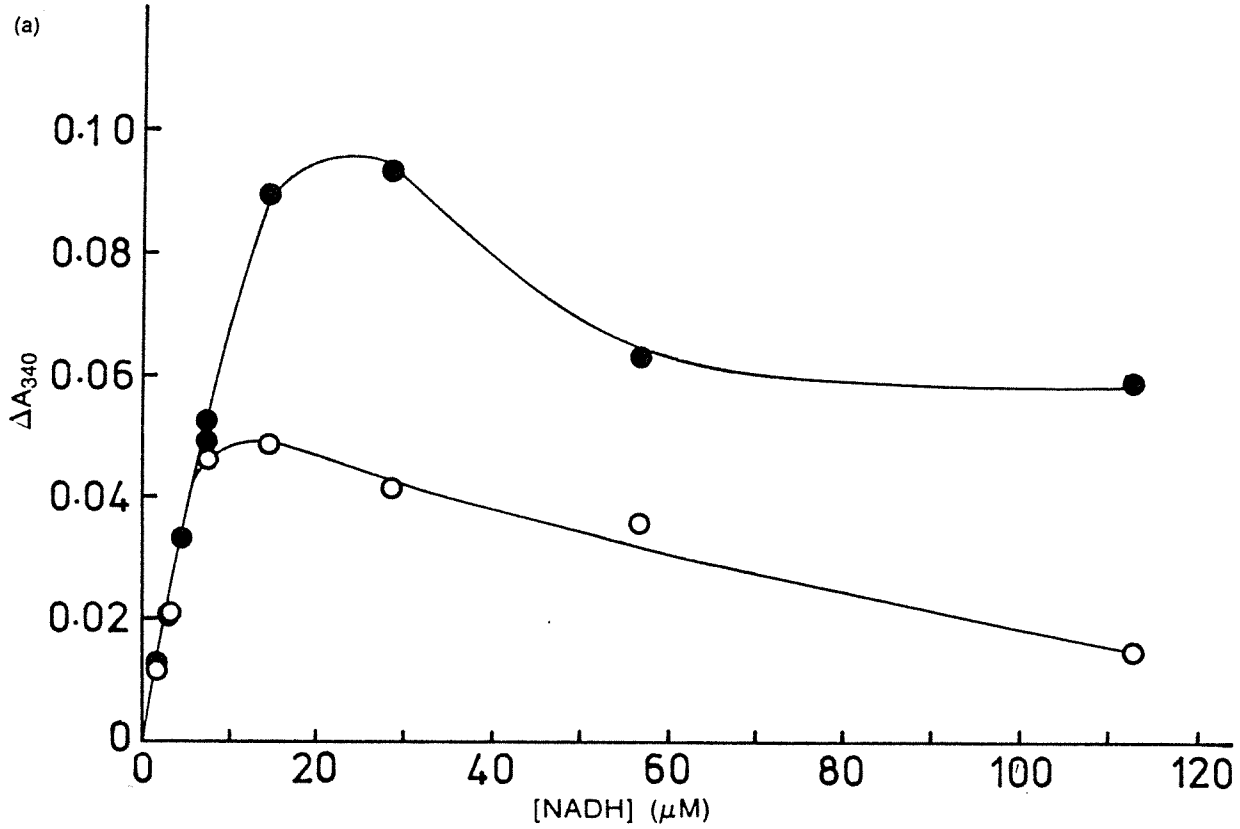
Solutions contained enzyme (3.13 μM), NADH (16.8 μM) and propionic anhydride (initially 130 μM) in electrolyte solution.

Trace A. Propionic anhydride was added last

Trace B. Propionic anhydride was added to the electrolyte solution. Enzyme and NADH were added simultaneously after 15 seconds.

Trace C. Propionic anhydride and NADH were mixed in electrolyte solution. Enzyme was added after 15 seconds.

Figure 6:5 THE EFFECT OF NADH ON HYDROLYSIS OF PROPIONIC ANHYDRIDE



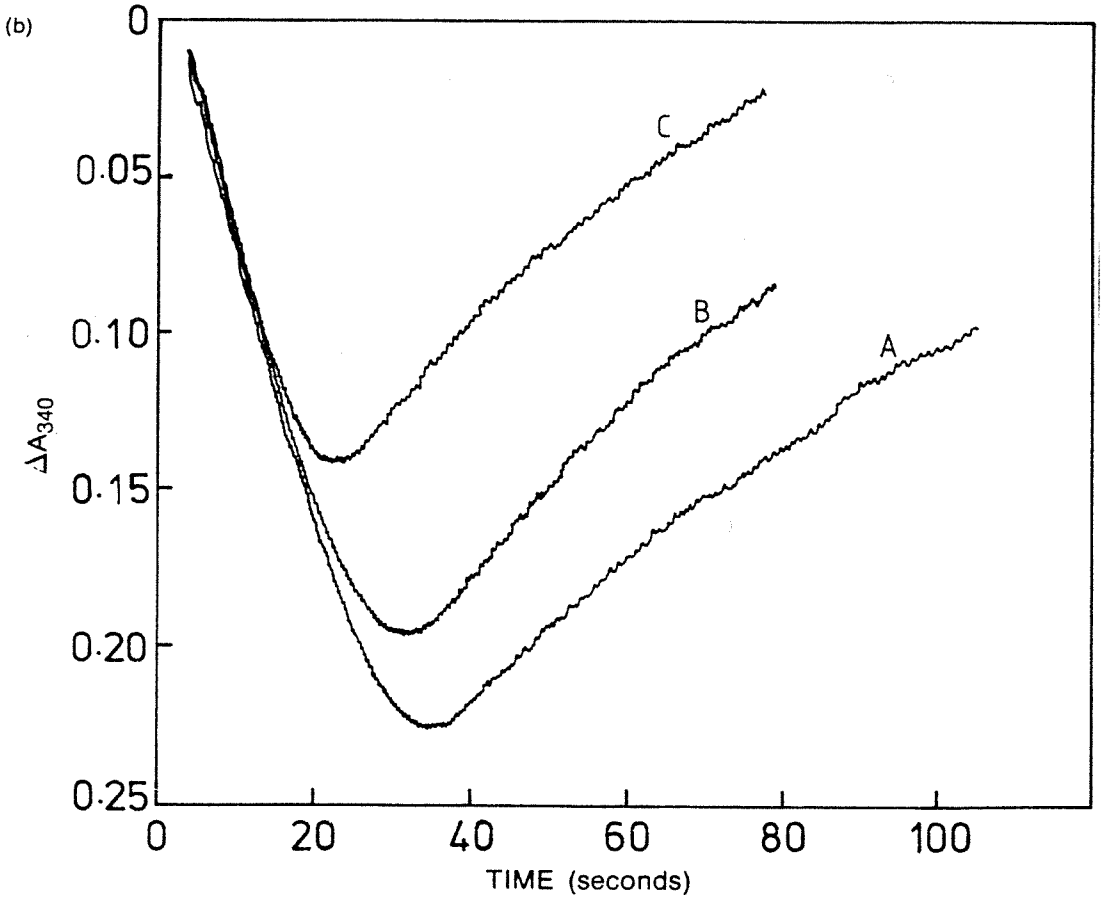


Figure 6:6

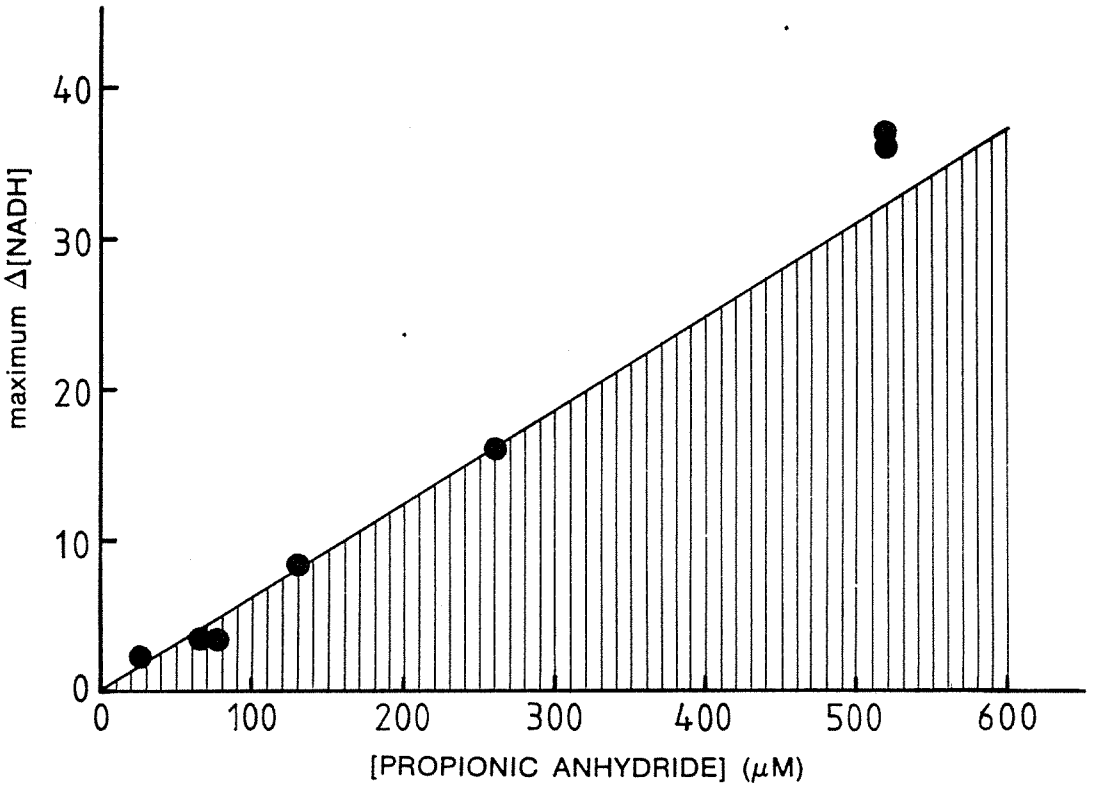


Figure 6.6 Optimum Utilisation of NADH as a Function of the Propionic Anhydride Concentration. The plot shows the highest NADH concentration at which the maximum proportion (91%) of the NADH was oxidised during the reaction. For combined concentrations of propionic anhydride and NADH which fell in the shaded area, 91% of the available NADH was oxidised during the reaction. For concentrations outside this area, a smaller proportion of the NADH was oxidised as the propionic anhydride hydrolysed too rapidly for the reaction to proceed normally. (The enzyme concentration in these experiments was 2-3 μM in all cases.)

proportion of the total NADH was oxidised. This plot is also important in terms of experimental design, as it is clear from the Figure that for concentrations of propionic anhydride and NADH which lie in the area under the line (shaded), then NADH is the species limiting the extent of the reverse reaction, whereas at concentrations which lie in the area above the line, the amount of propionic anhydride is limiting, because of the increased rate of hydrolysis produced by the NADH. Thus, at lower initial propionic anhydride concentrations, it was necessary to use lower NADH concentrations to avoid excessive curvature of the absorbance trace due to rapid propionic anhydride hydrolysis.

It is also apparent from these results that measurements of t_1 and t_2 or t were not particularly useful, except in a qualitative fashion or under certain conditions. In cases in which separate measurements of t_1 and t_2 could be made, t_1 corresponded to the time at which 90% of the NADH was used up (and was therefore a function of the initial concentration of NADH) whereas t_2 corresponded to the time at which the dehydrogenase reaction rate became significant and was therefore a function of the rate of propionic anhydride hydrolysis (and hence also a function of the buffer, NADH and enzyme concentrations), the initial propionic anhydride concentration, and probably also the amounts of NAD^+ and propionaldehyde produced by the reverse reaction. Reactions for which only a single time measurement, t, could be made represented those cases in which substantial amounts of NADH remained at the time the dehydrogenase reaction became favoured, adding an additional complicating factor so that this time measurement probably does not correspond exactly to either t_1 or t_2 of the previous case, although it clearly resembles t_2 rather than t_1.

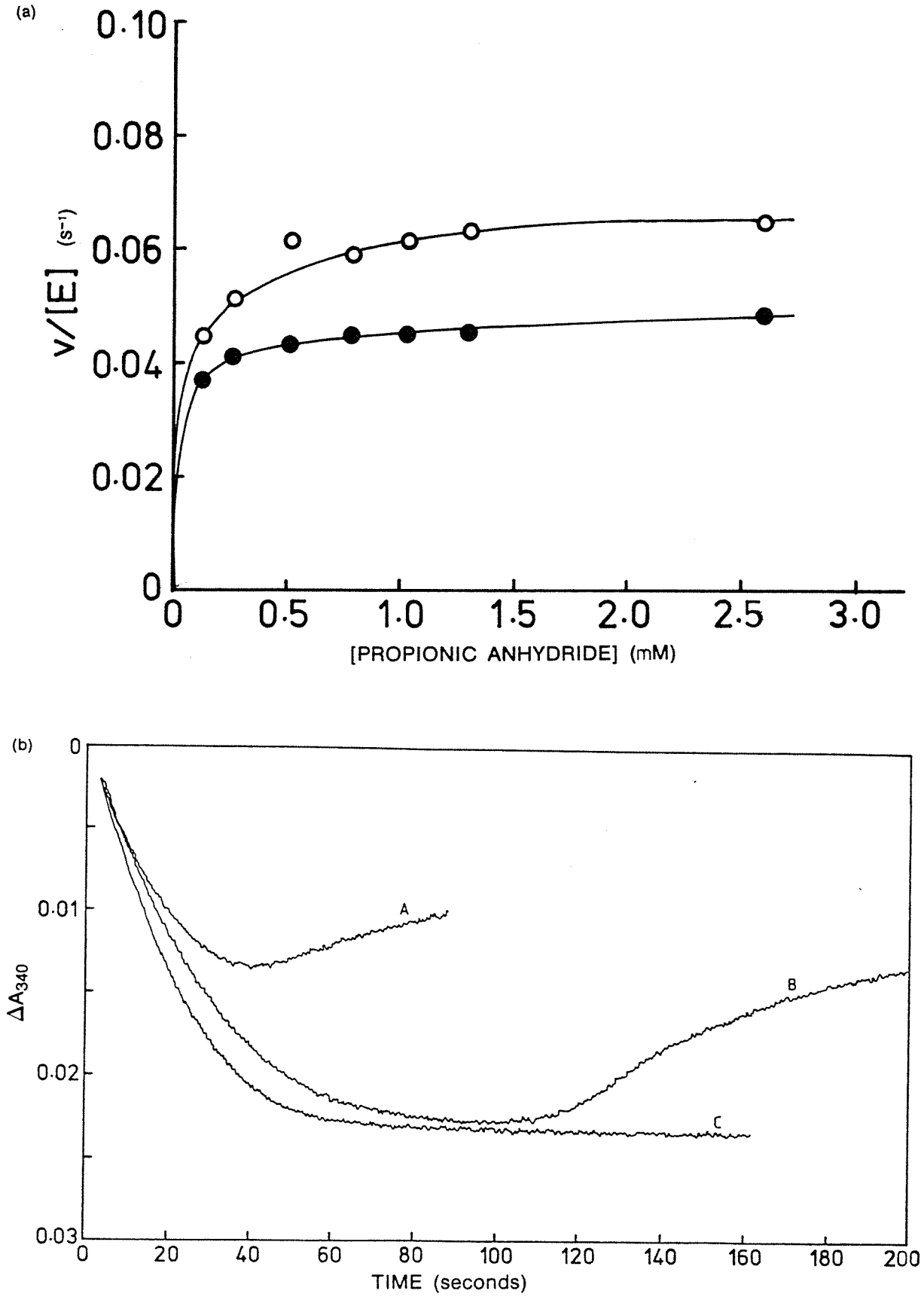
6.3.2 The Effect of Propionic Anhydride Concentration on the Reverse Reaction

The effect of propionic anhydride concentration on the initial rate of the reverse reaction is shown in Figure 6.7a & b. Figure 6.7b also shows clearly that in cases in which the maximum proportion (90%) of the NADH was oxidised, the propionic anhydride which remained unhydrolysed inhibited the subsequent dehydrogenase reaction. It was not possible to obtain accurate data at low propionic anhydride concentrations, despite numerous attempts. Propionic anhydride was removed from solution so quickly that even initial rates were

Figure 6.7 The Effect of the Propionic Anhydride Concentration on the Reverse Reaction.

6.7 (a) The dependence of the reaction rate on the propionic anhydride concentration is shown. Solutions contained 2.7 μM enzyme and 90 μM NADH in electrolyte solution. Both the initial rate (O) and the rate of the linear part of the reaction trace (●) are shown as hysteresis was observed in the first 10-20 seconds of the reaction.
6.7 (b) Reaction traces showing the effect of the propionic anhydride concentration on the progress of the reverse and subsequent dehydrogenase reactions. Reaction traces solutions contained enzyme (2.1 μM) and NADH (3.6 μM) in electrolyte solution. Propionic anhydride concentrations were 26 μM (A), 65 μM (B), and 520 μM (C).

Figure 6:7 THE EFFECT OF PROPIONIC ANHYDRIDE CONCENTRATION ON THE REVERSE REACTION



difficult to obtain and were largely inaccurate, due to the time required for conventional mixing (normally 4-7 seconds).

In addition there was no NADH concentration that could be used to produce a maximum rate at all levels of propionic anhydride. As observed in the previous section there was an optimum NADH concentration at each propionic anhydride level. Catalysis of propionic anhydride hydrolysis became progressively more significant at lower anhydride concentrations, interfering with the reverse reaction.

All propionic anhydride concentrations used in these experiments represented a substantial initial excess of this reagent over other reacting species and consequently the initial rates were all close to saturating rates. However as most of the propionic anhydride present was removed, not by the reverse reaction, but by rapid hydrolysis, the concentrations of propionic anhydride quoted only apply to initial rate measurements and can not be used quantitatively for the interpretation of any other measurements (such as ΔA_{340}, t_1, t_2, t). The involvement of reaction substrates and products and the enzyme itself in catalysis of this hydrolysis caused many experimental complications and limitations in interpretation of the results.

An attempt was made to determine approximately how fast propionic anhydride hydrolysed in solutions containing reaction species. Details of this experiment are given in chapter 7 (7.3.1.3).

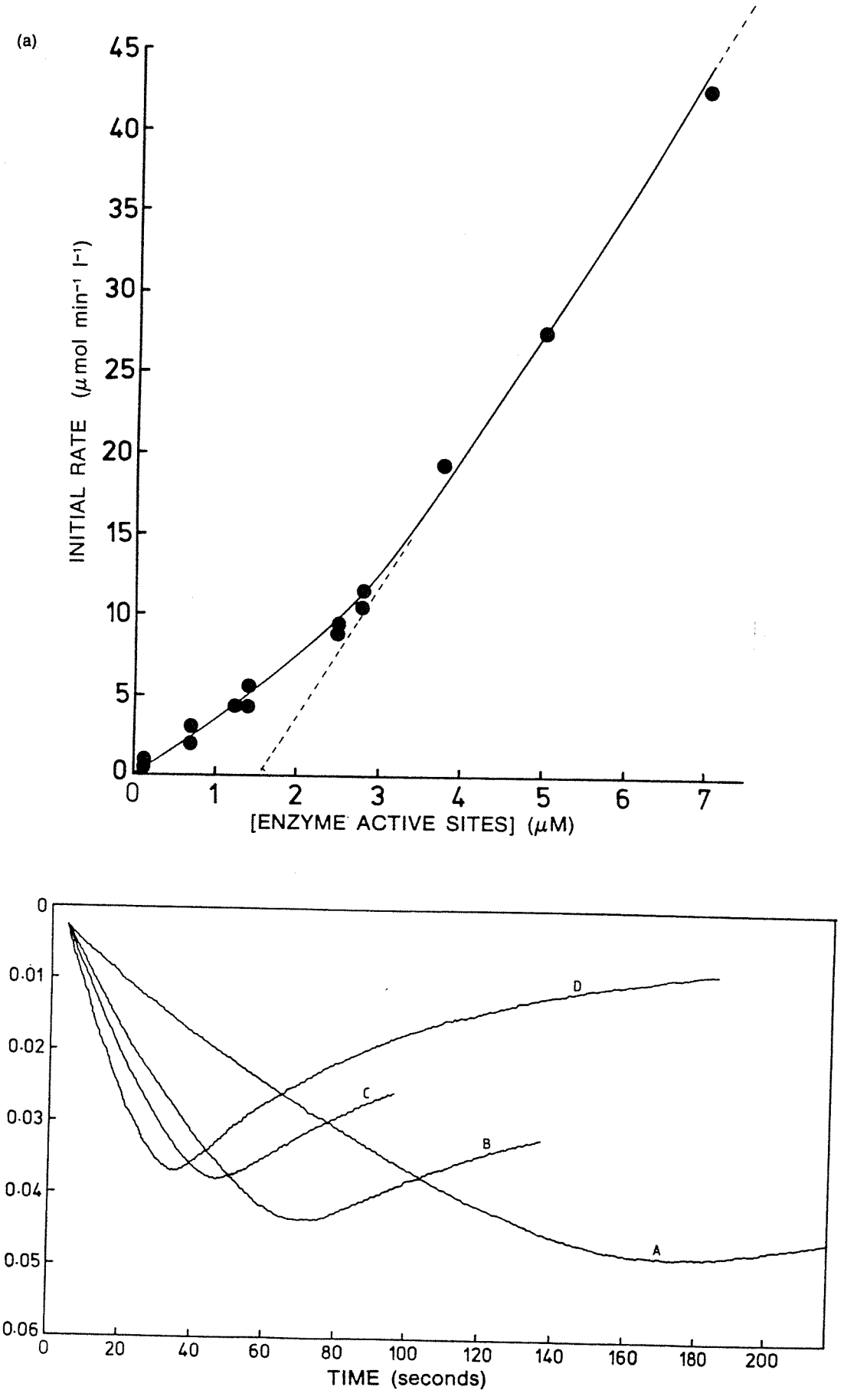
6.3.3 The Effect of Enzyme Concentration on the Reverse Reaction

As observed in chapter 5, the initial rate of the reverse reaction was dependent on the enzyme concentration. At low enzyme concentrations (below 1.0 μM), a linear dependence was observed. However, at higher enzyme levels there were departures from linearity towards faster rates (Figure 6.8a). Upon close re-examination of Figure 5.2b it was apparent that this data could also be fitted to a curve instead of a straight line.

This increase in specific activity at higher enzyme concentrations is very similar to that observed recently by L.F. Blackwell (personal communication) for the dehydrogenase reaction (2.1.3.5). As for the dehydrogenase activity, the effect was particularly notable upon dilution of the enzyme stock concentration prior to experiments at low enzyme concentrations, and therefore appears to involve some form of cooperativity at high enzyme concentrations. A k_{cat} value of 0.085 s^{-1}

Figure 6.8 The Effect of Enzyme Concentration on the Reverse Reaction.
6.8 (a) The dependence of the reaction rate on the enzyme concentration is shown. Reaction solutions also contained NADH (24 μM) and propionic anhydride (130 μM).
6.8 (b) Reaction traces showing the effect of the enzyme concentration on the progress of the reverse and subsequent dehydrogenase reactions. Reaction solutions contained NADH (24 μM) and propionic anhydride (130 μM) in electrolyte solution. Enzyme concentrations were 1.2 μM (A), 2.52 μM (B), 3.77 μM (C), and 5.02 μM (D).

Figure 6:8 THE EFFECT OF ENZYME CONCENTRATION ON THE REVERSE REACTION



was determined from the slope of a straight line fitted through the steepest part of the curve (dotted line, Figure 6.8a), and this value is very similar to that obtained previously from the double reciprocal plot of the NADH dependence of the reverse reaction (Figure 6.2c).

The values of k_{cat} obtained in these two experiments are much slower than those obtained previously from similar experiments carried out in 0.2 M pH 7.3 phosphate buffer (Chapter 5, refer Figure 5.2b). Since saturating concentrations of NADH and propionic anhydride were used in each case it must be assumed that the reaction rates were faster in the buffer solutions or that the enzyme concentration was incorrectly estimated in one of these experiments. As discussed in Chapter 2, accurate determination of the enzyme concentration is not a simple matter, in view of the change in specific activity of the enzyme with enzyme concentration which is observed (as above, Figure 6.8a). Since there is some evidence (see Figure 6.14b) that the rate of the reverse reaction was higher at higher buffer concentrations, either or both of these factors may account for the discrepancy between the values of k_{cat}.

A series of absorbance traces for reverse reactions at three different enzyme concentrations is shown in Figure 6.8b. The increase in the reaction rate at higher enzyme concentrations was clearly observed. In addition, a decrease in t_1, the time required to reach the minimum absorbance, was apparent at higher enzyme levels, presumably, in this case, due to faster hydrolysis of the anhydride, as the NADH was not all oxidised and this is consistent with enzyme-catalysed hydrolysis of the propionic anhydride.

6.3.4 The Effect of pH on the Reverse Reaction with Propionic Anhydride

The results of reverse reactions carried out at various pH values within the range 5-10 are given in Table 6.1. The initial rate data is also plotted as a function of pH in Figure 6.9. Solutions contained 2.5 μM enzyme 21 μM NADH and 2.6 mM propionic anhydride.

Figure 6:9 THE EFFECT OF pH ON THE REVERSE REACTION

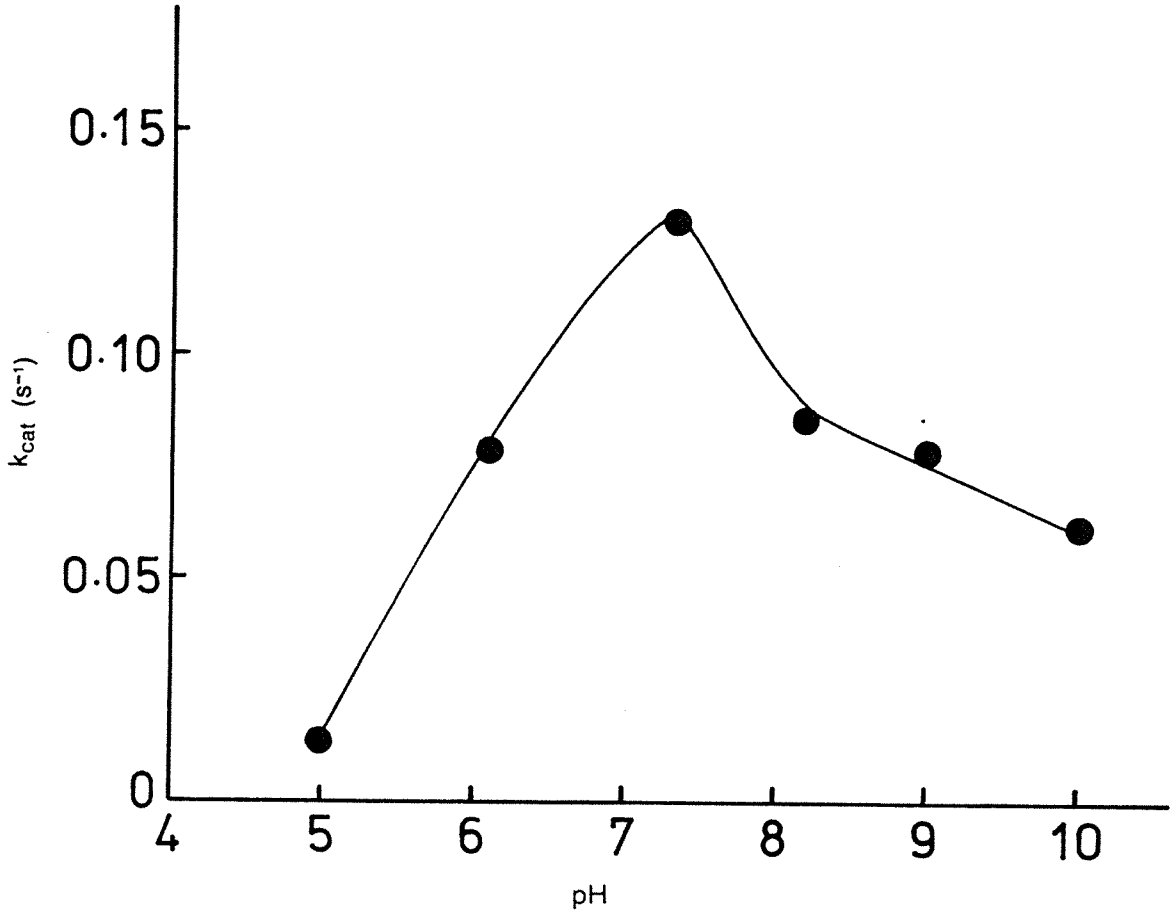


Figure 6.9 The Effect of pH on the Rate of the Reverse Reaction. Reaction solutions contained enzyme (2.5 μM), NADH (22 μM), and propionic anhydride (260 μM). Buffers were all of constant ionic strength (0.1 M) and were prepared as described in Methods, Chapter 4.

TABLE 6.1

The Effect of pH on the Steady State Reverse Reaction

pH	k_{cat}	t_1	ΔNADH
5.2	0.013	66	1.53
6.2	0.077	146	16.4
7.3	0.129	96	20.6
8.2	0.084	132	21.2
9.3	0.077	140	20.6
10.0	0.060	149	20.2

[E] = 2.52μM, [NADH] = 22 mM, [propionic Anhydride] = 260 μM

Over the pH range 6-10 there was not a very marked difference in reaction rates, as expected from the linearity of absorbance traces in previous experiments at high propionic anhydride concentrations. The highest rate obtained was at pH 7.3, but the amounts of NADH oxidized at each pH were also very similar. However at pH 5.2, both the reaction rate and the extent of the reaction (measured in terms of the decrease in NADH concentration) were markedly reduced. The pH profile of the reverse reaction was similar to that obtained in Chapter 4 for the dehydrogenase reaction, indicating that both reactions probably involve the same intermediates.

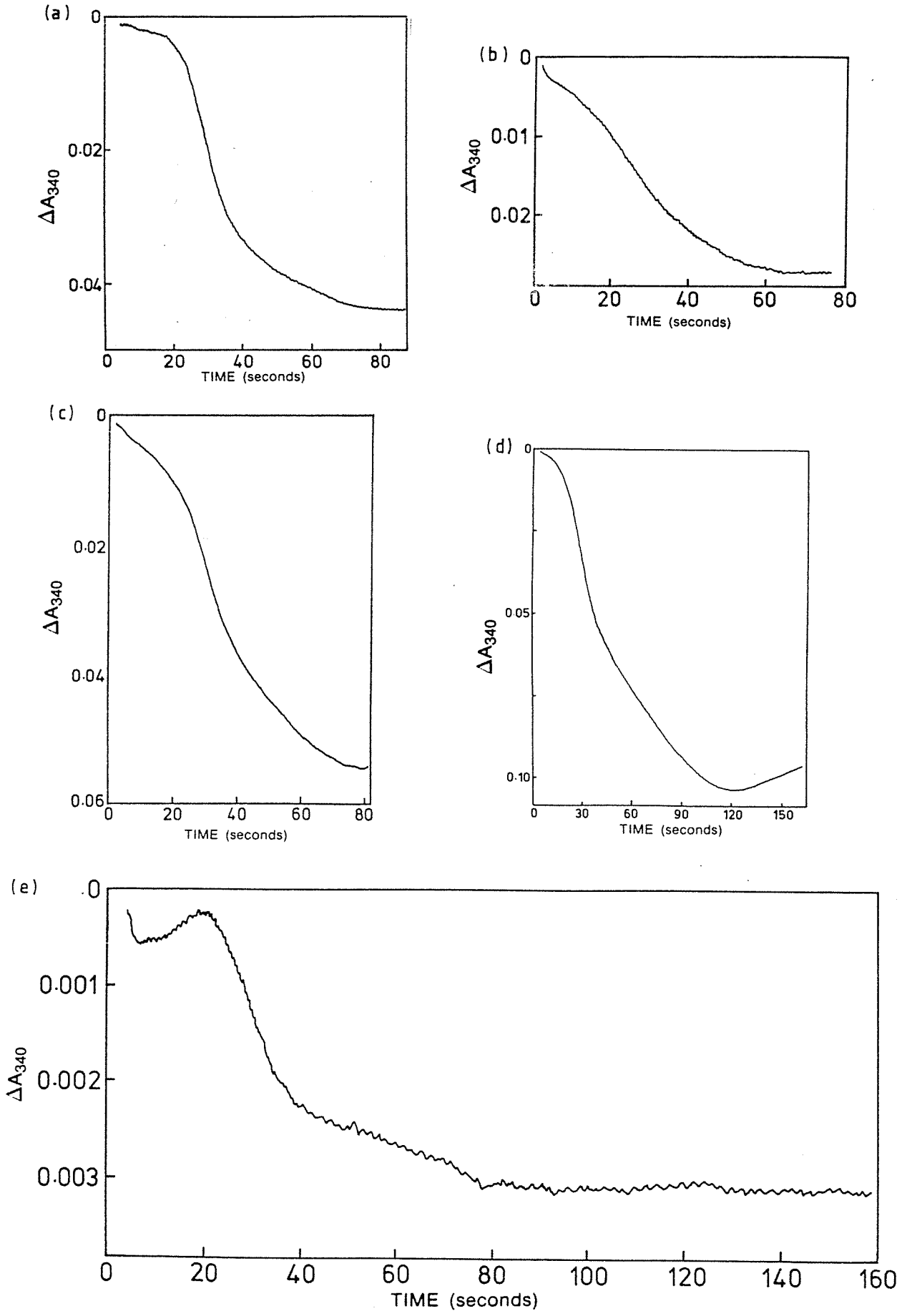
6.3.5 Lag Phases

Lag phases were observed in the initial stages of many reverse reactions, but their duration and occurrence was highly variable (Figure 6.10). Provided no lag phase was observed, agreement to within 1-2 seconds for time measurements (t_1, t_2, t) and to within 1-2% for ΔA_{340} was typical for duplicate reactions, but in marked contrast to this general very high reproducibility of reverse reactions, when lag phases were observed reproducibility for duplicate experiments was very poor. Lag phases were observed most frequently at an initial propionic anhydride concentration of 130 μM. Some effect of NADH was also noted, but quantitative results could not be obtained. When the concentrations of all reagents present in reaction conditions which repeatedly exhibited lag phases were doubled, no lag phase was observed.

Figure 6.10 Lag Phases observed during Reverse Reactions.
Reaction traces showing examples of the lag phase which was frequently observed in the reverse reactions. The concentrations of reactants used in each reaction are given below.

Figure	[E](μM)	[NADH](μM)	[Propionic Anhydride](μM)
a	3.1	8.4	260
b	3.0	8.4	130
c	3.1	16.8	260
d	2.8	34.0	260
e	2.8	0.34	260

Figure 6:10 LAG PHASES OBSERVED DURING REVERSE REACTIONS



The lag phases were observed in either NADH absorbance or fluorescence and also in the stopped flow experiments in both modes. For reverse reactions which involved lengthy lag phases, the time t_1 was correspondingly longer than similar reactions in which the lag was absent or very short. This indicated that the lag phase was a true feature of the reverse reaction; i.e. a process preceding the establishment of the final steady state. In some cases (e.g.Figures 6.10a & d) a rapid decrease in absorbance was observed immediately following the lag, which was not the normal steady state. Also, very rarely, at very low NADH concentrations other processes were observed preceding the lag. Figure 6.10e shows that in these cases there was an initial rapid decrease in A_{340}, then an increase back to the original absorbance level, followed by a normal reverse steady state decrease in absorbance.

This strongly suggests that the lag phase actually involves a competition between the reverse reaction and the dehydrogenase reaction at some point on the reaction pathway (see Scheme 6.1), and before the steady state is established.

Scheme 6.1

$$E.NADH \rightleftharpoons E.NADH.acyl \rightleftharpoons E.NAD^+.Ald \rightleftharpoons E.NAD^+ \rightleftharpoons E$$

Because of the poor reproducibility for duplicate experiments involving lag phases, it seems likely that the conditions in which such competition arises are somewhat unusual, and that the system is very sensitive to slight changes in reagent concentrations or ratios. It is significant that the conditions in which such increases in absorbance were observed involved NADH concentrations very similar to the enzyme concentration, or single turnover conditions (e.g. Figure 6.10e) in which there was more enzyme than NADH.

Considerable care was taken to ensure that mixing conditions were as consistent as possible and that all volumes were accurately measured. The reproducibility of experiments in which lags were not observed indicated that these conditions were being met. Because of the high temperature coefficient for the enzymic reactions, cuvettes containing electrolyte solution were pre-incubated in a waterbath at

25°C. Each time a pronounced lag phase was observed, the reaction was immediately repeated at the same concentrations of all reagents in an attempt to reproduce the same lag. Although the solutions were all allowed sufficient preincubation time for thermal equilibration, it is likely that in these duplicated experiments, the enzyme had been diluted in solution for shorter times than in the previous experiments. L.F. Blackwell (personal communication) has very recently observed a reduction in the specific activity of the enzyme upon dilution. This reduction appears to occur slowly and could possibly be the reason for the variability of lag phases. No other explanation is available at this stage.

Reproducibility of experiments involving lag phases was considerably improved for stopped flow experiments, in which several reactions could be studied by mixing from the same bulk solutions and in which the concentration of the enzyme (and consequently enzyme intermediates) was higher. These results are presented in section 6.3.10.

6.3.6 Inhibition Studies of the Reverse Reaction

6.3.6.1 The Effect of Added NAD^+ or Propionaldehyde

The effect on the reverse reaction of premixing NAD^+ or propionaldehyde with enzyme and NADH before the addition of propionic anhydride is shown in Table 6.2 and Figure 6.11.

TABLE 6.2

The Effect of Added NAD^+ and Propionaldehyde on the Reverse Reaction

Reagent	[NADH] μM	[Anhydride] μM	v/v°	$\Delta A/\Delta A^\circ$	T/T°
NAD^+ (8 μM)	8	130	0.82	0.68	0.78
NAD^+ (2 mM)	28	260	0.34	0.21	0.80
Prop[a] (100 μM)	28	260	0.45	0.40	0.71
Prop (20 mM)	28	260	0.17	b	b

$\Delta A = \Delta A_{340}$

a Prop is propionaldehyde

b not determined

Figure 6.11

6.11 (a) The Effect of Added Propionaldehyde on the Progress of the Reverse Reaction.

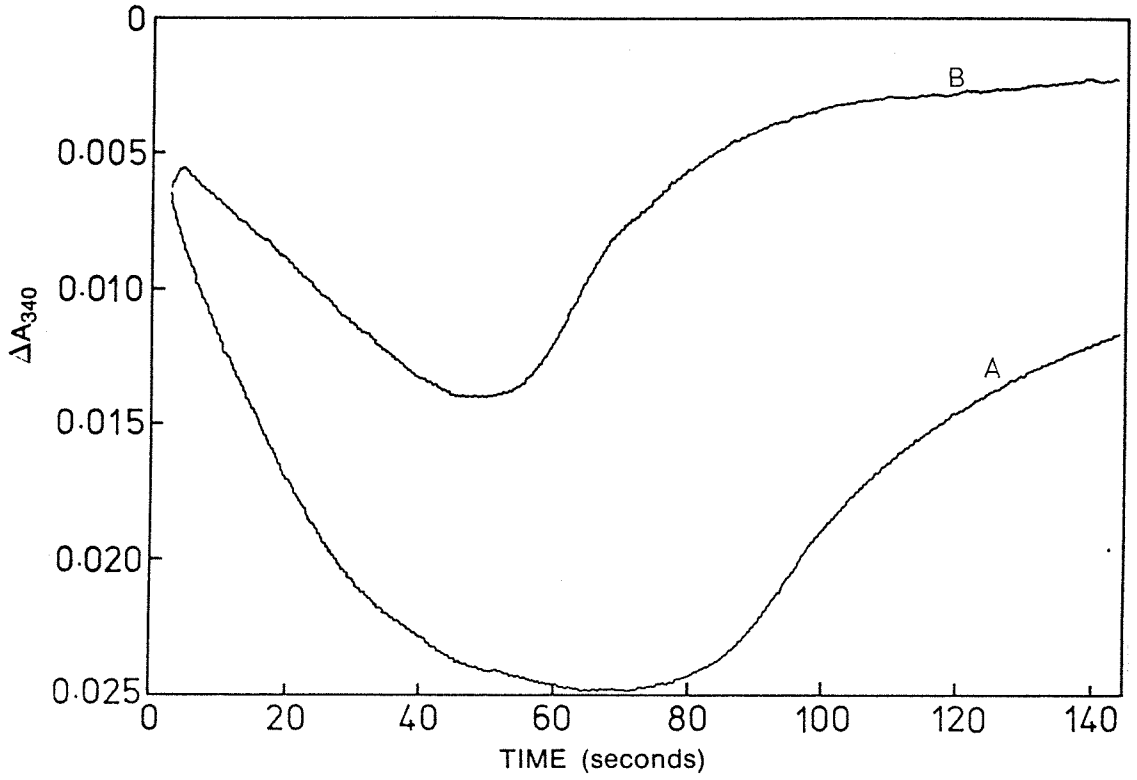
Reaction traces for the control (no added propionaldehyde) experiment (A) and a reverse reaction (B) in which propionaldehyde (100 μM) was premixed with enzyme (3.3 μM), NADH (3.9 μM) and propionic anhydride (130 μM).

6.11 (b) The Effect of Added NAD^+ on Progress of the Reverse Reaction.

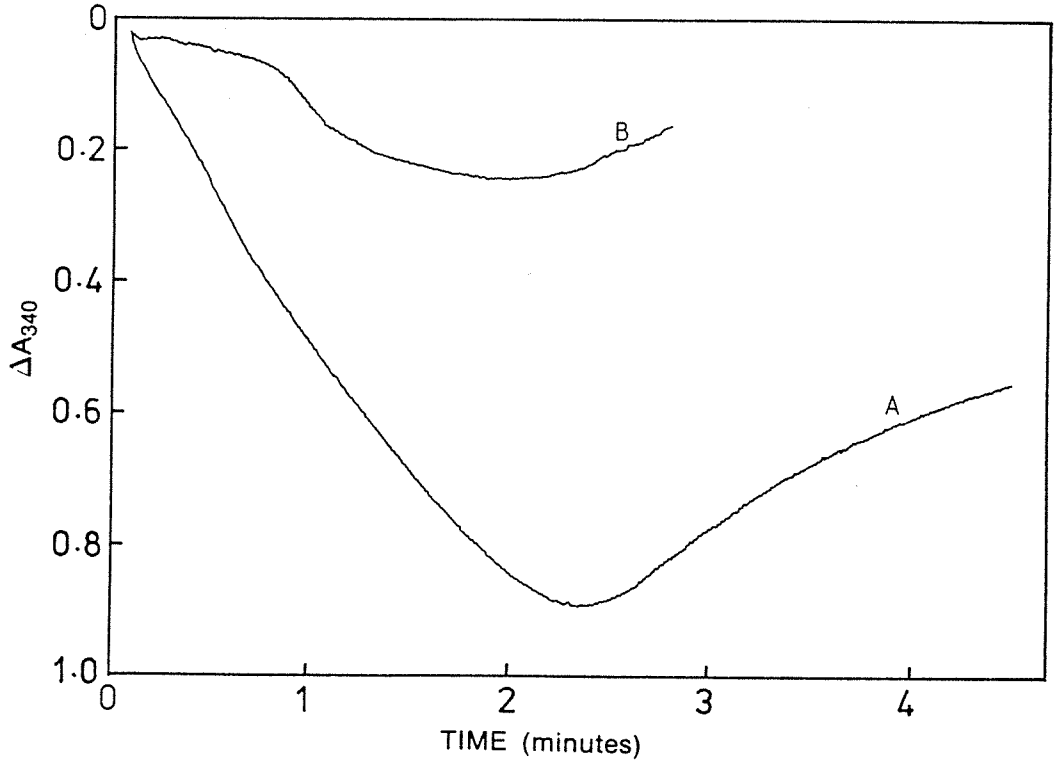
Reaction traces for the control (no added NAD^+) experiment (A) and a reverse reaction (B) in which NAD^+ (2 mM) was premixed with enzyme (2.7 μM), NADH (28 μM) and propionic anhydride (260 μM).

Figure 6:11

(a) THE EFFECT OF ADDED PROPIONALDEHYDE ON THE REVERSE REACTION



(b) THE EFFECT OF ADDED NAD^+ ON THE REVERSE REACTION



In all cases, inhibition of the reaction rate was observed, with accompanying decreases in ΔA_{340} and t. However, even very high concentrations of propionaldehyde (20 mM) did not totally inhibit the reverse reaction. As shown in Figure 6.11, there were complex absorbance changes in the initial stages of the reaction, the most notable of these being a a marked increase in the occurrence and appearance of the lag phase. Lag phases involving an increase in absorbance were frequently observed when either NAD^+ or propionaldehyde was present, as is apparent in the absorbance trace shown in Figure 6.11a, providing further evidence that the increase in absorbance involves competition with steps in the dehydrogenase pathway. These lag phases made it very difficult to obtain quantitative and reproducible data. The reaction rates given in Table 6.2 were all determined for the rate immediately following the lag.

A simple interpretation of these results is impossible because the amounts of the various substrates for the competing reverse and dehydrogenase reactions are changing throughout the reaction, but the accompanying hydrolysis of the propionic anhydride prevents the establishment of equilibrium between the two. The situation is particularly complicated for premixed propionaldehyde (100 μM, Figure 6.11a), since it can only bind to the enzyme.NAD^+complex (MacGibbon et al. 1977a, c). Thus inhibition by propionaldehyde cannot be simple competitive, since although propionic anhydride and propionaldehyde presumably both bind at the active site, propionaldehyde does not bind to the enzyme.NADH complexes. However, propionaldehyde will be able to react as soon as an enzyme.NAD^+ intermediate is formed (Scheme 6.1), and it is possible that, after a single turnover of the reverse presteady state intermediates, propionaldehyde could force the reaction in the opposite direction, producing NADH and a consequent increase in absorbance, as was observed. Failing this, a temporary build-up of Enzyme.NAD^+.Prop and Enzyme.NADH.acyl intermediates could occur, preventing release of products, and this would be observable as a lag phase. Following this however, a steady state must be established and the reaction proceeds in the reverse direction, since, although the propionaldehyde and propionic anhydride concentrations were comparable (100 μM and 130 μM respectively), there was more NADH present than NAD^+. The further the reaction proceeds, however, the more NAD^+ that will be produced and the greater the competition between the two reactions, until the dehydrogenase reaction becomes favoured as the

propionic anhydride hydrolyses.

The observation of a slow reverse reaction at high propionaldehyde concentrations (20 mM, compared to the propionic anhydride concentration of 260 mM used) is consistent with the above results (and a similar slight increase in A_{340} was observed initially), but it appears to exclude the possibility that propionaldehyde, when present at high concentrations), binds to the active site of the Enzyme.NADH complex, as proposed by Duncan (1985).

In the presence of NAD^+ (2 mM), the reaction proceeded despite the large excess of NAD^+ over NADH (Figure 6.11b). Since NAD^+ was premixed with enzyme and NADH, most of the enzyme-bound NADH would have been displaced by the NAD^+, so it appears that reaction proceeds via the small amount of enzyme.NADH that is present. As for the experiments involving premixed propionaldehyde, there is the possibility of lag phases or increases in absorbance in the initial stages of the reaction, since release of NAD^+ will presumably be inhibited by the high concentration of free NAD^+, and the added complication that this NAD^+ will increase the rate of hydrolysis of the propionic anhydride. Inhibition by NAD^+ is therefore most probably competitive in nature (as for the dehydrogenase reaction, MacGibbon et al., 1977a) with both NAD^+ and NADH competing for enzyme binding sites. However, the more favourable binding constant for NADH (0.83 μM^{-1} compared with 0.125 μM^{-1}, MacGibbon et al., 1977a) means that NAD^+ is not a very powerful inhibitor of the reverse reaction (in constrast to NADH, which is a powerful inhibitor of the dehydrogenase reaction).

6.3.6.2 PCMB, Disulphiram, Chloral Hydrate, and Glyoxylic Acid

The effect of a range of PCMB concentrations on the reverse reaction is shown in Figure 6.12a. Solutions contained 2.6 mM propionic anhydride, 100 µM NADH and 2.7 µM enzyme. Results are expressed relative to the control rate, measured in the absence of PCMB. Inhibition of the reverse reaction was clearly observed. These results may be compared with those given in chapter 3 (Figures 3.9, 3.10) for the effect of PCMB on the dehydrogenase activity at high and low propionaldehyde levels, and on the esterase activity of the enzyme. The inhibition observed clearly resembled that seen for the high propionaldehyde and esterase reactions, but there was no activation as seen at low propionaldehyde concentrations. A Scatchard plot of the data (3.4.5) is shown in Figure 6.12b for which a K_D value of 1.01 µM,

Figure 6:12 THE EFFECT OF PCMB ON THE REVERSE REACTION

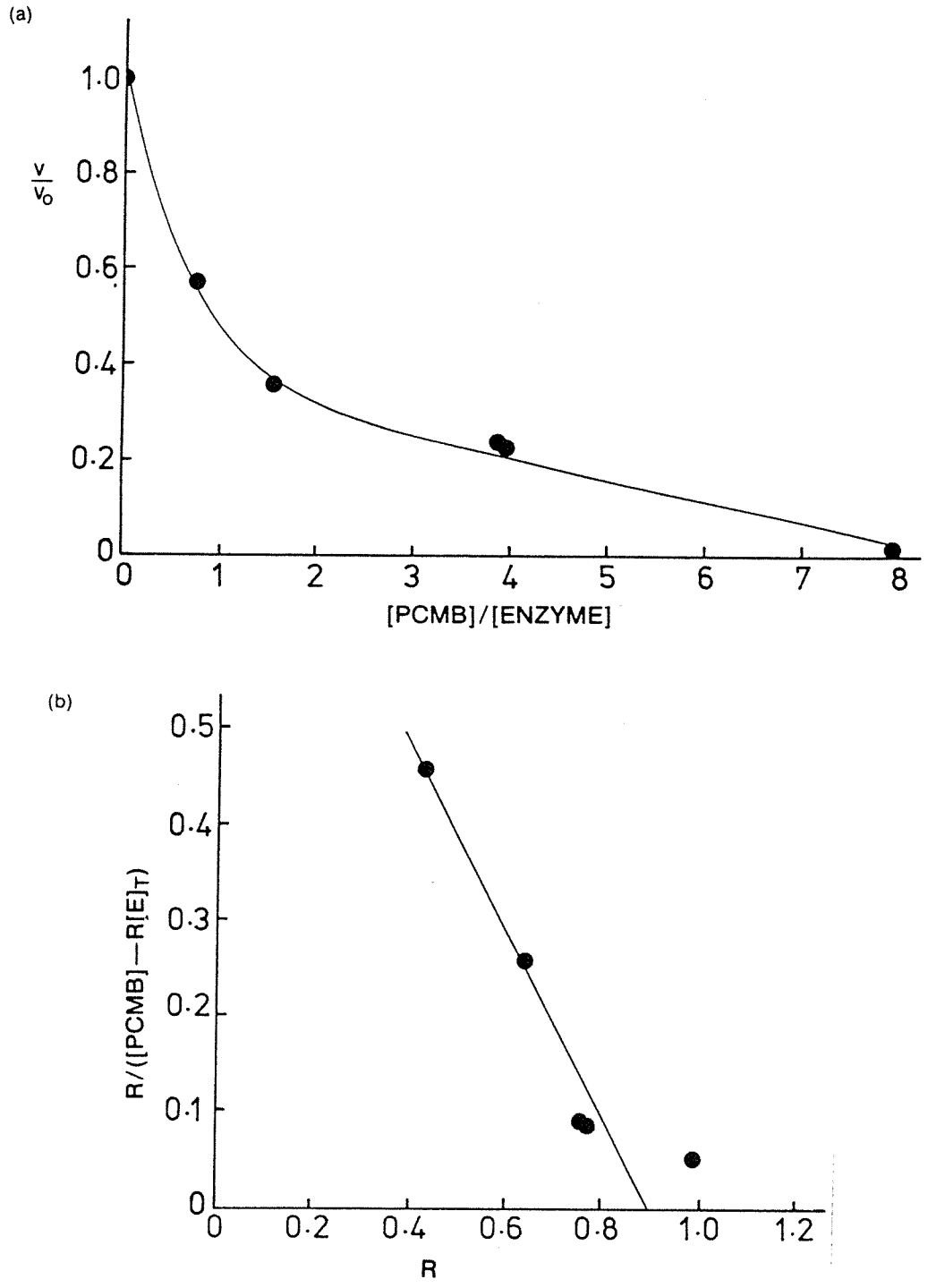


Figure 6.12 The Effect of PCMB on the Reverse Reaction

6.12 (a) The reaction rate (v) in the presence of PCMB relative to the control rate (v^o) is shown as a function of the PCMB:enzyme active site concentration ratio. Reaction solutions contained 2.6 mM propionic anhydride, 100 µM NADH and 2.7 µM enzyme.

6.12 (b) A Scatchard plot of the data is shown, where $R = 1 - v/v^o$. Values for K_D of 1.01 µM and for n of 0.89 were obtained.

and a value for n of 0.89 were obtained (if n=1 then K_D=1.14). These results may be compared to those obtained in chapter 3 (3.4.5, 3.4.6) for inhibition of the esterase reaction and the enzyme dehydrogenase reaction by PCMB at high propionaldehyde levels. PCMB has been shown (Chapter 3) to bind to the second of the two thiol groups revealed in the work of Kitson & Loomes (1985). Clearly at low propionaldehyde levels, since activation occurs, it cannot be binding to an active site sulphydryl group under these conditions. Thus it is unlikely that the inhibition of the reverse reaction involves PCMB binding at the active site, but the exact mechanism by which PCMB produces inhibition cannot be determined from these data alone.

The effects of disulphiram, chloral hydrate and glyoxylic acid on the reverse reaction were each investigated individually. Reverse reaction solutions contained 1.76 μM enzyme , 21 μM NADH, and 130 μM propionic anhydride. The enzyme was dialysed before use as described previously (3.2) to remove 2-mercaptoethanol. The modifier substance was added to enzyme premixed with NADH and the reverse reaction initiated by the addition of propionic anhydride. Reaction parameters were compared with those obtained from a control reverse reaction and the results are expressed relative to the control.

Disulphiram (10.7 μM) premixed with enzyme and NADH before the addition of propionic anhydride produced inhibition of the reverse reaction rate, just as is observed (Kitson, 1975, 1978) for the dehydrogenase reaction, a relative rate of 0.041 being obtained. The extent of inhibition was very similar to the relative reaction rate of 0.040 obtained for the low propionaldehyde assay using the same amounts of enzyme and disulphiram. Disulphiram, therefore, appears to produce the same effect on both reactions, probably by modifying the reactive cysteine recently identified in the horse and human liver enzymes (Hempel et al., 1982, 1984). The result, therefore, may be taken to indicate that this group is a functional group near or at the active site group as suggested by Hempel. Since, 4% of the enzymic activity still remains and hence it may also be concluded that modification of this cysteine residue causes inhibition indirectly by blocking access of substrates to the active site or by causing or blocking an essential conformational change. Chloral hydrate (333 μM) had very little effect on the reverse reaction. A relative rate of 0.80 was obtained. The absorbance amplitude ΔA_{340} was unaffected.

Glyoxylic acid (71 mM) had no detectable effect on the reverse

reaction rate or ΔA_{340}. Since neither of these steady state inhibitors has any marked effect on the reverse reaction, they cannot be blocking access to the active site functional group in the E.NADH and *E.NADH complexes. It has already been suggested that glyoxylic acid does not bind in the P_1 binding site and the present data suggest that chloral hydrate cannot be binding in the active site in the presence of NADH either.

6.3.7 Dehydrogenase Activity Following the Reverse Reaction

Although the maximum dehydrogenase rate following the reverse reaction was measured, interpretation of these results was complicated by the number of species involved in the reactions. It has been shown that the dehydrogenase reaction occurred as soon as the propionic anhydride concentration dropped below that necessary to cause inhibition. As discussed above, considerable amounts of NADH frequently remained at this time. Thus the rate of the following dehydrogenase reaction was affected by the concentrations of NAD^{+} and propionaldehyde produced in the reverse reaction (equimolar amounts of each) and the NADH remaining in the solution. Also, because the solutions were unbuffered and as exact propionic anhydride concentrations during reactions could not be determined, the true pH and amounts of residual propionic anhydride at the time of maximum dehydrogenase activity were unknown. Therefore no attempt was made to make a detailed analysis of the rate information. However, general trends appeared to be in agreement with those expected from studies of the conventional dehydrogenase reactions, suggesting that no major modification of the enzyme had occurred, despite the high concentrations of such a powerful acylating agent.

An inhibitory effect of NADH was noted, as reported by MacGibbon et al. (1977a). Figure 6.13 shows the effect of initial NADH concentration on the maximum dehydrogenase rate at two different propionic anhydride concentrations. Maximum activity was observed at an initial NADH concentration of 5 μM for 78 μM propionic anhydride, and at 18 μM NADH for an initial propionic anhydride concentration of 260 μM. Study of the ΔA_{340} data shows that these amounts of NADH corresponded to the maximum amounts of NADH which could be oxidised at these propionic anhydride concentrations. It is, however, interesting to note that at these NADH concentrations, utilisation of available

Figure 6:13

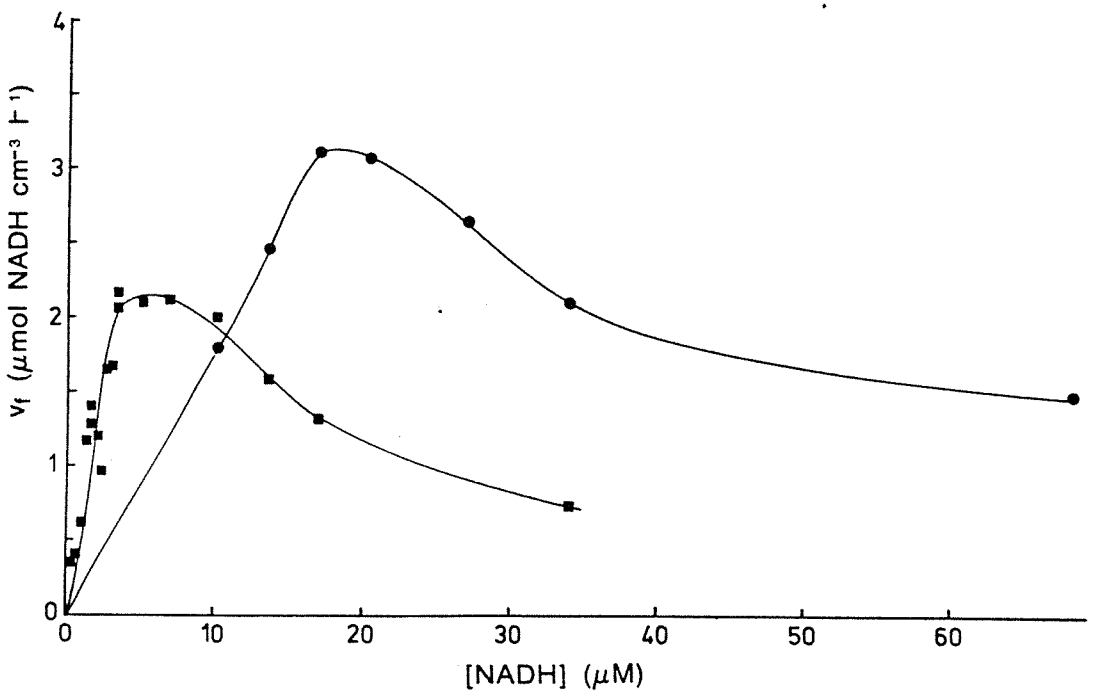


Figure 6.13 The Effect of the Initial NADH Concentration on the Maximum Rate of the Dehydrogenase Reaction following the Reverse Reaction. The enzyme concentration was 2.8 μM. Experiments were carried at two propionic anhydride concentrations, 78 μM (■) and 260 μM (●).

NADH was only approximately 50%. Thus maximum dehydrogenase activity was observed under conditions in which the concentrations of NAD^+, NADH, and propionaldehyde were all equal. Since the dissociation constants for NAD^+ and NADH are 8.0 μM and 1.2 μM respectively, more of the enzyme should have been present as E.NADH complexes than as $E.NAD^+$ complexes and the rate of the dehydrogenase reaction should have been lower than under conditions in which almost all of the NADH had been oxidised. Since total acid production should be the same in both cases, it can only be assumed that the residual propionic anhydride concentration is different in the two cases to account for the different rates, as NADH has been shown to be a very powerful inhibitor of the dehydrogenase reaction (MacGibbon et al., 1977a).

6.3.8 Reverse Reactions with Other Anhydrides

A decrease in absorbance at 340 nm was observed for all the anhydrides tested. The results are summarised in Table 6.3. In each case, addition of propionaldehyde (20 mM) to the reaction solution after several minutes produced an increase in A_{340} back to the original level, confirming that the decrease in absorbance represented oxidation of NADH.

In the case of the three anhydrides derived from monoprotic carboxylic acids, i.e. acetic, propionic and butyric (n-butanoic) anhydrides, spontaneous increase in A_{340} was observed following the reverse reaction and hydrolysis of the anhydride, consistent with utilisation of the NAD^+ and aldehyde products once conditions became favourable for the dehydrogenase reaction.

The reported half-life of maleic anhydride in pure water, 0.23 minutes (Rivett & Sidgwick, 1910) is very short, but despite this practical difficulty, a slow reaction was observed (Table 6.3).

TABLE 6.3

Reverse Reaction Rates for a Variety of Anhydrides

Anhydride	v_i	Hydrolysis in Water	
		k^a	t^b
	s^{-1}	$s^{-1} \times 10^3$	min
acetic anhydride	0.017[c]	2.625[d]	4.5
propionic anhydride	0.055	0.269[e]	42.9
butyric anhydride	0.048	0.783[e]	14.8
DEPC	0.009[c]	0.0035[f]	120.0
maleic anhydride	0.004	2.648[g]	0.23
succinic anhydride	0.005[c]	26.485[g]	4.5

[E] = 3.1 μM, [NADH] = 20 μM

a k is the rate constant for hydrolysis in water at 25°C.

b t is the half life of the anhydride in water at 25°C

c reactions carried out in 30 M buffer at pH 7.0

d Rivett & Sidgwick, (1910a), Gold, (1947)

e Wilsdon & Sidgwick, (1913)

f Melchior & Fahrney, (1970)

g Rivett & Sidgwick, (1910b)

Figure 6.14a shows absorbance traces for acetic anhydride reactions in electrolyte and buffer solutions. Figure 6.14b shows the results of a similar experiment using propionic anhydride and various concentrations of buffer. Opposite effects were observed for these two substances, despite their chemical similarity, as the extent of the reverse reaction of acetic anhydride was very severely limited in unbuffered soluions, whereas propionic anhydride reactions could not be carried out in phosphate buffer, as discussed in Chapter 5. Satisfactory data could not be obtained for acetic anhydride or succinic anhydride in the absence of buffer and DEPC produced complex absorbance changes unless buffer was present.

In pure water, the half-life of butyric anhydride is considerably shorter than that for propionic anhydride, (Table 6.3). However, as shown in Figure 6.15, butyric anhydride is hydrolysed more slowly than propionic anhydride in reaction solutions.

Figure 6:14

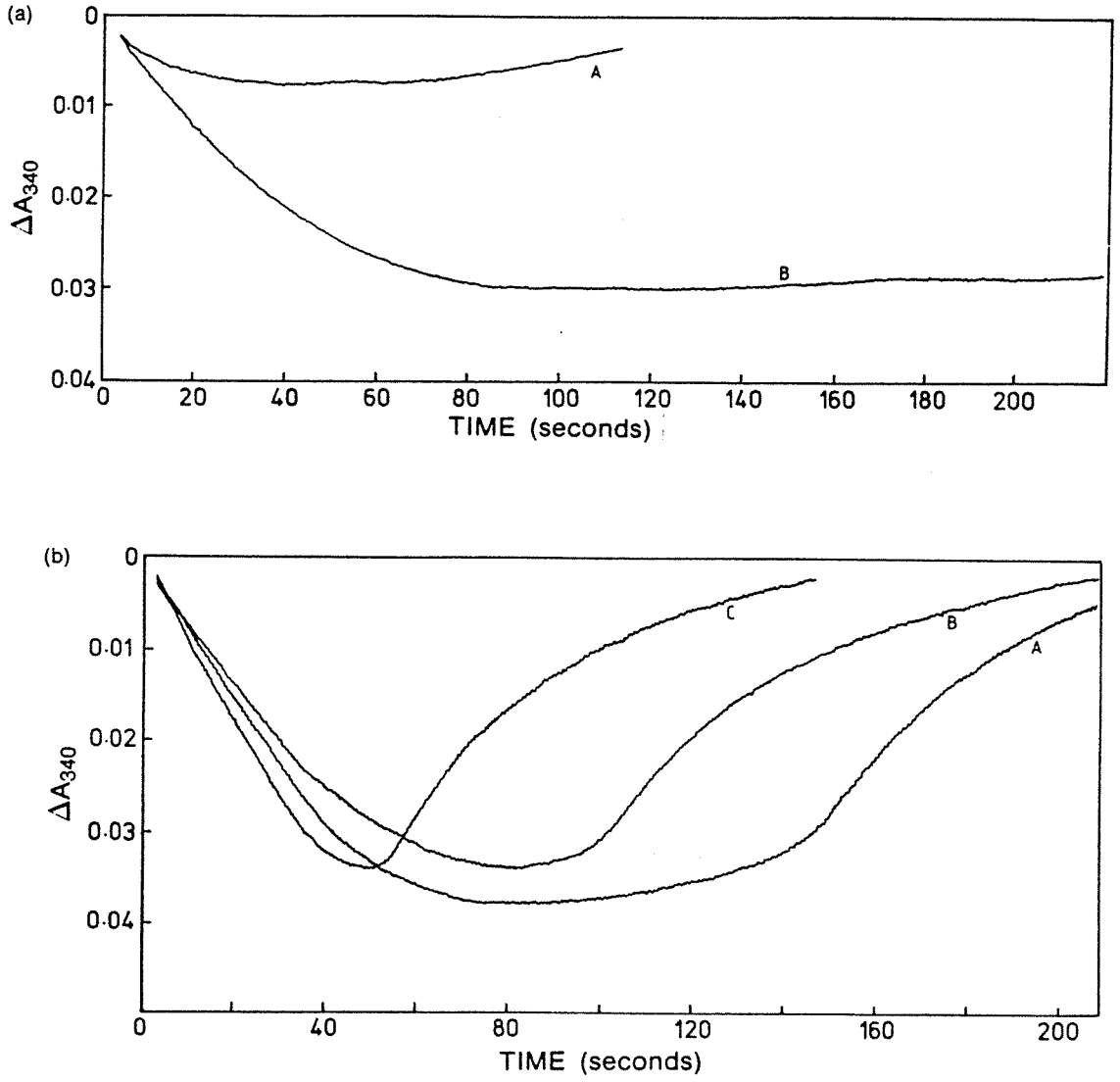


Figure 6.14 A Comparison of the Effects of Buffer on the Progress of Reverse Reactions with Acetic Anhydride and Propionic Anhydride.

6.14 (a) Reaction traces for reverse reactions with acetic anhydride (1.6 mM) in electrolyte solution (A) and 0.1 M pH 7.0 phosphate buffer (B) are shown. Both solutions contained 2.5 μM enzyme and 21 μM NADH.

6.14 (b) Reaction traces for reverse reactions with propionic anhydride in electrolyte solution to which phosphate buffer (pH 7.0) was added are shown. All solutions contained 3.1 μM enzyme and 8.4 μM NADH, and the buffer concentrations were 1.0 mM (A), 50 mM (B) and 250 mM (C).

Figure 6:15 A COMPARISION OF REVERSE REACTIONS INVOLVING PROPIONIC AND BUTYRIC ANHYDRIDES

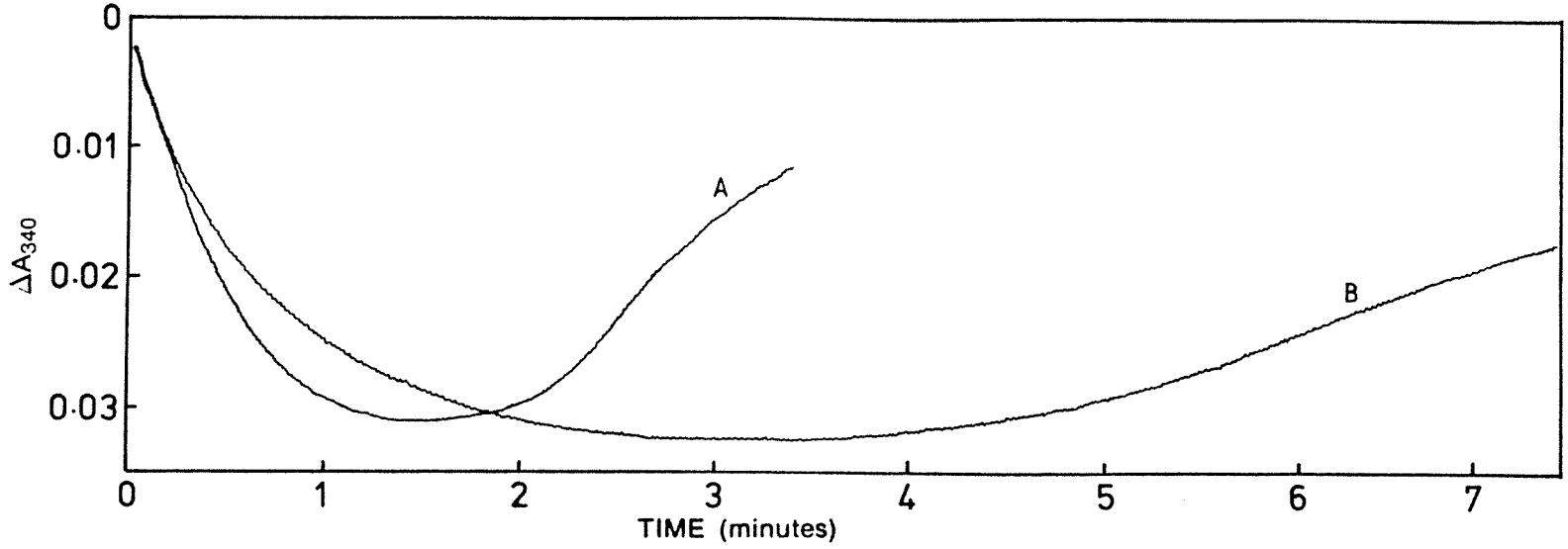


Figure 6.15 A Comparison of Reverse Reactions with Propionic and Butyric Anhydrides.

Reaction traces for propionic anhydride (A) and butyric anhydride (B) are shown. Solutions contained 3.1 µM enzyme and 9.5 µM NADH and the anhydride concentration was 260 µM in each case.

When acetic or propionic anhydride was added to a reacting system containing succinic or maleic anhydride, an immediate increase in rate was observed, indicating that acetic anhydride and propionic anhydride most probably displace the other reagents, and therefore that each of these reacts reversibly with the enzyme.

6.4 PreSteady State Results

6.4.1 Nucleotide Fluorescence

When enzyme premixed with NADH was rapidly mixed with a solution containing NADH and propionic anhydride a rapid first order decrease in nucleotide fluorescence was observed (Figure 6.16). This was followed by a steady state decrease in fluorescence. At saturating concentrations of NADH and propionic anhydride, the rate constant for this process was 30 ± 10 s^{-1}, and the relative amplitude of the process was 0.5 ± 0.1 compared to the formal enzyme concentration. At very low propionic anhydride concentrations, which could be produced only by utilising the fact that hydrolysis of the propionic anhydride occurred in reaction solutions, a dependence of the rate constant and amplitude on the anhydride concentration was observed (Figure 6.17), but the exact concentrations of these solutions could not be determined due to their method of preparation (6.2.3). However, within the detection limits of the stopped flow instrumentation used, a 2-3 fold decrease in the rate constant and a 3-4 fold decrease in the amplitude were observed as the propionic anhydride concentration decreased. This suggests that saturation kinetics are involved and hence that an enzyme.NADH.acyl complex is being formed.

At high propionic anhydride concentrations it was observed that a maximum amplitude for this fast process was obtained. It was also noted that on rare occasions at high concentrations of NADH a total inversion of the fluorescence signal was observed, presumably due to fluorescence quenching.

The effect of pH on this rapid process was studied and the results are shown in Table 6.4. However, no significant pH dependence was observed in the pH range 6-9. At pH 5.0, no presteady state process was observed, which was consistent with the results obtained for NADH burst experiments at pH 5.0 (4.3.3), At pH 10, although a rapid process

Figure 6:16 PRESTEADY STATE PROCESS IN REVERSE REACTION

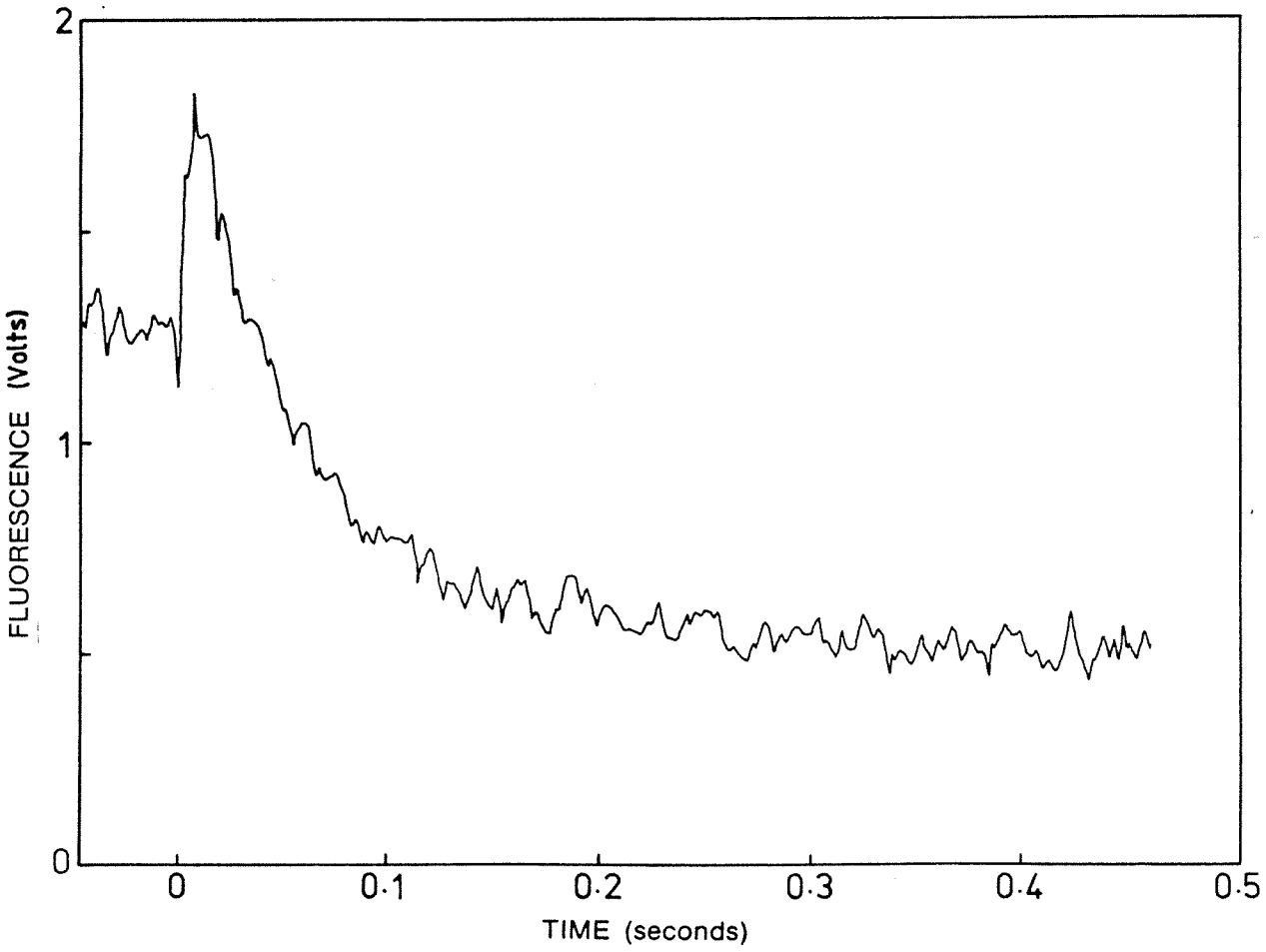


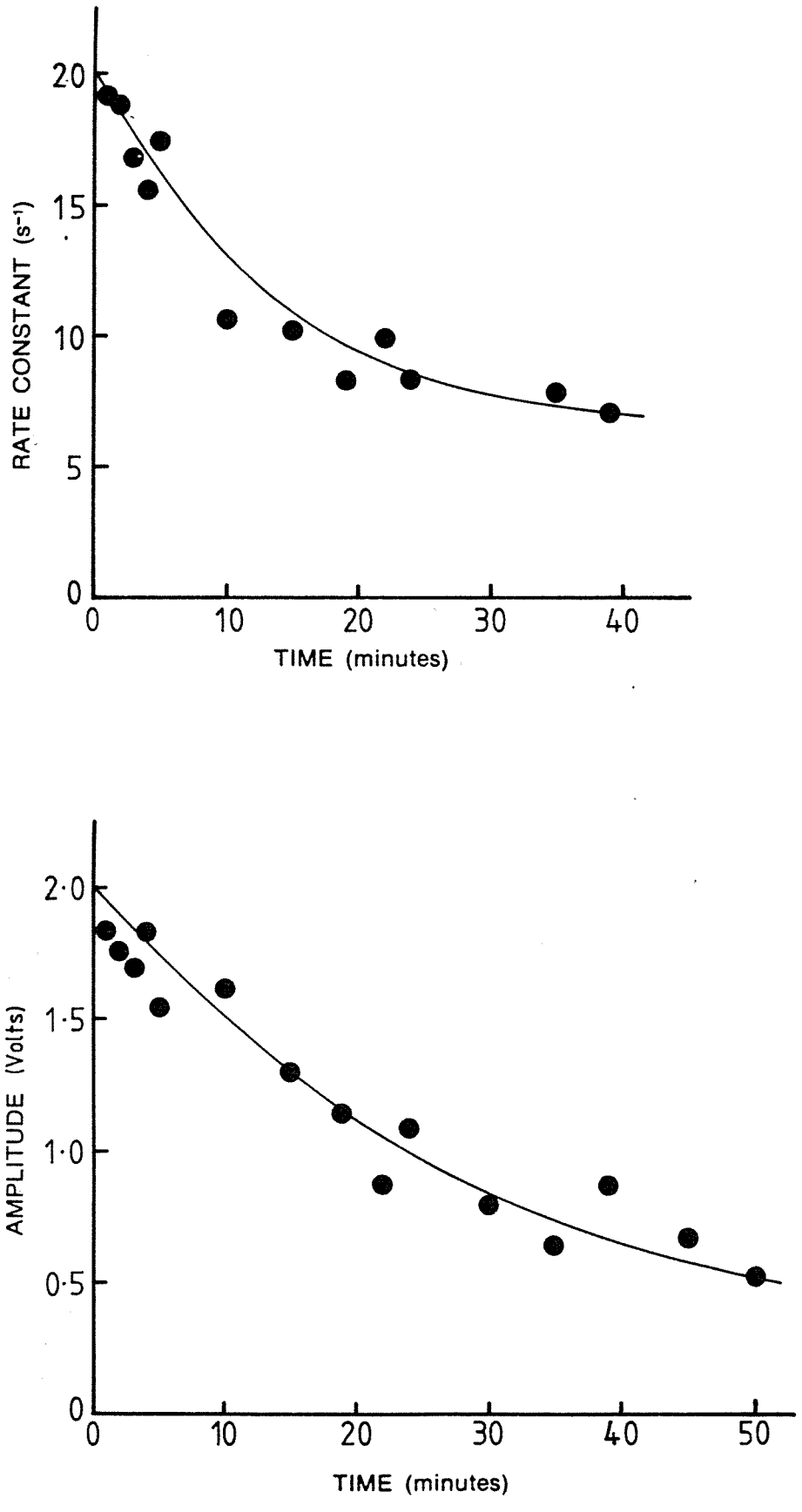
Figure 6.16 PreSteady State Decrease in Fluorescence observed in Reverse Reactions.

A solution of enzyme (10 M) and NADH (20 M) in electrolyte solution was rapidly mixed with a solution containing propionic anhydride (600 M) and NADH (12 M) in the stopped flow spectrophotometer. The rate constant obtained for this process was 20.4+0.5 s^{-1}. For the amplitude of this process, 1 V corresponds to an enzyme concentration of 4.3 M.

Figure 6.17 The Effect of the Propionic Anhydride Concentration on the PreSteady State Reverse Process.

A solution containing enzyme (5 μM) and NADH (20 μM) was rapidly mixed with NADH (11 μM) and propionic anhydride (320 μM) in the stopped flow spectrophotometer. The time between the time of the run and the time of preparation of the propionic anhydride solution was measured as repeated mixings from the same solutions were made. The rate constant (a) and the amplitude (b) of the presteady state process were calculated for each successive run until the signal to noise ratio prevented the fitting of the data.

Figure 6:17 THE EFFECT OF PROPIONIC ANHYDRIDE CONCENTRATION ON THE PRESTEADY STATE REVERSE PROCESS



was observed, this involved an apparent increase in fluorescence which appeared to be merely an inversion of the normal signal (as described in the previous paragraph). Attempts to eliminate this inversion were unsuccessful.

TABLE 6.4

The Effect of pH on the PreSteady State Reverse Process

pH	k s^{-1}	A[a]
5.0	b	b
6.0	31±2	0.45±0.05
7.0	47±5	0.50±0.05
8.0	47±3	0.65±0.05
8.7	56±12	0.46±0.05
10.0[c]	45±4	0.40±0.05

[E] = 5.02μM before mixing

[NADH] = 60 μM, 51 μM in the second syringe (see methods)

[propionic anhydride] = 390 mM before mixing

a relative to the amplitude of the NADH burst

b no burst was observed

c an increase in absorbance was observed

The steady state reactions following this rapid process could also be studied using the stopped flow apparatus (Figure 6.18a). Results obtained were consistent with those gained by conventional steady state methods by either absorbance or fluorescence measurements. However, due to the enhanced fluorescence of the E-NADH species, apparently steeper slopes (in terms of photomuliplier voltages) were obtained for the increase in nucleotide fluorescence following hydrolysis of the anhydride than for the corresponding absorbance measurements (Figure 6.18b). Thus the true rate of this dehydrogenase reaction was calculated by adjusting these apparent rates using the fluorescence enhancemant factor reported by MacGibbon et al. (1977b). A transition point was frequently noted at which this steeper slope abruptly changed to a slower apparent rate (Figure 6.18c), presumably

Figure 6.18 Stopped Flow Traces of Reverse Reactions.

Enzyme and NADH were rapidly mixed with propionic anhydride and NADH in each case. Traces b, c and d were all obtained after much of the propionic anhydride had hydrolysed in the solutions. The initial concentrations (immediately after mixing) of the respective solutions are shown below.

	a	b	c	d
[E] (M)	5	5	5	5
[NADH] (M)	36	10	16	15
$[(RCO)_2O]$ (mM)	2.6	0.26	2.3	0.32

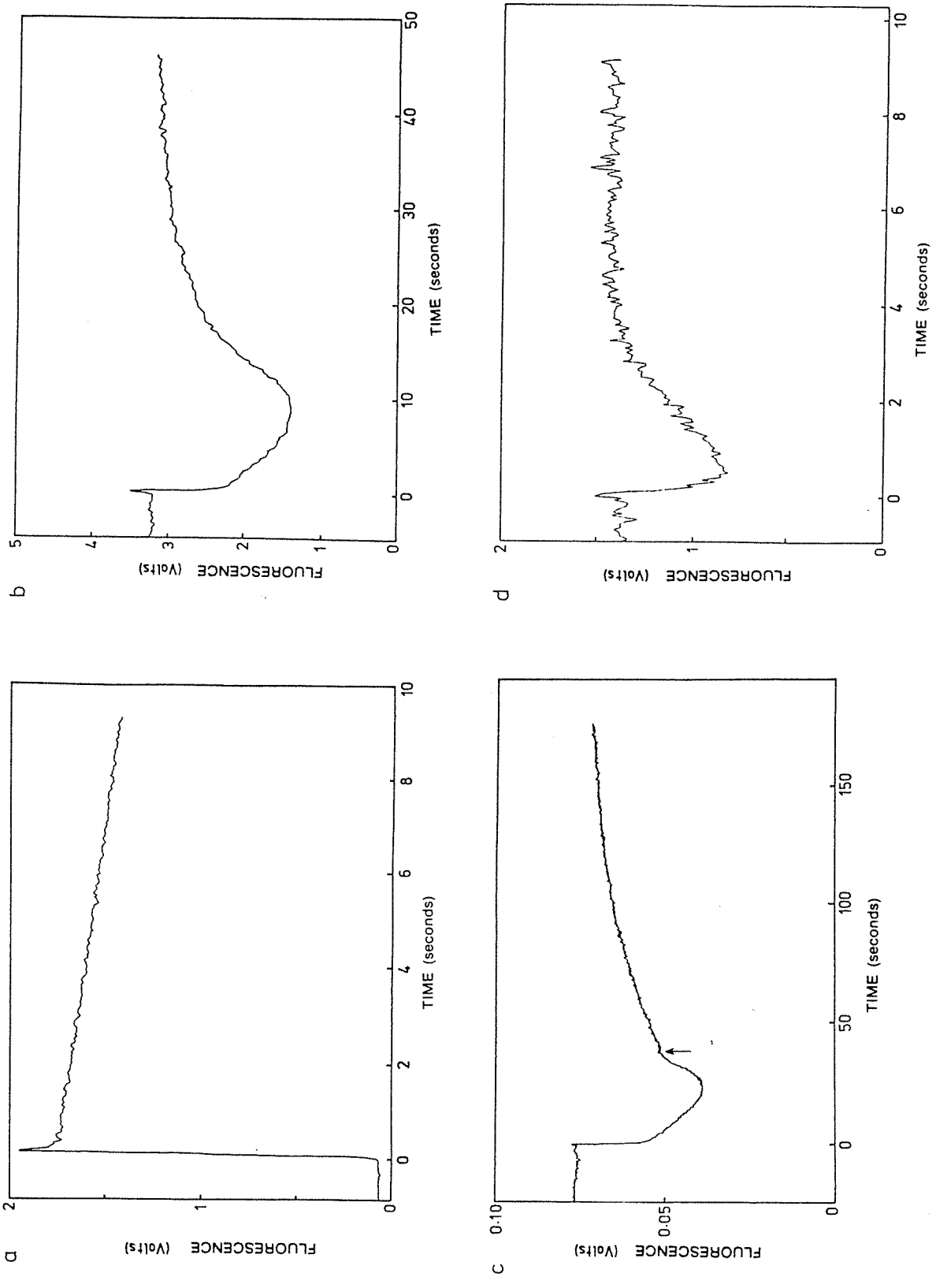

a
FLUORESCENCE (Volts)
TIME (seconds)
b
FLUORESCENCE (Volts)
TIME (seconds)
c
FLUORESCENCE (Volts)
TIME (seconds)
d
FLUORESCENCE (Volts)
TIME (seconds)

Figure 6:18

indicating a change from production of E.NADH to steady state production of free NADH. This suggests that at the turnover point, a significant proportion of the enzyme must be in an enzyme.NAD or free enzyme form. The rapid reverse process was observed to occur even in cases of low propionic anhydride concentrations in which no steady state reverse reaction was observed (Figure 6.18d) This most probably occurs because there is insufficient propionic anhydride left to establish a steady state, and therefore only a single turnover of the reverse rection occurs.

6.4.1.1 Other Anhydrides

A presteady state process was observed for all the other anhydrides studied (Table 6.5). The rate constants of the various anhydrides appeared to fall into three groups. The fastest, with rate constants around 30 s^{-1}, included acetic, propionic and maleic anhydrides. The second group with rate constants close to 14 s^{-1} includes butyric and succinic anhydrides. For diethylpyrocarbanate a much slower rate constant of 0.62±0.05 s^{-1} was obtained (Figure 6.19). Amplitudes for this process were all reasonably similar, varying between 30% and 60% of the NADH burst amplitude for the normal dehydrogenase reaction, which was used as a control.

Figure 6:19 REVERSE REACTION OF DIETHYLPYROCARBONATE : PRESTEADY STATE PROCESS

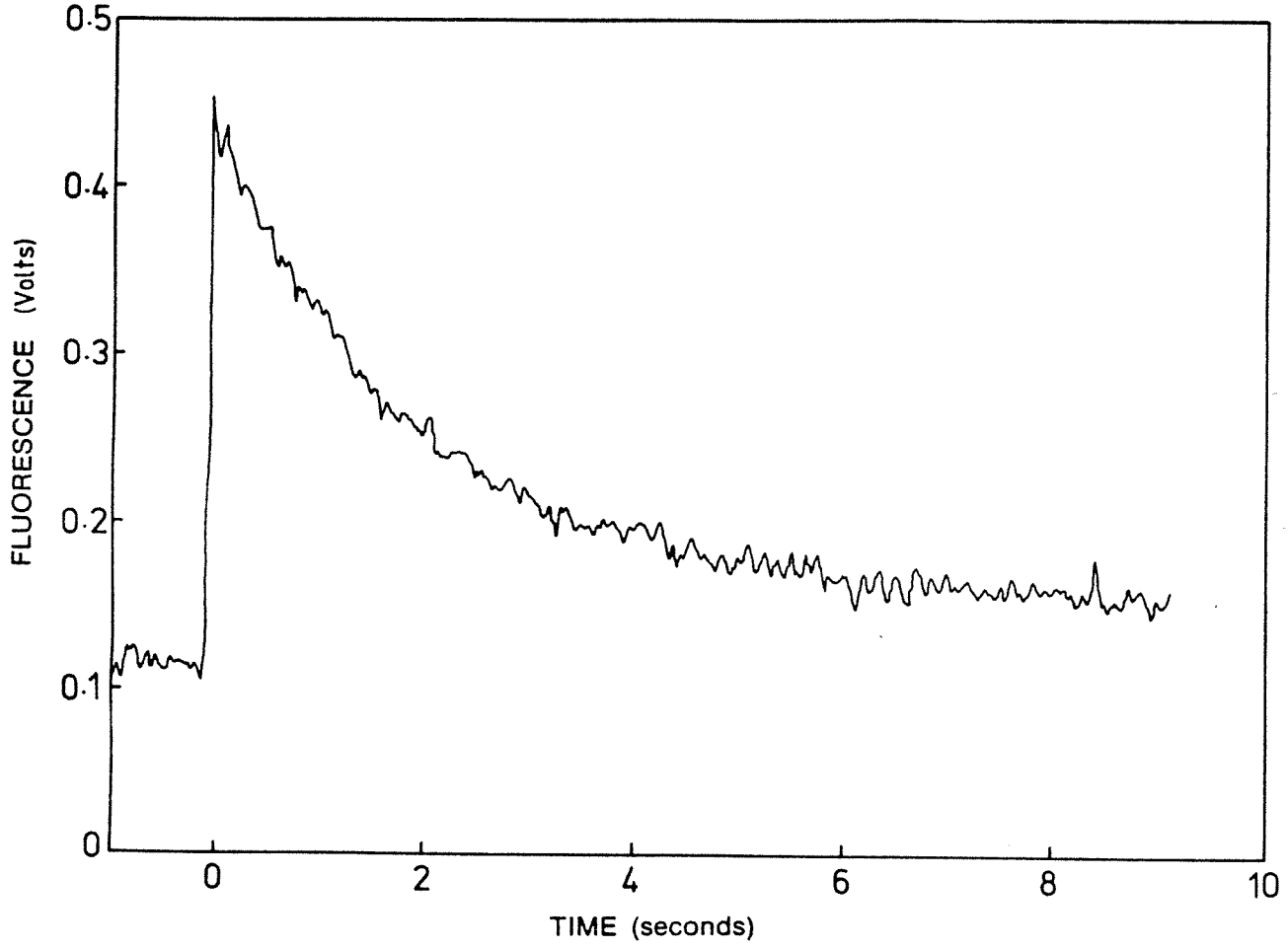


Figure 6.19 The PreSteady State Process in the Reverse Reaction of Diethylpyrocarbonate.

Enzyme (10 M) and NADH (24 M) in one syringe was pushed against NADH (20 M) and DEPC (0.8 mM) in the other. The rate constant determined from this trace was 0.062 s^{-1}. This trace represents accumulated (catted) data from four separate runs. For the amplitude 1 Volt corresponds to an enzyme concentration of 2.4 M.

TABLE 6.5

The PreSteady State Process:

Rate Constants for a Variety of Anhydrides

Anhydride	[Anhydride]	k	A*
	mM	s^{-1}	μM
acetic anhydride	1.6	11±3	0.3
propionic anhydride	1.6	30±4	0.6
	0.8	34±4	0.5
butyric anhydride	0.8	14±3	0.4
DEPC	1.6	0.62±0.2	0.4
maleic anhydride	1.6	34±4	0.3
succinic anhydride	0.8	14±3	0.6

[E] = 10 μM before mixing

[NADH] = 24 μM in the enzyme syringe

15 μM in the anhydride syringe

Anhydride Concentrations in the Table are those after mixing

* relative to the amplitude of the NADH burst in fluorescence at pH 7.0

6.4.2 Absorbance

No presteady state reduction in A_{340} was observed under the conditions used. However absorbance measurements are much less sensitive, so considerably higher enzyme concentrations are required for presteady state studies of this enzyme and a ten-fold excess of NADH over enzyme concentration was used to ensure that the majority of the enzyme had NADH bound. Such high levels of NADH made it impossible to observe any fast phase that may have been present as the voltage backoff required meant that only low sensitivity settings could be used. Since fluorescence studies indicate that the amplitude of any expected fast phase is only half that of the NADH burst in the dehydrogenase reaction, which is itself very difficult to study in the absorbance mode, no conclusion about the occurrence or otherwise of a

rapid transient decrease in absorbance can be made at this stage. Future experiments using very low concentrations of NADH (essentially single turnover conditions for NADH) are planned to resolve this question.

6.4.3 Protein Fluorescence

A decrease in protein fluorescence was observed when a solution containing enzyme (13 μM) and NADH (60 μM) was pushed against a solution of propionic anhydride (3.9 mM) and NADH (51 μM) (Figure 6.20a). A rate constant of 25±10 s^{-1} was obtained for this process, which was similar to that obtained in nucleotide fluorescence studies, suggesting that both fluorescence changes arise as a result of the same process. An increase in fluorescence followed this process and the final fluorescence level reached was considerably higher than the initial level (Figure 6.20b).

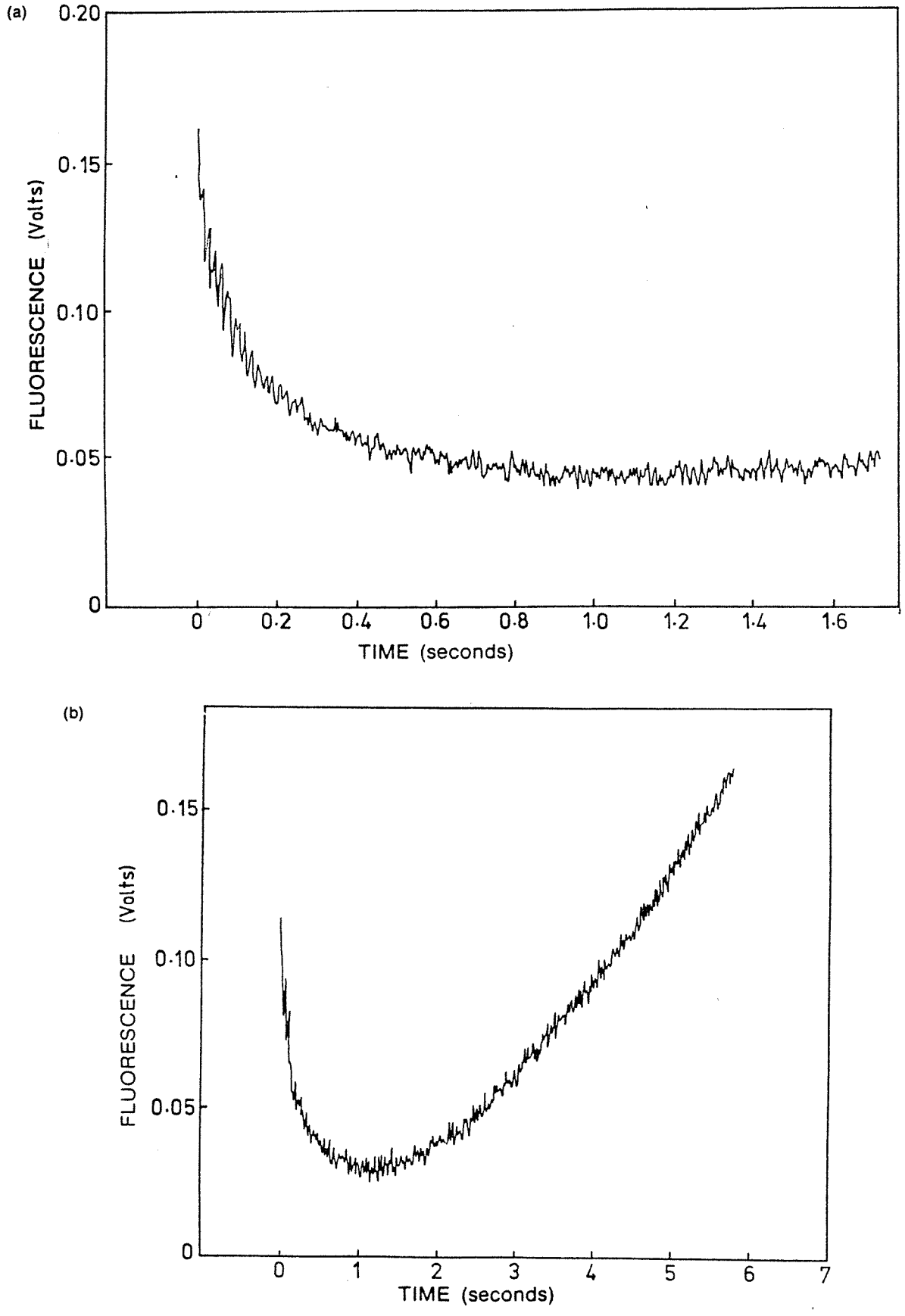
When NADH was not premixed with the enzyme, a much larger decrease in fluorescence (by a factor of about ten) was observed. Once again, this process was followed by an increase in fluorescence, but in this case, the final fluorescence level was similar to the initial fluorescence.

MacGibbon et al. (1977c) have shown that the protein fluorescence is quenched upon binding of either NAD^+ or NADH, but the quenching effect is greater for NAD^+ than for NADH. Therefore the decrease in protein fluorescence when enzyme was not premixed with NADH is consistent with NADH binding. However, when enzyme was premixed with NADH, with sufficient NADH present in the other syringe to prevent dissociation during mixing, the decrease in protein fluorescence appears to indicate that there is rapid conversion of enzyme.NADH to enzyme.NAD^+ intermediates. The subsequent increase in fluorescence, which occurs during the time scale of the reverse steady state reaction, is consistent with release of free NAD^+ as a product. Because the dissociation constant for NAD^+ is greater than that for NADH, the final proportion of free enzyme will be greater than that at the start of the reaction, and the final fluorescence level will also be higher, as was observed. For the alternative mixing condition, the fluorescence decrease would be expected to be biphasic (as was observed, although accurate estimates of the decay constants could not be made from the data available at this stage) and the

Figure 6.20 Changes in Protein Fluorescence associated with the Reverse Reaction.

A solution of enzyme (13 μM) and NADH (60 μM) was pushed against a solution of propionic anhydride (3.9 mM) and NADH (51 μM). Protein fluorescence was monitored at 340 nm following excitation at 280 nm.

Figure 6:20 CHANGES IN PROTEIN FLUORESCENCE ASSOCIATED WITH THE REVERSE REACTION



final fluorescence level would not be expected to be greater than the initial fluorescence, which was due to free enzyme.

6.4.4 Binding Studies in Nucleotide Fluorescence

When NADH was not premixed with the enzyme, study of any reverse reactions in nucleotide fluorescence were complicated by the concurrent fluorescence changes due to NADH binding to enzyme. However, a study of the manner in which propionic anhydride affected this binding was possible.

When enzyme in one syringe was mixed rapidly with a solution of NADH and propionic anhydride, biphasic traces were obtained, as for NADH binding in the absence of anhydride. However, the total amplitude of the binding processes was reduced to 40% of the control amplitude (Figure 6.21). The amplitude of the slow process was decreased by 40-50%, the fast process decreased by 70-80%. The apparent first order rate constants, λ_s and λ_f, appeared to be both increased, λ_s by 200-300%, λ_f by 150-200%. The alternative mixing condition in which enzyme was premixed with propionic anhydride produced the same results. Similar results were obtained using acetic anhydride.

Following the modified biphasic binding of NADH, a steady state decrease in fluorescence was observed (Figure 6.22a), consistent with the steady state reverse reaction involving NADH, enzyme and propionic anhydride. On longer time scales (Figure 6.22b, c), following the hydrolysis of the anhydride, an increase in fluorescence was observed, the final fluorescence value reached being higher than that reached after NADH binding (Figure 6.22c).

At very low propionic anhydride concentrations no reverse steady state process was observed, only a binding of NADH followed, some seconds later by an increase to a higher level of fluorescence. These processes were apparently independent of one another (Figure 6.22d), as the second process was controlled by the concentration of propionic anhydride remaining in solution after the binding process. In an experiment in which a small amount of anhydride was added to the syringe containing NADH and many consecutive traces obtained from repeatedly mixing the same solutions, It was observed that as the propionic anhydride hydrolysed although the same final fluorescence was obtained in each case the amplitude of the NADH binding process increased until the trace closely resembled the control binding

Figure 6:21 THE EFFECT OF PROPIONIC ANHYDRIDE ON NADH BINDING

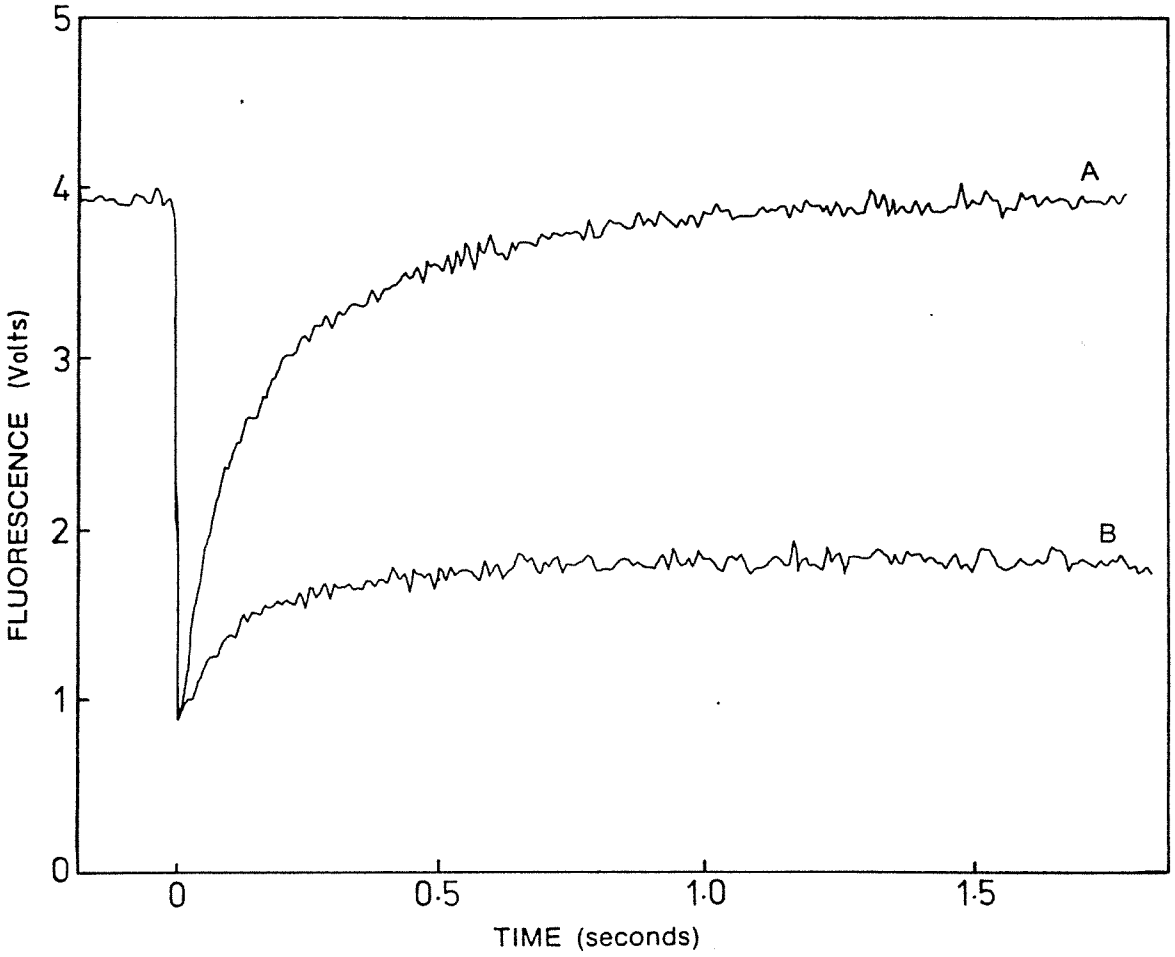


Figure 6.21 The Effect of Propionic Anhydride on the Binding of NADH. Stopped Flow traces for NADH binding in the presence (B) and absence (A is the control) of propionic anhydride. Enzyme (10 μM) from syringe A was pushed against a solution of NADH (100 μM) and propionic anhydride (5.0 μM) from syringe B.

Figure 6.22 Changes in Nucleotide Fluorescence following NADH Binding. A solution containing enzyme (10 M) was pushed against a solution of NADH and propionic anhydride in each case. The concentrations of NADH and propionic anhydride immediately after mixing are shown below.

	a	b	c	d
[NADH] (M)	50	50	50	50
$[(RCO)_2O]$ (M)	2300	300	200	200

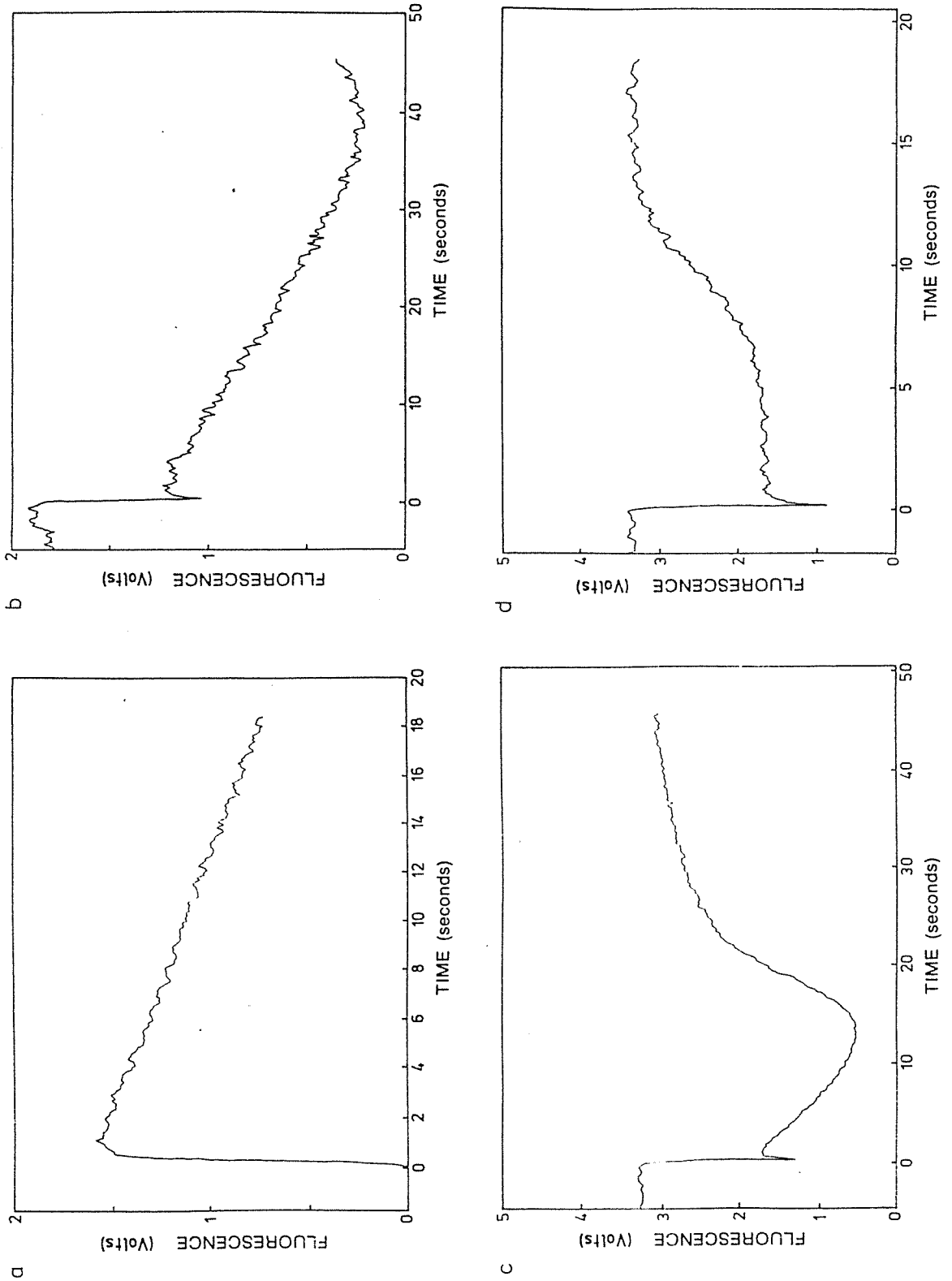


Figure 6:22 NUCLEOTIDE FLUORESCENCE CHANGES FOLLOWING NADH BINDING IN THE PRESENCE OF PROPIONIC ANHYDRIDE

trace as the propionic anhydride concentration approached zero. The final fluorescence, therefore, appeared to be the same as for NADH bound to enzyme (i.e. 5.6 times the fluorescence of free enzyme). Such a series of traces is shown in Figure 6.23a. Figure 6.23b shows the same type of experiment for the case in which enzyme was premixed with NADH (as in the previous section, 6.3.8.2).

An experiment was carried out to determine whether or not the total amplitude of the reverse processes was the same for the two alternative mixing conditions. Enzyme solution (10 μM) was pushed against a mixture of NADH (20 μM) and propionic anhydride (520 μM) and the difference in fluorescence between the minimum (after the reverse steady state) and the final maximum fluorescence level was compared with the fluorescence amplitude when enzyme, premixed with NADH, was pushed against propionic anhydride. A control experiment showed that dissociation of NADH on dilution by mixing was insignificant compared to the fluorescence changes observed in the reverse reaction and correction for this was therefore not necessary. Within experimental error, the total amplitude was the same in each case (Figure 6.24), indicating that the loss of amplitude in the binding experiments was due to the occurrence of the rapid reverse process immediately after the binding processes. Since the NADH binding was still biphasic, this is evidence that the reaction can only proceed after a conformational change.

6.4.4.1 The Effect of Diethylpyrocarbonate on the Binding of NADH

When a solution containing enzyme (5 μM) was rapidly mixed with a solution containing NADH (60 μM) and DEPC (1.6 μm) in electrolyte solution at pH 7.0 a biphasic binding was again observed (Figure 6.25a). In comparison with the binding of NADH in the presence of propionic anhydride, the total amplitude of this process was only slightly less than the total amplitude of the control experiment. Once again, the apparent rate constants for the two phases appeared to have increased, λ_s to 1.9 times the control rate and λ_f only slightly increased by a factor of 1.2. A first order decrease in fluorescence with a rate constant of $0.49 \pm 0.08\ s^{-1}$ followed the initial binding (Figure 6.25b). These results were consistent with the evidence that the loss of amplitude was due to the transient reverse process, which in the case of DEPC, is much slower than for all the other anhydrides studied, so that the individual processes are observed in this case.

Figure 6.23 The Effect of the Hydrolysis of Propionic Anhydride on NADH Binding and the Reverse Reaction.

6.23 (a) The stopped flow traces obtained when samples from a solution of enzyme (10 μM) were repeatedly mixed with samples from a solution of NADH (20 μM) and propionic anhydride (initially 520 μM) over a period of 30-40 minutes are shown. Traces are numbered in the order in which they were obtained.

6.23 (b) The stopped flow traces obtained when samples of a solution of enzyme (10 μM) and NADH (20 μM) were repeatedly mixed with samples of a solution of propionic anhydride (initially 520 μM) in the stopped flow spectrophotometer over a period of 30 minutes are shown. Traces are numbered in the order in which they were obtained.

Figure 6:23

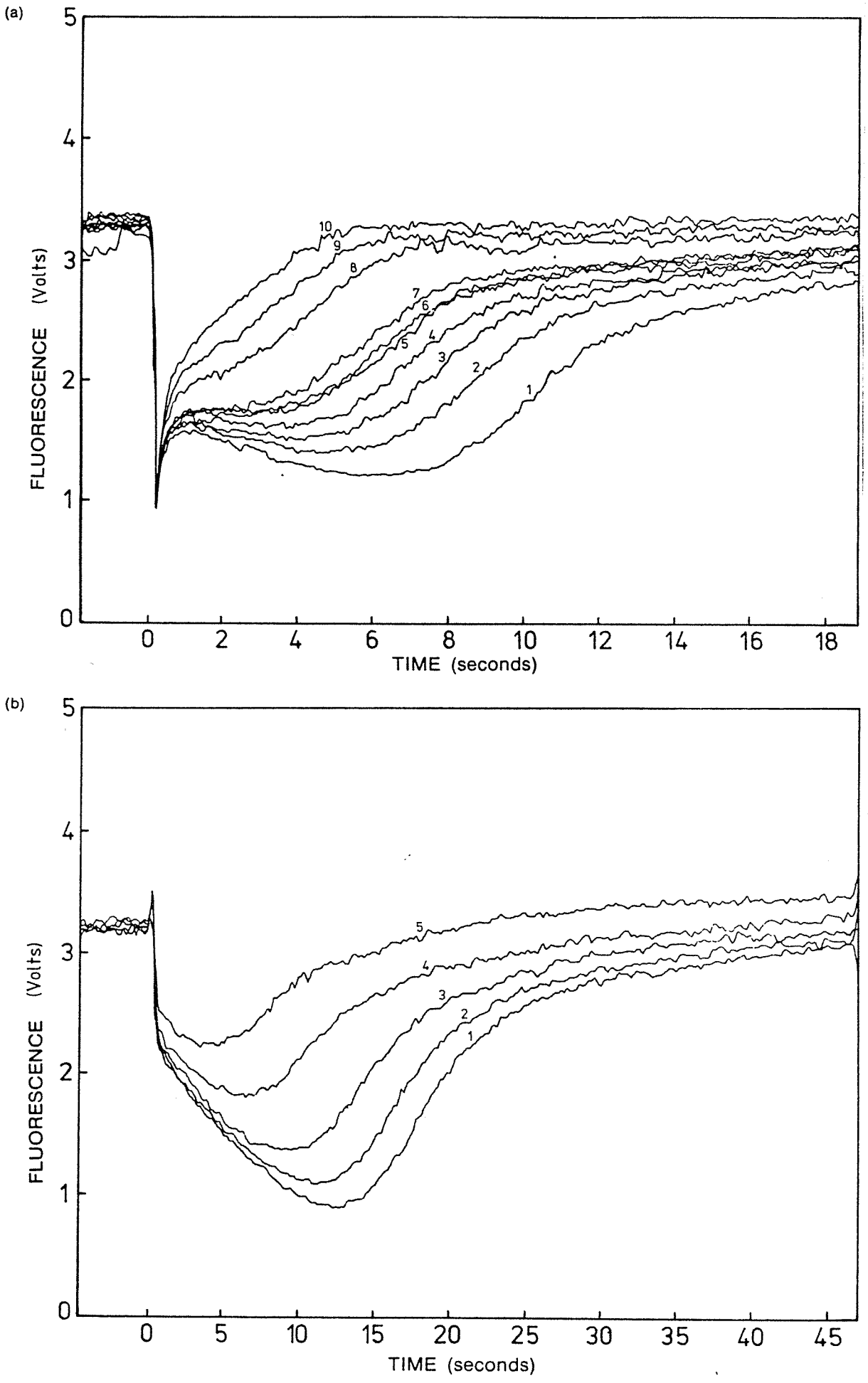

(a)
FLUORESCENCE (Volts)
TIME (seconds)
10
9
8
7
6
5
4
3
2
1
(b)
FLUORESCENCE (Volts)
TIME (seconds)
5
4
3
2
1

Figure 6:24

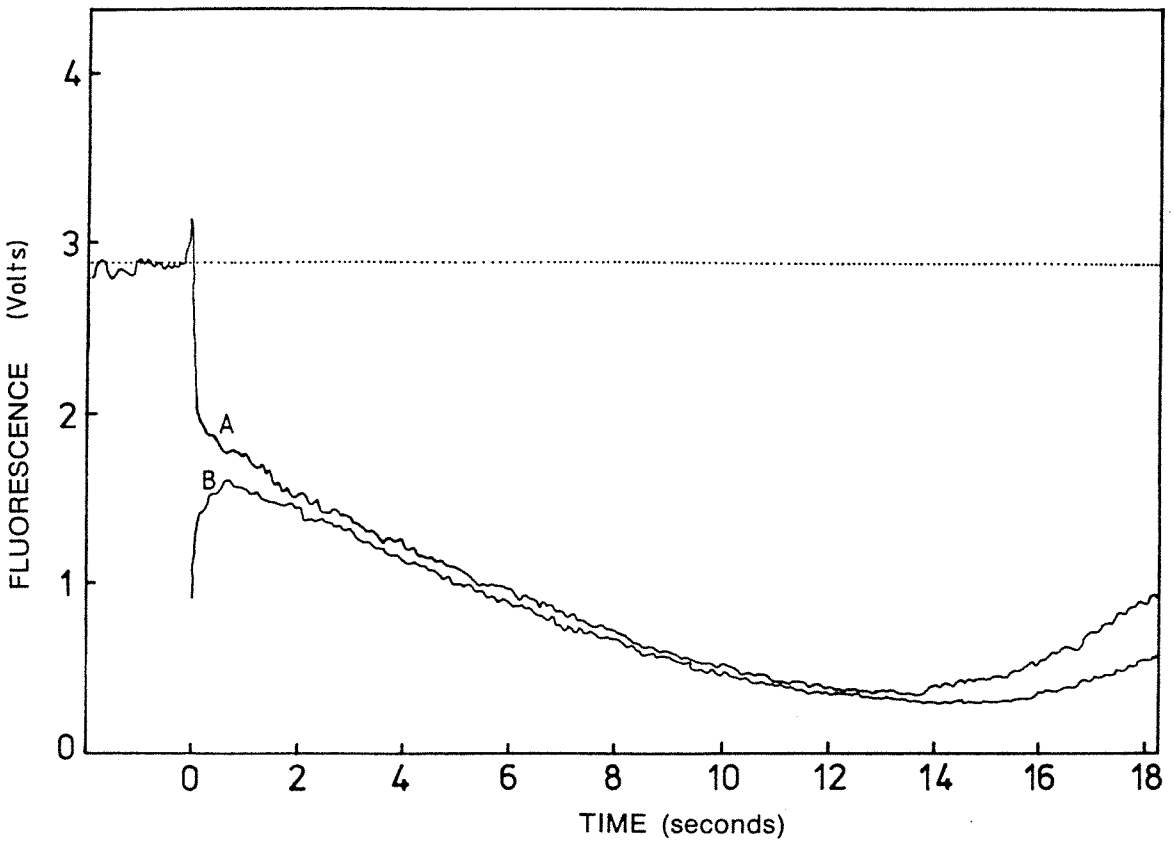


Figure 6.24 A Comparison of the Amplitudes of Changes in Nucleotide Fluorescence during the NADH Binding and Presteady State Reverse Processes.

Trace A: Enzyme (10 μM) premixed with NADH (20 μM) was pushed against propionic anhydride (520 μM).

Trace B: Enzyme (10 μM) was pushed against NADH (20 μM) and propionic anhydride (520 μM).

These reactions were carried out as soon as possible after the preparation of the propionic anhydride solution to minimise the extent of hydrolysis so that the propionic anhydride concentrations were comparable in each case.

Figure 6:25

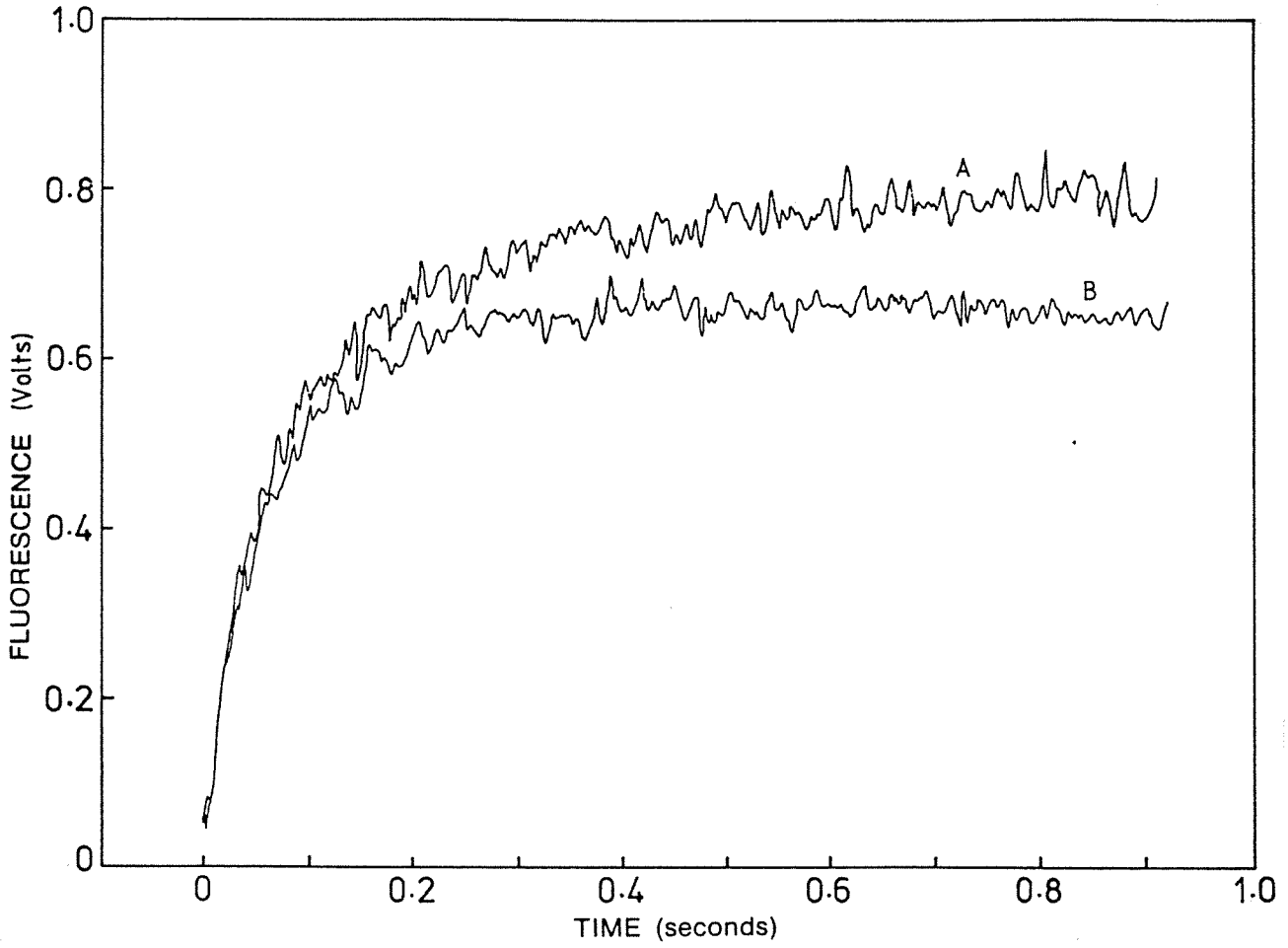


Figure 6.25 The Effect of DEPC on the Binding of NADH to the Enzyme. A solution containing enzyme (5 μM) was rapidly mixed with a solution containing NADH (60 μM) and DEPC (1.6 mM). The NADH binding processes and subsequent fluorescence changes were recorded over several time frames. In Figure 6.25 (a) both the traces for the control experiment in the absence of DEPC (A) and the binding of NADH in the presence of DEPC (B) are shown.

6.4.5 Lag Phases

Lag phases were a major feature of the steady state reverse reaction with propionic anhydride as observed using the stopped flow spectrometer. Both NADH fluorescence and 340 nm absorbance traces frequently exhibited marked and often lengthy lag phases. Typical traces of NADH fluorescence showing pronounced lags are shown in Figure 6.26. These lag phases followed the rapid transient decrease in fluorescence discussed in section 6.3.8.1 (Figure 6.26a), but on very long time scales the transient process often did not show. Reliable data for these lag phases were much more readily obtained using the stopped flow spectrometer than by use of a conventional steady state spectrophotometer or fluorimeter and reproducibility was markedly improved.

Lag phases were also observed when enzyme was not premixed with NADH, as described in section 6.3.9.

The length of the lag phase depended on the concentrations of NADH, propionic anhydride and enzyme and also probably upon the pH of the solution. When the concentrations of other reagents were kept constant, increasing amounts of either NADH or propionic anhydride increased the length of the lag phase, whereas increasing the enzyme concentration caused a shortening of the lag. Additionally, when the ratio of NADH to enzyme was constant, higher concentrations of these reagents at a constant propionic anhydride concentration produced longer lags. Results are shown in Table 6.6 and Figure 6.27. The length of the lag phase is expressed in terms of T, which was measured as indicated in the inset to Figure 6.26, T being obtained from the intercept of extrapolated tangents to the slope of the lag phase and the normal steady state rate which followed.

At high concentrations of both enzyme and NADH, the transient decrease in fluorescence was followed by an increase in the fluorescence signal to a peak value, followed by a hysteretic decrease which resembled a lag phase. The maximum fluorescence was not observed to be higher than the original fluorescence level. Examples are shown in Figure 6.28. Very slight increases in fluorescence appear to be apparent in Figures 6.27a Traces A & D.

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Figure 6.27 The Effect of Enzyme and Substrate Concentrations on the Lag Phase. Enzyme and NADH were mixed with NADH and propionic anhydride in each case. Reactions were recorded over two time frames in each case. The concentrations of each substance immediately after mixing are shown below.

	a	b	c	d
[E] (μM)	6	6	6	6
[NADH] (μM)	18	36	56	90
$[(RCO)_2O]$ (mM)	3.0	3.0	3.0	3.0

Figure 6:26

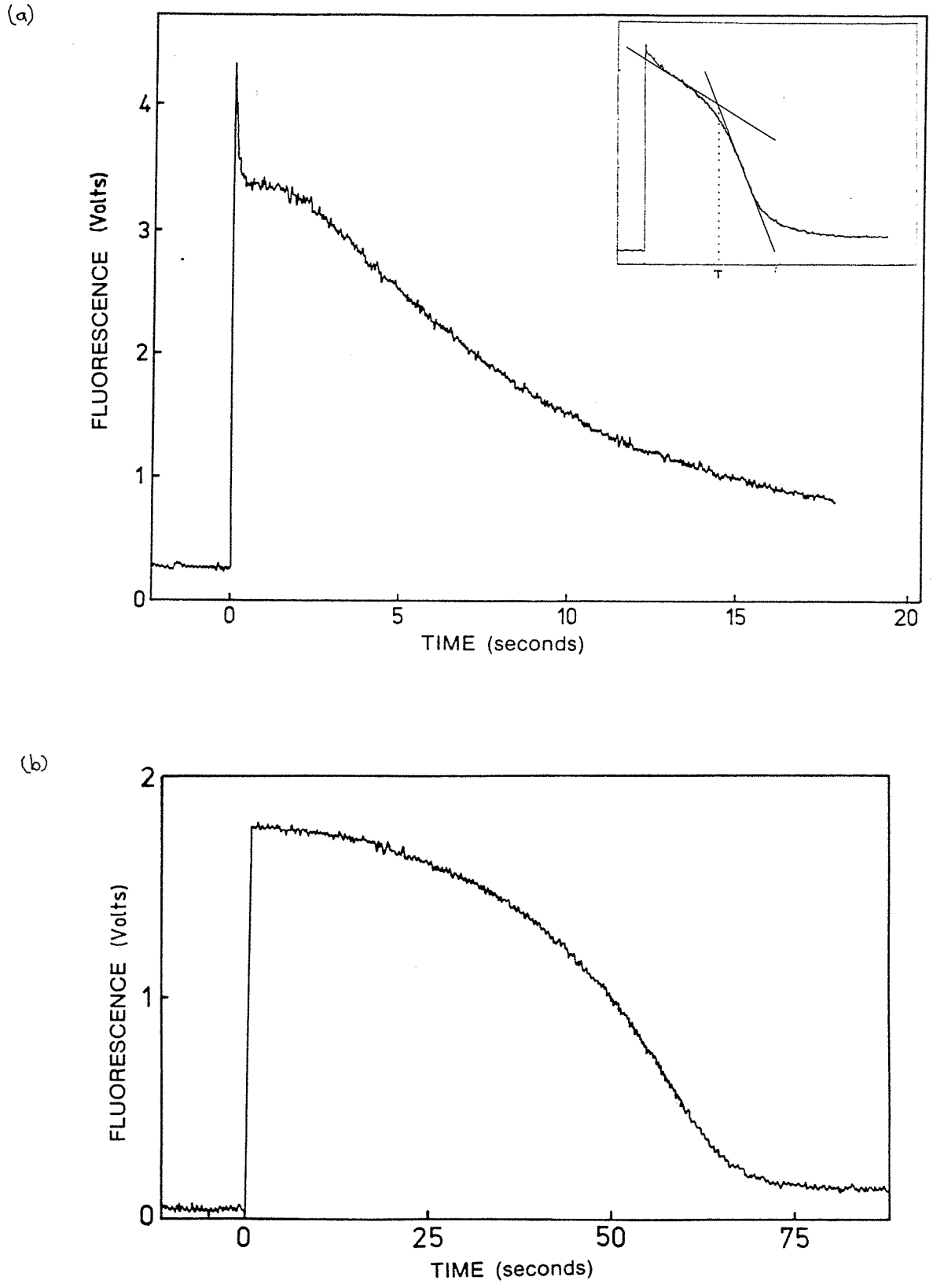


Figure 6.26 Lag Phases observed in Stopped Flow Experiments
Enzyme and NADH were rapidly mixed with NADH and propionic anhydride. The concentrations of each species in solution immediately after mixing were (a) enzyme (6.0 μM) NADH (20 μM) and propionic anhydride (3 mM) and (b) enzyme (5.0 μM) NADH (45 μM) and propionic anhydride (3.9 mM).

Figure 6:27

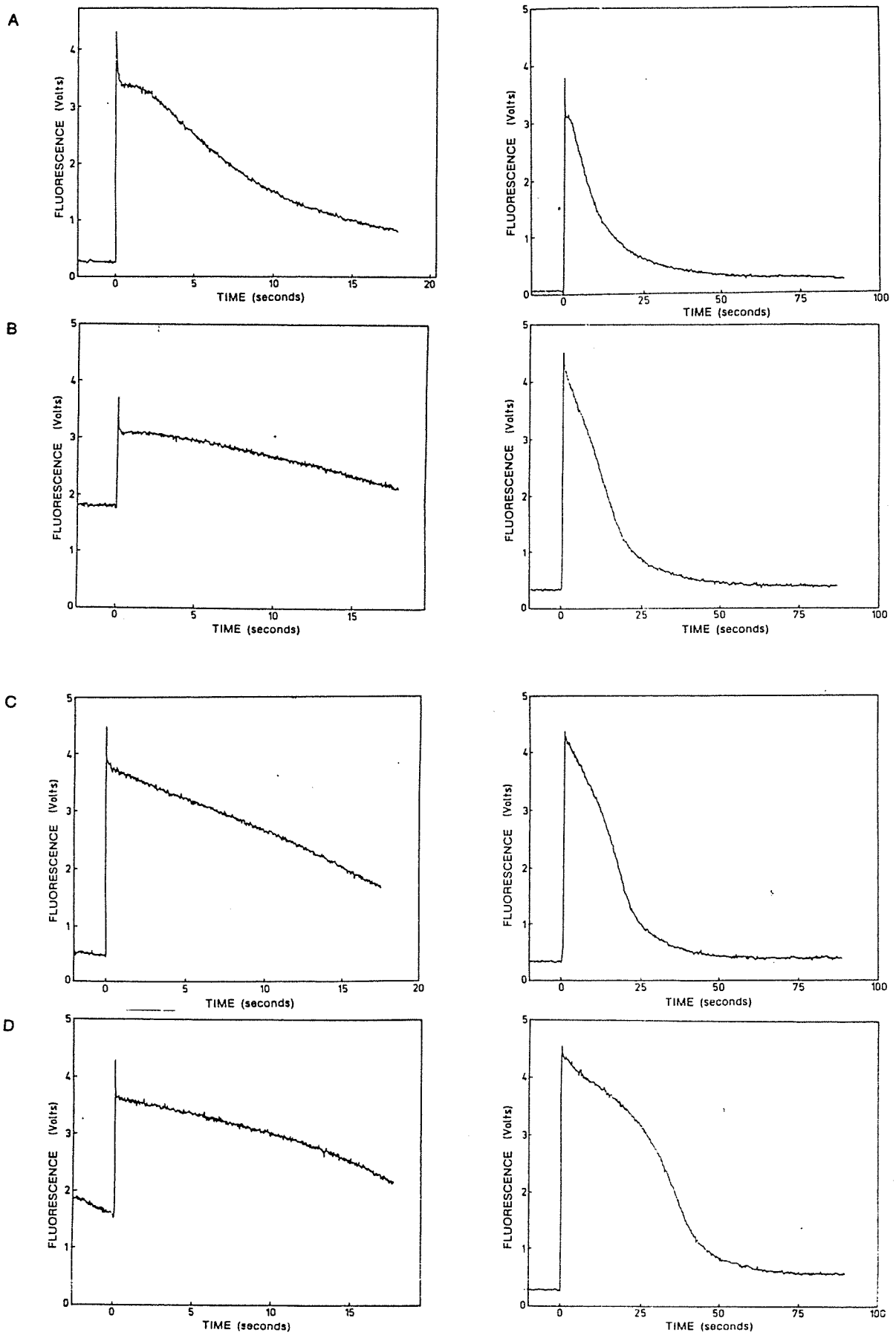

A
B
C
D
FLUORESCENCE (Volts)
TIME (seconds)
0
1
2
3
4
5
0
5
10
15
20
0
25
50
75
100

Figure 6:28

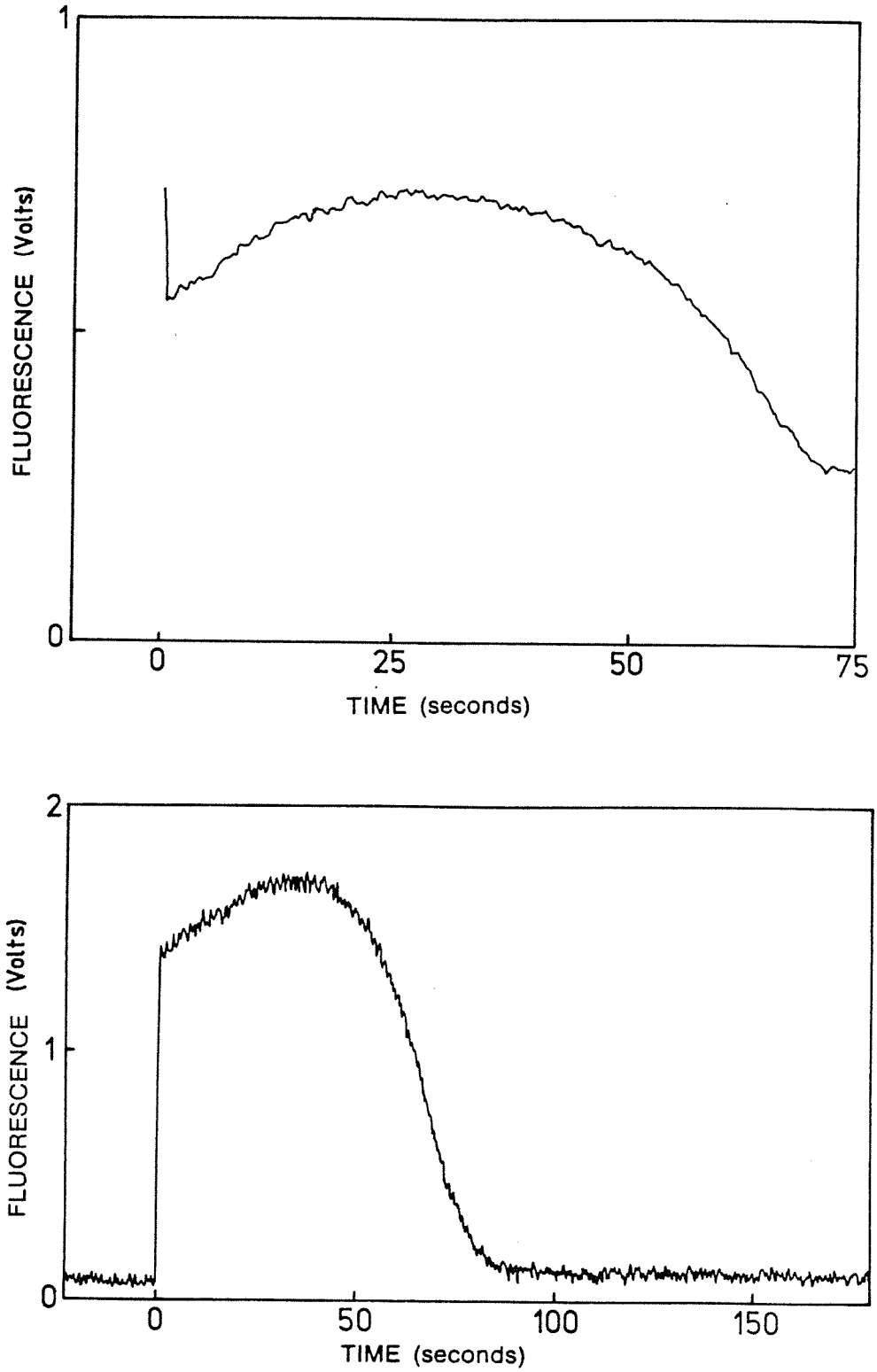


Figure 6.28 Lag Phases exhibiting an Increase in Fluorescence.
Enzyme and NADH were mixed with NADH and propionic anhydride in each case. The concentrations of each substance immediately after mixing were (a) enzyme(7 μM) NADH (60 μM) and propionic anhydride (3.6 mM) and (b) enzyme (10 μM) NADH (50 μM) and propionic anhydride (3.9 mM).

TABLE 6.6

Lag Phases

[NADH]	[E]	[Anhydride]	T
μM	μM	mM	s
(a) Vary NADH Concentration			
9	5.2	2.35	4
18	5.2	2.35	10
36	5.2	2.35	27
54	5.2	2.35	44
(b) Vary Propionic Anhydride Concentration			
36	5.2	0.39	-
36	5.2	0.78	1
36	5.2	2.35	27
(c) Vary Enzyme Concentration			
36	5.0	3.9	45
36	7.5	3.9	20
36	10.0	3.9	12
(d) Vary [E] and [NADH] at constant [NADH]/[E] = 9.0			
22.5	2.5	3.9	16
45	5.0	3.9	30
90	10.0	3.9	47

Enzyme (14 μM) was premixed with both NADH (80 μM)and NAD^+ (20 μM) and rapidly mixed with NADH (73 μM), NAD^+ (20 μM) and propionic anhydride (7.8 mM) in order to determine whether the presence of a small amount of NAD^+ premixed with enzyme would eliminate this increase in fluorescence. However, this had no effect on the lag phases and an increase in fluorescence following the transient process was still observed.

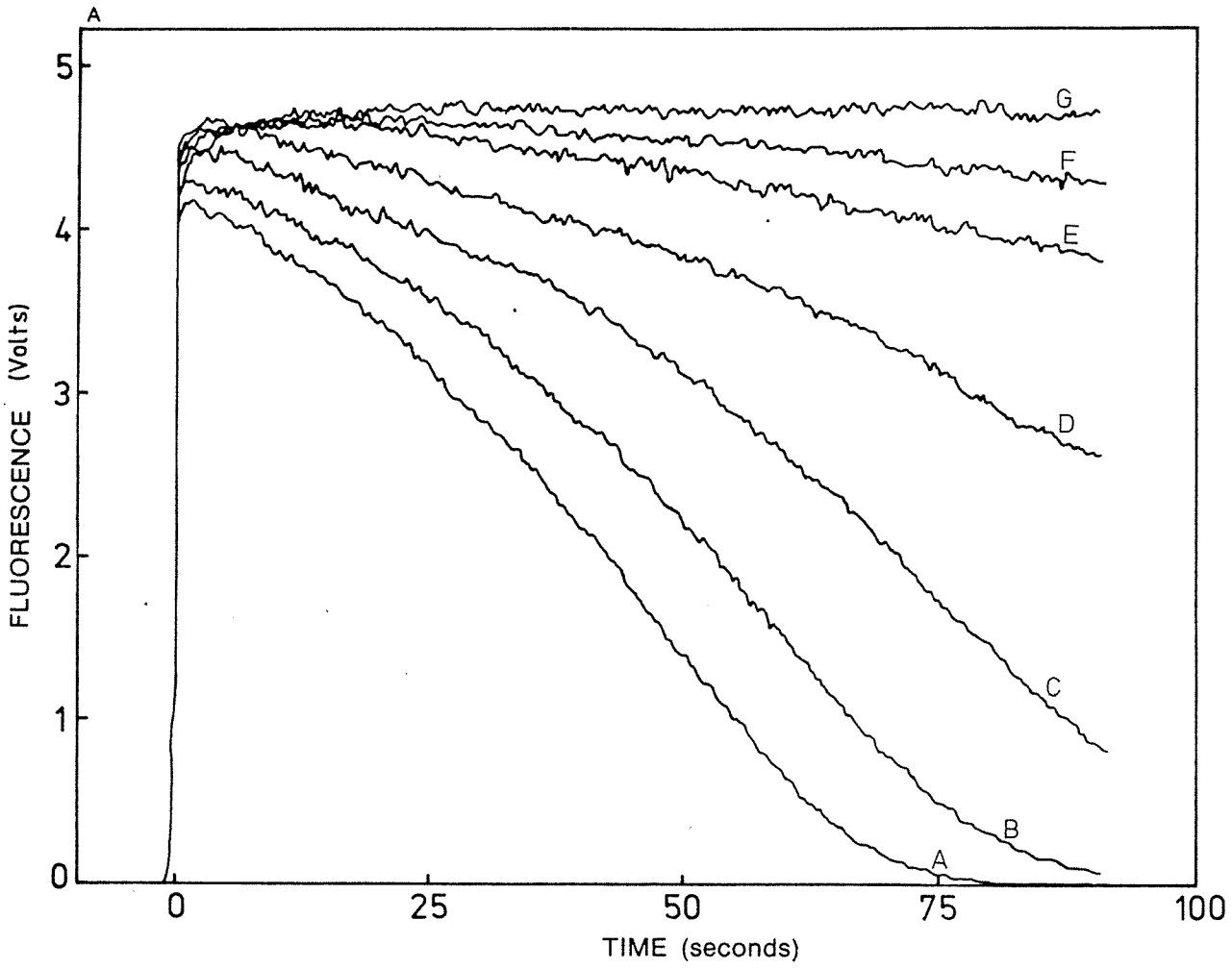
The pH of the solution may also affect the lag phase. For successive mixings from the same initial bulk solutions, longer lag phases were generally observed for later mixings despite lower concentrations of propionic anhydride, which, according to the results above should shorten the lag time. As these solutions had very low buffer capacity, hydrolysis of the propionic anhydride caused a lowering of the pH of these solutions in addition to the drop in the anhydride concentration. Figure 6.29a shows such a set of sequential

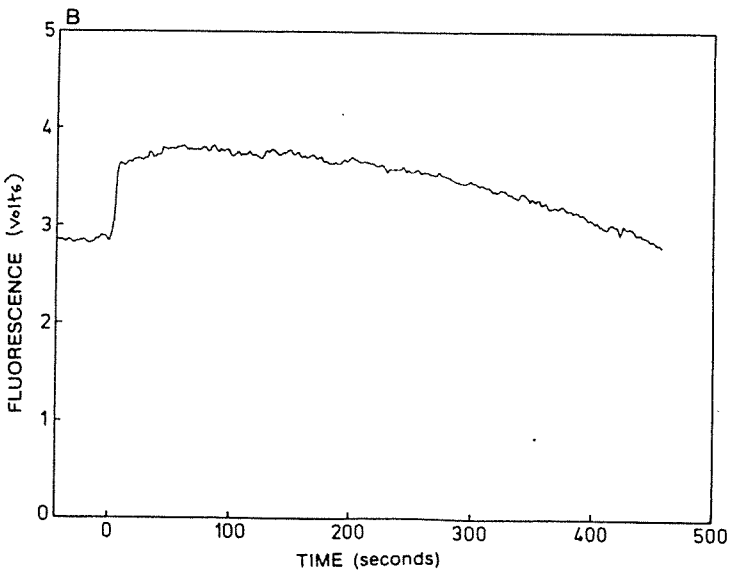
Figure 6.29 The Appearance of a Lag Phase and Increase in Fluorescence following NADH Binding.

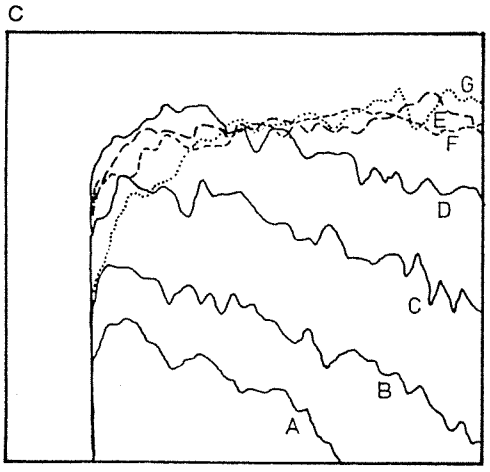
6.29 (a) Enzyme (5 μM) was repeatedly mixed with samples from a solution containing NADH (50 μM) and propionic anhydride (1.9 mM).

6.29 (b) The same experiment, but on a much longer time scale, demonstrating that the lag phase is present.

6.29 (c) An enlargement of a section of the traces in Figure 6.29a, showing the gradual appearance of an increase in fluorescence following the binding process.


A
FLUORESCENCE (Volts)
TIME (seconds)
G
F
E
D
C
B
A


B
FLUORESCENCE (Volts)
TIME (seconds)


C
G
E
F
D
C
B
A

traces in which enzyme (10 μM) was rapidly mixed with NADH (100 μM) and propionic anhydride (3.9 mM). The lag phase became longer and longer for later traces. This was not merely a loss of the steady state process in the final traces. As shown in Figure 6.29b observation on a very much longer time scale demonstrated that there was really a lag phase followed by a faster steady state. Figure 6.29c is a five-fold enlargement of the initial section of Figure 6.29a showing the appearance of a slow increase in fluorescence. This was either due to an effect of pH on the NADH binding causing a great decrease in λ_s or alternatively, the appearance of the increase in fluorescence observed as a part of the lag such as those in Figure 6.28.

6.5 DISCUSSION

A k_{cat} value of 0.16 s^{-1} per active site was obtained for the reaction of cytoplasmic aldehyde dehydrogenasewith propionic anhydride in the presence of NADH. Similarly, a value of 0.044 s^{-1} per active site for k_{cat} was obtained for acetic anhydride. These values are of the same order of magnitude as for oxidation of the propionaldehyde and acetaldehyde and demonstrate that all steps in the reaction mechanism are reversible once an enzyme-NADH-acyl intermediate is formed. Previous explanations for the irreversibility of the oxidation of aldehydes have been based on assumptions that the reversal of this process is themodynamically unfavourable and that carboxylic acids are unable to bind to the enzyme. This latter assumption is almost certainly correct, as it has been clearly demonstrated that there is no reversal of the reaction when acetic acid and NADH are mixed with enzyme, yet when more powerful acylating agents are used, the reverse reaction proceeds readily. Thus any thermodynamic arguments can really only be applied to the acylation process, which involves binding of acid and an accompanying loss of a water molecule, a step which is bypassed by the use of an acid anhydride as the acylating agent.

The K_m value of 4.0 μM for NADH obtained from the reverse reaction was higher than the dissociation constant of 1.2 μM (MacGibbon et al., 1977a) for NADH binding to enzyme. However, for reactions involving more than one substrate and more than one intermediate, K_m values are a function of the dissociation constants and inhibition constants for NADH and NAD^+ and are therefore likely to be higher than the corresponding dissociation constant. Thus the oxidation of NADH

apparently involves the same active site as the oxidation of aldehydes and hence is its microscopic reverse. The various anhydrides must therefore acylate the active site group which can then accept the hydride ion from NADH to form NAD^+ and the hemiacetal form of the aldehyde.

Recently, von Bahr Lindstrom et al. (1985) have demonstrated that a histidine residue is strictly conserved in the sequences of aldehyde dehydrogenases from human liver cytoplasm, human liver mitochondria and horse liver mitochondria, in a position close to the cysteine residue identified as the one which is selectively modified by iodoacetamide (Hempel et al., 1981, 1984, 1985) In the case of the sheep liver cytoplasmic isoenzyme, this cysteine residue has been shown to be one of a pair of reactive cysteines. (Kitson & Loomes, 1985).If this histidine residue is catalytically essential, then the close proximity, at least in the primary structure, of these reactive sulphydryl groups may explain the effectiveness of thiol reagents as modifiers of the enzymic activity.

The mechanism by which higher concentrations of NADH caused inhibition of the steady state rate is not known, but it is clearly linked to the hysteresis which became apparent at the same NADH concentrations. The initial rates were faster than the final linear rates, indicating that the inhibition arises as a result of a slow transition process. The rate constant for this process appeared to be about 0.03 s^{-1} and was independent of the NADH concentration.

Inhibition of the rate of the esterase reaction at high concentrations of NADH has also been observed (MacGibbon et al., 1978b), but in this reaction NADH is not an essential substrate as it is for the anhydride reaction, and different catalytic sites have been proposed for the esterase and dehydrogenase reactions (Blackwell et al., 1983a,b). NADH (and NAD^+) cause activation of the rate of enzyme-catalysed ester hydrolysis up to a maximum at an NADH level that is dependent on the initial concentration of ester.

On the basis of these results, it has been proposed (MacGibbon et al., 1978b) that there may be a second, low affinity, binding site for NAD^+ and NADH that is not directly involved in the catalytic activity, but which either overlaps with the esterase binding site, or modifies the enzyme so that the binding of p-nitrophenylacetate is adversely affected. In the case of the reverse reaction, it is possible that NADH binding at this second site produces a slow transition to a less

active form of the enzyme.

Although there was an optimum NADH concentration for each initial propionic anhydride concentration, this arises largely because of the involvement of NADH as a catalyst of propionic anhydride hydrolysis and produces an effect on the extent of the reaction rather than on the initial rate. The increased curvature of absorbance traces at high NADH levels limited the usefulness of the data and it became increasingly difficult at low propionic anhydride concentrations to determine at which NADH concentrations inhibition of the initial rate became significant. However, there was no real evidence to suggest that there was any such inhibition at NADH concentrations below 30 μM and the curvature of the traces made it impossible to detect any of the hysteretic effects which were observed at higher propionic anhydride concentrations. Thus despite apparent similarities, the NADH dependences of the esterase and anhydride reactions are not entirely analogous, but both results indicate the probable presence of a second NADH binding site. Catalysis of the hydrolysis of propionic anhydride by NADH, enzyme, enzyme-cofactor complexes, and phosphate has been clearly demonstrated and this limits the amount of information which can be gained about the reverse reaction. In particular, the concentration of propionic anhydride present at the turnover point from reverse to dehydrogenase reactions or at the time the dehydrogenase reaction rate is a maximum is unknown at present. In principle, it should be possible to gain some information about the rate of anhydride hydrolysis from logarithmic plots of the approach to the minimum absorbance level. However this rate of approach is often governed by a number of factors, including the concentrations of NADH, NAD^+ and propionaldehyde and the pH, in addition to the propionic anhydride concentration. Thus interpretation of these data is likely to be difficult and has not been attempted in this study.

There are two possible processes which could produce the rapid transient decrease observed in the stopped flow experiments. It is possible that the presteady state process involves a decrease in fluorescence, unaccompanied by any detectable absorbance changes. Since for enzyme premixed with NADH, most of the enzyme has NADH bound in either the E.NADH form or the conformationally altered *E.NADH form, both of which exhibit enhanced fluorescence of NADH, such a decrease in fluorescence must result from a reduction in the fluorescence enhancement of one or both of these species upon acylation or

alternatively, the displacement of NADH from one of these enzyme forms. Alternatively, it is possible that hydride transfer occurs during the presteady state phase of the reaction so that the fluorescence decreases as E.NADH.acyl is converted to E.NAD^+.Ald (Scheme 6.1).

Although it is not certain at this stage which of these occurs, there is strong evidence to favour the latter "reverse burst" proposal. The rate constant of the process which produced a decrease in protein fluorescence for enzyme premixed with NADH pushed against propionic anhydride and NADH was the same, within experimental error, as that observed in nucleotide fluorescence. As discussed previously, this change in protein fluorescence was consistent with hydride transfer, whereas it is not obvious that acylation of enzyme intermediates would cause a reduction in protein fluorescence. In addition, the increase in fluorescence which was sometimes observed following the "burst" (Figure 6.28) was also observed in absorbance studies (Figure 6.10e). Since such an increase in A_{340} suggests an increase in the NADH concentration in the solution, it is strong evidence that the preceding rapid transient process involves NAD^+ production. The observation that such increases in fluorescence and absorbance were also always observed when either NAD^+ or propionaldehyde (but not both) was added to reverse reaction mixtures (Figure 6.11a) provides further support for the suggestion that the transient is a burst in NAD^+ production.

The alternative proposal in which an E.NADH.acyl intermediate with reduced fluorescence is produced also appears to be inconsistent with the proposed mechanism for the dehydrogenase reaction. If the E.NADH.acyl intermediate produced after hydride transfer did not exhibit any enhancement of fluorescence with respect to free NADH, then the increase in fluorescence observed during the burst would have to occur during a subsequent step in the mechanism such as acyl-enzyme hydrolysis, but this is clearly contrary to the data obtained at low pH in Chapter 4.

In previous studies of the reverse reaction, the observation of an isotope effect of 4.0 when [4A-^{2}H]NADH was used instead of NADH for the cytoplasmic enzyme in the steady state led Hart & Dickinson (1978a) to conclude that hydride transfer is partially rate limiting in the steady state production of NAD^+. On the basis of this result, Hart & Dickinson (1978a) predicted a maximum rate of 0.83 s^{-1} for hydride transfer in the reverse direction. Clearly this result is inconsistent with the above proposal that the presteady state phase (governed by a

rate constant of about 30 s^{-1}) includes the hydride transfer step. Since there is good evidence in support of rapid hydride transfer, possible reasons for the inconsistency must be considered.

The [4A-^{2}H]NADH used by these authors was produced from NAD^+ by enzymic reduction using yeast alcohol dehydrogenase, followed by denaturation of this enzyme. Any NAD^+ which remained in the solution would have inhibited the reaction of acetic anhydride and NADH and could well have been misinterpreted as an apparent isotope effect. It is clear that further isotope studies are required to resolve this question, and studies of both the presteady state and steady state reactions using [4A-$_2$H]NADH are planned.

The fluorescence amplitudes obtained in the reverse burst experiments both with and without premixed NADH were reduced by 40-60% of the amplitudes of the burst in NADH production for the dehydrogenase reaction and in normal NADH binding experiments respectively. The loss of amplitude in the NADH binding experiments in the presence of propionic anhydride was shown to be equal to the decrease in nucleotide fluorescence in experiments in which NADH was premixed with enzyme, but the binding process was still observed to be biphasic.

It is therefore proposed that the isomerisation of enzyme.NADH complexes also occurs in the presence of propionic anhydride, and that this is followed by rapid hydride transfer. When enzyme is premixed with NADH, a mixture of E, E.NADH and *E.NADH will be present in solution at the start of the reaction, but although all three forms of the enzyme may be acylated, the reverse burst process is too fast for any significant interconversion of these forms during the process. The amplitude of the reverse burst will therefore be determined by the amount of *E.NADH initially present, and the rate of the subsequent steady state may be partially controlled by the rate at which more *E.NADH can be formed.

When enzyme is not premixed with NADH, then NADH must first bind to the enzyme. This is followed by the isomerisation process, but as fast as *E.NADH forms, it will be removed by the reverse reaction and therefore the apparent amplitude of the binding process will be reduced proportionately. Since hydride transfer is rapid, the rate of the presteady state processes for this mixing condition will be governed by the rate of the two slower processes, NADH binding and isomerisation, and the observed fluorescence changes would be biphasic, as was observed. The observation of distinct biphasic NADH binding and

reverse burst processes for reactions involving DEPC, for which the reverse burst process is much slower, supports this proposal.

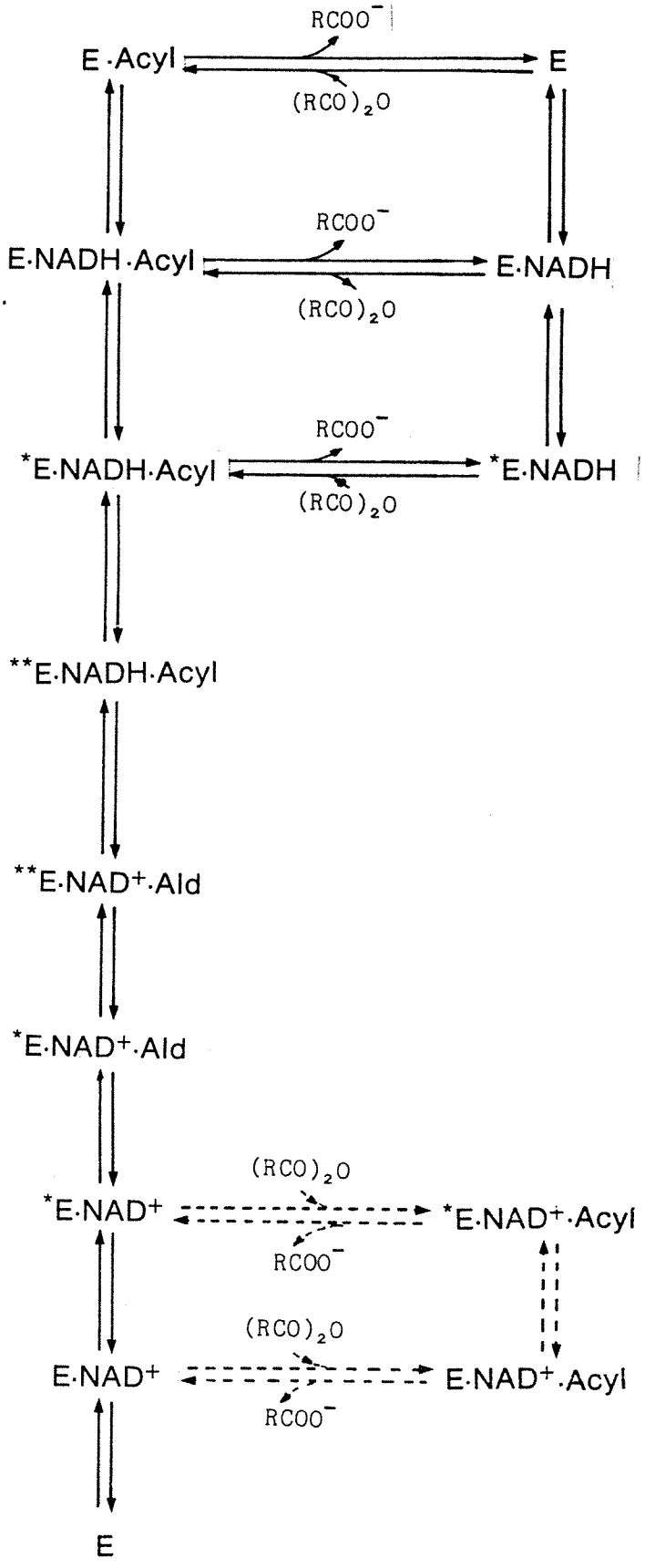
The increase in the apparent rate constants, λ_f and λ_s, for NADH binding in the presence of propionic anhydride indicates that propionic anhydride can probably acylate at or near the active site of more than one form of the enzyme. Acylation of a number of non-catalytic groups also undoubtably occurs, but it is assumed that such acylation does not have a dramatic effect on the activity of the enzyme. (The effects of acylation are investigated further in Chapter 7.) Since λ_f and λ_s are functions of the rate constants for both NADH binding and the isomerisation process, it is not certain whether the rates of both processes were increased. The amplitude of the faster process (NADH binding) was significantly decreased and this probably indicates that the rate of this process was increased (2.1.4). This would mean that propionic anhydride was reacting with the free enzyme. The observation of a reverse burst when NADH was premixed with the enzyme indicates that propionic anhydride also acylates the *E.NADH form directly, and it is therfore assumed that the intermediate E.NADH complex is also acylated. It is not yet known if the rate of the isomerisation of these enzyme.NADH.acyl complexes is faster than for the corresponding enzyme.NADH complexes. These results also demonstrate that propionic anhydride binding probably occurs very rapidly. Substrate addition for the reverse reaction does not therefore appear to be ordered, in contrast with the dehydrogenase reaction.

The proposed mechanism for the reverse reaction is given in Scheme 6.2. Two isomeric enzyme.NADH.acyl complexes are included in this mechanism on the basis of symmetry, as presumably the two conformational rearrangements which are thought to occur in the presteady state phase of the dehydrogenase reaction must have their counterparts in the reverse reaction (i.e. each conformational change must be reversed at some later step in the mechanism before free enzyme is regenerated).

Hart and Dickinson (1978a) reported the same steady state reaction rates for both acetic and butyric anhydrides for the mitochondrial isoenzyme. However, for the cytoplasmic aldehyde dehydrogenase, different steady state reaction rates were obtained for reverse reactions using different anhydride substrates.

As shown in chapter 5, steady state reaction rates for the cytoplasmic enzyme for propionic anhydride were 3.6 times the rates for

Scheme 6.2


RCOO⁻
E·Acyl
E
(RCO)₂O
RCOO⁻
E·NADH·Acyl
E·NADH
(RCO)₂O
RCOO⁻
*E·NADH·Acyl
*E·NADH
(RCO)₂O
**E·NADH·Acyl
**E·NAD⁺·Ald
*E·NAD⁺·Ald
(RCO)₂O
*E·NAD⁺
*E·NAD⁺·Acyl
RCOO⁻
(RCO)₂O
E·NAD⁺
E·NAD⁺·Acyl
RCOO⁻
E

acetic anhydride at the same anhydride concentration. The steady state rates for butyric and propionic anhydrides were very similar, whereas those for the two cyclic anhydrides and for DEPC were very much slower. There is insufficient information to determine why the reaction with acetic anhydride is so much slower than for propionic anhydride, since it might be expected that acetic anhydride should bind to the enzyme more rapidly and also that hydride transfer should occur more rapidly for this species, but both the presteady state and steady state rates were slower than for propionic anhydride.

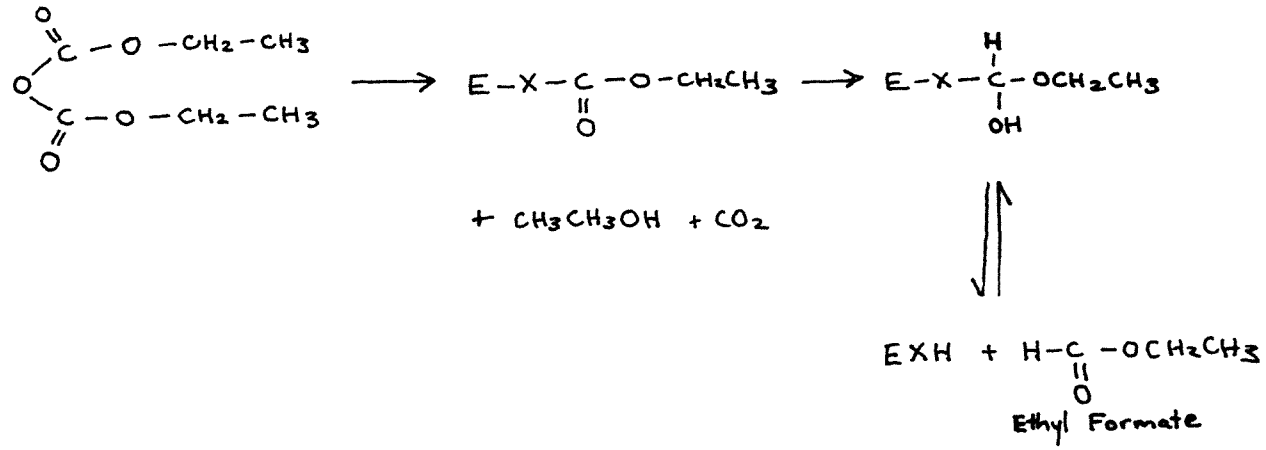
Since an increase in A_{340} was observed when excess propionaldehyde was added to reacting solutions of each of the the anhydrides studied, this suggests that NADH is oxidized to NAD^+ in each case. In chapter 5 it was established that acetaldehyde was a product of the reaction involving enzyme, NADH and acetic anhydride. The increase in A_{340} which followed the reverse reaction once the appropriate anhydride hydrolysed suggests that propionaldehyde and butyraldehyde are produced from propionic and butyric anhydrides respectively.

The structures and probable reaction products of DEPC and maleic and succinic anhydrides are shown in Scheme 6.3. Succinic and maleic anhydrides react with amino groups of proteins and also reversibly with sulphydryl, tyrosyl, and imidazole groups and, for both of these reagents, acylation of an amino group results in a change in the charge at the site of acylation (Means & Feeney, 1971 pp 74-77). DEPC reacts with histidine, both amino and tyrosyl groups and possibly also with sulphydryl groups.

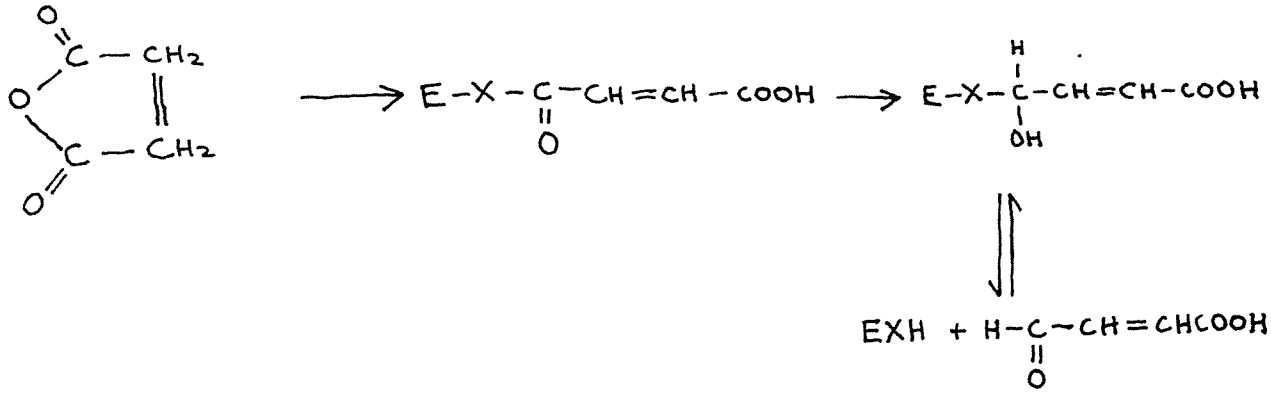
The frequent observation of a lag phase and associated absorbance and fluorescence increases following the reverse burst indicate that at least two distinct enzyme intermediates which are directly observable are formed under some circumstances before a steady state production of aldehyde is established. No complete analysis of the nature and causes of the lag phase can be made at this stage with the limited amount of information available and the problems of reproducibility under some conditions. However, a possible mechanism by which some aspects of the lag phase could arise can be proposed based on the reaction sequence shown in Scheme 6.2.

It was proposed above that, during the reverse burst process, only the *E.NADH form of the enzyme is involved. Because the isomerisation of *E.NADH and E.NADH occurs relatively slowly compared to the reverse burst, there is a temporary depletion of *E.NADH and no steady state

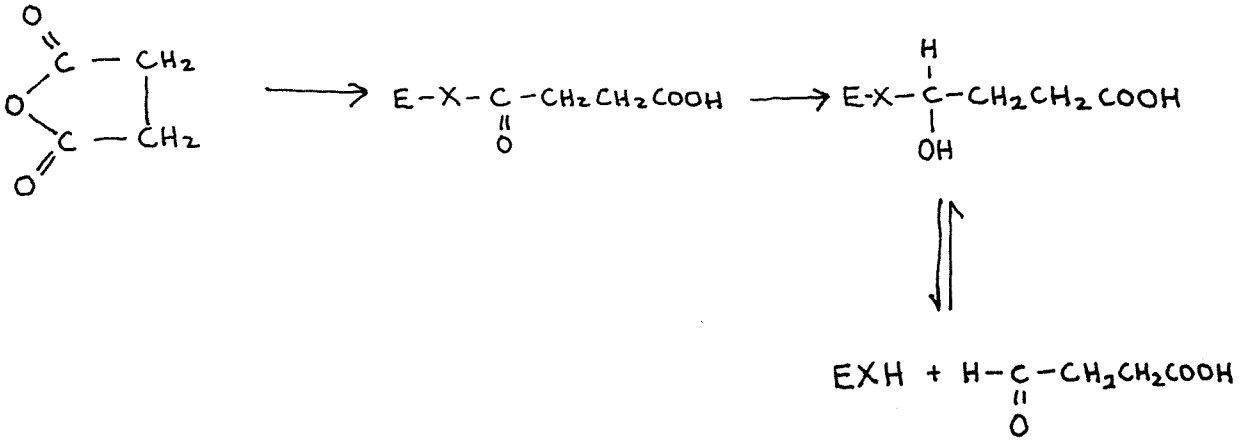
DIETHYLPYROCARBONATE



MALEIC ANHYDRIDE



SUCCINIC ANHYDRIDE



SCHEME 6.3

can be established immediately following the burst. In the temporary absence of any steady state reaction flux, it is proposed that the **E.NAD.Ald intermediate undergoes hydride transfer back to reform **E.NADH.Acyl, presumably because this complex is more thermodynamically stable than the *E.NAD.Ald intermediate (Scheme 6.2). The increase in absorbance and fluorescence back to the initial level which is sometimes observed is a result of this reversal of the hydride transfer. According to this scheme the steady state production of NAD^+ and aldehyde is not established until sufficient *E.NADH can be produced from E.NADH to maintain the reaction. The actual steady state rate may therefore be partially controlled by the isomerisation rate, but is probably also limited by the rate of one or more steps in the pathway following hydride transfer, possibly dissociation of E.NAD^+ for which a rate constant of about 1.6 s^{-1} has been proposed previously by MacGibbon et al. (1977c). Propionaldehyde or NAD^+ present at the start of the reaction would be expected to increase the likelihood of hydride transfer back to **E.NADH.Acyl, thus producing an increase in absorbance or fluorescence as was experimentally observed. However, although this scheme explains why an increase in absorbance or fluorescence occurs after the presteady state process, it is not clear why such an increase should not be observed in every case, nor why increased amounts of NADH and propionic anhydride should increase the length of the lag phase. Since there is evidence that propionic anhydride can acylate the active site of at least two (and probably three) enzyme forms, it may also acylate the enzyme.NAD^+ complexes. Any such acylation would be reversible, resulting in hydrolysis of the propionic anhydride, but the presence of enzyme.NAD^+.acyl complexes would be expected to have a temporary inhibitory effect on the reverse reaction which may also appear as a lag phase.

An acceptable explanation of the lag phase must include the reasons for the increase in absorbance (and fluorescence), the dependence of the length of the lag on the concentrations of enzyme, NADH and propionic anhydride and also the reasons why lag phases are not always observed and why the lag phase is sometimes followed by a very fast decrease in absorbance. Any such explanation is likely to include more than just a single process, and more experimental work is certainly required before the exact conditions which produce the lag phases are known.

6.6 CONCLUSION

The normal irreversibility of the enzyme dehydrogenase reaction appears to be due to failure of carboxylic acids to acylate the enzyme. However, acylation by a variety of anhydrides occurred readily and the simple acid anhydrides such as acetic, propionic and butyric anhydrides appeared to have a high affinity for the enzyme, reasonably similar to that of aldehyde substrates such as propionaldehyde.

However study of the reverse reaction with simple acid anhydrides is complicated by the accompanying hydrolysis of the anhydride. Classical kinetic studies are therefore largely inappropriate, and approach to an equilibrium between the dehydrogenase and reverse reactions is impossible. Because very high concentrations of anhydride were required, this substrate was almost always initially present in excess, which limited the amount of quantitative information which could be gained. Despite these limitations, it was possible to gain a large amount of useful information about the reverse reaction using propionic anhydride as the substrate.

On the basis of these results, it is proposed that, following acylation of the enyme by propionic anhydride, the reverse reaction is the microscopic reverse of the usual dehydrogenase mechanism. Substrate addition does not appear to be ordered for the reverse reaction, as the results indicate that either NADH or propionic anhydride is able to bind in the absence of the other. Similarly, in Chapter 4, it was concluded that release of products during the dehydrogenase reacation was not necessarily ordered.

The presteady state processes which were observed depended on the mixing conditions, but in each case the hydride transfer step appeared to be very rapid, and to occur in the presteady state phase of the reaction. When enzyme was preincubated with NADH, the presteady state process appeared to involve only the conformationally rearranged *E.NADH complex. When enzyme was not premixed with NADH, the presteady state phase included the two NADH binding processes previously reported by MacGibbon *et al*. (1977b) in addition to hydride transfer. The observation of lag phases probably indicates that more than one slow process controls the steady state rate. One of these steps is probably the conformational change which follows NADH binding, even though this step preceeds hydride transfer, and the other is possibly dissociation of NAD^{+} from the enzyme.

CHAPTER 7

THE EFFECTS OF ANHYDRIDES ON ALDEHYDE DEHYDROGENASE

7.1 INTRODUCTION.

In the previous two chapters a variety of anhydrides were used as substrates for the reverse reaction of aldehyde dehydrogenase. Attempts to produce a reverse reaction with less powerful acylating agents had previously proved unsuccessful.(MacGibbon et al., 1977a). Since all the anhydrides tested are substrates for the reverse reaction, they must react at the active site and therefore be able to act as inhibitors of the normal dehydrogenase reaction. The possibility of these reactive anhydrides reacting with other groups on the enzyme and also indirectly modifying the enzyme activity cannot however be excluded.

Acetic anhydride has been widely used as an acetylating agent (Fraenkel-Conrat, 1959, Means & Feeney, 1971, 69-71) and both succinic and maleic anhydrides are useful reagents for the modification of amino groups in proteins. Similarly, possible acylation of reactive groups on the enzyme by propionic and butyric anhydrides must be considered. In addition to reaction with amino groups, all of these reagents can react reversibly with sulphydryl, tyrosyl and imidazole groups of proteins (Means & Feeney, 1971, 69-77). Diethylpyrocarbonate (ethoxyformic anhydride) has been widely used as a specific reagent for the acylation of imidazole groups of histidine residues in proteins (Melchior & Fahrney, 1970, Holbrook & Ingram, 1973, Hegyi et al., 1974, Dickenson & Dickinson, 1975, Choong et al., 1977).

It was therefore decided to carry out an investigation of the effects of these reagents on the dehydrogenase activity of the enzyme. Since the identity of the reactive group in the enzyme active site has not yet been established (chapters 3 & 4, Buckley & Dunn, 1982), it was of particular interest to study the effect of diethylpyrocarbonate on the enzyme, to determine whether or not the enzyme contains a catalytically essential imidazole group. Weiner (1985) has recently shown that the horse liver enzyme is inhibited by DEPC, two histidines per mole of protein being acylated with loss of 80% of the enzymic activity.

Since the sulphydryl reagent PCMB produced markedly different effects on the enzyme activity at high (20 mM) and low (100 μM) concentrations of propionaldehyde (Chapter 3) it was considered

essential to study the effects of these acylating agents at both propionaldehyde concentrations.

A study of the effect of these anhydrides on the NADH burst was also undertaken.

7.2 METHODS.

7.2.1 Steady State Experiments.

7.2.1.1 The Effect of Propionic Anhydride on the Steady State Reaction

The effect of propionic anhydride on the dehydrogenase activity of the enzyme was determined using the standard high (20 mM) and low (100 µM) propionaldehyde assays as described previously (2.2.1), with some modification. To minimize hydrolysis of the propionic anhydride, assays were carried out in 0.1 M Na_2SO_4, 0.1 M $NaNO_3$ solutions instead of 25 mM buffer, and at pH 7.0 instead of pH 7.6. NAD^+ solutions were all prepared in doubly distilled deionised water and the pH was adjusted to 7.0 using 0.1 M NaOH.

Varying amounts of a solution of propionic anhydride in acetonitrile were added to assay mixtures and the reaction rate was measured by monitoring the production of NADH, observed as an increase in absorbance at 340 nm, using an Aminco DW 2a Spectrophotometer. Because of the rapid hydrolysis of propionic anhydride in the presence of enzyme and NAD^+, (6.3.2), assays were carried out in the following way unless otherwise stated. Solutions containing enzyme (1-4 µM) and NAD^+ (1 mM) were prepared at 25°C. Propionic anhydride (10 µM - 26 mM) was added, followed within 10 seconds by the addition of propionaldehyde (100 µM or 20 mM) to initiate the dehydrogenase reaction. Good reproducibility was obtained by using Hamilton syringes to add 10-100 µl of propionic anhydride solution to the assay mixture. The reaction rates in the presence of propionic anhydride were compared with the control rate, determined under the same conditions, but in the absence of anhydride. The enzyme active site concentration was always determined using standard assays at pH 7.62 in 25 mM phosphate buffer (2.2.1).

The effect of propionic anhydride upon the dehydrogenase reaction at non-saturating levels of NAD^+ and propionaldehyde was also investigated.

Propionic anhydride was added to solutions containing enzyme (3.13 μM), NAD^+ (8.0 μM) and propionaldehyde (8.0 μM) in varying amounts to give anhydride concentrations of 13-210 μM. In all other respects reaction conditions were the same as those described above. Control experiments were conducted under the same conditions but in the absence of propionic anhydride and the initial reaction rate was measured as a tangent to the A_{340} versus time curve.

7.2.1.2 The Effect of Maleic Anhydride on the Enzyme Steady State Activity.

The effect of maleic anhydride on enzyme activity was determined using the standard assays at pH 7.62 in 25 mM phosphate buffer. Under the assay conditions used in the previous section for propionic anhydride, precipitation of the enzyme occurred at high concentrations of maleic anhydride (≧ 1 mM). The standard assay at pH 7.6 was used in preference to high ionic strength assays so that the results could be related to most of the established work and because of the problems with rapid buffer-catalysed hydrolysis of the propionic anhydride encountered in Chapter 5 when phosphate buffer of a high concentration was used.

7.2.1.3 The Effect of Diethylpyrocarbonate on the Steady State Activity

The effect of DEPC on the enzyme activity was determined at both low and high concentrations of propionaldehyde , using the standard assays at pH 7.6. Various mixing orders were employed, with DEPC added to enzyme in the presence or absence of premixed NAD^+ and propionaldehyde . The effect of DEPC concentration on the enzymic activity at low levels of propionaldehyde was determined at pH 6.1 by using 35 mM pH 6.1 acetate buffer to replace the pH 7.6 phosphate buffer used in the standard assays. The final buffer concentration was 25 mM in all cases. Reaction rates were compared with the control rate obtained in the absence of DEPC. DEPC solutions were prepared in A.R. grade absolute ethanol and the concentrations of stock solutions of DEPC were determined by measuring the change in absorbance at 233 nm when a solution containing DEPC was added to a solution of 10 μM histidine. A value for the extinction coefficient, ε, at 233 nm of 3200 $l.mol^{-1}.cm^{-1}$ was used (Melchior & Fahrney, 1970).

7.2.2 PreSteady-State Experiments.

NADH burst experiments were carried out as previously described (2.3.4) except that all solutions contained 0.1 M Na_2SO_4 and 0.1 $NaNO_3$ (at pH 7.0) instead of the 25 mM phosphate buffer pH 7.62 used previously. Buffer contributions from stock enzyme and NADH solutions amounted to a final buffer concentration of 2-5 mM, as described in chapter 6 (6.2.1). This low buffer concentration was necessary to minimize the rate of hydrolysis of some anhydrides, and thus the same conditions were used for all the anhydrides for the purposes of direct comparison. Burst experiments were conducted at both high (20 mM) and low (100 μM) propionaldehyde concentrations with a variety of premixing conditions. A solution of anhydride in acetonitrile was added to either or both reservoir syringes immediately prior to introduction into the drive syringes of the stopped flow spectrometer.

Control experiments without anhydride were carried out for each enzyme sample and at both high and low propionaldehyde levels for each set of experiments.

7.3 RESULTS

Steady State Experiments

All reaction rates are given as relative rates, i.e. rates expressed relative to the control rate, measured under the same conditions but in the absence of anhydride.

7.3.1 The Effect of Propionic Anhydride on the Enzyme Activity

7.3.1.1 Low Propionaldehyde Levels.

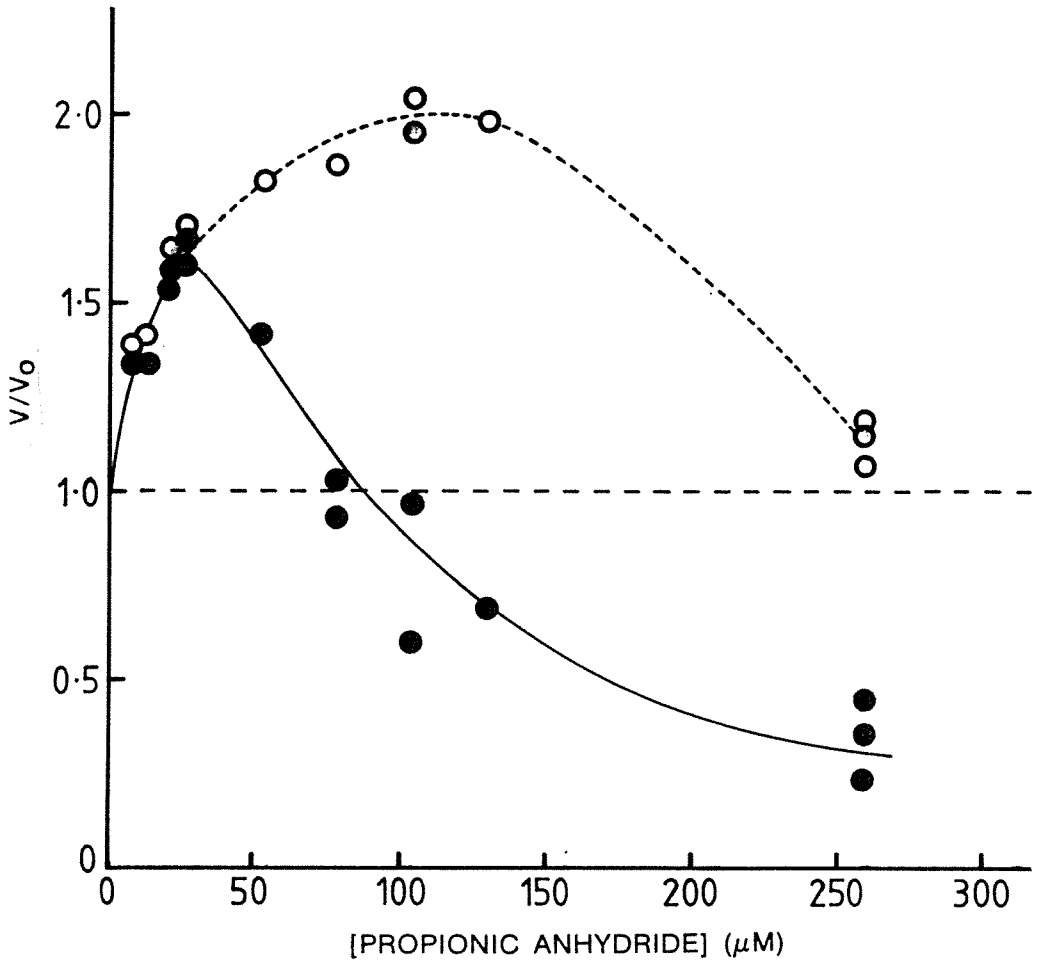
The effect of a range of propionic anhydride concentrations on the dehydrogenase activity of aldehyde dehydrogenase at low propionaldehyde levels is shown in Figure 7.1a. In curve A, initial rates, measured as tangents to the A_{340} versus time curve, are shown. Activation of the steady state rate was observed, reaching a maximum of 1.6 times the control rate at a propionic anhydride concentration of 26 μM. This represents an apparent propionic anhydride to enzyme active site ratio of 7.4. At higher concentrations of propionic anhydride, less activation was observed, until at 90 μM the initial rate was the same as the control rate. Inhibition of the steady state rate was then

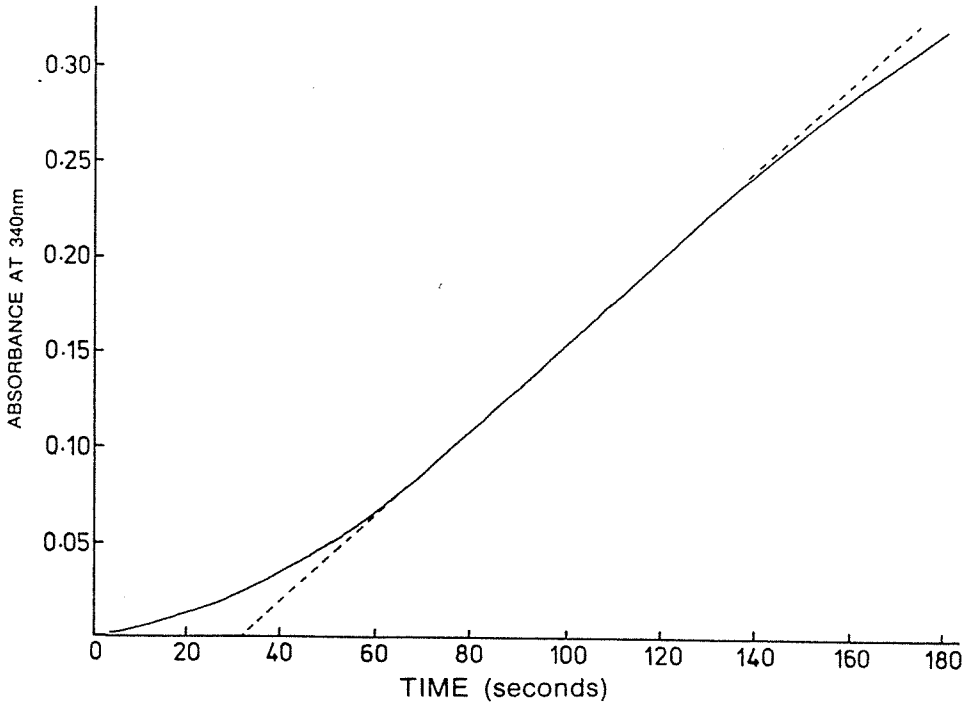
Figure 7.1 The Effect of Propionic Anhydride on Enzyme Activity at Low Concentrations of Propionaldehyde.

7.1 (a) Varying amounts of a solution of propionic anhydride in acetonitrile were added to enzyme (3.53 μM) premixed with NAD^+ (1 mM) and propionaldehyde (100 μM) was added within 10 seconds. Both the initial (●) and maximum (○) rates of the subsequent dehydrogenase reaction are shown relative to the control rate measured in the absence of propionic anhydride. An amount of pure acetonitrile was also added to solutions so that the total amount of this substance was the same in each case.

7.1 (b) Curvature of the absorbance versus time trace was observed as shown. Both the initial rate (measured as a tangent to the initial part of the trace, and the maximum rate (obtained from the linear part of the trace) were measured. The solution contained enzyme (3.53 μM), NAD^+ (2 mM), propionaldehyde (100 μM) and propionic anhydride (104 μM).

Figure 7:1 THE EFFECT OF PROPIONIC ANHYDRIDE ON DEHYDROGENASE ACTIVITY AT LOW PROPIONALDEHYDE CONCENTRATIONS





observed at concentrations of propionic anhydride greater than this, the rate decreasing to a relative value of 0.3 at a propionic anhydride concentration of 260 μM.

As shown in Figure 7.1b the reaction rate (measured as a tangent to the A_{340} vs time curve) was not constant, but increased to a maximum value, which was maintained for 60 seconds or more, and then subsequently decreased. In addition to the initial rate measurements, this maximum rate was determined for each assay. These are shown in Figure 7.1a, Curve B. In comparison with the initial rates, no inhibition of the maximum steady state rate was observed within the concentration range used. A maximum activation of 2.0 was obtained for an apparent initial propionic anhydride concentration of 105 μM. However, the actual concentration of propionic anhydride at the time this maximum rate was achieved would have been significantly lower than the initial concentration, due to hydrolysis of the propionic anhydride. Since the time required to attain the maximum rate increased as the initial propionic anhydride concentration increased, it is most likely that activation is only observed within a true concentration range of 0 - 100 μM (as indicated by the initial rate data).

At very high propionic anhydride levels (1.3 mM) a slow decrease in absorbance at 340 nm with time was now observed. A similar decrease in A_{340} also occurred in solutions containing only enzyme and propionic anhydride. If similar experiments at 1.3 mM propionic anhydride were carried out in 25 mM phosphate buffer solution at pH 7.6 (used to promote more rapid hydrolysis of the propionic anhydride), a decrease in A_{340} was again observed. This was followed by an increase in absorbance back to the original level, suggesting that the decrease is a result of a reverse reaction due to the presence of enzyme-bound NADH (as noted previously in chapter 6, (6.3.1)) The extent of this decrease in absorbance corresponded to 0.2 mole NADH/mole enzyme active sites.

As certain concentrations of both PCMB and propionic anhydride have been shown to cause activation of the steady state rate at low propionaldehyde levels, an experiment was attempted in order to determine whether these effects were additive or mutually exclusive. Enzyme was first dialysed extensively as described previously (3.2) to remove 2-mercaptoethanol. Propionic anhydride (26 μM) was added to a solution of enzyme (1.76 μM) and NAD^+ (1 mM), and propionaldehyde (100 μM) was added 10 seconds later. PCMB (4.24 μM) was added after

60 seconds, by which time the reaction rate in the presence of anhydride was at its maximum. An increase in the reaction rate was observed upon addition of PCMB. Results are shown in Table 7.1. and indicate that these effects are additive, at least to some extent. However, the total activation produced in the presence of both reagents was still less than the maximum activation possible with either of these reagents individually, suggesting that the activating site (if there is only a single one) was not saturated in either of the control experiments. Although unequivocal conclusions cannot be made at this stage, these results suggest that propionic anhydride and PCMB act at different sites to produce activation.

TABLE 7.1

The Combined Effects of Modifiers on the Steady State Reaction

Modifier	Concentration	v/v_o [b]	v/v_a [c]
	μM		
PA[a]	26	1.32	-
PCMB	4	1.72	-
PA[a] + PCMB[d]	4,26	1.65	1.25
PA[a]	26	1.20	-
Disulphiram	10	0.040	-
PA[a] + Disulphiram[d]	10,26	0.046	0.042

a PA is propionic Anhydride

b v_o is the control rate in the absence of any modifiers

c v_a is the anhydride activated rate

d added after 60 seconds

The effect of disulphiram (10.7 μM) on the anhydride low propionaldehyde assay was determined and the results are also shown in Table 7.1. Inhibition by disulphiram was observed, the extent of inhibition (95% relative to the activated rate in the absence of disulphiram) being the same for the activated assay as for the control assay (96.0%). It appears that the slight activation caused by the propionic anhydride was not immediately removed by disulphiram, although the difference in rate was small and hence may not be significant.

7.3.1.2 High Propionaldehyde Levels.

The effect of propionic anhydride on the dehydrogenase assay at high propionaldehyde levels is shown in Figure 7.2, for a range of propionic anhydride concentrations. The initial and maximum rates were measured as described above.

There was some evidence for slight activation at the lowest propionic anhydride concentration used, but in general inhibition of the steady state reaction rate was observed. Very high concentrations of propionic anhydride were needed to cause significant inhibition. The relative rate was only reduced to 0.5 at a propionic anhydride concentration of 390 μM, which corresponds to a 100 fold excess of this reagent with respect to the enzyme active site concentration. The reaction rate was not reduced to zero even at 26 mM, the maximum practical concentration obtainable for solubility of the propionic anhydride. Precipitation of the enzyme occurred after 15 minutes at this high concentration of propionic anhydride as expected if polyacylation is occurring. However, the propionaldehyde concentration in all of these assays was 20 mM, which is comparable to the highest concentration of propionic anhydride, so that if both propionaldehyde and propionic anhydride compete for the same site or sites and have similar affinities for these sites, very high propionic anhydride concentrations would be required for significant inhibition.

7.3.1.3 Non-Saturating Substrate Levels.

The effect of propionic anhydride on the enzyme was also investigated under conditions in which the concentration of NAD^+ and propionaldehyde were both 8 μM. These concentrations of substrate were chosen to approximate the amounts of these reagents produced at the completion of a reverse reaction such as that shown in Figure 7.3a. In this example, all the available NADH was oxidised after 80 seconds. An increase in A_{340} was not observed until a further 70 seconds had elapsed following hydrolysis of most of the propionic anhydride. As observed previously (5.3, 6.3) and above for the low propionaldehyde assay, high concentrations of propionic anhydride appear to totally inhibit the dehydrogenase reaction at low propionaldehyde concentrations. However upon removal of most of the propionic anhydride by hydrolysis, dehydrogenase activity was observed for solutions containing enzyme NAD^+ and propionaldehyde.

This experiment was designed primarily to establish approximately how rapidly propionic anhydride hydrolyses under conditions similar to

Figure 7:2 THE EFFECT OF PROPIONIC ANHYDRIDE ON DEHYDROGENASE ACTIVITY AT HIGH PROPIONALDEHYDE CONCENTRATIONS

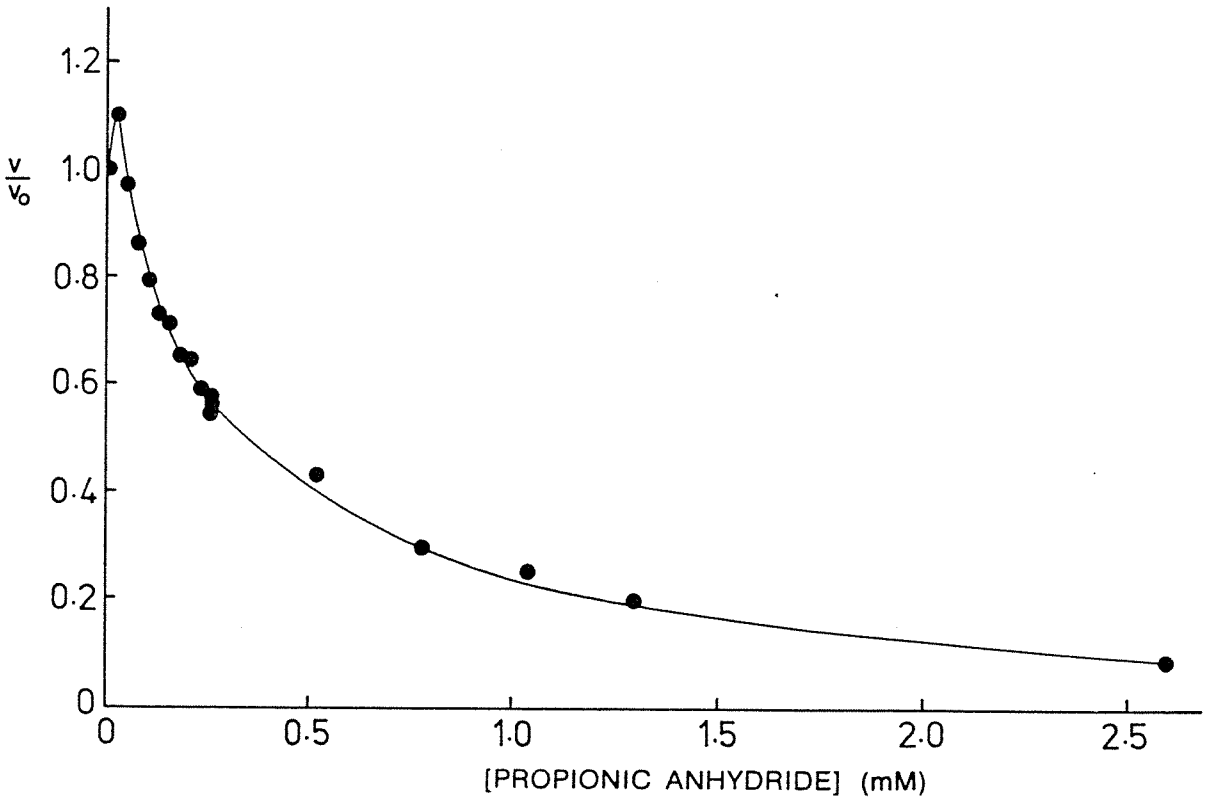


Figure 7.2 The Effect of Propionic Anhydride on Enzyme Activity at High Concentrations of Propionaldehyde.

Varying amounts of a solution of propionic anhydride in acetonitrile were added to a solution of enzyme (3.87 μM) premixed with NAD^+ (2 mM) and propionaldehyde (20 mM) was added within 10 seconds. The initial rate of the subsequent reverse reaction is shown relative to the control rate in the absence of propionic anhydride.

the actual reverse reaction conditions, because no direct determination could be made (6.3.2). The time for which the dehydrogenase activity was suppressed (the lag time) was expected to reflect the amount of propionic anhydride added initially. Such a dependence was observed experimentally and is shown in Figure 7.3b.

In many cases an initial decrease in A_{340} was also apparent, particularly at high propionic anhydride concentrations. This was consistent with the results obtained at 100 µM propionaldehyde in section 6.2.1.1. However the curvature of the traces caused by this phenomenon made measurements of the lag time difficult. Measurements were therefore made by constructing a tangent to the steepest part of the curve (representing the maximum reaction rate) and a horizontal tangent to the lowest point on the lag (or inhibited region). The time (t_{lag}) at which these tangents intersected was measured as shown in Figure 7.3b (inset). The dependence of the lag time on the propionic anhydride concentration is shown in Figure 7.4a and it can be seen that the relationship between t_{lag} and the initial propionic anhydride concentration was not linear, but that disproportionately longer times were required at higher anhydride concentrations before significant dehydrogenase activity was observed. However, it must be noted that these results do not represent the actual rate of hydrolysis of propionic anhydride, but the time required until the maximum dehydrogenase rate was observed. This is therefore probably a measurement of the time taken to reach a certain specific propionic anhydride concentration, but it may be also influenced by other factors, such as the pH of the solution, in which case a simple interpretation of the dependence of this lag time on concentration would be impossible.

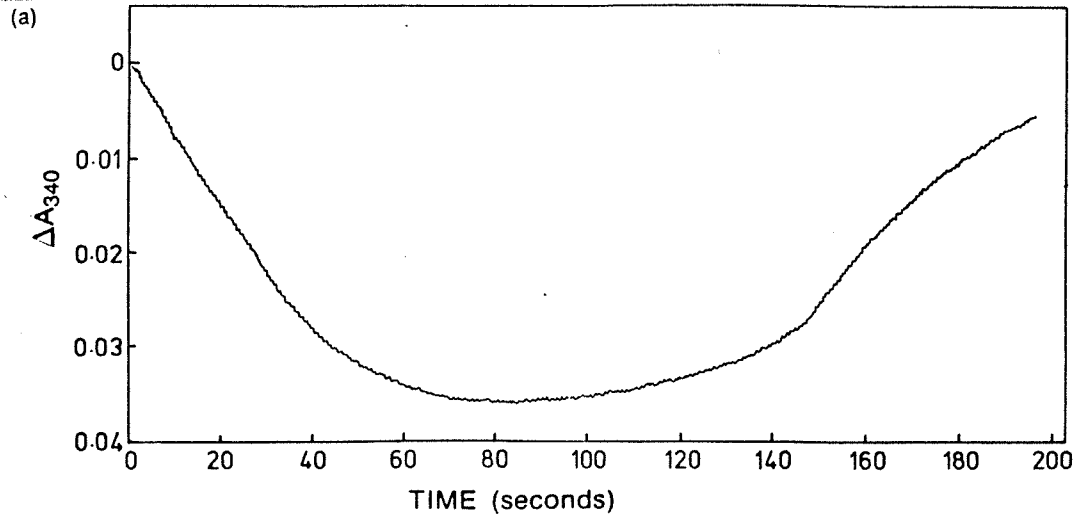
In addition to the lag times, the maximum reaction rate following the lag was measured. A comparison of the increase in A_{340} in Figure 7.3a with those in Figure 7.3b shows a clear similarity in the general shape of the curves indicating that the conditions in these assays did in fact closely approximate the conditions applying in the minimum absorbance region of reverse reactions.

Comparison of the maximum rates and the control rate, shows that activation was observed, and that the activation remains after the inhibitory effect is removed, as was observed for the experiments at 100 µM propionaldehyde. The rates obtained in this experiment are shown in Figure 7.4b, expressed relative to the control rate. Once again, the true propionic anhydride concentration would have been much

Figure 7.3 The Effect of Propionic Anhydride on Enzyme Activity at Non-Saturating Concentrations of NAD^+ and Propionaldehyde .

7.3 (a) The progress curve for a reverse reaction in which enzyme (3.13 μM) NADH (8 μM) and propionic anhydride (260 μM) were mixed.

7.3 (b) Varying amounts of propionic anhydride in acetonitrile were added to solutions containing enzyme (3.13 μM) and NAD^+ (8.0 μM). Propionaldehyde (8.0 μM) and propionic anhydride were added simultaneously. The maximum rate of the dehydrogenase reaction and the lag time, t_{lag}, were measured as shown in the inset, and are shown in Figure 7.4.



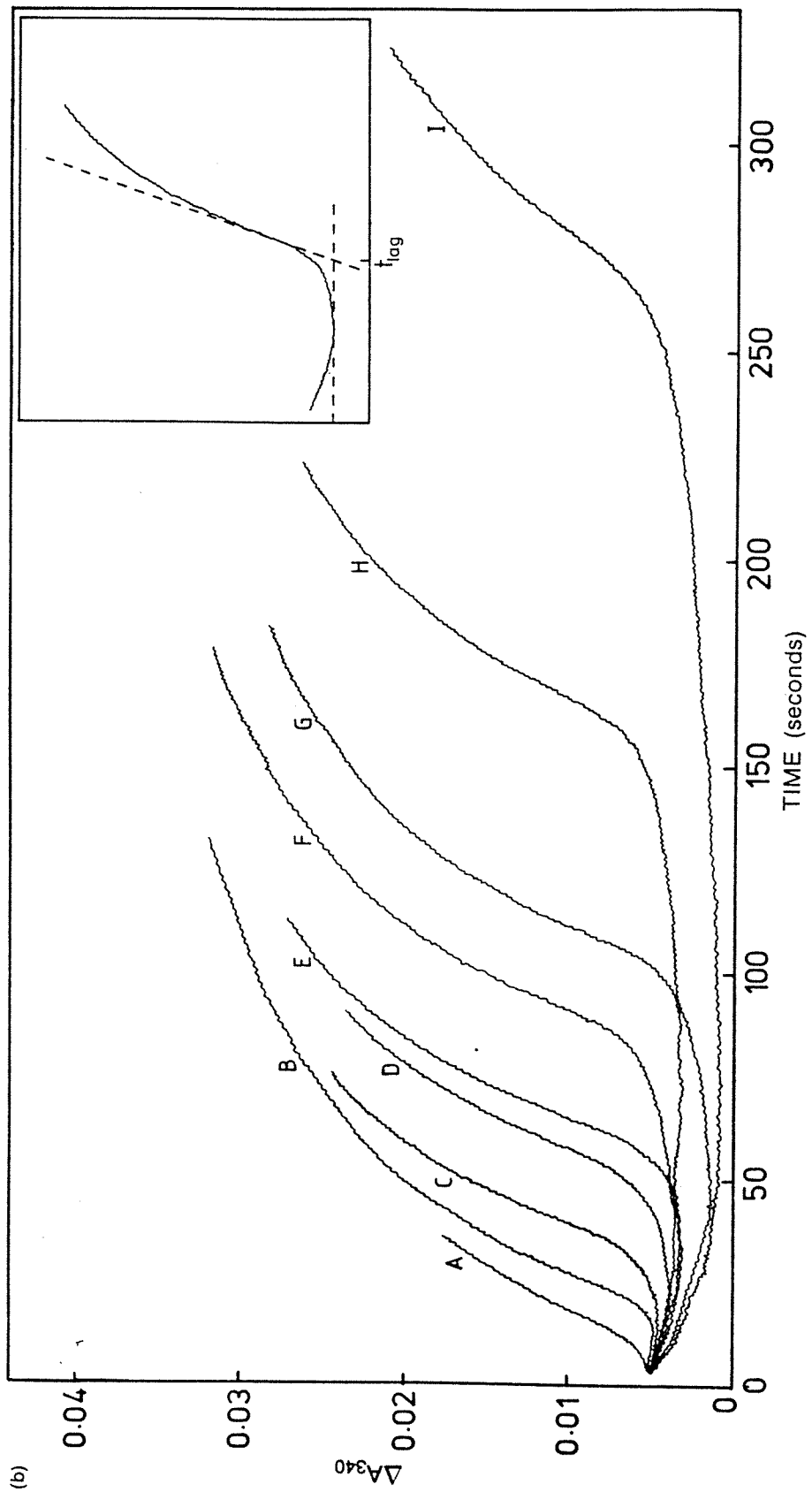
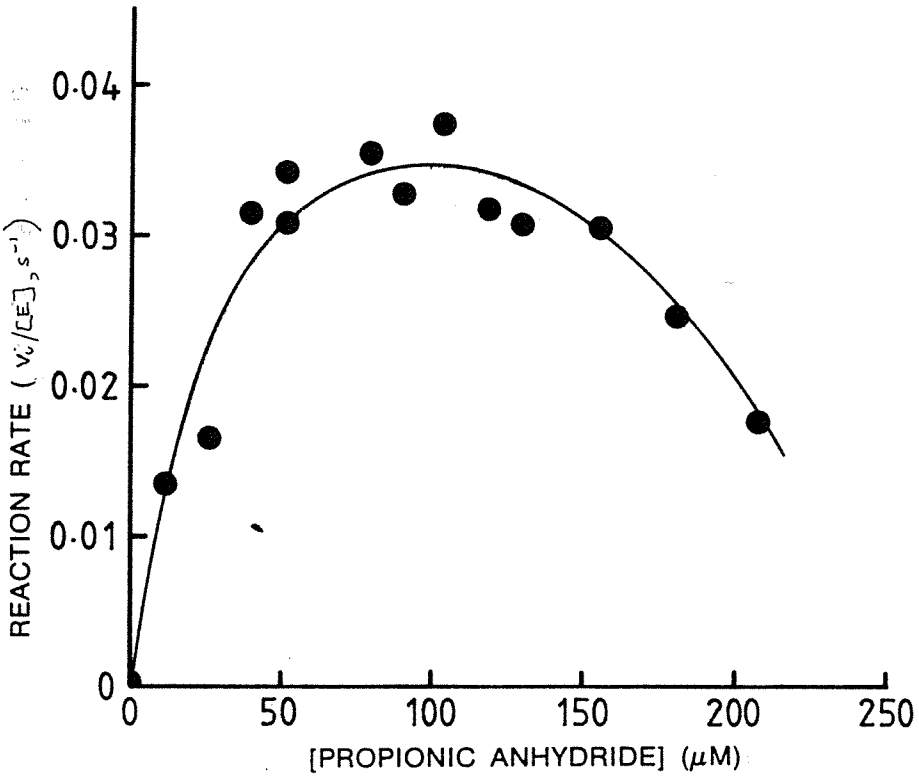
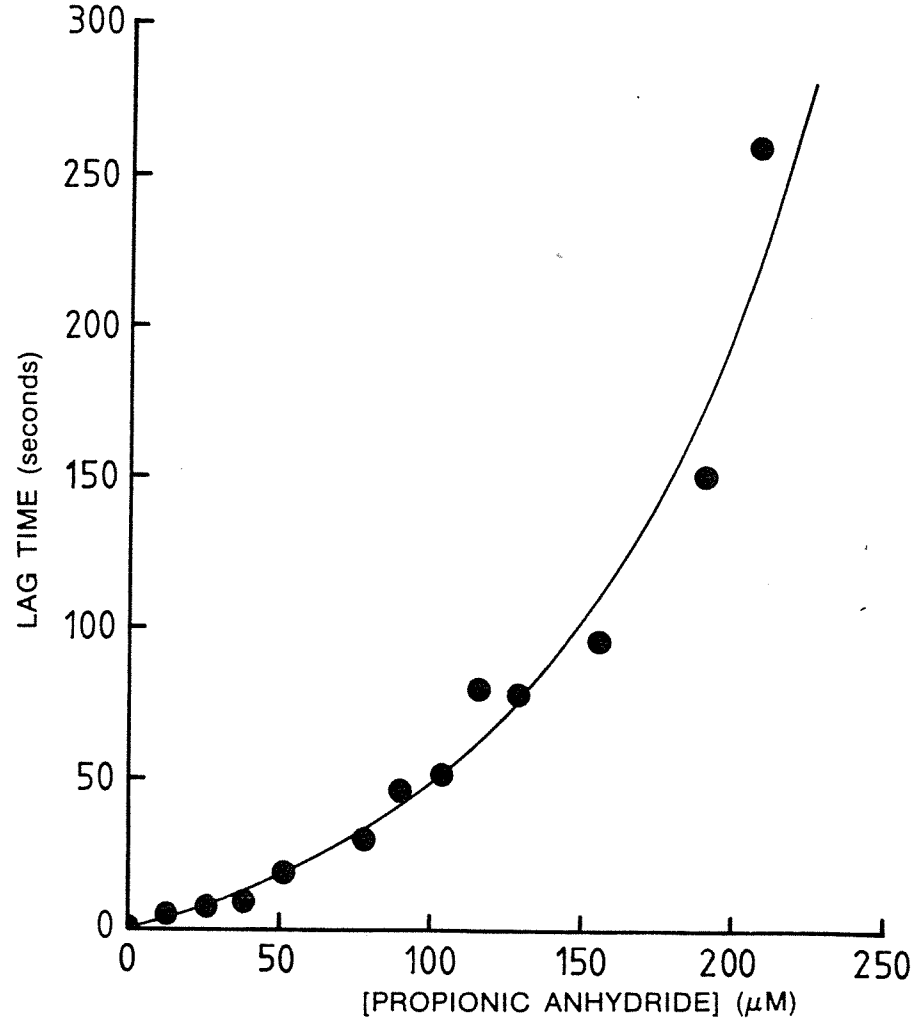

(b)
0.04
0.03
0.02
0.01
0
ΔA340
0
50
100
150
200
250
300
TIME (seconds)
A
B
C
D
E
F
G
H
I
tlag

Figure 7:3

Figure 7.4 The Dependence of the Lag Time and the Relative Reaction Rate on the Propionic Anhydride Concentration at Non-Saturating Concentrations of NAD^+ and Propionaldehyde.
The maximum rate (7.4(a)) of the reaction (relative to the control rate) and the delay time t_{lag} (7.4(b)) before this maximum rate was achieved were measured from the data shown in Figure 7.3(b).

Figure 7:4


0·04
0·03
0·02
0·01
0
REACTION RATE ($v_0/[E]_t, s^{-1}$)
0
50
100
150
200
250
[PROPIONIC ANHYDRIDE] (μM)


300
250
200
150
100
50
0
LAG TIME (seconds)
0
50
100
150
200
250
[PROPIONIC ANHYDRIDE] (μM)

lower than the initial concentration used in the Figure, and thus the real concentration range giving activation of the steady state rate is much lower than that indicated in Figure 7.4b. There was inhibition of the maximum rate (compared to the control rate) at the two lowest propionic anhydride concentrations used in these experiments, whereas no such effect was observed at a propionaldehyde concentration of 100 µM (Figure 7.1). Inhibition was also observed at the highest propionic anhydride concentration used in these experiments (208 µM).

7.3.2 Diethylpyrocarbonate.

The effect of a range of DEPC concentrations on the enzyme was determined using both the low and high propionaldehyde assays. However, numerous order of mixing effects were observed by L. Deady (personal communication) when DEPC was used as a modifier of aldehyde dehydrogenase. Similar effects were also observed in the present study. Therefore it was necessary to determine the most suitable mixing conditions before proceeding to a more detailed study.

A series of assays was carried out with a variety of different mixing conditions, at pH 7.6 and at both high and low concentrations of propionaldehyde .

The effect of adding DEPC last to assay solutions at pH 7.6 is shown in Figure 7.5. This pH was used initially so that the reaction rates in the presence of DEPC could be compared with the rates of the standard assay. DEPC was added last, after first determining the reaction rate (control) of the unmodified enzyme at each propionaldehyde concentration. Curvature of the absorbance trace was observed, a time dependent decrease in activity being observed at both propionaldehyde levels. Rate constants of $0.035\pm0.005\ s^{-1}$ at the low propionaldehyde level and of $0.030\pm0.005\ s^{-1}$ at the high propionaldehyde level were determined from difference logarithmic plots of the approach to the final inhibited rate, as described previously (3.4.4.3). Although no attempt has been made at this stage to determine the effect of DEPC concentration on this slow process, the same process appears to be involved in the inhibition at both concentrations of propionaldehyde, as these rate constants are the same within experimental error.

The results of mixing the reagents in different orders are shown in Table 7.2. These results show that the earlier the DEPC was added, the lower the initial remaining activity and the lower the degree of

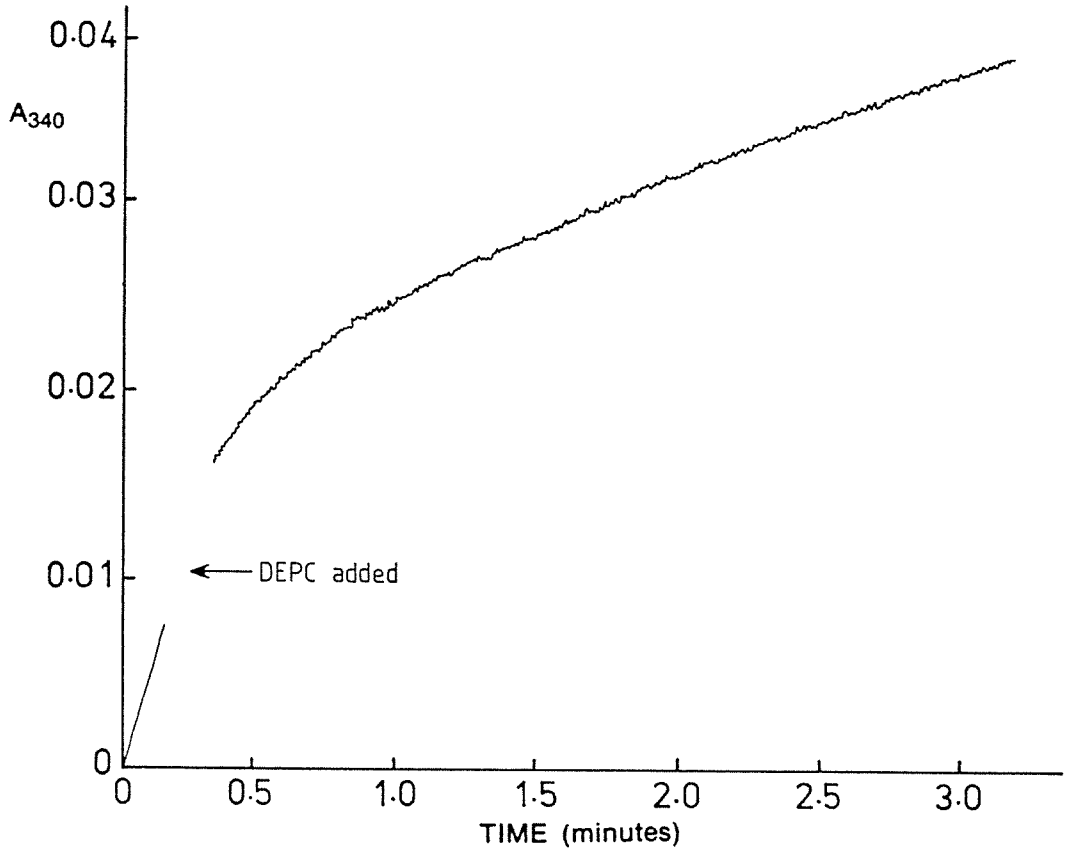
Figure 7.5 The Effect of DEPC on the Enzyme Activity at pH 7.6

7.5 (a)The effect of adding DEPC (260 M) last to an assay solution containing enzyme (1.6 M) NAD^+ (1 mM) and propionaldehyde (100 M). The control rate was obtained from the slope of the curve prior to addition of DEPC.

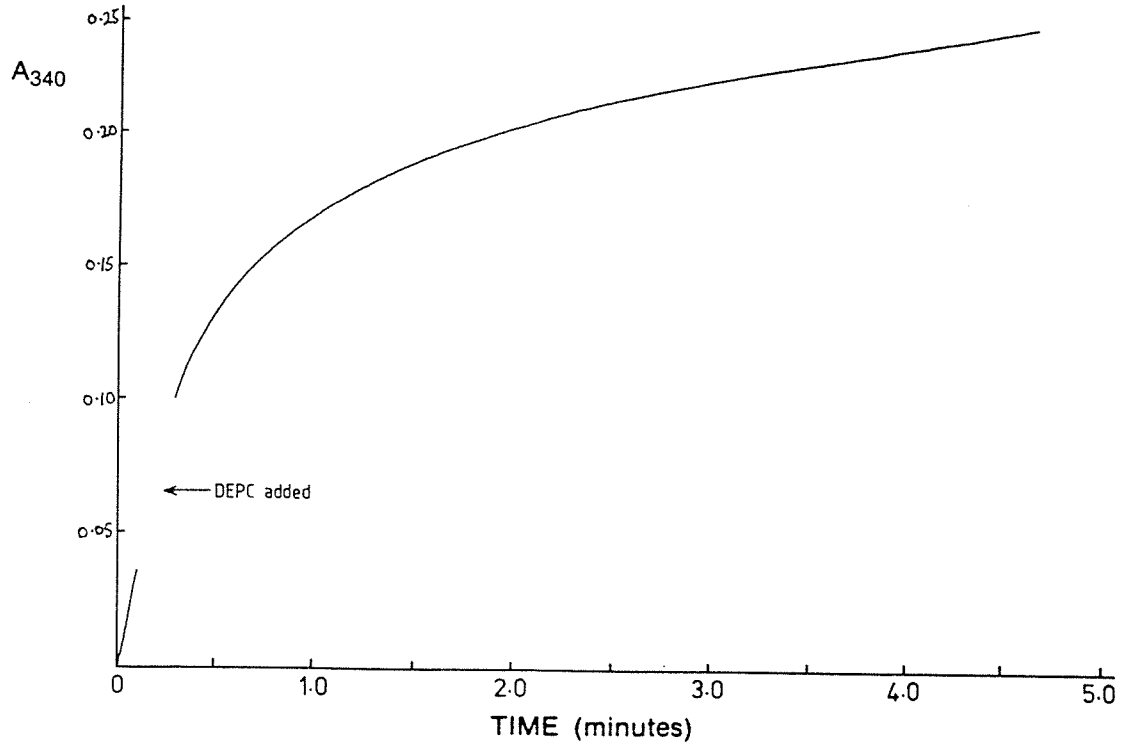
7.5 (b)The effect of adding DEPC (130 M) last to an assay solution containing enzyme (2.39 M) NAD^+ (1 mM) and propionaldehyde (20 mM). The control rate was obtained from the slope of the curve prior to addition of DEPC.

Figure 7:5 THE EFFECT OF DEPC ON DEHYDROGENASE ACTIVITY

(a) LOW PROPIONALDEHYDE LEVELS



(b) HIGH PROPIONALDEHYDE LEVELS



curvature of the trace. Final rates were largely independent of the order of mixing but were dependent on the DEPC concentration. The only mixing condition for which this did not apply was that in which enzyme was premixed with DEPC in the absence of NAD^+. In this case, although the initial rate was inhibited, marked curvature of the A_{340} trace was observed even after incubation times of 5 minutes suggesting that a histidine residue is more accessible in the enzyme.NAD^+ complex than in the free enzyme. Very high DEPC concentrations were used in these experiments, yet the enzyme activity was not reduced to zero under any of the conditions used. Weiner et al. (1955) also reported that the activity of horse liver aldehyde dehydrogenase was not reduced to zero in the presence of DEPC.

TABLE 7.2

The Effect of Mixing Conditions on Inhibition by DEPC

Order of Mixing	[DEPC] mM	v_i/v_o	v_f/v_o
(a)Low Propionaldehyde Levels			
E, NAD^+, Prop, DEPC	0.26	0.45	0.05
E, NAD^+, DEPC, Prop	0.26	0.12	0.09
E, NAD^+, DEPC, Prop	0.78	0.12	0.005
E, DEPC, NAD^+, Prop	0.26	0.10	0.09
E, NAD^+, DEPC, 5 min, Prop	0.78	0.007	0.007
E, DEPC, 5 min, NAD^+ & Prop	0.78	0.06	d
(b)High Propionaldehyde Levels			
E, NAD^+, Prop, DEPC	0.26	0.68	0.08
E, NAD^+, DEPC, Prop	0.26	0.17	0.09
E, NAD^+, DEPC, Prop	0.78	0.12	0.04
E, DEPC, NAD^+, Prop	0.26	0.17	0.14
E, NAD^+, DEPC, 5 min, Prop	0.78	0.01	0.02
E, DEPC, 5 min, NAD^+ & Prop	0.78	0.04	0.01

a v_i is the initial rate, v_f, the final rate and v_o the control rate at the same level of propionaldehyde

b Prop is propionaldehyde

c NAD^+ and propionaldehyde added simultaneously

d reaction reversed, a decrease in absorbance was observed

The order of mixing effects appeared to be a result of a slow reaction between DEPC and the enzyme. A final constant rate was achieved after 90-180 seconds.

Therefore for subsequent assays at pH 7.6, DEPC was added to solutions containing enzyme and NAD^+. Propionaldehyde (100μM or 20 mM) was then added after 2.0 minutes and the reaction rate was measured. Downward curvature of the A_{340} trace was not observed under these conditions, even at the lowest DEPC concentrations used, suggesting that this delay allowed sufficient time for complete reaction of DEPC with the enzyme.

The effect of a range of DEPC concentrations on the enzyme activity at high concentrations of propionic anhydride at pH 7.6 is shown in Figure 7.6. Assay solutions containing enzyme (1.73 μM), NAD^+ (1 mM) and DEPC (0.6 - 30 μM) in 25 mM pH 7.6 phosphate buffer were incubated at 25°C for two minutes before the addition of propionaldehyde (20 mM). Reaction rates are expressed relative to the control rate in the absence of DEPC. Final reaction rates are shown, as there was some degree of upwards hysteresis observed.

Inhibition of the steady state rate was observed througout the entire concentration range. However, even a 14 fold excess of DEPC over enzyme did not reduce the enzyme activity to zero. It was possible to fit the first 10 points to a straight line with an intersect of 6.4±1 μM as shown. This represents a DEPC to enzyme active site concentration ratio of 3.7±0.5.

The effect of DEPC concentration on the enzyme activity at low propionaldehyde concentrations was determined at pH 6.1 and at a temperature of 30°C and the results are shown in Figure 7.7. Inhibition was again observed at all DEPC concentrations. Although the activity was not reduced to zero within the concentration range used, small amounts of DEPC produced a significant reduction in activity, and extrapolation of a straight line through the data points at low DEPC concentrations produced an intersect of 1.2 μmol $DEPC.l^{-1}$, which corresponded to a DEPC/enzyme active site ratio of 0.85 (or 1.7, if the higher value of kcat is used).

Figure 7:6

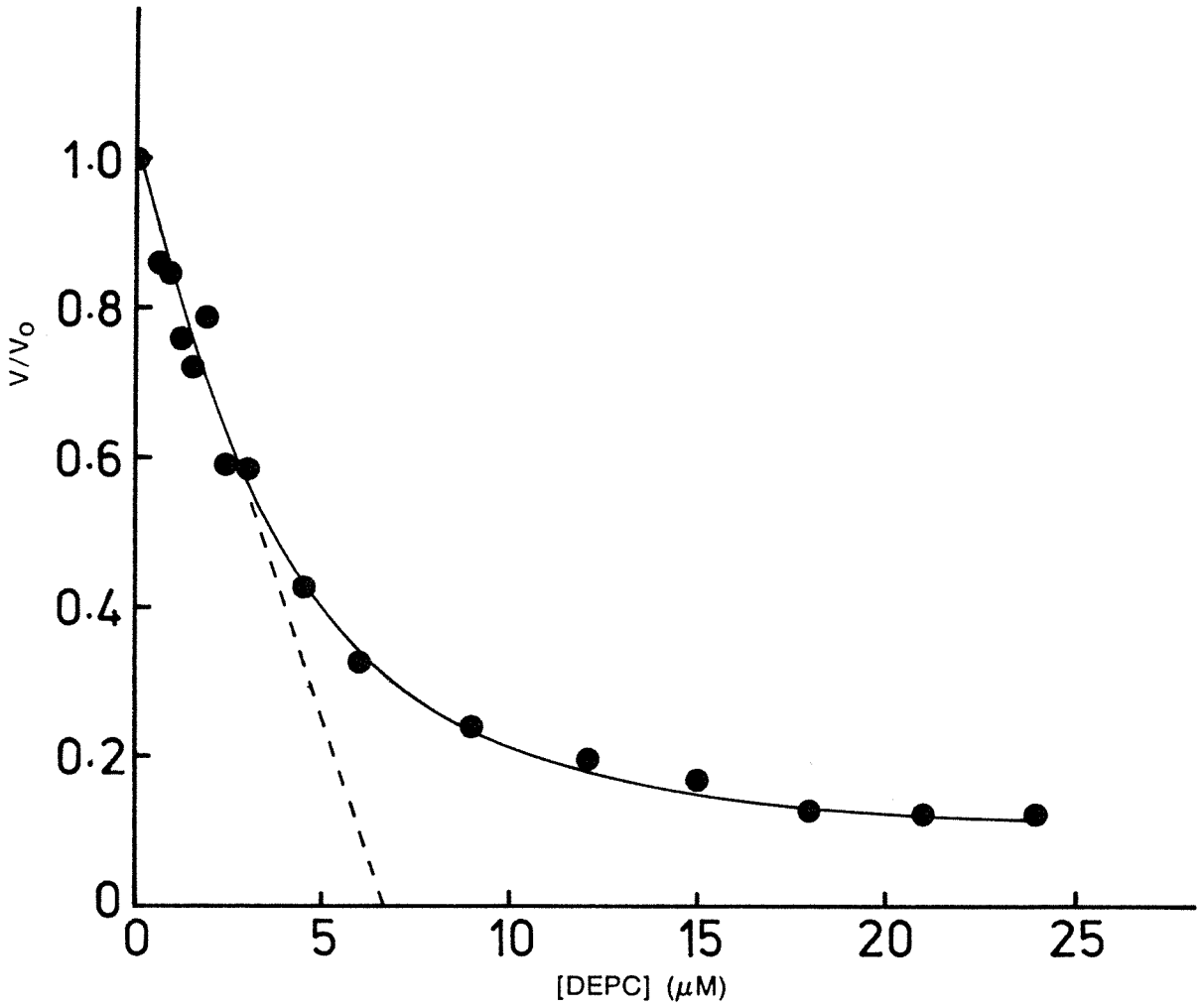


Figure 7.6 The Effect of DEPC on Enzyme Activity at High Concentrations of Propionaldehyde at pH 7.6.

Assay solutions contained enzyme (1.73 μM), NAD^+ (1 mM) and propionaldehyde (20 mM) and varying concentrations of DEPC. DEPC was premixed with enzyme and NAD^+ and propionaldehyde was added after 2.0 minutes.

Figure 7:7

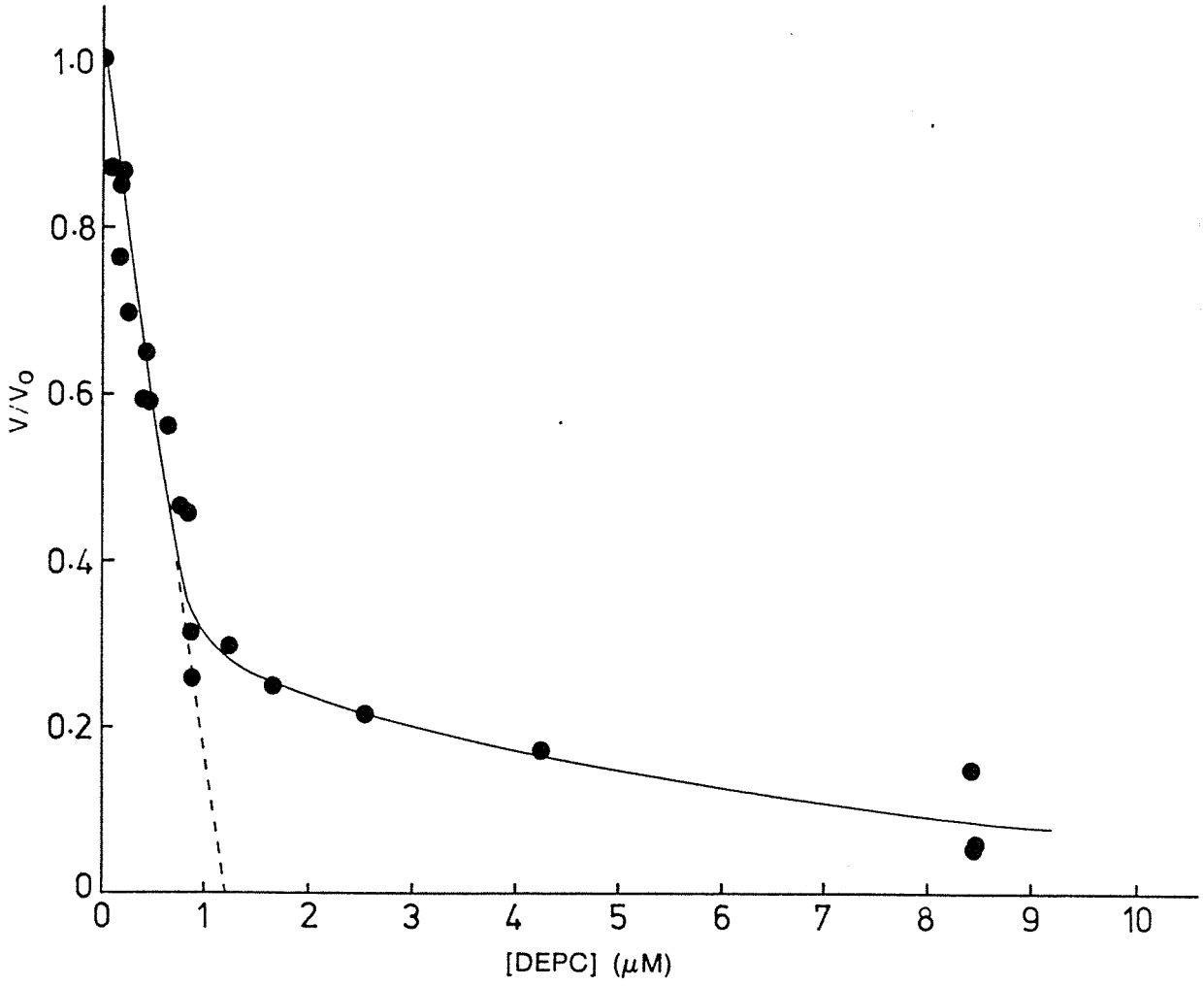


Figure 7.7 The Effect of DEPC on Enzyme Activity at Low Concentrations of Propionaldehyde at pH 6.1. DEPC was added to a cuvette containing enzyme (1.4 μM) premixed with NAD^+ (1 mM) and propionaldehyde (100 μM) was added within 15-30 seconds. These assays were carried out at 30°C.

7.3.3 Maleic Anhydride.

The effect of maleic anhydride (2.6 mM) on the low propionaldehyde assay was determined using an enzyme concentration of 2.5 μM. Maleic anhydride was added last after first determining the control rate. The initial relative rate after mixing was 0.25 (Figure 7.8a). However, this increased over a three minute period until the final rate was the same as the control rate.

Slight differences in the extent of the inhibition which occurred upon mixing the assay reagents in different orders could be attributed to hydrolysis of the maleic anhydride in reagent solutions. (The half-life of maleic anhydride in water is 0.23 minutes (Rivett & Sidgwick, 1910b)).

The effect of this reagent on the high propionaldehyde assay was completely different, as shown in Figure 7.8b. Concentrations of maleic anhydride and enzyme were the same as those used for the low propionaldehyde assay above. The initial rate was the same as the control rate, showing that there was no observable effect of the reagent within the time of mixing. However, the final rate was 0.26 relative to the control rate, thus showing significant inhibition.

7.4 PreSteady State Results.

7.4.1 The Effect of Propionic Anhydride on the NADH Fluorescence Burst.

7.4.1.1 NADH Burst at Low Propionaldehyde Levels.

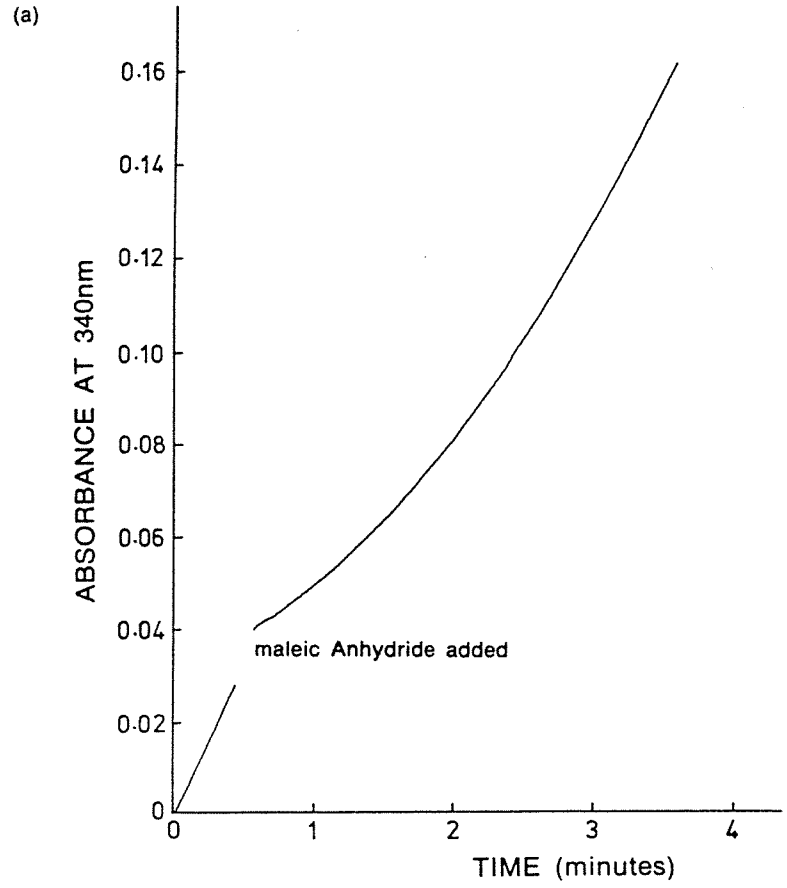
These results are summarized in Table 7.3a. The rate constants and amplitudes of any bursts observed are expressed relative to the control burst rate constant and amplitude for that experiment.

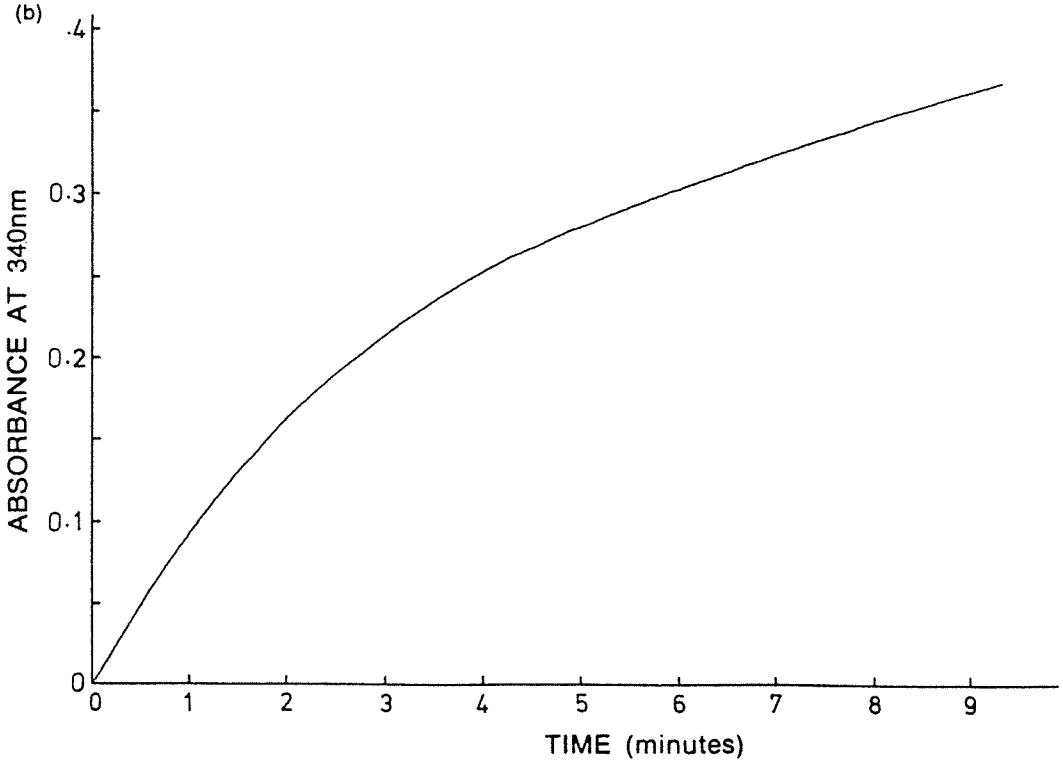
If the enzyme was premixed with propionic anhydride at concentrations which caused activation of the steady state rate (at low propionaldehyde levels) then regardless of the presence or absence of premixed NAD^+, no burst was seen. If enzyme was not premixed with propionic anhydride then a very small burst (7.5% of the control amplitude) was observed with a rate constant apparently greater than the control rate constant. However, for this mixing condition, at very high propionic anhydride levels (4.7 mM), no burst was observed either. As observed previously in steady state experiments (7.3.1.1), a

Figure 7.8 The Effect of Maleic Anhydride on the Enzyme Activity.

7.8 (a)The effect of adding maleic anhydride (2.6 mM) last to an assay solution at a low concentration of propionaldehyde (100 M) is shown. The solution also contained enzyme (2.5 M) and NAD^+ (1 mM). Although the reaction rate in the presence of maleic anhydride was initially only 25% of the control rate, the final rate was the same as the control rate.

7.8 (b)The effect of adding maleic anhydride (2.6 mM) last to an assay solution at a high concentration of propionaldehyde (20 mM) is shown. The solution also contained enzyme (2.5 M) and NAD^+ (1 mM). In this case the initial rate was the same as the control rate, but decreased to a final rate which was 26% of the control rate. The solution also contained enzyme (2.5 M) and NAD^+ (1 mM).


(a)
ABSORBANCE AT 340nm
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
maleic Anhydride added
0
1
2
3
4
TIME (minutes)


(b)
ABSORBANCE AT 340nm
·4
0·3
0·2
0·1
0
0
1
2
3
4
5
6
7
8
9
TIME (minutes)

decrease in fluorescence was observed as an apparent reverse reaction occurred, despite the absence of added NADH.

TABLE 7.3

The Effect of Propionic Anhydride on the NADH Burst

(a)NADH Burst at Low Propionaldehyde Concentrations

Mixing Conditions	[PA][i] mM	k/k^{o}[a]	A/A^{o}[b]	v/v^{o}[c]
E,NAD^+/Prop[d],PA[e]	0.31[f]	1.5	0.075[h]	0.67-5.5
E,NAD^+,PA/Prop	0.53[f]	g	g	0.67-3.6
E,PA/NAD^+,Prop	0.51[f]	g	g	0.61
E,NAD^+/Prop,PA	4.7	g	g	reverse

(b)NADH Burst at High Propionaldehyde Concentrations

Mixing Conditions	[PA] mM	k/k^{o}	A/A^{o}	v/v^{o}
E,NAD^+/Prop,PA	0.45[f]	1.5	0.28	0.36
E,NAD^+,PA/Prop,PA	0.42[f]	0.30	0.18	0.43
E,NAD^+/Prop,PA	4.7	2±1	0.054[h]	0.37
E,Prop/NAD^+,Prop,PA	0.31[f]	0.45	0.42	0.40

a k is the burst rate constant.

b A is the burst Amplitude.

c v is the steady state rate; initial and final rates are shown.

d Prop is propionaldehyde.

e PA is propionic anhydride.

f Levels of propionic anhydride were similar to those which produced activation of the steady state rate in conventional experiments.

g No burst was observed.

h These bursts were of very small amplitude and the rate constants could not be determined with great accuracy.

i The concentrations of propionic anhydride given refer to the initial concentration in the individual drive syringes.

These effects on the burst were reversible. In experiments in which propionic anhydride was premixed with enzyme and NAD^+ in one syringe, but in which the other syringe contained only propionaldehyde, there was no burst initially (despite the halving of the concentration of propionic anhydride on mixing). However successive runs from the same bulk solutions showed that the burst quickly returned, each subsequent run having a greater amplitude than the previous one until a

normal full amplitude burst was restored. (Figure 7.9a). On addition of more propionic anhydride to the same enzyme-NAD^+ solution the entire process was repeated. This reappearance of the burst was rapid. For propionic anhydride premixed with enzyme in the presence of NAD^+, the burst was completely restored after 10 minutes. However, for propionic anhydride premixed with enzyme only, the return of the burst occurred considerably more slowly, taking approximately 30 minutes for reappearance of the full burst and in the case of propionic anhydride premixed with propionaldehyde, longer than 60 minutes.

These results are in agreement with the previous results regarding hydrolysis of propionic anhydride (5.3.2, 5.4), which is markedly catalysed by the enzyme, and provided a useful method of determining whether or not changes in the burst produced by propionic anhydride were reversible.

At activating levels of propionic anhydride, even in the absence of a burst, steady state activity was observed. The rates determined were initially 60-70% of the control rate, but increased to an activated rate up to 5.5 times the control rate and then slowly decreased (Figure 7.9b).

7.4.1.2 NADH Burst at High Propionaldehyde Concentrations

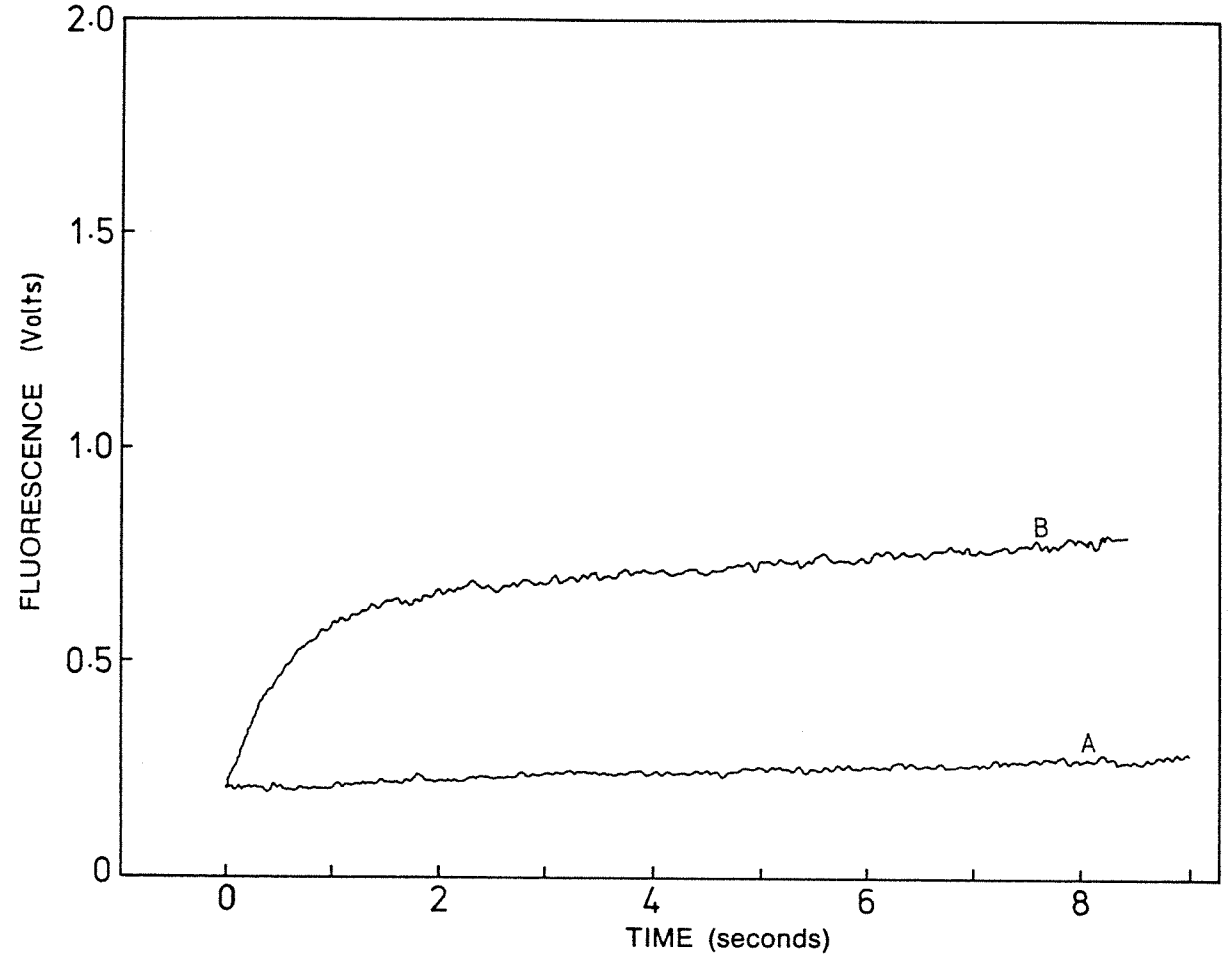
These results are summarised in Table 7.3b and were significantly different from those obtained with low concentrations of propionaldehyde. The amplitude of the burst was greatly reduced by the addition of propionic anhydride but was never eliminated totally, as was the case for certain mixing conditions at low propionaldehyde as described above.

At activating levels of propionic anhydride, and when the propionic anhydride was premixed with enzyme and NAD^+, the amplitude of the burst was reduced to 18% of the control amplitude and the rate constant to 30% of the control rate constant (Figure 7.10a). A decrease in the burst rate constant was also observed for enzyme premixed with 20 mM propionaldehyde. The effect on the burst was less if the propionic anhydride was not premixed with enzyme and NAD^+, in which case the amplitude was 28% of the control amplitude and the rate constant apparently increased by a factor of 1.5 (Figure 7.10b). However, the burst amplitude was so small in these cases, that accurate fitting of the data was difficult because of an unfavourable signal to noise ratio and therefore the apparent increase in these rates may not be significant. Improved fitting by the catting procedure was

Figure 7.9 The Effect of Propionic Anhydride on the NADH Dehydrogenase Burst at Low Concentrations of Propionaldehyde.

7.9 (a) A solution containing enzyme (10 M), NAD^+ (2 mM) and propionic anhydride (530 M) was rapidly mixed with a solution of propionaldehyde (100 M) in the stopped flow spectrophotometer. No burst (B) was observed initially, although a steady state rate production of NADH was observed. For repeated mixing from the same solutions, the burst was gradually restored, the amplitude of each successive run being larger than the previous one, until a full amplitude burst with the same amplitude and rate constant as the control was restored (A).

7.9 (b) Even in the absence of a burst, slow production of NADH was observed. This 'steady state' rate was initially 70% of the control rate, but increased to an activated rate (refer results). All concentrations in this experiment were initially the same as those above.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1·5
1·0
0·5
0
FLUORESCENCE (Volts)
B
A
0
2
4
6
8
TIME (seconds)

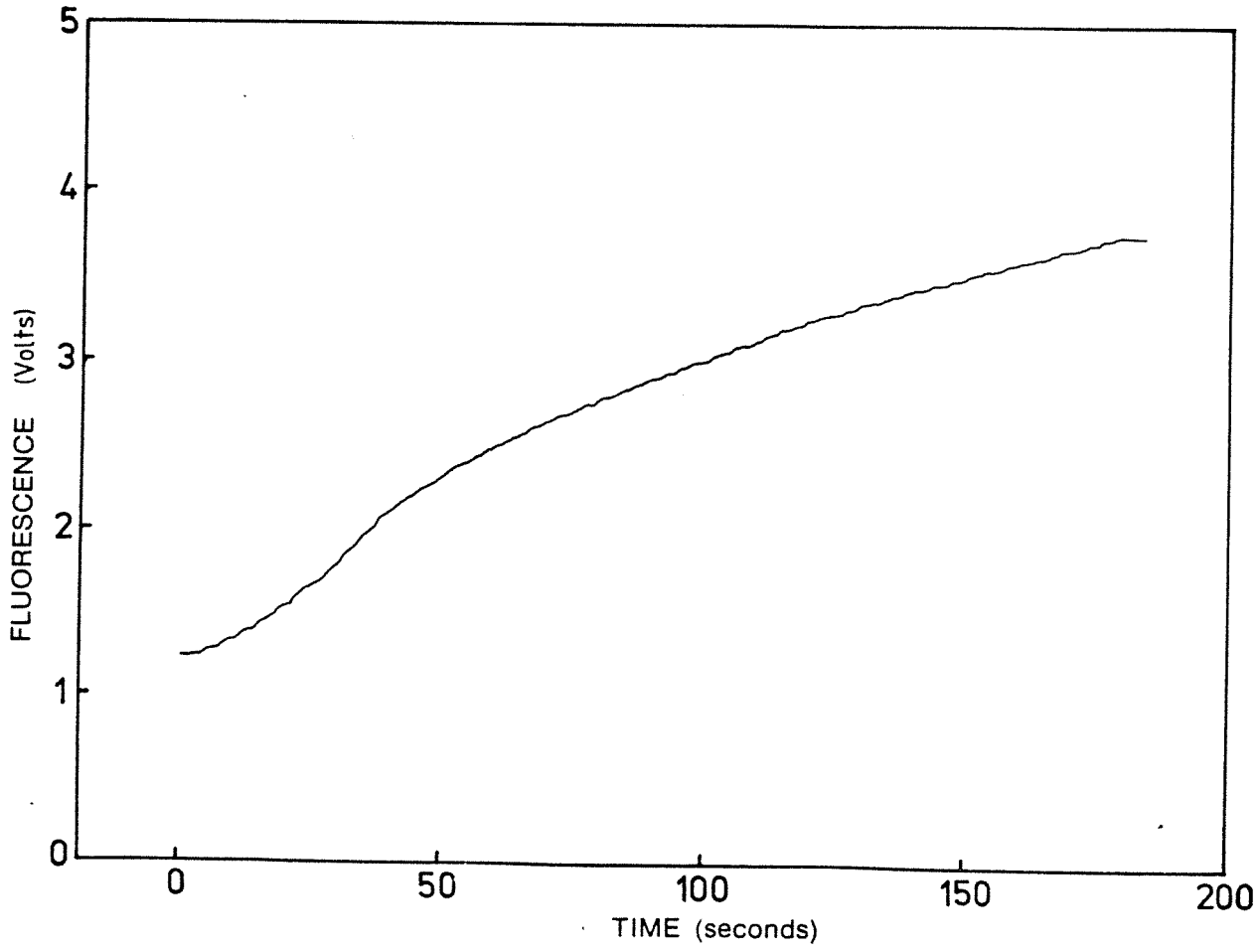
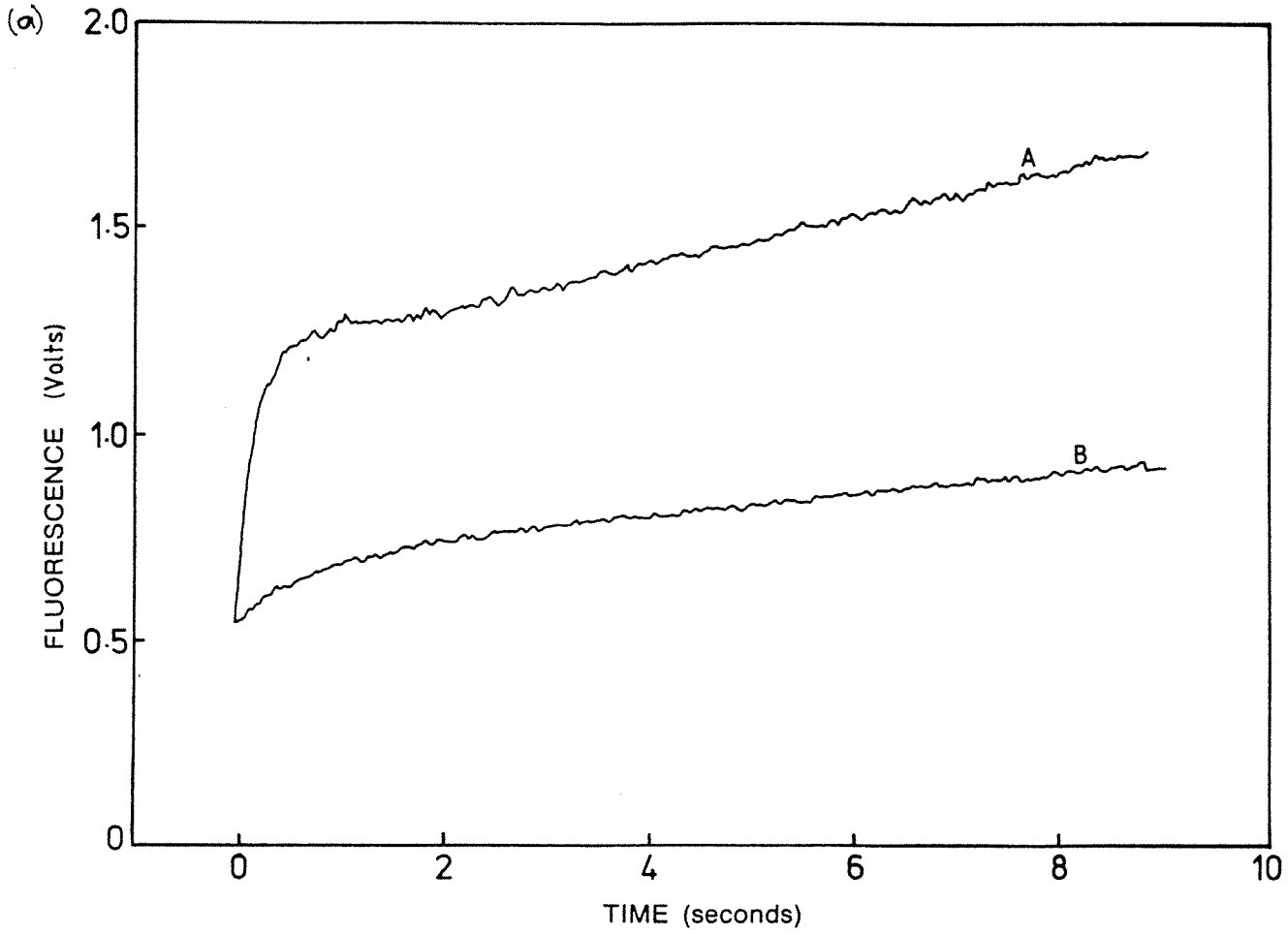
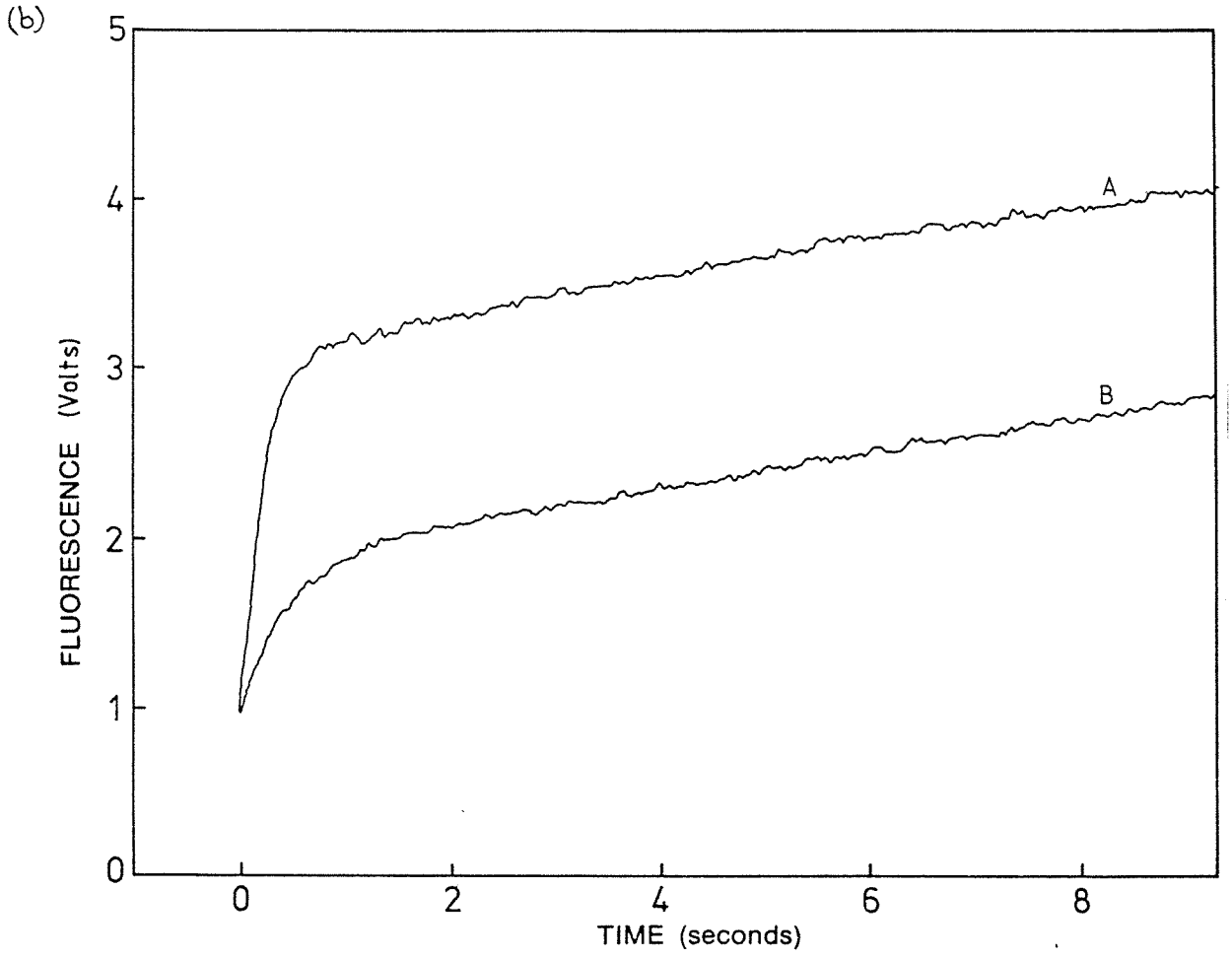

5
4
3
2
1
0
FLUORESCENCE (Volts)
0
50
100
150
200
TIME (seconds)

Figure 7.10 The Effect of Propionic Anhydride on the NADH Burst at High Concentrations of Propionaldehyde.

7.10 (a) A solution containing enzyme (10 M), NAD^+ (2 mM) and propionic anhydride (420 M) was rapidly mixed with a solution containing propionaldehyde (20 mM). Both the control (A) and the propionic anhydride modified (B) burst processes are shown. The burst rate constant was reduced to 30% of the control, and the amplitude was reduced to 18% of the control amplitude.

7.10 (b) A solution containing enzyme (10 M) and NAD^+ (2 mM) was rapidly mixed with a solution of propionic anhydride (450 M) and propionaldehyde (20 mM). Both the control (A) and the propionic anhydride modified (B) burst processes are shown. In this case, the amplitude was only reduced to 28% of the control amplitude, and the rate constant appeared to be increased by a factor of 1.5.


(a)
2·0
1·5
1·0
0·5
0
FLUORESCENCE (Volts)
A
B
0
2
4
6
8
10
TIME (seconds)


(b)
5
4
3
2
1
0
FLUORESCENCE (Volts)
A
B
0
2
4
6
8
TIME (seconds)

impossible due to the changing propionic anhydride concentration.

At much higher propionic anhydride concentrations the burst was very small (5.4% of control amplitude) but still not completely eliminated. Steady state rates following the burst were all reduced to 30-40% of the normal high propionaldehyde steady state rate (i.e. to rates similar to those obtained at low propionaldehyde concentrations).

7.4.2 Other Anhydrides.

The effect of DEPC and maleic anhydride on the low propionaldehyde NADH burst is shown in Table 7.4.

TABLE 7.4

The Effect of DEPC and Maleic Anhydride on the NADH Burst at Low Concentrations of Propionaldehyde

Mixing Conditions	[Anhydride][h] μM	k/k^{o} [a]	A/A^{o} [b]	v/v^{o} [v]
E,NAD^+/Prop,DEPC	2.0	1.42[c]	0.48	d
E,NAD^+/Prop,DEPC	4.0	1.58[c]	0.38	d
E,NAD^+,DEPC/Prop,DEPC	4.0	e	e	0.013
E,NAD^+,MA[f]/Prop	1.6	e,g	e	0.020

a k is the burst rate constant.

b A is the burst amplitude.

c v is the steady state rate following the burst.

d The burst was followed by an exponential decrease in fluorescence.

e No burst was observed

f MA is maleic anhydride.

g A slow exponential increase in fluorescence with a rate constant of 0.13 s^{-1} was observed.

h The concentrations of anhydrides given refer to the initial concentration in the individual drive syringes.

As was the case for propionic anhydride, when either anhydride was premixed with the enzyme, no burst was observed. However, slow steady state rates were obtained in each case, but differed markedly for the

two reagents. No return of the normal burst was observed in either case after a 30 minute period. For maleic anhydride, there was a gradual appearance over a 10 minute period of a slow burst process (Figure 7.11) with a rate constant of $0.13s^{-1}$

In experiments in which DEPC was not premixed with the enzyme, a burst of reduced amplitude was observed (Figure 7.12a). The rate constant for the burst was increased by a factor of 1.5, but a decrease in fluorescence was observed following the burst (Figure 7.12b,c,d). The apparent rate constant for this process, obtained by fitting a single exponential to that portion of the curve, was $0.73\pm0.09s^{-1}$ at 2 mM DEPC and $1.0 \pm 0.3\ s^{-1}$ at 4 mM DEPC. An increase in fluorescence followed this process (Figure 7.12d), showing that a steady state production of NADH was occurring. Doubling the DEPC concentration also produced a further reduction of the burst amplitude (Figure 7.12e), although both concentrations of DEPC represent a large excess of this reagent.

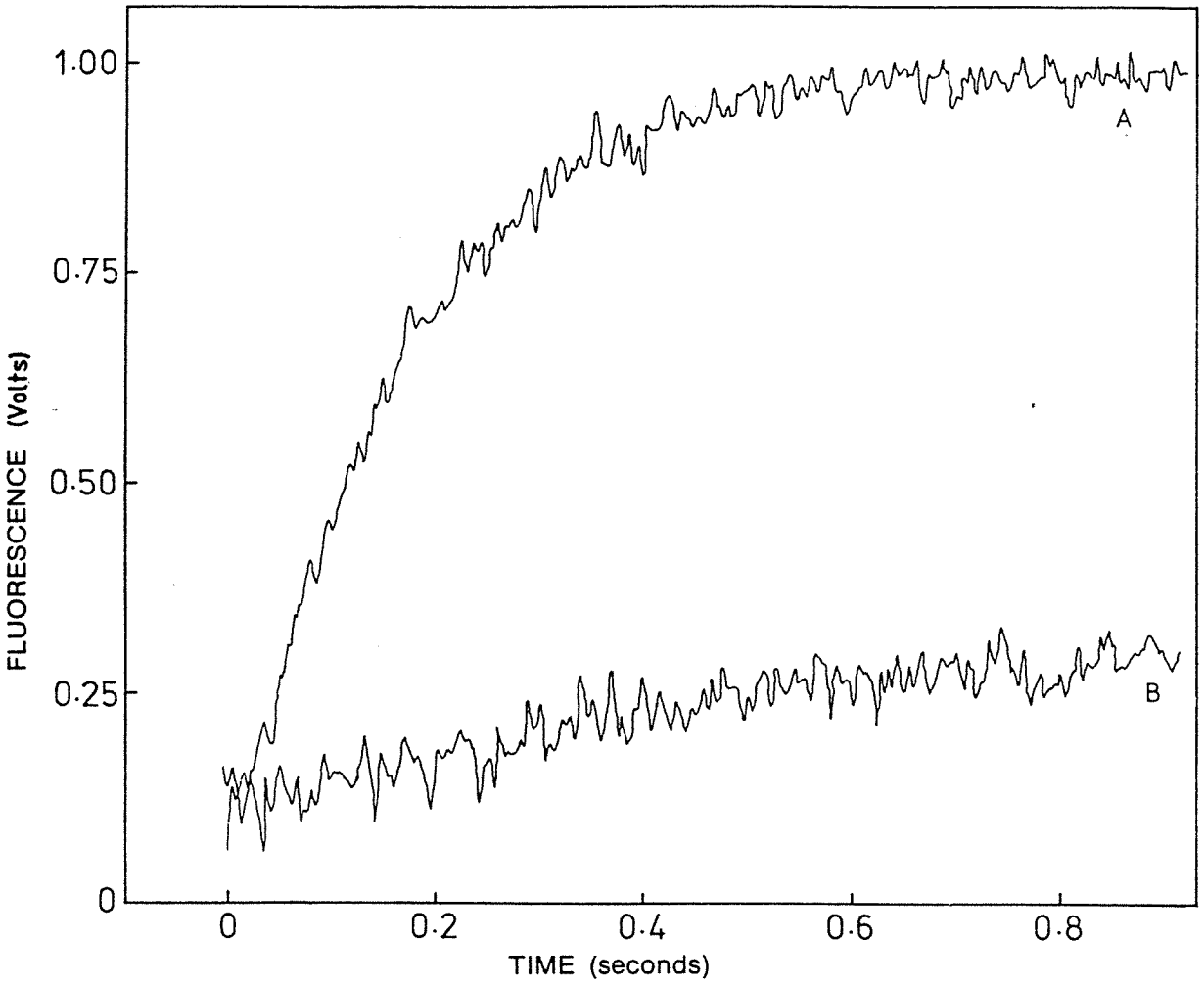
7.5 DISCUSSION

The effect of propionic anhydride on the steady state rate is complex and appears to involve acylation of at least two sites which influence the activity, producing activation at one site and inhibition at the other. Since enzyme, (particularly the enzyme.NAD^+ complex) has been shown to catalyse the hydrolysis of propionic anhydride, the possibility that hydrolysis occurs after acylation at one or both of these sites must also be considered. At the high levels of propionic anhydride which it was necessary to use in most experiments, it is probable that acylation of a large number of groups of the enzyme occurs. Although many of these may not have any effect on the activity they represent possible additional sites for hydrolysis of propionic anhydride since acylation of cysteine, serine or histidine residues by propionic anhydride is reversible, the acylatled residues being susceptible to spontaneous hydrolysis.

At a propionaldehyde concentration of 100 μM, which was just sufficient to saturate the high affinity (P_1) propionaldehyde binding site (see section 2.1.3), the rate of oxidation of propionaldehyde changed as the propionic anhydride hydrolysed. Propionic anhydride concentrations lower than 26 μM activated the initial rate relative to the control rate and there was a slight increase in this rate to a constant value which was maintained for 1-4 minutes before decreasing

Figure 7.11 The Effect of Maleic Anhydride on the NADH Burst at Low Concentrations of Propionaldehyde.

A solution of enzyme (10 M) NAD^+ (2 mM) and maleic anhydride (1.6 mM) was pushed against a solution of propionaldehyde (100 mM). No burst was initially observed but a slow exponential increase (pictured) was observed in later runs. An apparent rate constant of 0.13 s^{-1} was obtained for this process. Although the trace appeared to be biphasic, no faster process was observable on a shorter time scale.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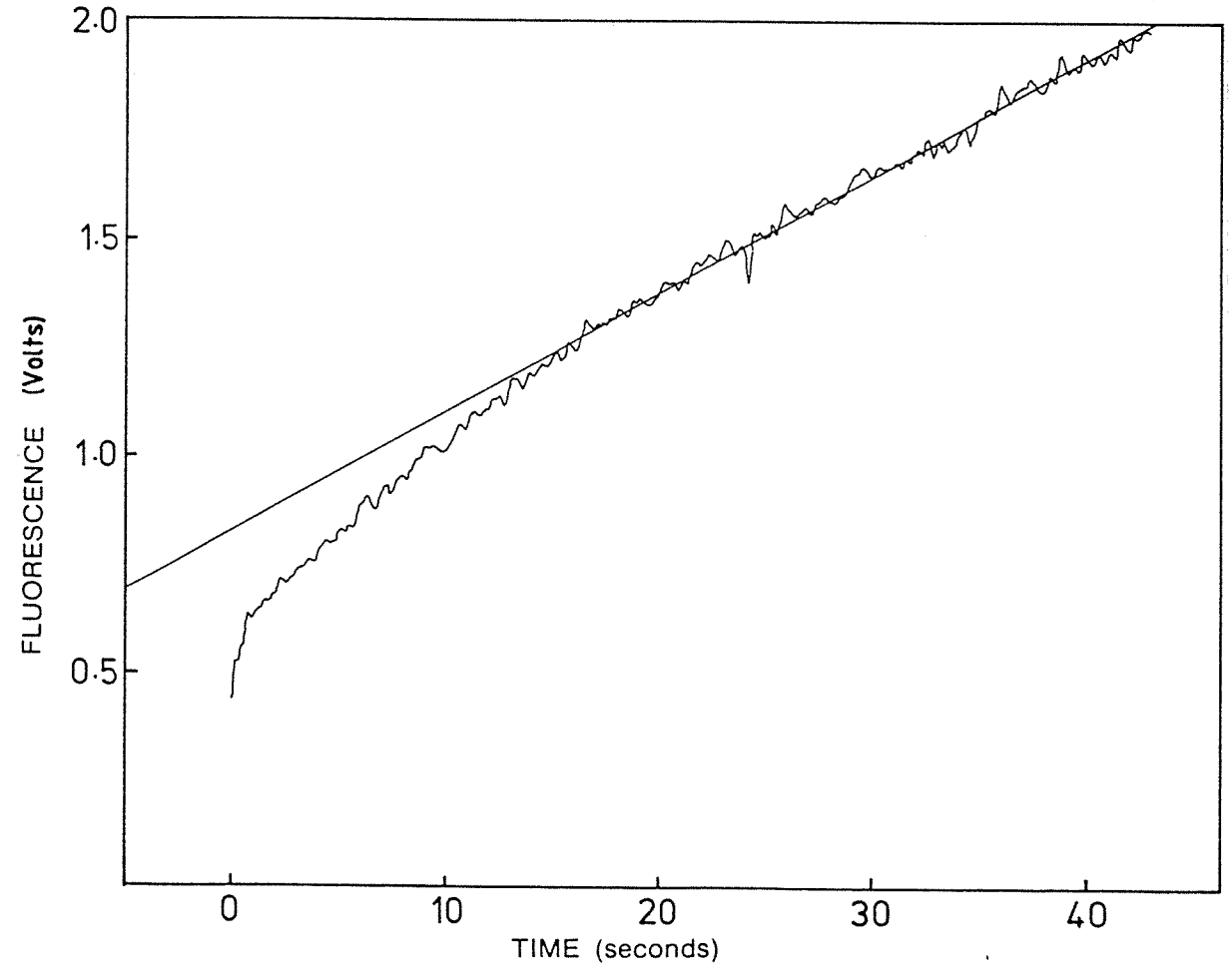
0·75
0·50
0·25
0
FLUORESCENCE (Volts)
A
B
0
0·2
0·4
0·6
0·8
TIME (seconds)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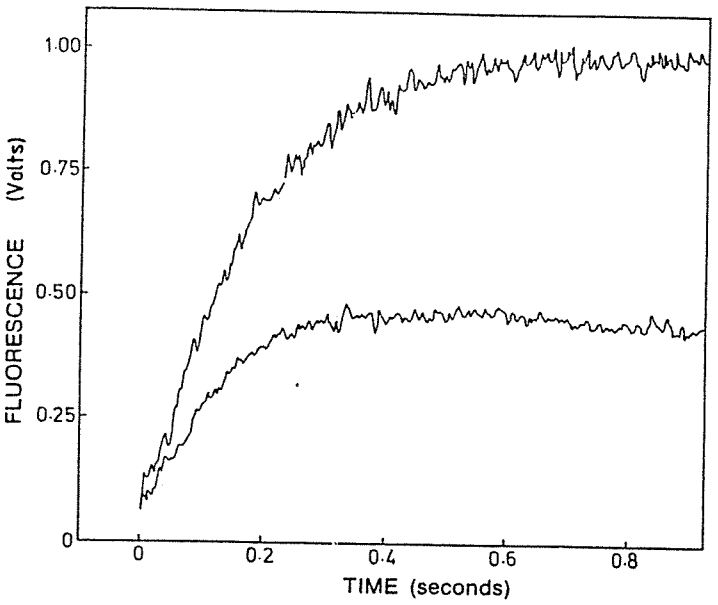
1·5
1·0
0·5
FLUORESCENCE (Volts)
0
10
20
30
40
TIME (seconds)

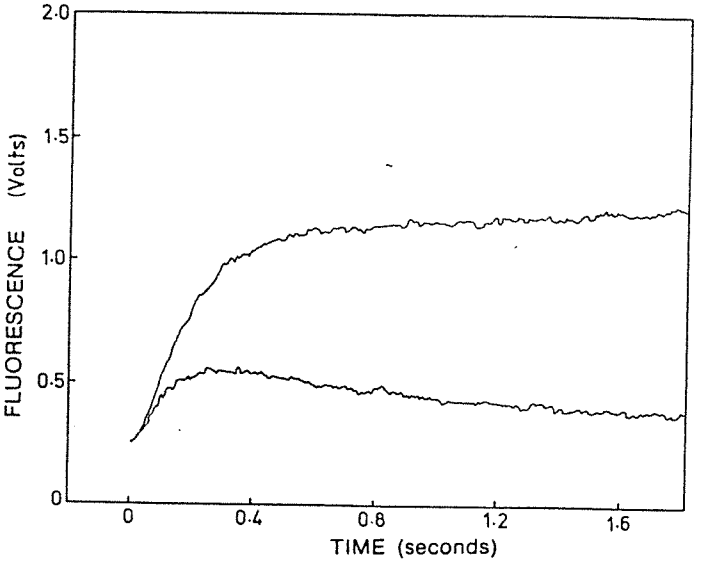
Figure 7.12 The Effect of DEPC on the NADH Burst at Low Concentrations of Propionaldehyde.

A solution of enzyme (4.8 M) and NAD^+ (1 mM) was rapidly mixed with a solution containing propionaldehyde (100 M) and DEPC (2.0 mM). The reaction is shown over three different time frames. In (a) and (b), both the control burst (A, no DEPC present) and the burst in the presence of DEPC are shown. A value of 0.73 s^{-1} was obtained for the apparent decay constant of the decrease in fluorescence which follows the burst (c).

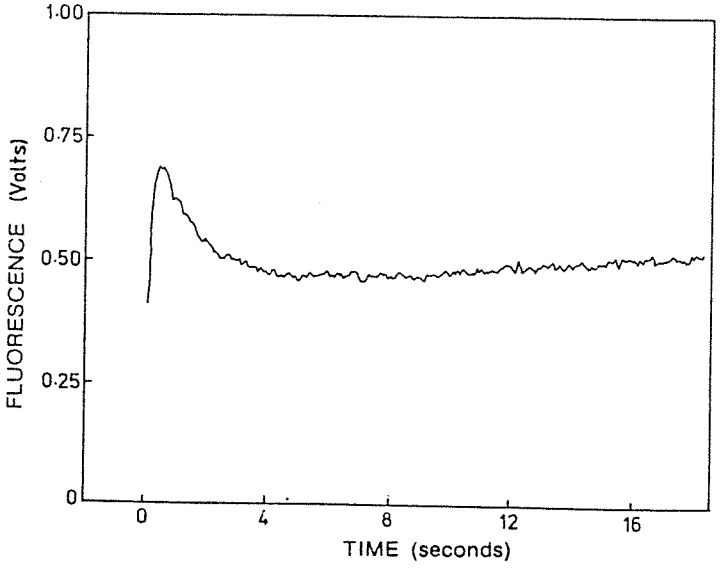
(a)


1·00
0·75
0·50
0·25
0
FLUORESCENCE (Volts)
0
0·2
0·4
0·6
0·8
TIME (seconds)

(b)


2·0
1·5
1·0
0·5
0
FLUORESCENCE (Volts)
0
0·4
0·8
1·2
1·6
TIME (seconds)

(c)


1·00
0·75
0·50
0·25
0
FLUORESCENCE (Volts)
0
4
8
12
16
TIME (seconds)

slowly (apparently because of depletion of propionaldehyde). This clearly indicates that propionic anhydride is acylating a group other than the active site functional group otherwise inhibition would result. At a propionic anhydride concentration of 52 μM, the initial rate was significantly lower than the maximum rate (as shown in Figure 7.1a).

Although the initial rate was inhibited relative to the control rate at propionic anhydride concentrations greater than 100 μM, an increase to an activated rate occurred as the propionic anhydride hydrolysed in the solution. Thus the lowest propionic anhydride concentration at which inhibition was seen was 80-100 μM, which was the same as the initial propionaldehyde concentration.

The simplest explanation for these results is that propionic anhydride acylates the active site of the enzyme.NAD^+ complex with a Km similar to that for propionaldehyde. This of course would inhibit the dehydrogenase reaction by forming a temporary dead-end complex. If propionic anhydride binds at the P_1 site of the $^*E.NAD^+$ complex (Scheme 2.2, 2.1.3), then there will be direct competition with propionaldehyde for the site. However, if propionic anhydride is also able to bind to the active site group of the free enzyme or the $E.NAD^+$ complex (which seems unlikely if the site is inaccessible to propionaldehyde in these forms) then the inhibition will not be simple competitive. Since it was always necessary to use relatively large amounts of propionic anhydride because of the high rate of hydrolysis of this reagent, it was not possible to carry out classical inhibition studies at different non-saturating concentrations of propionaldehyde and propionic anhydride to determine the type of inhibition. The observation of activation at lower propionic anhydride concentrations also complicates the interpretation of classical inhibition studies. However there was evidence that inhibition resulted mainly from competition with propionaldehyde for the active site since very high propionic anhydride concentrations totally inhibited the reaction until the propionic anhydride level was reduced by hydrolysis.

When non-saturating concentrations of NAD^+ and propionaldehyde were used, the maximum rate at the lowest propionic anhydride concentrations (13-26 μM) was inhibited when compared with the control rate. The propionaldehyde concentration in these experiments was 8 μM and hence competition by propionic anhydride is feasible. These results suggest therefore that the site of inhibition (probably the active site) has a somewhat higher affinity for propionic anhydride

than the activating site (although the difference in affinities is unlikely to be very large). Also the affinities of propionaldehyde and propionic anhydride for the active site must be of similar orders of magnitude as discussed above. This suggestion is consistent with the results obtained for the reverse reaction which also indicated that the affinities of propionaldehyde and propionic anhydride for the active site were not vastly different.

The inhibition observed at high concentrations of propionaldehyde is unlikely to be simple and also probably involves a number of processes. At low propionic anhydride concentrations, propionic anhydride will be totally unable to bind at the active site because of the competitive inhibition which undoubtedly occurs. When propionic anhydride cannot react with the active site it would presumably react with other groups on the enzyme. Therefore activation similar to that observed at low propionaldehyde concentrations (Figure 7.1a) might be expected at very low propionic anhydride concentrations (as seen in Figure 7.2) It is unlikely that the inhibition observed in Figure 7.2 is caused by propionic anhydride binding at the active site even at concentrations of about 2 mM given the probable similarity in affinities for propionic anhydride and propionaldehyde for the enzyme.NAD^+ complex. Thus, it is likely that the inhibition is caused by competition for the second (or P_2) propionaldehyde binding site, removing the activatory effect of high propionaldehyde concentrations. Therefore the propionic anhydride appears to be acylating at least three groups on the enzyme. Since the inhibition is greater than 70% of the high propionaldehyde control rate it is clear that binding of propionic anhydride in the P_2 binding domain leads to an enzyme.NAD^+.P_1Ald.P_2acyl form (where aldehyde binds in the P_1 site and the P_2 site is acylated) which is less active than enzyme.NAD^+.P_1Ald.P_2Ald.

Inhibition of the enzyme by propionic anhydride was completely reversible. At low propionaldehyde concentrations, as the propionic anhydride hydrolysed, an activated rate was usually finally obtained. Only at propionic anhydride concentrations greater than 200 μM was activation not seen, and this is considered to be due to a decrease in the pH of the unbuffered solution. Since the enzyme activity has been shown to be greatest at pH 6-7 (4.3.1.2), a decrease in pH to below 6.0 would result in a lowering of the activity which would mask any activation. It is, however, also possible that irreversible acylation of other sites may occur at high levels of propionic anhydride.

Acetic anhydride is known to react rapidly with amino and tyrosyl groups of proteins (Means & Feeney, 1971 pp 69-71) and it is assumed that propionic anhydride reacts in the same way. Acylation of amino groups is irreversible under normal conditions, and although acylation of tyrosyl groups is easily reversible under alkaline conditions (or by the addition of hydroxylamine) such conditions do not apply in these experiments. Thus it is unlikely that the observed inhibition results from acylation of either lysine or tyrosine residues in the enzyme molecule. It seems reasonable therefore to exclude lysine as a possibility for the active site amino acid, as any enzyme-acyl complex involving lysine would be expected to be too stable to allow reaction to proceed in either the dehydrogenase or reverse directions by the mechanism proposed for aldehyde dehydrogenase. However since activation was still observed after the inhibition was removed and the resulting activated rate was usually constant for 60 seconds or more, it is likely that the acylation of the activating site is irreversible, which suggests that a lysine or tyrosine residue may be involved in this process.

Although both propionic anhydride and PCMB have been shown to cause activation of the enzymic activity at low concentrations of propionaldehyde, it is most likely that activation by propionic anhydride and activation by PCMB are each produced at different sites and possibly by different mechanisms. The apparent irreversibility of activation by propionic anhydride suggests that a lysine (or possibly a tyrosine) group is involved. Activation by PCMB, however, clearly involves a sulphydryl group. PCMB appears to cause activation by binding at the high propionaldehyde (P_2) binding site. As a result of inhibition studies, Blackwell et al., (1983a,b) have proposed that hydrolysis of p-nitrophenylacetate also occurs at or near the P_2 site. However since activation by propionic anhydride appears to be irreversible, the group which is acylated is unlikely to be the same one as that which is involved in the hydrolysis of p-nitrophenylacetate. Furthermore, since the activating effects of PCMB and propionic anhydride appeared to be additive (7.3.1.1) it is likely that there are two activation sites.

In experiments involving activation, although the initial rate was activated at low propionic anhydride concentrations (Figure 7.1a) the extent of the activation was actually greater at propionic anhydride concentrations at which activation was only observed after some of the anhydride had hydrolysed (Figure 7.1a curve B, Figures 7.3b & 7.4b).

This suggests that the activation is the result of a slow process (slow binding or a conformational change following binding), but interpretation of this data is difficult because of the possibility of confusion of time-dependent processes with changes produced as a result of decreases in the propionic anhydride concentration and the pH of the solution. Therefore the exact process producing activation cannot easily be identified.

DEPC is known to react with amino and imidazole groups of proteins (Means & Feeney, 1971, 80-83) and reaction with other groups, such as tyrosine, tryptophan, and cysteine, may also be possible at high pH values. However, the reagent should be specific for histidine at pH 6.0 as other groups (including the ε-amino groups of lysine residues) should be protonated at this pH value. Studies carried out by L. Deady (personal communication) of the spectral changes which occurred when DEPC and enzyme were mixed at pH 6.1 clearly demonstrated that DEPC does react specifically with histidine groups of the enzyme at this pH value and probably acylates 1-2 histidine residues per active site, as found also for the horse liver enzyme by Weiner et al. (1985).

It is not yet known if a histidine residue is catalytically essential and further investigations are still required, but there is evidence that the enzyme may contain an essential histidine group. As observed previously (6.3.8) DEPC acts as a substrate for the reverse reaction at pH 7.0 and therefore must react at the active site under those conditions, since the reverse reaction does appear to be a microscopic reversal of the normal enzyme mechanism.

A histidine group is acylated at pH 6.0, as shown by the difference spectrum of Deady (personal communication) and this must still occur at pH 7.6. It is not possible, however, to completely eliminate the possibility that a sulphydryl group is also acylated by DEPC at the higher pH and hence it cannot be definitely be definitely concluded that the site of acylation is a histidine residue. However, there is some evidence that a reverse reaction with DEPC as the substrate occurs at pH 6.0, although complex absorbance changes (very similar to those of Figure 6.10e) were observed, making interpretation difficult. If this can be further substantiated, then the active site group long held to be a sulphydryl must in fact be a histidine . In this respect it is interesting that von Bahr-Lindstrom et al. (1985) have shown that a histidine residue is very strongly conserved in the primary structures of the human and horse liver cytoplasmic and mitochondrial aldehyde dehydrogenases in the section of the protein

which also contains the disulphiram-sensitive thiol group.

It has recently been suggested by Duncan (1985) that the activation observed at high concentrations of propionaldehyde does not involve a second binding site for propionaldehyde, but is caused by formation of an *E.NADH.Ald complex in which propionaldehyde binds weakly in the active site. Dissociation of NADH from this complex then occurs rapidly, bypassing the slow isomerisation process which controls the reaction rate at low concentrations of propionaldehyde. Although there is evidence indicating that such a complex is formed (Dickinson, 1985, P.D. Buckley and L.F. Blackwell, personal communication), it is not yet known whether propionaldehyde binds at the active site (as suggested by Duncan, 1985) rather than at a separate (P_2) site as proposed by Blackwell et al. (1983a,b,) but evidence from pH studies (Chapter 4) is strongly in favour of the latter proposal.

If DEPC was reacting with an active site histidine (as suggested by its participation in the reverse reaction) and if propionaldehyde was able to bind to the active site of the *E.NADH complex (as suggested by Duncan, 1985 and Dickinson, 1985), then high concentrations of propionaldehyde would be expected to protect the enzyme against inhibition by DEPC. However, the rate constants for the decrease in activity when DEPC was added last to assay solutions were the same within experimental error for both high and low propionaldehyde concentrations, showing that this was not the case. In both cases, the DEPC was actually added to a working system (Figures 7.5a,b) and therefore most of the enzyme would be expected to have NADH bound, since the burst process normally observed at both propionaldehyde concentrations indicates a build up of NADH-containing enzyme intermediates, and the steady state rate is therefore controlled by the rate of processes involving such intermediates. The rate of binding of DEPC would be expected to be much slower if propionaldehyde was also able to bind at the active site of these enzyme.NADH complexes and thus was contrary to experiment. This suggests that either the histidine residue with which DEPC reacts does not reside in the active site, or else that propionaldehyde binds at a site other than the active site of the binary enzyme.NADH complexes. Although neither possibility can be definitely excluded at this stage, on the basis of these inhibition studies alone there is a considerable amount of other evidence (section 4.4) to suggest that the two-site model of Blackwell et al. (1983a,b) is correct.

Maleic anhydride was observed to produce two distinctly different

effects, depending on the propionaldehyde concentration. At propionaldehyde levels sufficient to saturate only the P_1 site, reversible inhibition of the steady state rate was observed within the time of mixing. At high propionaldehyde concentrations there was no immediate inhibition, suggesting that the inhibition was competitive with propionaldehyde and probably involves reaction of maleic anhydride at the catalytic site since this substance is also a substrate for the reverse reaction. However, whereas the inhibition observed at low propionaldehyde concentrations was completely removed as the maleic anhydride hydrolysed, a slow decrease was observed in the steady state rate at high propionaldehyde concentrations to a final rate which was very similar to the normal steady state rate at low propionaldehyde levels. As the hydrolysis of this anhydride is very rapid, it is unlikely that this slow process involves slow binding of maleic anhydride as was the case with DEPC. It seems more likely that under these conditions maleic anhydride binds very quickly and irreversibly at a second non-catalytic site and produces a slow conformational change in the enzyme structure which results in removal of the activating effect of high concentrations of propionaldehyde.

Inhibition of the enzymic activity at high propionaldehyde concentrations by PCMB was attributed to reaction with a thiol group in the vicinity of the P_2 binding domain (3.4). Since maleic anhydride is known to react with thiol groups (as well as amino, histidine and tyrosyl groups) it may also bind to the thiol group in the P_2 binding domain and both substances may act in the same mannner. Although there is insufficient information at this stage to determine the exact nature of the inhibition produced by maleic anhydride. these results suggest that propionaldehyde binds at two distinct sites as it not possible to interpret these results in terms of reaction at a single active site.

At low concentrations of propionaldehyde, the NADH fluorescence burst was completely eliminated if enzyme was premixed with propionic anhydride, DEPC, or maleic anhydride, but a steady state production of NADH was still observed. Premixed NAD^+ was apparently not essential for loss of the burst in the case of propionic anhydride (Table 7.3), but this has not yet been tested for the other two anhydrides. The effect of propionic anhydride on the burst was shown to be completely reversible, whereas maleic anhydride, which also hydrolyses very rapidly, appeared to produce more permanent changes, again suggestive of covalent modification (perhaps by reaction with a sulphydryl) as discussed above. The rate of hydrolysis of DEPC was presumed to be too

slow to determine whether modification by this reagent was reversible. In all cases a steady state rate was seen showing that these acylating agents were not simply inactivating the enzyme.

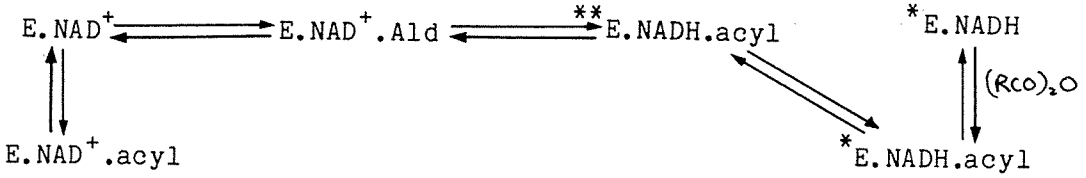
There are three possible ways in which the burst in NADH fluorescence could be removed by reaction with propionic anhydride without also eliminating the steady state reaction. Obviously, if propionic anhydride completely acylates the active site, both the burst and the steady state will disappear. It is possible that quenching of the fluorescence of the bound NADH results from acylation. Secondly, there could be a significant decrease in the rate of one or more of the steps in the presteady state phase of the reaction (Scheme 2.2) so that there is no build up of NADH-containing enzyme intermediates during the reaction. Thirdly, acylation at the active site of the *E.NADH species of Scheme 2.2, followed by a rapid reverse reaction might effectively cancel out the burst. Although fluorescence quenching may appear to be an acceptable explanation, elimination of the fluorescence enhancement normally observed for enzyme-bound NADH would only result in a reduction of the burst amplitude to 16-20% of its control value, and this would still have been easily detected. If fluorescence quenching is involved, then the fluorescence of all enzyme-bound NADH must be totally eliminated (or the wavelength of emitted radiation must be dramatically shifted) and this seems unlikely following reaction with a non-chromophoric reagent such as propionic anhydride.

The second explanation requires that propionic anhydride must affect the rate of at least two steps in the reaction mechanism, although not necessarily by reaction at a single site. The rate of production of NADH-containing enzyme intermediates must become slower than the production of free NADH, which is normally controlled by the rate of the isomerisation of the enzyme.NADH complexes. Since the activation of the steady state which is observed must be due to an increase in the isomerisation rate, then it is quite possible that there is a change in the rate-determining step.

Since the reaction rate in the absence of a burst was initially about 70% of the normal steady state rate, before slowly increasing to an activated rate, the activation may be the result of a second slow process independent of the loss of the burst, as suggested previously by the steady state results. The extent of activation in these experiments was much greater (up to 5.5 times the control rate) than that observed by conventional methods, but this may be more apparent than real if there are any changes in the fluorescence enhancement

factors in the presence of propionic anhydride..

In view of the fact that the reverse reaction occurs readily when enzyme.NADH complexes are mixed with propionic anhydride, the third explanation seems the most likely. According to this explanation, propionic anhydride would compete with propionaldehyde for the active site of the $E.NAD^+$ complex and would also bind to the active site of the $^*E.NADH$ species (Scheme 7.1)



Thus when propionic anhydride is not premixed with the enzyme, some (perhaps most) of the enzyme will be rapidly converted into $^*E.NADH$. However, as soon as this species is formed it can react with propionic anhydride in a rapid reverse reaction regenerating E, NAD^+ and aldehyde. The net effect is of course a much reduced burst amplitude. As the propionic anhydride is hydrolysed, spontaneously as well as by the $E.NAD^+$.acyl and $^*E.NADH$.acyl forms, more and more of the enzyme will be converted into the E.NADH form from which NADH can dissociate and the steady state reaction rate will increase with time (hysteresis) as observed experimentally.

When propionic anhydride is premixed with $E.NAD^+$ then most of the enzyme will be converted to the $E.NAD^+$acyl species and no burst will be seen. However a steady state will be ultimately observed as for the previous mixing condition as the propionic anhydride hydrolyses..

The activation of the steady state which is observed must result from an increase in the rate of isomerisation of the binary enzyme.NADH complexes (2.1.3, 3.5) This may be caused either by the pH decrease which accompanies propionic anhydride hydrolysis (refer section 4.3.3) or as a result of the fact that the NADH release steps now take place from an acylated enzyme species.

Although DEPC and maleic anhydride also appear to eliminate the burst, while retaining some steady state activity, there were notable differences between the three anhydrides. The gradual appearance of a slow exponential increase in fluorescence produced by maleic anhydride may be related to the slow process which removed the activating effect of high concentrations of propionaldehyde (Figure 7.8b), but requires further investigation before any definite

conclusions can be drawn.

The rate constant of 0.73 s^{-1} obtained for the exponential decrease in fluorescence which followed the reduced amplitude NADH burst (in cases in which a burst was observed) in the presence of DEPC was very similar to the rate constant obtained for the presteady state reverse process for DEPC (6.4.1.1) and this is consistent with the possibility that the reaction is also forced in the reverse direction by DEPC as soon as NADH is formed. Alternatively, DEPC may inactivate a proportion of the enzyme or alter the NADH dissociation constant, since the results shown in Figure 7.12 appeared to be very similar to the results obtained in burst experiments involving PCMB (3.4) Figures 3.11,3.12). Further experiments are required to determine whether both substances do in fact act in the same manner. Surprisingly, the same mixing conditions gave a significantly higher burst amplitude when high concentrations of propionaldehyde were involved in all the experiments (Table 7.3). No hystersis was observed following the burst, which makes the explanation given for the low propionaldehyde data untenable in this situation. Propionic anhydride would not be expected to bind to the active site of $^{*}E.NAD^{+}$ complex in the presence of 20 mM concentrations of propionaldehyde. Thus, the major effect of propionic anhydride would be expected to be exerted on the active site of the enzyme.NADH binary complexes, or at some other site possibly in the vicinity of the P_2 binding domain. An added complication for an understanding of the results is that the effect of high concentrations of propionaldehyde on the NADH displacement process (the combined isomerisation and dissociation steps) is unknown. There is some evidence to suggested (Chapter 4) that two pathways may operate under these conditions; one in which the isomerisation step is rate imiting and a second in which the rate of NADH release from E.NADH is rate limiting.

The results could perhaps be explained on the basis of two parallel pathways, one of which behaved in a similar fashion in the presence of propionic anhydride as the usual low propionaldehyde pathway. If the alternative pathway did not undergo a reverse reaction, but was modified in the presence of propionic anhydride, the data might be accounted for. Whatever the correct explanation, it is clear from the experiments with propionic anhydride that the enzyme is behaving very differently at high concentrations of propionaldehyde and more basic kinetic work is required before a full understanding can be reached.

Few definite conclusions can be made about the exact mechanism by which propionic anhydride causes activation of the enzyme activity at low concentrations of propionaldehyde, or how each of these substances affects the presteady state processes. The information which was obtained from experiments involving propionic anhydride was limited by the rapid rate of hydrolysis of this substance and the high concentrations which were necessary in these experiments. As a result, the concentration of propionic anhydride was always changing throughout a particular reaction, by processes other than the reaction itself. Also, the initial high levels of anhydride which were necessary probably resulted in acylation of a large number of groups on the enzyme, not merely those of relevance to the catalytic activity. It is unlikely that further worthwhile study of the effects of this reagent is possible unless a suitable buffer system that does not catalyse the hydrolysis of the propionic anhydride can be found. Using such a buffer the pH of the solution could be kept constant and meaningful initial velocity and inhibition patterns might be determined.

Preliminary studies involving DEPC and maleic anhydride, have both produced interesting results. Further studies of the effects of these reagents on the NADH burst under different mixing conditions and at different propionaldehyde concentrations may prove useful. Also studies of spectral changes at 243 nm as DEPC reacts with the enzyme may provide further information about the way in which these substances affect the enzyme activity, and the role of excess propionaldehyde as a modifier of the enzymic activity.

The effects produced by propionic anhydride, maleic anhydride and DEPC on the enzyme dehydrogenase activity are difficult to interpret in a simple way even with two sites. However, the data are consistent with a number of functional groups on the enzyme, such as sulphydryls, lysine, histidine and possibly tyrosine, whose modification leads to important kinetic effects. The question of the identitiy of the active site group which is acylated during the oxidation of aldehydes is still unresolved but circumstantial evidence is mounting in favour of an essential histidine residue.

APPENDIX I

CALCULATION OF THE CONCENTRATION OF FREE AND ENZYME-BOUND NADH IN SOLUTIONS CONTAINING ENZYME AND NADH

A value of 1.2 μM was reported by MacGibbon et al (1977a) for the dissociation constant of NADH bound to aldehyde dehydrogenase.
i.e.

$$K_D = 1.2 = \frac{[NADH]_{free}[E]_{free}}{[E.NADH]}$$

hence

$$\frac{([NADH]_o - [E.NADH])([E]_o - [E.NADH])}{[E.NADH]} = 1.2$$

and

$$[E.NADH]^2 - ([E]_o + [NADH]_o + 1.2)[E.NADH] + [E]_o[NADH]_o = 0$$

This equation has solutions:

$$[E.NADH] = \frac{([E]_o + [NADH]_o + 1.2) \pm \sqrt{([E]_o + [NADH]_o + 1.2)^2 - 4[E]_o[NADH]_o}}{2}$$

The solution containing the negative square root term is the one applicable to the physical situation, and the concentration of free NADH is then easily obtained:

$$[NADH]_{free} = [NADH]_o - [E.NADH]$$

APPENDIX II

ABBREVIATIONS

acyl	C R O
ADH	alcohol dehydrogenase
AlDH	aldehyde dehydrogenase
Bistris	2-[2-bis(hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol
DACA	p-dimethylaminocinnamaldehyde
DEAE	diethylamino...
DEPC	diethylpyrocarbonate (ethoxyformic anhydride)
E	enzyme
$E.NAD^+$	enzyme with NAD^+ bound
E.NADH	enzyme with NADH bound
$E.NAD^+.Ald$	enzyme with both NAD^+ and aldehyde bound
EDTA	Ethylenediaminetetraacetic acid
ε	molar extinction coefficient
F	fluorescence
ΔF	fluorescence difference
ΔF_{max}	maximum fluorescence difference
g	gram
g	unit of gravititational field
IEP	Isoelectric Point
K_a, K_b	Michaelis constants for species A,B
k_{cat}	turnover number
K_D	dissociation constant
K_{ia}	dissociation constant for species A
l	litres
λ	observed decay constant
LDH	lactate dehydrogenase
NAD^+	nicotinamide adenine dinucleotide
NADH	dihydronicotinamide adenine dinucleotide
P_1	High affinity propionaldehyde binding site
P_2	Low affinity propionaldehyde binding site
PAG	Polyacrylamide Gel
PCMB	p-(chloromercuri)benzoate
PEG	Polyethlyene glycol
PMB	p-(mercuri)benzoate

PNPA	4-nitrophenylacetate
R	fractional saturation of specified binding sites
s	seconds
SDS	sodium dodecylsulphate
TEA	triethanolamine hydrochloride
Tris	tris-(hydroxymethyl)aminomethane hydrochloride
v	reaction velocity (rate)
V_{max}	maximum reaction velocity

DEFINITIONS

Low Propionaldehyde	Concentrations of propionaldehyde (usually 100 μM) sufficient to saturate the high affinity propionaldehyde binding site.
High Propionaldehyde	Concentrations of propionaldehyde (usually 20 mM) sufficient to saturate the low affinity propionaldehyde binding site.
Electrolyte solution	A solution containing both 0.1 M Na_2SO_4 and 0.1 M $NaNO_3$ at pH 7.0

APPENDIX@III

CHEMICALS

Acetaldehyde	British Drug Houses (B.D.H.) Poole, England
Acetic Anhydride	May & Baker (M. & B.), Dagenham, England.
Acetonitrile	J. T. Baker Chemical Co., Philipsburg, New Jersey.
Ammonium Sulphate	B.D.H. (specially low in heavy metals)
Boric Acid	B.D.H.
n-Butanoic Anhydride	Aldrich Chemical Co., Milwaukee, U.S.A.
Chloral hydrate	B.D.H.
p-Chloromercuribenzoate	Sigma Chemical Co.
Diethylpyrocarbonate	B.D.H. (Biochemical)
EDTA	B.D.H.
Glycine	Prolabo Produits, Rhone
Glycylglycine	Recrystallized from Sigma
Glyoxylic Acid	Sigma
Imidazole	Sigma
Maleic Anhydride	B.D.H.
2-Mercaptoethanol	Baker
NAD"+	Sigma, Grade III and Grade AA 1
NADH	Sigma, Grade III
p-Nitrophenylacetate	Aldrich
Phenol Red	B.D.H.
Polyethylene Glycol	B.D.H
Propanal	Koch Light Laboratories, Colnbrook, Bucks., England.
Propionic Anhydride	Koch Light Laboratories.
Semicarbazide Hydrochloride	M. & B.
Sodium Acetate	Sigma
Sodium Barbitone	B.D.H
Sodium oxalate	M. & B.
Sodium Pyrophosphate	Sigma
Succinic Anhydride	Sigma
Tris	Baker (A.R.)

All other chemicals were of the highest purity available and were used without further purification.

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