STRUCTURAL AND FUNCTIONAL PROPERTIES OF SHEEP LIVER ALDEHYDE DEHYDROGENASE

A Thesis presented in fulfilment of the
requirements for the degree of
Doctor of Philosophy in Chemistry
at Massey University

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1988

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ABSTRACT

When aldehyde dehydrogenase reacts with disulfiram (a drug used in alcoholism therapy) a thiol group (A) is initially modified causing enzyme inactivation, and the label is subsequently displaced by a second thiol group (B). In Chapter 3 the effects of various other thiol-modifying agents on the dehydrogenase activity of mitochondrial aldehyde dehydrogenase were examined, and it was found that 4,4'-dithiodipyridine and 5,5'-dithiobis(1-methyltetrazole) are good activators. This implies that modification is at group B and not with the disulfiram-sensitive thiol, group A. On the other hand, methyl diethylthiocarbamyl disulfide and methyl 5-(1-methyltetrazolyl) disulfide are good inhibitors, just as they are of the cytoplasmic form. 2,2'-Dithiodipyridine has no effect on activity of the mitochondrial enzyme and suggests the involvement of groups other than groups A and B. Methyl 5-(1-methyltetrazolyl) disulfide may be the compound responsible *in vivo* for the unpleasant physiological response to alcohol (like that caused by disulfiram) observed during therapy with certain β-lactam antibiotics which contain a 1-methyltetrazole-5-thiol sidechain.

The remainder of this thesis concerns studies of the cytoplasmic isozyme. In Chapters 4 and 5 the research focuses on the esterase and dehydrogenase activities exhibited by this enzyme. Traditionally, these activities have presumed to be mediated by a common active site. However, in the literature over the past 10 years evidence has been presented which suggest they occur at two positionally distinct sites, called P1 and P2 for the dehydrogenase and esterase activities respectively. In the present study support for a common active site was provided by findings which suggest that modification of a certain thiol group by various reagents always leads to a concerted reduction in both these activities (Chapter 4). The intermediacy of a common active site was also supported by these findings, namely: (1) [^{14}C]acetaldehyde is produced from a reaction mixture containing enzyme, NADH, and 4-nitrophenyl [^{14}C]acetate (Chapter 5); and (2) the same catalytic nucleophile appears to be involved in the hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole and the oxidation of *trans*-4-N,N-dimethylaminocinnamaldehyde (Chapter 7).

Another area investigated in this study concerns the identity of the disulfiram-sensitive group. Disulfiram is a reagent which potently inhibits the cytoplasmic enzyme, and is inferred to react with Cys-302 on the basis that pre-modification

of the enzyme with disulfiram blocks the incorporation of [^{14}C]iodoacetamide. (The latter has been shown to label Cys-302 specifically.) In Chapter 6 the reaction between [^{14}C]iodoacetamide and enzyme previously modified with various thiol reagents was monitored. With a 2-fold excess of either disulfiram, methyl diethylthiocarbamyl disulfide, or methyl 2-pyridyl disulfide, the reaction rate with [^{14}C]iodoacetamide was slowed compared to the reaction rate with native enzyme. However, in all these cases (including the native enzyme) the total amount of bound radioactivity was the same after 24 hours. Only with a 4-fold excess of disulfiram or methyl diethylthiocarbamyl disulfide was the amplitude reduced significantly over this period. These results suggest that the disulfiram-sensitive group is not Cys-302.

Finally, a true dehydrogenase acyl intermediate was trapped using *trans*-4-N,N-dimethylaminocinnamaldehyde. The denatured species was digested and the active site peptide purified by gel filtration and HPLC. The chromophore was associated almost exclusively with a peptide identified as T5 in the known primary structure. Although, on chemical grounds, only Ser-74 (from T5) has the ability to act as a nucleophile, no direct evidence was found to prove a covalent link between this amino acid and the chromophore. Arguments for and against Ser-74 being the catalytic nucleophile are discussed.

ACKNOWLEDGEMENTS

This thesis was made possible by the contributions and advice of a number of people. Dr. Dave Harding and Dr. Margaret Brimble generously gave their time and inspiration in aspects of the peptide synthesis work, and Mr. Dick Poll and Ms Trisha Heaton freely offered their expertise. Mr. Julian Reid and Dr. Graeme Midwinter also helped with peptide sequencing. To everyone I am grateful.

Special thanks goes to all those people who have provided input into this work and have helped in the unfolding of this thesis. In particular, I would like to thank Mrs Jenny Trow for diagrams; Mark for sharing much more than my laboratory (... and flat, and food); and Neill for his informative comments on many occasions at the local. Not least I thank Karen for having to put up with my rather nocturnal habits during writing. I hope you all treat this acknowlegement as a token of my sincere appreciation.

Above all, I would like to express a particular debt of gratitude to my supervisors Dr. Trevor Kitson and Dr. Paul Buckley who have provided so much. They have not only furthered my understanding in this subject area, but have freely given their advice and support whenever I needed it. Thanks Trevor and Paul.

TABLE OF CONTENTS

CHAPTER page

CHAPTER page

CHAPTER page

LIST OF FIGURES

FIGURE page

FIGURE page

FIGURE page

TABLES

CHAPTER 1

INTRODUCTION

This thesis concerns the enzyme aldehyde dehydrogenase, the action of which is to remove various aldehydes from the body. One of its important roles is catalysing the breakdown of acetaldehyde produced during ethanol metabolism. In this Chapter the functional, kinetic, and structural aspects of this enzyme are reviewed in terms of the existing literature.

1.1 General Aspects of Ethanol Metabolism

Ingested alcohol can be removed from circulation in the blood in at least three ways (Li, 1977): (1) by the action of alcohol dehydrogenase; (2) by the action of catalase in the presence of excess hydrogen peroxide; and (3) by the microsomal ethanol-oxidising system (MEOS) in the presence of NADPH and oxygen.

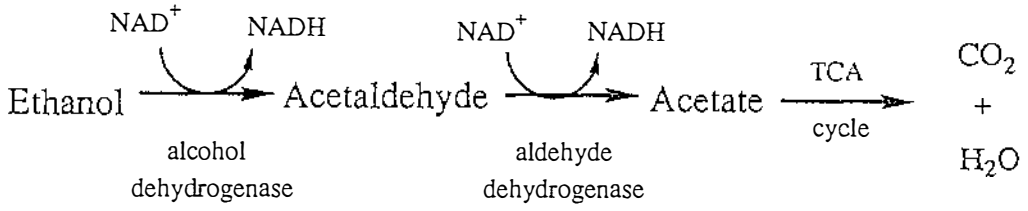
Under certain circumstances the microsomal ethanol-oxidising system can be significantly stimulated by either thyroid hormones or propylthiouracil (a thyreostatic drug used in the treatment of liver disease; Mereno *et al.*, 1981). However, there is widespread evidence to suggest that alcohol dehydrogenase is the chief enzyme responsible for alcohol metabolism *in vivo*. For instance, pyrazole is a potent inhibitor of alcohol dehydrogenase *in vitro* (Coleman & Weiner, 1973; Branden *et al.*, 1975) but not of catalase nor the MEOS. When administered to rats *in vivo* pyrazole causes a marked reduction in the rate of ethanol oxidation (Goldberg & Rydberg, 1969; Shimada *et al.*, 1987). Conversely, specific inhibitors of catalase have no significant effect on ethanol metabolism (Thurman *et al.*, 1975).

The functional, kinetic, and structural properties of alcohol dehydrogenase are particularly well studied (Jornvall, 1970a,b; Jornvall *et al.*, 1988; Jornvall & Harris, 1970; Eklund *et al.*, 1976; Branden *et al.*, 1975). This enzyme is composed of at least two subunits, exists in a variety of multiple molecular forms (Li, 1977; Eriksson, 1987; Bosron, 1985) and occurs predominantly in the liver. Mechanistically, alcohol dehydrogenase catalyses the reversible oxidation

of alcohols in an ordered pathway where coenzyme binds first. The reaction proceeds via coordination of the alcohol to a catalytically bound zinc atom which functions as a Lewis acid (Sund & Theorell, 1963).

The second stage of ethanol metabolism involves the removal of acetaldehyde, the product from alcohol dehydrogenase. The enzymes capable of this include aldehyde dehydrogenase, xanthine oxidase, and aldehyde oxidase. However, only aldehyde dehydrogenase has Michaelis constants low enough for the enzyme to be effective at physiological concentrations of acetaldehyde (Li, 1977). This enzyme is present in a variety of tissues and cells but is most abundant in the liver (Deitrich, 1966). For this reason, most acetaldehyde removal also occurs in the liver (Li, 1977).

The major route of ethanol metabolism discussed above can be summarised by the following Scheme:



Scheme 1.1

According to Scheme 1.1 potentially three mechanisms exist which can control the overall rate of ethanol metabolism, namely: (1) the oxidation of ethanol to acetaldehyde; (2) the oxidation of acetaldehyde to acetate; and (3) the rate at which reduced coenzyme is oxidised to NAD^+ (Crow, 1985).

Under certain conditions where aldehyde dehydrogenase is inhibited, the second possibility ((2) above) becomes significantly rate-limiting. In this case ethanol can be oxidised to acetaldehyde, but no further. In effect, the pathway is terminated at this point and the blood concentration of acetaldehyde rises significantly. This is exactly what happens when disulfiram (tetraethylthiuram disulfide, $(C_2H_5)_2NCSS\text{-}SCSN(C_2H_5)_2$) is administered *in vivo*. The build-up of acetaldehyde produces symptoms which include vomiting, flushing, nausea, palpitations, and tachycardia. These effects are well documented and are known collectively as the "Disulfiram-Ethanol Reaction" or DER (Kitson, 1977).

1.2 Factors which Affect Aldehyde Dehydrogenase in vivo

In addition to inhibiting aldehyde dehydrogenase disulfiram has also been found to suppress the level of norepinephrine (Kitson, 1977). One effect of this is prolonged hexobarbital-induced anaesthesia in disulfiram-treated rats (Nilsson *et al.*, 1987). Disulfiram also interferes with the normal metabolism of the biogenic aldehyde 3,4-dihydroxyphenylglycolaldehyde (a metabolite of norepinephrine). This substrate can normally be oxidised by aldehyde dehydrogenase to the acid or reduced to the corresponding alcohol by alcohol dehydrogenase or aldehyde reductase. However, under circumstances where oxidation through aldehyde dehydrogenase is blocked (as it is when disulfiram is administered) most flux enters a reductive pathway (Kitson, 1977).

This phenomenon also occurs with 3,4-dihydroxyphenylacetaldehyde and 5-hydroxyindole-3-acetaldehyde (the metabolites of dopamine and serotonin respectively) during alcohol metabolism. In this instance, however, the observed shift toward reduction is not because aldehyde dehydrogenase is intrinsically inhibited. Rather, it is due to competition by acetaldehyde for the remaining enzyme activity (MacKerell *et al.*, 1986a; Helander & Tottmar, 1987).

Known commercially as "Antabuse", disulfiram has been used since 1948 in deterrent therapy for the treatment of chronic alcoholism. It is the expectation that a full knowledge of the imminent consequences of a DER, should alcohol be consumed, will be enough to overcome a patient's desire to drink (Kitson, 1977). Although this is generally found to be the case there have been reports of patients who purposely swallow antabuse while drinking. Chevens (1953) suggests this may be a self-induced punishment for succumbing to alcohol or perhaps a last desperate attempt to stop drinking.

There are also a number of other compounds which elicit a response similar to disulfiram. These include calcium carbimide (or cyanamide) a drug introduced in 1956 (Fergusson, 1956) but with a somewhat milder effect than disulfiram (Brien *et al.*, 1978); several prodrug forms containing carbimide (Kwon *et al.*, 1986); coprine, found in the mushroom Coprinus atramentarius; pyrogallol (Peterson & Hjelle, 1982); nitrefazole (Brien & Loomis, 1985); and certain members of the cephalosporin family which have a methyltetrazolethiol

sidechain: moxalactam, cefamandole, and cefoperazone (Reeves & Davies, 1980; Portier, 1980; Neu & Prince, 1980).

A DER-like response is also known frequently to occur in a high proportion of Oriental people (Harada *et al.*, 1981, 1982). These individuals possess a mutant aldehyde dehydrogenase which has a significantly reduced catalytic capacity (Ferencz-Biro & Pietruszko, 1984) and this is the primary cause of the build-up in acetaldehyde. Furthermore, these subjects may possess an alcohol dehydrogenase isozyme whose kinetic behaviour differs significantly from that in normal subjects, and is due to a variant β_1 subunit - designated the β_2 (Oriental) subunit (Fong & Keung, 1987a,b).

Alcoholics too have an impaired capacity to metabolise acetaldehyde, and are also prone to elevated acetaldehyde levels (Jenkins *et al.*, 1984; Jenkins & Peters, 1980). However, in these cases the basis of aldehyde dehydrogenase inhibition is not due to a primary abnormality as it is with Orientals. Rather, reduced activity appears to be attributable to a depression in total hepatic aldehyde dehydrogenase content, and is directly mediated by alcohol consumption. This decline in activity is also seen in erythrocytic aldehyde dehydrogenase (Agarwal *et al.*, 1987) although, interestingly, no reduction is observed in human brain (Pietruszko *et al.*, 1981). In some instances this activity loss is reversible, and can return during a period of abstinence from alcohol (Jenkins *et al.*, 1984).

1.3 The Isolation and Subcellular Location of Aldehyde Dehydrogenase

Aldehyde dehydrogenase was first isolated by Racker (1949) from bovine liver, and since then from a variety of other sources, namely: yeast (Seegmiller, 1953; Steinman & Jakoby, 1967); the bacteria Acetobacter suboxydans (King & Cheldelin, 1956) and Pseudomonas fluorescens (Jakoby, 1958); rat, monkey, and bovine brain (Erwin & Deitrich, 1966); pig brain (Duncan & Tipton, 1971); rabbit liver (Maxwell & Topper, 1961) and kidney (Gillette, 1959); horse liver (Feldman & Weiner, 1972a); human liver (Kraemer & Deitrich, 1968; Blair & Bodley, 1969); and sheep liver (Crow *et al.*, 1974).

In the 1970's intensive research was carried out which examined both the subcellular location and functional properties of mammalian aldehyde dehydrogenase. Of great interest was the particular isozyme responsible for acetaldehyde oxidation. In rat liver three aldehyde dehydrogenases were reported by Horton & Barrett (1975) namely: a betaine aldehyde dehydrogenase predominantly in the cytosol, and two enzymes these authors designated ALDH-1 and ALDH-2 which accounted for roughly 80 % and 20 % respectively of the total activity. ALDH-1 was mitochondrial while ALDH-2 was microsomal in origin. Generally speaking, similar results were also obtained from other studies on rat liver (Tottmar *et al.*, 1973; Shum & Blair, 1972) although in one case 20 % of the total activity was found in the cytosol (Marjanen, 1973). However, as Tottmar *et al.* (1973) pointed out, this disparity may be due to contamination from mitochondrial and endoplasmic reticulum sources during homogenisation.

It is now known that ALDH-1 is exclusively mitochondrial in origin and has a very low K_M for acetaldehyde (< 10 μM). ALDH-2 is associated principally with the outer mitochondrial membrane and microsomes, and has a much higher K_M of 0.9-1.7 mM (Tottmar *et al.*, 1973). Subsequent purification and kinetic analysis of ALDH-2 from rat liver shows this enzyme to have markedly different properties from "conventional" aldehyde dehydrogenase. Membrane bound, this enzyme exists as large polymeric aggregates with a monomeric molecular weight of 51,000, and has a high affinity for long chain aliphatic aldehydes (Nakayasu *et al.*, 1978; Weiner, 1982). This is in contrast to ALDH-I which is a tetramer and has a high affinity for acetaldehyde (Feldman & Weiner, 1972a).

An interesting phenomenon observed with rat liver is the induction of cytoplasmic aldehyde dehydrogenase by phenobarbital in genetically selected strains (Koivula & Koivusalo, 1975; 1982). Likewise, different aldehyde dehydrogenases are also inducible by an array of xenobiotics and carcinogens (Koivusalo & Rautoma, 1987). All these aldehyde dehydrogenases are located in the cytosol, have very high K_M's for acetaldehyde, and are not detectable in normal livers. The latter aldehyde dehydrogenases differ from the phenobarbital NAD^+-dependent enzyme in that they are characterised by preferential oxidation of aromatic aldehydes with $NADP^+$ as cofactor. Recently, the cDNA which encodes the tumor-induced aldehyde dehydrogenase has been isolated and cloned (Jones *et al.*, 1987).

To summarise, studies on the subcellular distribution in rat liver indicate that most activity exists in the mitochondria with very little present in the cytosol. Furthermore, mitochondrial aldehyde dehydrogenase is the only isozyme in rat liver which possesses a micromolar K_M for acetaldehyde.

For human, horse, and sheep liver, however, this is not the case as significant amounts of a cytoplasmic aldehyde dehydrogenase exist which also has a high affinity for acetaldehyde. Crow *et al.* (1974) report the cytoplasmic and mitochondrial activities to be roughly equal in sheep liver with both isozymes having micromolar Michaelis constants for glyceraldehyde. In human liver Koivula (1975) found the cytoplasmic fraction to contain 30 % of the overall activity and at least two separable isozymes. One of these possessed a low K_M for aldehydes (in the micromolar range) while the other had a K_M at the millimolar level. More than 50 % of the dehydrogenase activity was found to be mitochondrial, with 10 % (high K_M) present in the microsomes. Both betaine aldehyde and formaldehyde dehydrogenase were found in the cytosol.

In later studies, these aldehyde dehydrogenases from human liver were classified as ALDH-1, ALDH-2 (this is different from the ALDH-2 from rat liver) ALDH-3, and ALDH-4 (Harada *et al.*, 1980); or as E2, E1 (i.e. E2 = ALDH-1 and E1 = ALDH-2) E3, and E4 respectively in the nomenclature of Forte-M^{c}Robbie & Pietruszko (1985). E2 is mitochondrial, has a low K_M for acetaldehyde, and is insensitive to disulfiram. On the other hand, E1 is cytoplasmic in origin, has a slightly higher K_M (33.6 μM; Forte-M^{c}Robbie & Pietruszko, 1985) and is strongly inhibited by disulfiram. In all other respects, however, E1 and E2 are very similar: they are both homotetramers of almost equal subunit weight, have similar kinetic characteristics, and are present in all livers which have been investigated (Forte-M^{c}Robbie & Pietruszko, 1985).

In contrast to E1 and E2, E3 and E4 display completely different properties, and are classified as "high K_M" enzymes because of their millimolar Michaelis constants. E4 is a dimer with a subunit weight around 70,600, and is not precipitated by antibodies raised against E1 and E2. The characterisation of E3 is more difficult because it is not detectable in all livers. However, preliminary estimates indicate it may be polymorphic with a molecular weight similar to E4.

The relationship of E3 and E4 to the high K_M isozyme found in rat liver is currently unknown (Forte-M^{c}Robbie & Pietruszko, 1985).

For horse liver Eckfeldt *et al.* (1976) reported the isolation of two similar aldehyde dehydrogenases, F1 and F2, with molecular weights of 230,000 and 240,000 respectively. The first of these, F1, has a K_M for acetaldehyde of 70 μM and is now known to correspond to E1 above. Similarly, F2 is identical to E2. However, these workers found no evidence for the existence of high K_M cytosolic enzymes.

1.4 The Site of Acetaldehyde Oxidation

One generalisation that can be made from Section 1.3 is that low K_M mitochondrial aldehyde dehydrogenase has been found in all species that have been studied. The cytoplasmic compartment, however, contains a mixture of high and low K_M enzymes which vary considerably between species (MacGibbon *et al.*, 1978a; Koivula, 1975). This implies an important role for the mitochondria during alcohol metabolism, especially when it is considered that rat liver contains hardly any cytoplasmic aldehyde dehydrogenase at all. The literature has focussed on rat liver (Hedlund & Kiessling, 1969; Hassinen *et al.*, 1970; Lindros *et al.*, 1972) and the findings presented below do indeed suggest that at low acetaldehyde concentrations oxidation occurs predominantly in the mitochondria.

Firstly, using perfused rat liver and isolated rat hepatocytes Parrilla *et al.* (1974) demonstrated that below concentrations of 400 μM, DL-cycloserine has no effect on aldehyde oxidation. DL-cycloserine is an inhibitor of certain transaminative steps in the malate-aspartate cycle, and limits the rate at which reducing equivalents (NADH) can enter the mitochondria. The lack of inhibition with DL-cycloserine led these authors to conclude that acetaldehyde oxidation occurs predominantly in the mitochondria. On the other hand, at higher concentrations of acetaldehyde (0.4-10 mM) the cytosol does assume a more prominent role. Under these conditions, acetaldehyde uptake is enhanced (with a K_M of 1.1 mM) but now strongly inhibited by DL-cycloserine.

Secondly, formaldehyde can be oxidised in rat liver by both mitochondrial aldehyde dehydrogenase and by a cytosolic glutathione-dependent formaldehyde dehydrogenase (Uotila & Koivusalo, 1974, 1987; Koivula & Koivusalo, 1975; Cinti *et al.*, 1976). Recently it has been found that the glutathione depressing agents diethyl maleate and phorone decrease the rate of both formaldehyde and acetaldehyde oxidation in tandem. Both these reagents inhibit mitochondrial aldehyde dehydrogenase (Dicker & Cederbaum, 1985, 1986).

Finally, Eriksson *et al.* (1975) compared the oxidation rate of acetaldehyde between rats treated with phenobarbital and control groups. However, despite a significant increase in the level of high K_M cytoplasmic aldehyde dehydrogenase in the reactor group, no corresponding increase was observed in the oxidation rate.

Although the above studies clearly demonstrate a prominent mitochondrial role, rat liver may nonetheless represent an extreme case for two reasons: (1) most activity is localised in the mitochondria anyway; and (2) the cytosolic fraction that is present (even when induced by phenobarbital) displays an exceedingly high K_M for acetaldehyde compared to the low K_M mitochondrial form (Horton & Barrett, 1975). Considering this, it is perhaps not surprising that under physiological concentrations of acetaldehyde - which are typically in the micromolar range (Parrilla *et al.*, 1974) - mitochondrial aldehyde dehydrogenase is almost exclusively assayed.

This may not be the case, however, for other species. As mentioned previously, horse, sheep, and human liver all contain significant quantities of a low K_M cytoplasmic aldehyde dehydrogenase which, in the main, has very similar properties to mitochondrial aldehyde dehydrogenase (Greenfield & Pietruszko, 1977; Hempel *et al.*, 1982b). Consequently, the possibility exists that the cytoplasm plays a more prominent role. However, while this may be true to some extent, the DER-like response experienced by certain Oriental people after small amounts of alcohol (Section 1.2) clearly demonstrates the importance of the mitochondria in ethanol derived acetaldehyde metabolism.

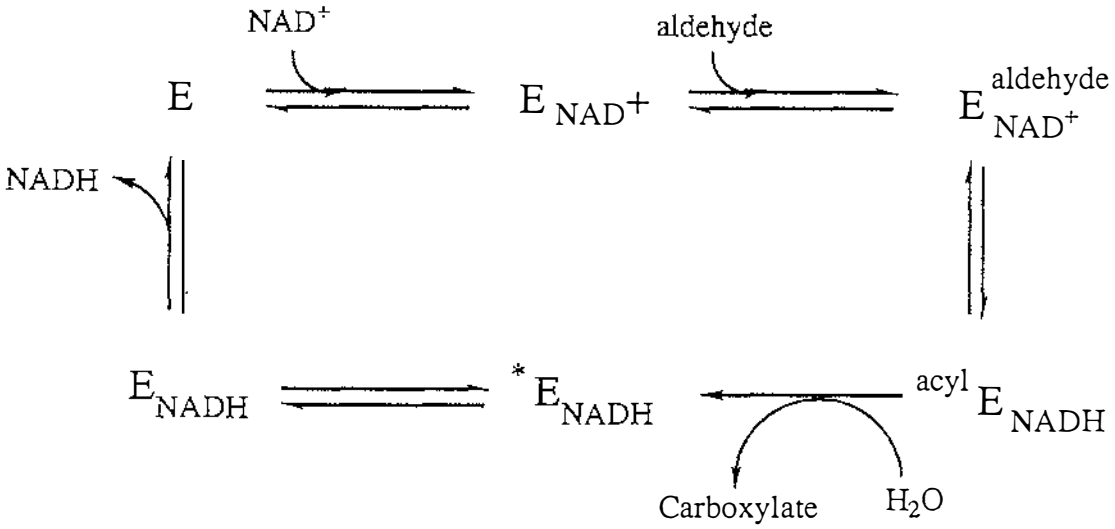
These individuals possess a phenotypically different aldehyde dehydrogenase from that found in normal subjects (Yoshida *et al.*, 1984; Agarwal *et al.*, 1981;

Ikawa *et al.*, 1983). Thus while studies demonstrate the presence of similar amounts of functional cytoplasmic aldehyde dehydrogenase in both typical and atypical livers, the properties of the Oriental mitochondrial enzyme differ drastically from those of its normal counterpart (Ferencz-Biro & Pietruszko, 1984).

The basis of this is a mutation where Glu-487 is substituted for Lys-487, and this occurs in a region of the primary structure which is otherwise well conserved between species (Hempel *et al.*, 1984b; Hempel & Jornvall, 1987). The consequence of this exchange is a protein which still exists in the mitochondria (M^{c}Michael *et al.*, 1986) but has a catalytic activity only 20-30 % of that in normal liver (Ferencz-Biro & Pietruszko, 1984; Harada *et al.*, 1985). Presumably, it is this reduced activity in the mitochondria which is responsible for the DER-like syndrome. How the presence of Lys-487 specifically affects the kinetic mechanism of aldehyde oxidation is not clear. Generally, however, the cause of inhibition is known to occur either consequent with or after hydride transfer (see later). This is because the affinity for aldehyde and coenzyme is largely unaffected but the maximum velocity, V_{MAX}, is dramatically reduced (Ferencz-Biro & Pietruszko, 1984).

1.5 On the Mechanism of Aldehyde Oxidation

Aldehyde dehydrogenase catalyses the oxidation of a wide variety of aldehyde substrates in an ordered bi-bi mechanism with NAD^+ binding first (Scheme 1.2).



Scheme 1.2

At low aldehyde concentrations, and for all NAD^+-dependent aldehyde dehydrogenases that have been studied, it is generally agreed the mechanism proceeds according to Scheme 1.2 (Blackwell *et al.*, 1987; Dickinson, 1986; Hart & Dickinson, 1978b; MacGibbon *et al.*, 1977a,b,c). Evidence supporting the above Scheme comes from a variety of studies including steady state experiments with propionaldehyde, product inhibition studies, and dead-end inhibition studies (MacGibbon *et al.*, 1977b; Sidhu & Blair 1975b).

The kinetic ordering is also confirmed by studies on the associated esterase activity exhibited by this enzyme. Feldman & Weiner (1972b) and Sidhu & Blair (1975a) report a stimulation of 4-nitrophenyl acetate hydrolysis by NAD^+ and NADH with activation constants of 117 μM and 3.5 μM respectively. In contrast, propionaldehyde (in the absence of coenzyme) is found to be a competitive inhibitor with a dissociation constant, K_I, of 0.49 mM (Feldman & Weiner, 1972b). As this value is over 1000 times that calculated for the binding of propionaldehyde to the enzyme from the dehydrogenase data, Feldman & Weiner (1972b) conclude that NAD^+ binds preferentially, and in doing so dramatically lowers the binding constant for aldehyde. The basis of this may be

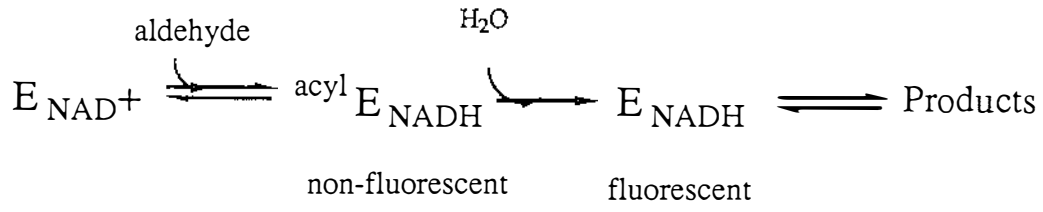
a conformational change induced by coenzyme, and may have the effect of exposing the aldehyde binding domain (Bennett *et al.*, 1982; Hart & Dickinson, 1982).

The rate-limiting step in Scheme 1.2 is known to occur after hydride transfer as a burst in the production of NADH is generally observed for most aldehyde substrates (4-nitrobenzaldehyde is an exception at high conentrations). Also, no deuterium isotope effect is observed with [1,2,2,2-^{2}H]acetaldehyde or [1-^{2}H]propionaldehyde in the forward direction (MacGibbon *et al.*, 1977b,c; Hart & Dickinson, 1982; Dickinson, 1985).

Potentially, the rate-limiting step can constitute either acyl enzyme hydrolysis, NADH dissociation, or a combination of these. There is little question that under certain circumstances acyl hydrolysis is clearly rate-limiting. For example, *trans*-4-N,N-dimethylaminocinnamaldehyde is a substrate for aldehyde dehydrogenase, albeit a poor one. This aldehyde is useful from a kineticist's point of view because the appearance and disappearance of the transient acyl species, $^{acyl}E_{NADH}$, can be monitored spectroscopically at 464 nm. Stopped flow experiments with this substrate show that at pH 10.5 the time course of absorbance at this wavelength passes through a maximum; i.e. the acyl-enzyme builds up significantly, but then rapidly decays as the steady state is reached. As the pH is gradually lowered, however, deacylation becomes progressively slower and the intermediate survives for several minutes. In the limit (below pH 6) acylation becomes limiting (Dunn & Buckley, 1985).

Unfortunately, with conventional substrates such as propionaldehyde and acetaldehyde evidence concerning the formation and subsequent hydrolysis of the acyl ternary species relies on more indirect methods of analysis. Hart & Dickinson (1982) report that stopped flow experiments with propionaldehyde and *trans*-cinnamaldehyde give fluorescent and absorbance traces which differ significantly from one another. Thus while the steady state is entered rapidly in the absorbance trace, the fluorescent time course displays a reproducible lag phase between the initial burst and the final steady state.

Dickinson (1986) interprets this as consistent with two NADH containing species, both having identical absorption chararteristics but markedly different fluorescent properties (Scheme 1.3)



Scheme 1.3

where for convenience all steps occurring before acyl-enzyme formation are not detailed. The first of these species, $^{acyl}E_{NADH}$, is presumed to have little or no fluorescence and is identified as the ternary acyl intermediate resulting from hydride transfer. Irreversible hydrolysis of this liberates the second species, E_{NADH}, which has an enhanced fluorescence compared with free NADH.

According to this model the observed lag in the fluorescence trace is directly attributable to the decay in non-fluorescent $^{acyl}E_{NADH}$ to fluorescent E_{NADH}. The absorbance time course, however, does not display a lag phase because both these species have identical absorbancy properties at 340 nm. Consequently, the lag caused by hydrolysis is effectively masked (Dickinson, 1986; Dickinson & Haywood, 1986). Supporting this theory is the observed dependence of the rate constant for the absorbance transient on the propionaldehyde concentration, but the apparent independence of the fluorescent rate constant. The dependence of the former is because formation of $^{acyl}E_{NADH}$ is directly affected by the rate of aldehyde binding, which in turn is determined by the aldehyde concentration. The independence of the latter is because the fluorescent rate constant reflects only the hydrolysis rate, a step not directly related to aldehyde concentration (Dickinson, 1986).

It is apparent from the foregoing that acyl-enzyme hydrolysis is much faster than NADH dissociation otherwise no lag phase would be observed, only a direct entry into the steady state[1]. Dickinson & Haywood (1986) calculate the rate

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1 - Under these conditions hydrolysis will be partially rate limiting. As the steady state will now be largely governed by this step no lag will occur.

constant from the fluorescent transient to be around 3 s^{-1}, a value that is roughly comparable with the hydrolysis rate constant obtained from simulation studies (Blackwell *et al.*, 1987; Bennett *et al.*, 1982).

The rate-limiting step for aldehyde oxidation (when acyl hydrolysis is relatively fast) is controlled by the relatively slow displacement of NADH (Blackwell *et al.*, 1987; MacGibbon *et al.*, 1977a). As Scheme 1.2 shows this is a two step process involving a slow isomerisation of $^{*}E_{NADH}$, followed by actual release of coenzyme. This relatively slow displacement of NADH is also found to be rate limiting for other dehydrogenases including lactate dehydrogenase, alcohol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase at high pH (Dunn, 1985; Dalziel, 1975; Fersht, 1977). Interestingly, a wealth of literature exists which suggests that coenzyme binding to alcohol dehydrogenase is also biphasic, incorporating a slow conformational change which is rate limiting just as it is for aldehyde dehydrogenase (Branden *et al.*, 1975; Dunn, 1985). However, the isomerisation step for aldehyde dehydrogenase is slower than that for alcohol dehydrogenase (0.25 s^{-1} compared to 3 s^{-1})[2].

One area of research that has long been confusing is the apparent disparity between the rate constant for isomerisation (0.25 s^{-1}, Blackwell *et al.*, 1987) and the value of k_{cat}. Intuitively, the two should be similar because k_{cat} is merely a function of the rate constants involved in the breakdown of $^{acyl}E_{NADH}$. If the isomerisation rate is very slow relative to all other processes then k_{cat} will limit to this step.

Although this is currently believed to be the case (Blackwell *et al.*, 1987) historically there has been much uncertainty and confusion concerning the correct value of k_{cat}. For example, Blackwell *et al.* (1983) found a value of 0.082 s^{-1} for k_{cat} at low concentrations of propionaldehyde (<100 μM) whereas Hart & Dickinson (1982) obtained a value of 0.4 s^{-1}. This descrepancy is

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2 - The value of 3 s^{-1} represents the first order rate constant for alcohol oxidation and it is inferred that the isomerisation step is also around this value. This assumption is based on a large number of supportive kinetic studies (Branden *et al.*, 1975).

difficult to reconcile but appears to stem partially from the different methods these authors used to express enzyme concentration; i.e. an active site versus a tetrameric interpretation respectively. k_{cat} is defined as

$$k_{cat} = V_{MAX} / [E_T]$$

Under saturating conditions the intermediate E_{NADH} exists predominantly in the steady state and limits to the total enzyme concentration E_T when all the preceeding steps are fast. V_{MAX} represents the maximum rate of breakdown from this complex.

Clearly, if aldehyde dehydrogenase has more than one active site per tetramer then the "active site" and "tetrameric" concentrations will differ proportionately and will affect k_{cat} accordingly. In addition to this, Blackwell *et al.* (1987) suggest the disparity may be due to a hitherto unrecognised phenomenon in which the active site concentration is halved when enzyme stock is prediluted in reaction assays. Consequently, the observed k_{cat} of 0.082 s^{-1} is obtained by dividing V_{MAX} by a site concentration which is too high, and the correct value will be approximately twice this.

1.6 Non Michaelis-Menten Behaviour

At high substrate concentrations the simplified mechanism of Scheme 1.2 breaks down and activation or inhibition is observed depending on the concentrations of coenzyme and aldehyde. Substrate inhibition occurs at high concentrations of propionaldehyde when NAD^+ is present in limiting amounts (Hart & Dickinson, 1982). Under these conditions NAD^+ is non-saturating and free enzyme (E in Scheme 1.2) predominates in the steady state. Inhibition results from formation of the abortive species, E^{prop}, which has a decreased affinity for NAD^+ compared to native enzyme (Hart & Dickinson, 1982).

Substrate activation, in contrast, is observed at saturating levels of NAD^+ and at high concentrations of a variety of aldehydes (Erwin & Deitrich, 1966; Hart &

Dickinson, 1978b; MacGibbon *et al.*, 1977b; Dickinson & Haywood, 1987). The degree of activation increases with the concentration of aldehyde and reaches a maximum rate at approximately 20 mM for propionaldehyde. Typically, double reciprocal plots with aldehyde as the variable parameter deviate from the idealised straight line predicted from Scheme 1.2, and gradually curve downwards toward the abscissa. For propionaldehyde this curvature is apparent at only relatively high concentrations (>1 mM) whereas for acetaldehyde it is evident throughout the entire substrate range (MacGibbon *et al.*, 1977a).

Substrate activation can be explained in a number of ways, one of which is the presence of two similar isozymes which have different V_{MAX} and K_M parameters. However, MacGibbon *et al.* (1977a) show that theoretical curves based on the assumption that these presumed enzymes behave independently do not agree well with the experimental curves found with acetaldehyde.

Blackwell *et al.* (1983) suggest an alternative mechanism where high concentrations of aldehyde bind to $^{acyl}E_{NADH}$ (Scheme 1.2). As the rate of acyl hydrolysis from this species was thought to largely control the steady state, activation could be explained in terms of an increased rate of this step. In the limit, dissociation of NADH from E_{NADH} would become rate-limiting.

A different model for substrate activation was proposed by Hart & Dickinson (1982) and involves abortive complex formation between the aldehyde and E_{NADH}. In this case, activation occurs because dissociation of NADH from the resulting ternary species is faster than from E_{NADH}. Evidence supporting the existence of abortive complexes includes stopped flow studies which show a decrease in fluorescence when enzyme/NADH is rapidly mixed with NADH/-propionaldehyde. Furthermore, the fluorescence spectrum of enzyme/NADH changes with varying concentrations of propionaldehyde (Dickinson, 1985; Dickinson & Haywood, 1987).

The concept of abortive formation can be generalised further to encompass other phenomena including substrate inhibition. For instance, assay systems containing the substrate 4-nitrobenzaldehyde exhibit a hysteresis effect where the rate of NADH production accelerates throughout the time course (Dickinson

& Haywood, 1987). The explanation again involves complex formation between E_{NADH} and aldehyde, except that inhibition now occurs because of a decreased dissociation rate of NADH. Throughout the time course because the concentration of this substrate declines so too does the amount of abortive complex. This explains the accelerating rate (Dickinson & Haywood, 1987).

Stopped flow experiments also support this mechanism if the assumption is made that the abortive complex from E_{NADH} and 4-nitrobenzaldehyde has little or no fluorescent properties. When enzyme/NAD^+ is mixed with 20 μM 4-nitrobenzaldehyde a small burst in fluorescence is observed, consistent with rapid formation of E_{NADH} followed by a slower release of NADH. However, at higher concentrations of this substrate (144 μM) no burst in fluorescence is seen at all. Under these conditions 4-nitrobenzaldehyde binds to E_{NADH} as soon as it is formed, and in doing so quenches the fluorescence (Dickinson, 1986).

1.7 The Present Study

It is well known that the dehydrogenase activity of cytoplasmic aldehyde dehydrogenase is strongly inhibited by disulfiram, a drug used in the treatment of alcoholism (Kitson, 1978, 1981, 1982a). Equally documented, however, is the relative absence of inhibition when disulfiram is added to assays containing mitochondrial aldehyde dehydrogenase (Allanson & Dickinson, 1984; MacKerell *et al.*, 1985). The differences in these activities and their implications are interesting from two perspectives.

Firstly, on the basis of circumstantial evidence such as the susceptibility of cytoplasmic aldehyde dehydrogenase to disulfiram and also by analogy to the mechanism of glyceraldehyde-3-phosphate dehydrogenase (Harris & Waters, 1976) it has been commonly assumed that the disulfiram-sensitive group is catalytically essential. However, if disulfiram modifies the same presumed catalytic thiol in both isozymes, then strong inhibition should also be observed for mitochondrial aldehyde dehydrogenase. As this is obviously not the case it potentially casts doubt on a "disulfiram-catalytic thiol" hypothesis.

Secondly, from a physiological perspective a dilemma exists in that disulfiram has only a slow inactivating effect on mitochondrial aldehyde dehydrogenase *in vitro*. Yet it is this isozyme that is thought to mediate the removal of acetaldehyde and, presumably therefore, it must be ultimately affected by disulfiram *in vivo*. In the present study Chapter 3 deals with these issues in more detail by investigating the effects of various thiol modifiers and disulfiram-like analogues on the dehydrogenase activity of mitochondrial aldehyde dehydrogenase.

The second major focus of the present research concerns the one-site/two-site controversy over the dehydrogenase and esterase activities of aldehyde dehydrogenase. Traditionally it has been assumed that both aldehyde oxidation and 4-nitrophenyl acetate hydrolysis are mediated by a common active site. That was, however, until a two-site model was formulated which proposed these activities to occur at distinct active sites (Blackwell *et al.*, 1983). Since then there has been much debate in the literature over the validity of these models, and in particular over the interpretation of the kinetic evidence used to support the two-site scenario (Duncan, 1985).

In this study the one-site/two-site debate is approached in two ways. Firstly, Chapter 4 deals with this indirectly by examining the effects that various modifiers have on the esterase activity of cytoplasmic aldehyde dehydrogenase. The aim is to compare these results with the corresponding behaviour observed with the dehydrogenase activity in the expectation that this will provide useful information concerning the one-site/two-site argument. Secondly, Chapter 5 takes a more direct line by investigating the reversibility of the esterase pathway in the presence of NADH. Detection of dehydrogenase substrates in such a system would provide convincing experimental support for a common active site.

The final area of research in the present study concerns the identification of crucial residues, and particularly the identification of the peptide containing the catalytic nucleophile. The identity of this group is as yet completely unknown, although circumstantial evidence does suggest it may be Cys-302. When this amino acid is modified a dramatic loss in activity occurs. It is specifically labelled by iodoacetamide (Hempel *et al.*, 1982a; Hempel & Pietruszko, 1981;

Pietruszko *et al.*, 1982) and is closely positioned to the coenzyme binding site (von Bahr-Lindstrom *et al.*, 1985). Furthermore, it is implicated in the reaction with disulfiram (Pietruszko *et al.*, 1982) and is conserved in all primary structures that have been investigated (Hempel & Jornvall, 1987). Recently it has been found that Glu-268 is specifically modified by the substrate analogue bromoacetophenone (MacKerell *et al.*, 1986; Abriola *et al.*, 1987) and again this amino acid occurs in a region of the primary structure which is conserved between species.

In the present research, Chapter 6 examines the nature of the thiol modified by disulfiram in more detail by (a) labelling the cytoplasmic enzyme with [^{14}C]disulfiram, and (b) monitoring the reaction between [^{14}C]iodoacetamide and the cytoplasmic enzyme under various premodification conditions.

The major focus of this study, however, concerns the identity of the catalytic nucleophile. In Chapter 7 it is the aim to isolate and characterise the peptide which contains this residue by reacting the cytoplasmic enzyme with either *trans*-4-N,N-dimethylaminocinnamaldehyde or *trans*-4-N,N-dimethylamino-cinnamoyl imidazole. The first of these is an actual dehydrogenase substrate (Dunn & Buckley, 1985) and has the distinct advantage of possessing an intensely coloured chromophore. This can be used as a "marker" to follow the labelled peptide throughout the subsequent column steps. Likewise, *trans*-4-N,N-dimethylaminocinnamoyl imidazole also contains this chromophoric group, but unlike *trans*-4-N,N-dimethylaminocinnamaldehyde it is not an aldehyde, and has yet to be investigated as a potential substrate for this enzyme. Finally, Chapter 8 presents an overview of the one-site/two-site debate in the light of findings from Chapter 7.

CHAPTER 2

EXPERIMENTAL PROCEDURES

This Chapter outlines the experimental and preparative aspects of the present study. Specifically, it details the sources of compounds, the isolation of low K_M aldehyde dehydrogenase from sheep liver, general procedures such as instrumentation, and the synthesis of compounds referred to in subsequent Chapters.

2.1 Sources of Compounds

The chemicals used in the present study and where they were purchased are as follows: 4,4'-dithiodipyridine, 2,2'-dithiodipyridine, *trans*-4-N,N-dimethylaminocinnamic acid, *trans*-4-N,N-dimethylaminocinnamaldehyde, iodoacetamide, protamine sulphate (grade III), N-acetyl-L-cysteine, iodoacetic acid (sodium salt) 4-nitrophenyl acetate, β-NAD^+ (grade AA1), DEAE-Sephacel, DEAE-Superose CL-6B, Sephacryl S-200, methyl methanethiosulfonate: Sigma; 1,4-dithiothreitol, 2-mercaptoethanol: Merck; 5-mercapto-1-methyltetrazole sodium salt hydrate, 2-thiopyridone, 4-thiopyridone, Aldrich; sodium diethyldithiocarbamate: BDH; silica gel (100-200 mesh): Ajax Chemicals; DEAE-23-cellulose, CM-cellulose: Whatman.

All solvents and reagents were of AnalaR quality where available. Acetaldehyde was distilled before use. *trans*-4-N,N-Dimethylaminocinnamaldehyde was resublimed on a cold finger under vacuum before use. The radioactive chemicals [^{14}C]iodoacetamide and [^{14}C]acetic anhydride (donated by Dr. D. Harding) were obtained from Amersham International. [^{14}C]Iodoacetamide was diluted with unlabelled iodoacetamide to give a specific activity of 1.96×10^{12} cpm/mole. [^{14}C]Disulfiram was prepared (Kitson, 1976) and generously donated by Dr. T.M. Kitson. It had a specific activity of 4.15×10^{11} cpm/mole.

2.2 Enzyme Isolation

All experiments in the present study have used aldehyde dehydrogenase isolated from sheep. Livers were obtained from the local freezing works and were transported in ice. The time between slaughter and the commencement of enzyme isolation typically did not exceed one hour. All operations were carried out at 4 ^{o}C and distilled, deionised water was used throughout.

2.2.1 Cytoplasmic aldehyde dehydrogenase: This was purified according to the method of Dickinson *et al.* (1981). Approximately 1.5 kg of the best parts of six sheep livers was finely chopped and homogenised (using an Ultraturrax type T45) in 5 mM sodium phosphate buffer, pH 7.4, containing 0.3 mM EDTA and 0.1 % 2-mercaptoethanol. This was carried out in six batches and enough buffer was used so that the total volume was less than 4 litres. The homogenate was spun at 1500 rpm in a Sorvall RC2B centifuge using a GS3 head for 5 minutes to remove large insoluble material. The supernatant from this was then respun at 13,000 rpm for 15 minutes in a GSA head. For the first precipitation step the pH of the supernatant was reduced to pH 5.3 using 50 % acetic acid; the mixture was spun (13,000 rpm for 15 minutes). After re-adjusting the pH to 7.4 with 4 M ammonia, approximately 100 ml of protamine sulphate (10 mg/ml) was added to precipitate nucleic acids; insoluble material was again spun down. To the supernatant (which was now a deep brownish-red colour) ammonium sulphate was added at a concentration of 240 g per litre of extract. The mixture was left to stand for 15-20 minutes, and insoluble protein removed by centrifugation. Further ammonium sulphate was added (160 g/l) and the resulting precipitate which contained aldehyde dehydrogenase activity was spun down. During the ammonium sulphate fractionation procedures the pH was always kept above pH 7 with 4 M ammonia if necessary. The pellet was redissolved in 25 mM sodium phosphate buffer pH 7.4 + 0.3 mM EDTA + 0.1 % 2-mercaptoethanol, and dialysed against at least 3 x 4 litre changes of this buffer overnight.

The crude extract was loaded onto a DEAE-23-cellulose column (7.5 cm x 50 cm) equilibrated with 25 mM sodium phosphate, pH 7.4, (plus EDTA and 2-mercaptoethanol) and eluted with this buffer. Enzyme was collected by hand in approximately 100 ml fractions. Those containing activity were pooled and

precipitated by the addition of ammonium sulphate (400 g/l). After centrifugation, the pellet was dissolved in a small volume of 5 mM sodium phosphate (plus EDTA and 2-mercaptoethanol) and dialysed overnight against at least 4 x 4 litre changes of this buffer as before.

The crude extract was loaded onto a second DEAE-23-cellulose column (5 cm x 45 cm) equilibrated with 5 mM sodium phosphate, pH 7.4, + 0.3 mM EDTA + 0.1 % 2-mercaptoethanol. Before loading, the conductivity and pH of both the dialysis buffer and column eluate were double checked. After washing the column with this buffer for 1-2 hours, an ionic strength gradient was started in which 1 litre of 70 mM sodium phosphate, pH 7.4, was run into 1 litre of 5 mM sodium phosphate buffer, pH 7.4, (both containing EDTA + 2-mercaptoethanol). Fractions were collected automatically with an Ultrorac LKB fraction collector, and those fractions which contained aldehyde dehydrogenase activity were pooled and dialysed overnight against ammonium sulphate (400 g/l). Sufficient ammonium sulphate was added to compensate for the volume of extract.

The precipitate was spun down and redissolved in a minimal amount of 50 mM sodium phosphate buffer, pH 7.4, containing 0.3 mM EDTA and dithiothreitol (30 mg/l)[1]. After dialysis for a few hours against this buffer (to remove most of the ammonium sulphate) the enzyme solution was loaded onto a Sephacryl S-200 column (2 cm x 200 cm) equilibrated with this buffer. Fractions were collected automatically overnight. Those with activity were combined and dialysed overnight against several changes of 10 mM bis-tris buffer, pH 6.5.

The final purification stage involved a pH gradient step to remove any contamination with mitochondrial aldehyde dehydrogenase. The enzyme was loaded onto a DEAE-Sephacel column (2 cm x 14 cm) equilibrated with the above buffer. (Again the conductivity and pH were checked prior to loading.) The gradient was developed by running 400 ml of 10 mM sodium acetate buffer, pH 4.6, into 400 ml of 10 mM bis-tris, pH 6.5. Some mitochondrial aldehyde dehydrogenase eluted approximately half way through the gradient, and the major peak of cytoplasmic aldehyde dehydrogenase activity eluted at the end of

1 - All buffers from this point contained EDTA (0.3 mM) and dithiothreitol (30 mg/l)

the gradient. The fractions which contained most aldehyde dehydrogenase activity were pooled, precipitated, and spun down as described previously. The enzyme was then redissolved and dialysed against 50 mM sodium phosphate, pH 7.4, which had been thoroughly degassed. Enzyme stock was stored in this buffer at -20 ^{o}C at a concentration typically around 20 μM.

2.2.2 Mitochondrial aldehyde dehydrogenase (1): This was prepared essentially by the method of Hart & Dickinson (1977) as modified by Allanson & Dickinson (1984). Approximately 1.5 kg of sheep liver was finely chopped and divided into six portions. Each of these was then homogenised in 800 ml 5 mM sodium phosphate buffer, pH 7.4, containing 0.3 mM EDTA, 0.1 % 2-mercapto-ethanol, and 0.25 M sucrose. The homogenates were pooled and spun at 500 g for 10 minutes in a GS3 head. The supernatant was respun at 20,000 g for 30 minutes in a GSA head to sediment the mitochondria. After washing with 0.25 M sucrose the precipitate was resuspended in 500 ml of 0.1 M sodium citrate buffer pH 5.4 and homogenised again. The mitochondria were then disrupted by sonication using a MSE 100 Watt Ultrasonic Disintegrator. This was carried out in batches using two sonicating periods of 45 s. The pH was readjusted to pH 5.4 with 0.5 M citric acid and after 30 minutes all insoluble material was spun down. Aldehyde dehydrogenase was precipitated by ammonium sulphate (400 g/l) redissolved in 5 mM sodium phosphate buffer, pH 7.4, (with EDTA and 2-mercaptoethanol) and dialysed against at least 4 x 4 litre changes of this buffer overnight.

The subsequent purification procedure for mitochondrial aldehyde dehydrogenase was very similar to that described above for cytoplasmic aldehyde dehydrogenase with the exception of the following: for this preparation the first column step with DEAE-cellulose was omitted, and the dialysed extract loaded directly onto DEAE-Superose CL-6B. The ionic strength gradient consisted of running 400 ml of 70 mM sodium phosphate buffer, pH 7.4, into a constant reservoir containing 200 ml of 5 mM phosphate buffer, pH 7.4, (both containing 0.3 mM EDTA and 0.1 % 2-mercaptoethanol). An additional column step was also included in which the pooled material from the gradient step was dialysed against 5 mM sodium phosphate buffer, pH 6.0, and passed down a CM-cellulose column (1.5 cm x 15 cm) also equilibrated with this buffer. The enzyme eluted immediately after the void volume.

Mitochondrial aldehyde dehydrogenase (2): This was isolated as the contaminant in the pH gradient step from the cytoplasmic preparation. Although the relative activity was small compared to that of the major component and was also distributed over a large number of tubes, the absolute amount of mitochondrial aldehyde dehydrogenase present was significant enough for experiments to be performed. Enzyme collected in this way was completely insensitive to 20 μM disulfiram, as expected for the mitochondrial isozyme (Allanson & Dickinson, 1984).

2.2.3 The standard assay: The dehydrogenase activity of aldehyde dehydrogenase was measured by monitoring the production of NADH at 340 nm. Assays were prepared by the addition of 0.1 ml of a 30 mM NAD^+ stock (which was made up in the assay buffer) to 2.80 ml of 35 mM sodium phosphate buffer, pH 7.4, in a quartz cuvette. To this a small amount of the sample to be tested was added (typically 25 μl) on a glass nail and the solution incubated at 25 ^{o}C. The enzyme-catalysed reaction was initiated by the addition of 0.1 ml of a 30 mM acetaldehyde solution. (This stock solution was prepared by diluting freshly distilled acetaldehyde to the required volume with water.)

Enzyme activity was defined in terms of the concentration of NADH produced per minute in the undiluted sample in μmole l^{-1} min^{-1}. One unit of enzyme activity was that amount needed to catalyse the production of 1 μmole NADH per minute. Protein concentrations were calculated by assuming $^{1\%}Abs_{1cm}$ = 11.3 at 280 nm for cytoplasmic aldehyde dehydrogenase (on the basis of dry weight measurements; Dickinson *et al.*, 1981) and $^{1\%}Abs_{1cm}$ = 9.7 at 280 nm for mitochondrial aldehyde dehydrogenase (Allanson & Dickinson, 1984). Prior to all experiments performed in the present study aldehyde dehydrogenase was dialysed overnight against nitrogen-saturated 50 mM sodium phosphate buffer, pH 7.4, + 0.3 mM EDTA at 4 ^{o}C. This was to prevent possible interference from dithiothreitol.

The molecular weight of aldehyde dehydrogenase was taken to be 212,000 (Kitson, 1979). Protein yields and specific activities were measured and were in accordance with those obtained previously for the cytoplasmic (Dickinson & Berrieman, 1979) and mitochondrial enzyme (Hart & Dickinson, 1977).

2.3 General Procedures

2.3.1 Amino acid analysis: This was performed on a Beckman amino acid analyser 119BL. The sample (approximately 1 mg) was dissolved in approximately 0.5 ml of 6 M HCL containing 0.1 % phenol. The tube was evacuated, sealed, and heated at 100 ^{o}C for 24 hours. The hydrosylate was then reduced to dryness, redissolved in the starting buffer, and loaded.

2.3.2 Peptide sequencing: This was carried out on an Applied Biosystems 470A gas phase protein sequencer. The peptide sample was dissolved in either water or acetic acid and loaded. The corresponding phenylthiohydantoin (PTH) amino acid derivatives from each cycle were analysed on an attached Applied Biosystems 120A PTH analyser.

2.3.3 Radioactivity counting: This was done on a Packard (model 2002) liquid scintillation counter according to the method of Kitson (1978). The scintillation solvent was prepared by dissolving 0.06 g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene (P.O.P.O.P) and 1.75 g 2,5-diphenyloxazole (P.P.O) in a toluene/ethanol mixture (350 ml and 150 ml respectively). For counting, an aqueous aliquot of the sample (typically 0.1 ml) was added to 5 ml of this solvent.

2.3.4 Spectrophotometric instrumentation: Most measurements apart from those in Chapter 7 were performed on an Aminco DW-2a UV-VIS spectrophotometer with a slit width of 3.0 nm. In some instances a Gilford spectrophotometer model 260 was also used. Here, the monochromator slit width was manually adjusted to give a control readout of 0.000. In Chapter 7 measurements were carried out on a Hewlett-Packard HP8452a single beam diode array spectrophotometer. This instrument contains a deuterium lamp which covers the wavelength range 190 nm-820 nm with a spectral bandwidth of 2 nm. The wavelength accuracy and reproducibility are reported as +- 2 nm and < 0.001 AU respectively. Spectral and kinetic information were stored on floppy disk as either wavelength or time based files, and recalled when needed.

2.4 Synthesis

2.4.1 trans-4-N,N-Dimethylaminocinnamoyl imidazole (Weber et al., 1986): *trans*-4-N,N-Dimethylaminocinnamic acid (0.25 g) was dissolved in 25 ml of a dimethylformamide/tetrahydrofuran mixture (1:4 v/v) at 0 ^{o}C. One equivalent of triethylamine (0.18 ml) was added, then one equivalent of the coupling reagent isobutyl chloroformate (0.17 ml, freshly distilled). After approximately 15 minutes the mixture was quickly filtered to remove triethylamine hydrochloride and two equivalents of imidazole were added (0.18 g); the solution was stirred overnight at 4 ^{o}C. After this period the solvent was removed and the solid extracted with toluene at 50 ^{o}C. This was then cooled to 10 ^{o}C and filtered. *trans*-4-N,N-Dimethylaminocinnamoyl imidazole was precipitated with hexane and recrystallised from ethyl acetate and hexane.

trans-4-N,N-Dimethylaminocinnamoyl imidazole (similar to Breaux & Bender, 1976): To 0.02 g of *trans*-4-N,N-dimethylaminocinnamic acid was added approximately one equivalent of dicyclohexylcarbodiimide (both dissolved in acetonitrile). After stirring at room temperature for two hours an equimolar amount of imidazole was then added. The solution was stirred overnight and then filtered to remove dicyclohexylurea. The solvent was removed and the product recrystallised from ethyl acetate and hexane.

In both the above procedures *trans*-4-N,N-dimethylaminocinnamoyl imidazole crystallised to give yellow-orange needles with a melting point of 164-167 ^{o}C (compare 164-167 ^{o}C; Breaux & Bender, 1976). The identity was also confirmed spectroscopically (λ_{MAX} = 424 nm) and by mass spectrometry (found m^{+} = 241.1, predicted 241.3).

2.4.2 N-Acetyl-Ile-Gly-Ser-Pro-Trp-Arg-NH_2: This peptide was prepared by Dr. D. Harding according to an automated procedure similar to that of Merrifield (Stewart & Young, 1984). Each complete cycle consisted of three steps: (1) removal of the *tert*-butyloxycarbonyl (Boc) group to liberate the terminal amine salt; (2) neutralisation with triethylamine; (3) coupling of the next amino acid via dicyclohexylcarbodiimide. The following quantities of amino acids were

used: Boc-Arg^{Tos}, 1.05 g; Boc-Trp, 1.33 g; Boc-Pro, 1.10 g; Boc-Ser^{Bzl}, 1.2 g; Boc-Gly, 0.7 g; Boc-Ile, 0.9 g. The solid support used was benzyhydrylamine (resin) so that subsequent cleavage of the peptide with HF would liberate an amidated terminal arginine. All steps included 10 % anisole and 1.5 % 2-mercaptoethanol.

Purification and desalting of the hexapeptide was performed on a G-15 Sephadex column (4 cm x 55 cm) in 30 % acetic acid. Fractions were collected and subsequently freeze-dried. Results from amino acid analysis revealed the following proportions: Ile, 0.75; Gly, 1.0; Ser, 0.74; Pro, 0.87; Trp, (destroyed); Arg, 0.97. Purification of this peptide by HPLC showed the peak position was identical to that found with the thermolysin redigested fragment of T5 (see Chapter 7).

2.4.3 4-Nitrophenyl [14-C]acetate (Fife, 1965): Acetic anhydride (28.5 mCi/mmole) was equilibrated overnight with 0.82 ml glacial acetic acid. Half of this was used to prepare the acid chloride by refluxing with an equimolar amount of thionyl chloride (freshly distilled) in approximately 2-3 ml of anhydrous ether. This was cooled and then added dropwise to an equimolar solution of 4-nitrophenol and pyridine in excess anhydrous ether. This was stirred for 2-3 hours and left to stand overnight (a calcium chloride drying tube was attached throughout this period). After filtering to remove pyridine hydrochloride, the solvent was evaporated (under nitrogen) and the residual brown oil recrystallised from an ether-hexane mixture. The white product obtained was confirmed to be identical with authentic 4-nitrophenyl acetate on the basis of tlc (the elution solvents used were toluene:ethyl acetate, 8:2 v/v) and also on the rate of appearance of 4-nitrophenoxide from the enzyme-catalysed hydrolysis reaction. 4-Nitrophenyl acetate labelled with ^{14}C in the carbonyl group had a specific activity of 9.39 x 10^{10} dpm/mole.

2.4.4 Mixed disulfides: Methyl 2- and methyl 4-pyridyl disulfide were prepared according to Kitson & Loomes (1985c). One equivalent of either 2- or 4-thio-pyridone was reacted with NaOH in distilled water. Methyl methanethio-sulfonate was added (an equimolar amount) and an immediate white cloudiness

formed which separated as a pale yellow oil. After shaking at room temperature for half an hour the product was extracted into CH_2Cl_2 and dried over $MgCl_2$. The product was purified by chromatography on silica gel using a solvent system of toluene/ethyl acetate (8:2 v/v, Kimura *et al.*, 1982).

Methyl 5-(1-methyltetrazolyl) disulfide and methyl diethylthiocarbamyl disulfide were prepared in essentially the same manner (Kitson, 1986a) except that sodium 1-methyltetrazole-5-thiolate and sodium diethyldithiocarbamate were directly reacted with methyl methanethiosulfonate. Furthermore, it was found that a molar excess of 1-methyltetrazole-5-thiolate was needed (typically 7-fold) to drive the equilibrium predominantly towards product formation.

Methyl diethylthiocarbamyl disulfide was purified on silica gel with a solvent system of hexane/CH_2Cl_2 (6:4 v/v, freshly distilled).

2.5 Results

The results of the column steps for a typical enzyme preparation are shown in Figures 2.1, 2.2, and 2.3. For the mitochondrial enzyme the method detailed in Section 2.2.2 differs slightly from that of Hart & Dickinson (1977). After resuspension in 0.1 M citrate buffer, pH 5.4, the homogenate was blended before sonication. It was found that when this step was included there is a much higher yield in activity.

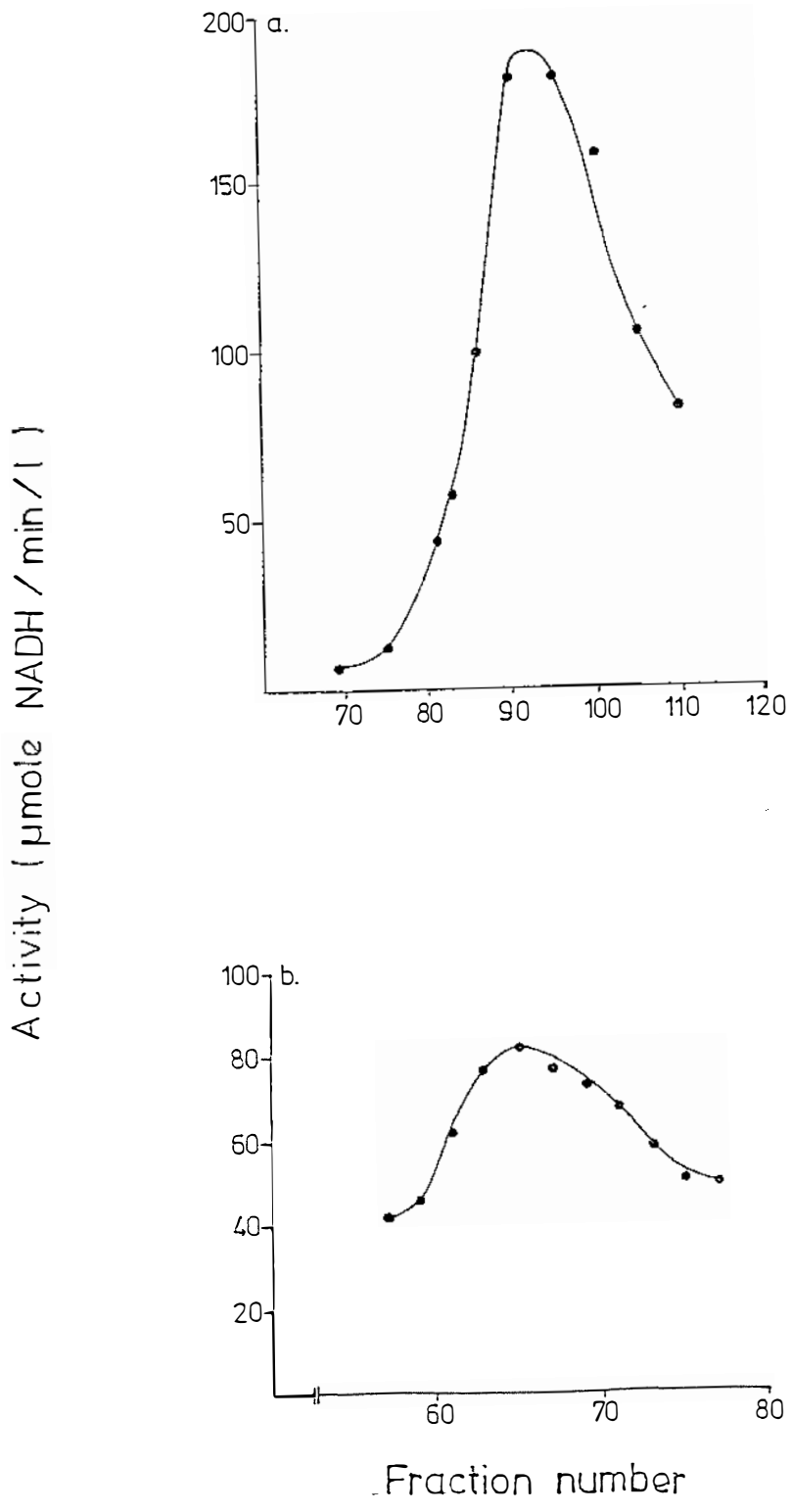
The elution profiles for the ionic strength gradient column step for both cytoplasmic and mitochondrial preparations are shown in Figures 2.1a and 2.1b. The main difference between the two is the smaller total activity of the mitochondrial enzyme. This agrees with the findings of Allanson & Dickinson (1984) who found the cytosol to contain 3-4 times more activity than the mitochondrial compartment. Figure 2.2a shows a typical profile from the Sephacryl column. At this stage in the isolation of the cytoplasmic enzyme, the residual activity of the pooled fractions in the presence of 20 μM disulfiram varies from one preparation to the next, but is usually around 10 %. Most noticeable, however, is the sensitivity of the mitochondrial fractions toward 20 μM disulfiram (Figure 2.2b). This is similar to the findings of Hart &

Dickinson (1977) who found a 50 % reduction in activity with enzyme at this stage of the purification, showing that there is still considerable contamination with the cytoplasmic enzyme.

Finally, Figure 2.3 shows the elution profile from the final pH gradient column for the cytoplasmic preparation. This was first introduced by Dickinson *et al.* (1981) and demonstrates, quite clearly, the effectiveness of this method in removing contaminating mitochondrial aldehyde dehydrogenase. The profile in Figure 2.3 is governed mainly by the non-linear pH gradient. Early in the time course the pH changes very slowly, and the mitochondrial isozyme elutes over a large number of tubes. These fractions are insensitive to 20 μM disulfiram. As the gradient progresses, however, the pH begins to fall more rapidly and the cytoplasmic activity elutes in a relatively sharp peak. Strong inactivation by disulfiram is now observed.

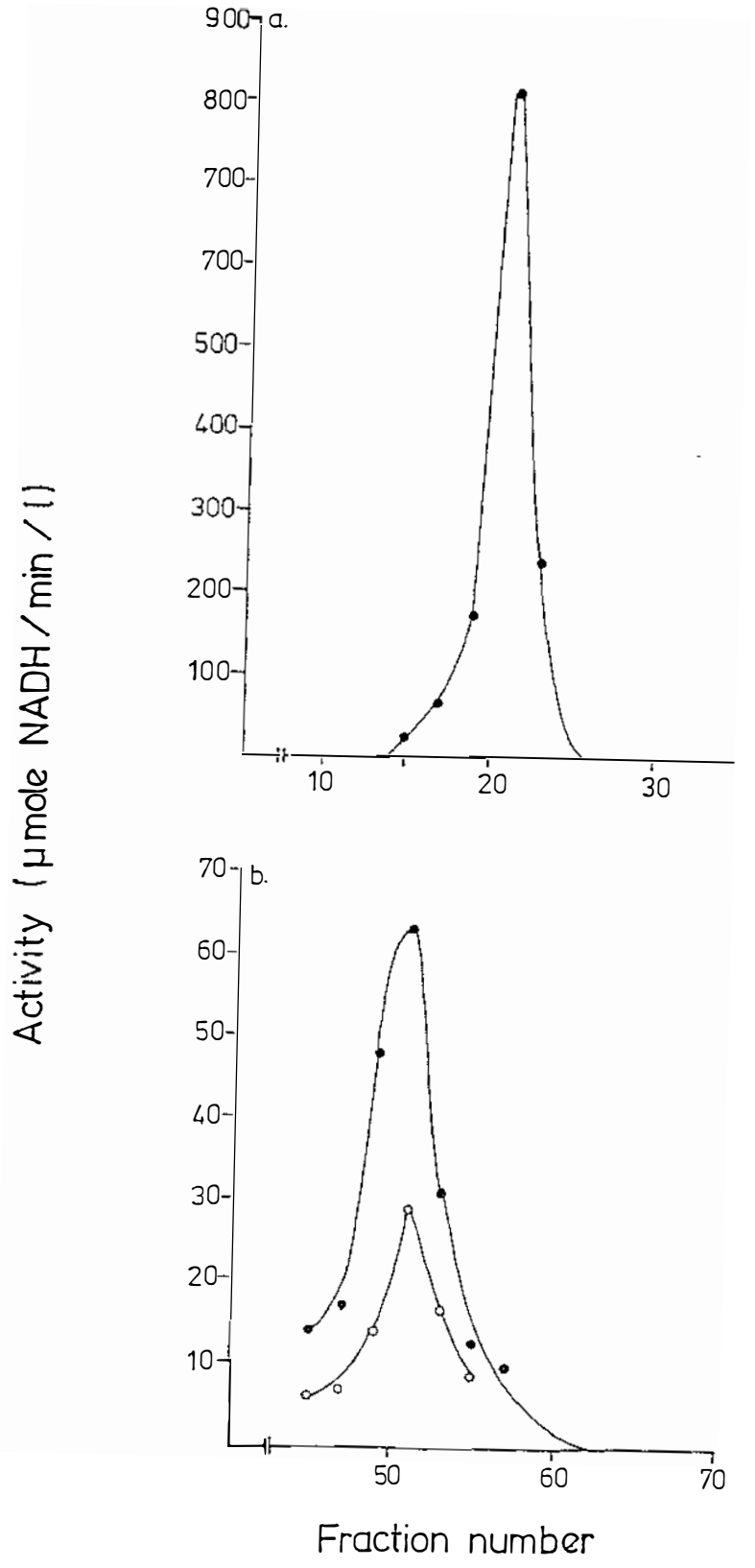
The DEAE-Sephacel pH gradient step was also used to purify the pooled fractions from Figure 2.2b for the mitochondrial preparation. However, it was found that detection of activity in the fractions was more difficult because of the much lower levels of this enzyme. Nevertheless, when those fractions containing activity were pooled and reconstituted in a much smaller volume of buffer, an almost complete insensitivity of the activity toward disulfiram was observed.

Figure 2.1 Elution Profiles for Aldehyde Dehydrogenase from the Ionic Strength Gradient Column



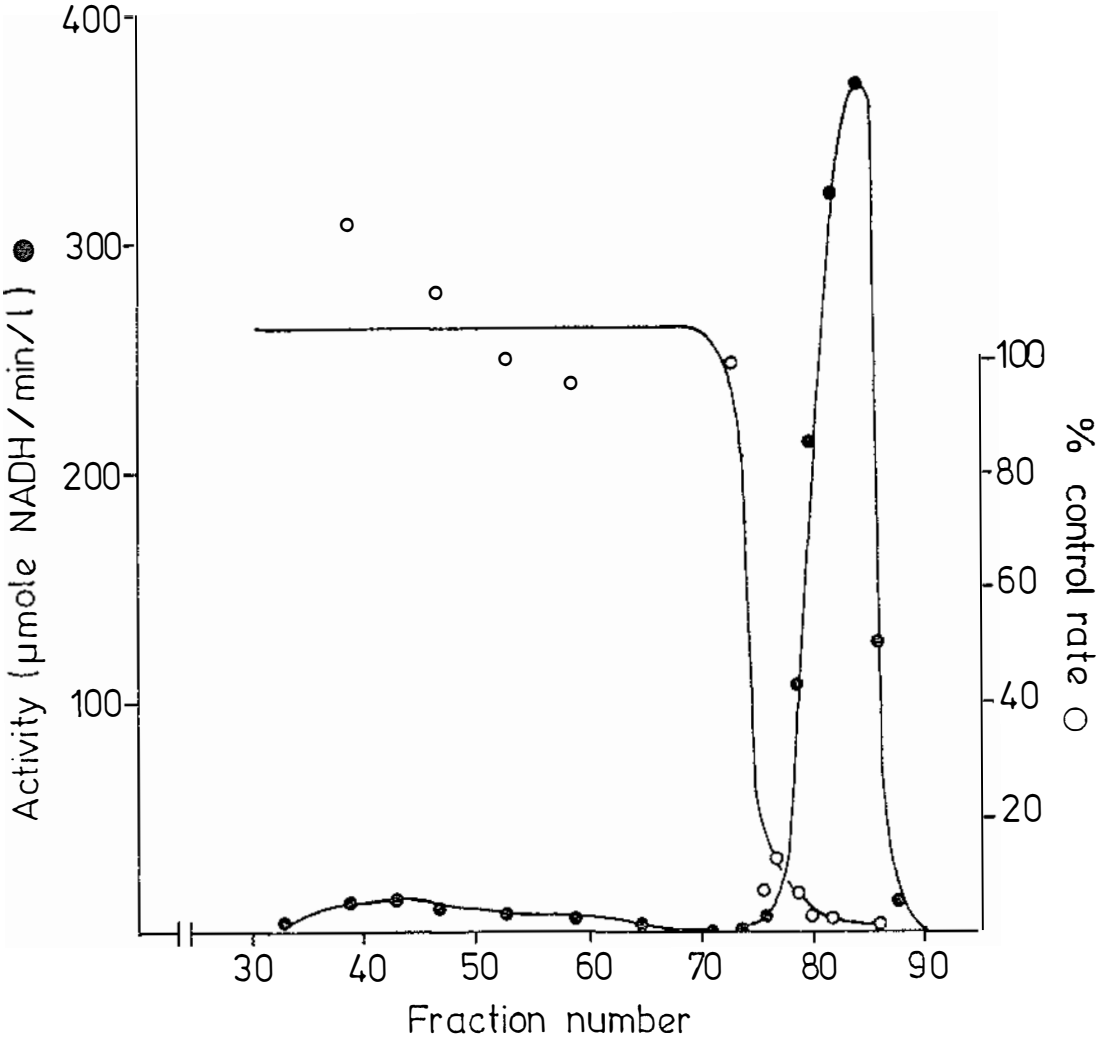
Pooled material from Figures 2.1a,b were loaded onto a Sephacryl S-200 column (2 cm x 200 cm) and eluted with 50 mM sodium phosphate, pH 7.4, + 0.3 mM EDTA and 0.1 % 2-mercaptoethanol. The activity profiles for both the cytoplasmic and mitochondrial isozymes were obtained according to the standard assay (Section 2.2.3) and are shown in Figures 2.2a and 2.2b respectively. For the latter, the dehydrogenase activity was measured in the presence (○) and absence (●) of 20 μM disulfiram.

Figure 2.2 Aldehyde Dehydrogenase Elution Profiles from Gel Filtration



The pooled material from Figure 2.2a was loaded onto a DEAE-Sephacel column (2 cm x 14 cm) equilibrated with 10 mM bis-tris, pH 6.5. The pH gradient involved running 400 ml of 10 mM sodium acetate, pH 4.6, into 400 ml of 10 mM bis-tris, pH 6.5. Buffers contained 0.3 mM EDTA + dithiothreitol (30 mg/l). Activity was measured according to the standard assay (Section 2.2.3) in the absence (●) and presence (○) of 20 μM disulfiram.

Figure 2.3 Separation of Mitochondrial and Cytoplasmic Aldehyde Dehydrogenase on DEAE-Sephacel



2.6 Discussion

Purification of aldehyde dehydrogenase

It is well documented that cytoplasmic aldehyde dehydrogenase is strongly inhibited by disulfiram *in vitro* (Dickinson & Berrieman, 1979) but its mitochondrial counterpart is relatively unaffected (Allanson & Dickinson, 1984). In the early literature, however, the degree of inhibition reported for these two isozymes has been confusing. For example, Kraemer & Deitrich (1968) found that human aldehyde dehydrogenase isolated from the soluble components of liver homogenates was inactivated by approximately 50 % at disulfiram concentrations of 100 μM. Similarly, Hart & Dickinson (1977) reported the same level of inhibition with aldehyde dehydrogenase isolated from sheep liver mitochondria.

Although these studies imply that both cytoplasmic and mitochondrial aldehyde dehydrogenase are equally affected by disulfiram, this conclusion is nevertheless incorrect. Instead, what these findings do demonstrate are the misleading conclusions which can be made from impure enzyme preparations. The preparations in both the studies described above contained similar proportions of cytoplasmic and mitochondrial aldehyde dehydrogenase. The 50 % reduction in activity in the presence of disulfiram merely reflects this inhomogeneity.

In the late 70's very pure enzyme preparations were obtained with the development of several new purification techniques. Kitson (1982c) found that cytoplasmic aldehyde dehydrogenase could be separated from mitochondrial aldehyde dehydrogenase by covalent chromotography on reduced thiopropyl-Sepharose 6B. This column step exploits the known susceptibility of the cytoplasmic isozyme to disulfiram and involves premixing this modifier with the enzyme before elution. When loaded, the thiolate groups on the resin displace the diethyldithiocarbamyl label, and in doing so covalently bind cytoplasmic aldehyde dehydrogenase. As mitochondrial aldehyde dehydrogenase is relatively unreactive with disulfiram, this enzyme simply elutes after the void volume. Bound material can then be liberated by adding 2-mercaptoethanol. Hempel *et al.* (1982b) also showed that the human liver enzymes E1 and E2 can be simultaneously purified and separated from each other on 5'-AMP Superose.

In the present study the methods of Dickinson *et al.* (1981) and Allanson & Dickinson (1984) are adopted; these both separate cytoplasmic and mitochondrial aldehyde dehydrogenase on the basis of their differing isoelectric points (5.2 and 5.6 respectively; Dickinson *et al.*, 1981). It is interesting to speculate on the relatively large amount of contamination in the mitochondrial fractions from the Sephacryl column (Figure 2.2b). Allanson & Dickinson (1984) found the cytoplasm to contain significantly more activity than the mitochondrial compartment. A small percentage of cross compartmental contamination from the cytoplasm in the early stages of isolation could, therefore, constitute a major contribution in later steps. In addition to this, ammonium sulphate precipitation procedures may selectively favour the increase in cytoplasmic contamination, because this isozyme is preferentially precipitated (Allanson & Dickinson, 1984).

Another alternative is that contamination is inherent within the subcellular compartment itself. It could be that the disulfiram sensitive component originates from the outer-mitochondrial membrane, and tenaciously follows the inner matrix aldehyde dehydrogenase throughout the separation procedures. For instance, this has been found to happen with mitochondrial aldehyde dehydrogenase isolated from rat liver. Horton & Barrett (1975) reported non-linear Lineweaver-Burk plots when the concentration of aldehyde was varied. Subsequent analysis found two mitochondrial-derived species ALDH-1 and ALDH-2 (Section 1.3) which had Michaelis constants of < 5 μM and 1.5 mM respectively.

If this explanation is accepted for the results in Figure 2.2b then it is also likely that this contaminant differs from ordinary low K_M cytoplasmic aldehyde dehydrogenase. Although both are inhibited by disulfiram, the former may resemble high K_M microsomal aldehyde dehydrogenase (Tottmar *et al.*, 1973) or E4 which is found in the cytoplasmic fraction of human liver (Forte-McRobbie & Pietruszko, 1985). The concentrations of acetaldehyde used to assay the mitochondrial fractions in the present study (1 mM) would certainly be high enough to detect the presence of such a species.

Finally, it is of interest to note the similar amounts of mitochondrial aldehyde dehydrogenase obtained from both purification methods (Section 2.2.2). This

may seem a little surprising considering the expectation that a procedure designed to isolate this isozyme should yield more activity than the contaminant pooled from a cytoplasmic preparation. However, the similarity may reflect the fragility of the mitochondria during the early stages of isolation. Release of mitochondrial aldehyde dehydrogenase during this period would account for the reduced yield in the mitochondrial fractions, and the consequent gain in the cytosol.

CHAPTER 3

MODIFICATION OF MITOCHONDRIAL ALDEHYDE DEHYDROGENASE

3.1 Introduction

The sensitivity of cytoplasmic aldehyde dehydrogenase towards disulfiram is well documented (Kitson, 1978, 1982a, 1983). When modified by this reagent *in vitro* the dehydrogenase activity of this enzyme is almost totally inhibited. (In very pure preparations the residual activity is around 2 %; Dickinson *et al.*, 1981.). The mixing order also has no effect; disulfiram is an equally effective inhibitor when it is premixed with enzyme either in the absence or presence of cofactor. When added to an ongoing assay immediate inactivation occurs within the time of mixing (except at very high aldehyde concentration; Kitson, 1985).

The effects of a wide variety of other thiol modifiers on cytoplasmic aldehyde dehydrogenase are also well researched. For instance, methyl diethylthiocarbamyl disulfide, 5,5'-dithiobis(1-methyltetrazole), and methyl 5-(1-methyltetrazoyl) disulfide all have some structural similarity to disulfiram. All are potent inhibitors of activity (Kitson & Loomes, 1985a; Kitson, 1986a).

In contrast to the above modifiers, others exist which activate the dehydrogenase activity of cytoplasmic aldehyde dehydrogenase. Both 2,2'-dithiodipyridine and 4,4'-dithiodipyridine initially react with aldehyde dehydrogenase in the presence of NAD^+ to form an activated enzyme derivative. In a subsquent slower process, however, the thiopyridyl label is displaced by a closely positioned enzymic thiol to liberate the corresponding thiopyridone chromophore, and to produce an enzyme disulfide. This latter species is inactive (Kitson & Loomes, 1985a,b).

Mitochondrial aldehyde dehydrogenase is also affected by certain thiol modifiers, but in some instances the resulting activity differs markedly from that of its cytoplasmic counterpart. Thus while this enzyme is rapidly inhibited by methyl diethylthiocarbamyl disulfide (as is the case with the cytoplasmic enzyme) modification by disulfiram is very slow (unlike the cytoplasmic isozyme; Allanson & Dickinson, 1984).

In this Chapter the behaviour of mitochondrial aldehyde dehydrogenase toward the above modifiers is examined, and compared to that found for the cytoplasmic enzyme. Any differences have the potential to provide insight into the possible catalytic role of the disulfiram-sensitive groups. In addition to this, particular interest is focussed on the effects of 5,5'-dithiobis(1-methyltetrazole). This reagent has been implicated as the metabolite responsible for the DER-like reaction caused by certain cephalosporin antibiotics which contain a 1-methyl-tetrazole-5-thiol sidechain (Kitson, 1987b).

3.2 Experimental

3.2.1 Modification experiments: To investigate the effects of various modifiers on the dehydrogenase activity of mitochondrial aldehyde dehydrogenase, assays were performed as follows (the values in brackets being the final concentrations in the assay).

To enzyme (0.083 μM) in 35 mM sodium phosphate buffer, pH 7.4, at 25 ^{o}C containing NAD^{+} (1 mM) 15 μl of the appropriate modifier solution in ethanol was added. After approximately 10 minutes acetaldehyde was added (1 mM) and the enzyme-catalysed reaction monitored by following the production of NADH at 340 nm. The total volume of the assay mixture in all experiments was 3 ml. In some instances a different mixing order was carried out. Here, the modifier was premixed with native enzyme in the absence of NAD^{+} and incubated for the same period as above. Substrates were then added as before. The activities in these modified assays were expressed as a percentage of the control rate.

3.2.2 Reaction of enzyme with 4,4'-dithiodipyridine and 2,2'-dithiodipyridine: Enzyme (2 μM) and NAD^+ (1 mM) were mixed at 25 °C in 3 ml of 35 mM sodium phosphate buffer, pH 7.4. After a baseline was run against a control, 15 μl of a 2,2'-dithiodipyridine solution in ethanol was added on a glass nail as quickly as possible (8.2 μM). The subsequent production of 2-thiopyridone was then followed spectrophotometrically at 342 nm. The procedure for 4,4'-dithiodipyridine was identical to the above except that the enzyme and modifier concentrations were 0.8 μM and 3.4 μM respectively, and absorbance was monitored at 324 nm.

The concentrations of 2,2'-dithiodipyridine and 4,4'-dithiodipyridine were double checked prior to each experiment by measuring the absorbance change when aliquots were added to a solution containing excess dithiothreitol.

3.2.3 Release of 2-thiopyridone and 4-thiopyridone from labelled enzyme: This was carried out in a similar manner to Kitson & Loomes (1985a). Duplicate enzyme solutions (14 μM) in 0.7 ml of 50 mM-sodium phosphate, pH 7.4, containing NAD^+ (1.6 mM) were incubated at 25 °C. After a few minutes a 9-fold excess of 4,4'-dithiodipyridine was added (15 μl of ethanol solution on a glass nail) and the mixtures incubated for a further 5-10 minutes. The reaction mixtures were then passed down separate Bio-Gel P-6 columns (11 cm x 0.8 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.4, at room temperature. Elution was also with this buffer, and took only a few minutes. A volume of 1 ml was collected immediately after the void volume. Prior to use the Bio-Gel P-6 columns were visually inspected for their ability to separate large and small molecules by using blue dextran and 4-nitrophenol. After elution, the enzyme (which was now labelled with a 4-thiopyridyl sidechain) was heated at 25 °C, and at specified time periods the absorbance due to 4-thiopyridone at 324 nm was measured. A control was used as a reference cell (this was treated identically with the reaction mixtures throughout the above procedures but had no 4,4'-dithiodipyridine added to it initially). In addition to this the activities of both reaction mixtures were measured by the standard assay (see Section 2.2.3) and compared to the control rate.

The above procedure was also performed with 2,2'-dithiodipyridine except that the starting enzyme and modifier concentrations were 19 μM and 140 μM

respectively, and the subsequent release of 2-thiopyridone was followed at 342 nm.

3.3 Results

3.3.1 The effect of modifiers on mitochondrial aldehyde dehydrogenase

Figure 3.1 shows the inhibitory effect of methyl 5-(1-methyltetrazolyl) disulfide on the dehydrogenase activity of mitochondrial aldehyde dehydrogenase. The major feature to note is the marked decline in activity as the modifier/tetramer ratio is increased. Also, it makes little difference whether this modifier is premixed with enzyme either in the presence or absence of NAD^+; the same extent of inactivation is observed. Furthermore, a small amount of residual activity is present (2 %) even with high concentrations of methyl 5-(1-methyl-tetrazolyl) disulfide.

Also included in Figure 3.1 are experimental points obtained with methyl diethylthiocarbamyl disulfide. This modifier has been shown previously to inhibit mitochondrial aldehyde dehydrogenase (MacKerell *et al.*, 1985) and as shown here the extent of inactivation is similar to methyl 5-(1-methyltetrazolyl) disulfide. This is perhaps not surprising considering they are structurally similar to one another, and both attach a methylthio sidechain to the enzyme.

There is one difference worth noting between the results presented here and those obtained under identical conditions for the cytoplasmic enzyme. In Figure 3.1 a 2-fold excess of both methyl diethylthiocarbamyl disulfide and methyl 5-(1-methyltetrazolyl) disulfide over the enzyme tetramer concentration reduce the activity by 40 %; and it is not until a 10-fold excess that activity falls to its lowest level. In contast, a 2-fold excess of either of these reagents would be sufficient almost completely to inactivate the cytoplasmic enzyme.

The effects on mitochondrial aldehyde dehydrogenase activity of other modifiers are shown in Figure 3.2. Perhaps the most striking observation is the strong activation by 5,5'-dithiobis(1-methyltetrazole). This totally contrasts with its behaviour towards the cytoplasmic enzyme (Kitson, 1986a) and with the action

of its mixed disulfide methyl 5-(1-methyltetrazolyl) disulfide (Figure 3.1). The order of mixing, however, does seem to have some effect on the extent of activation. When premixed with enzyme in the absence of NAD^+, 5,5'-dithiobis(1-methyltetrazole) is a poorer activator than when this cofactor is present. Also interesting is that enzyme activated by 5,5'-dithiobis(1-methyltetrazole) is not at all protected against subsequent inhibition from methyl 5-(1-methyltetrazolyl) disulfide. The extent of inhibition in this case (3 %) is similar to that if methyl 5-(1-methyltetrazolyl) disulfide is premixed with native enzyme (Figure 3.1).

The other major result in Figure 3.2 is the total absence of either activation or inhibition of mitochondrial aldehyde dehydrogenase activity by 2,2'-dithiodipyridine. Like 5,5'-dithiobis(1-methyltetrazole) this again is contrary to its activating effect on the cytoplasmic enzyme (Kitson, 1982b). The lack of effect of 2,2'-dithiodipyridine cannot simply mean that the reagent does not react with the enzyme, as is demonstrated by the results in Figure 3.3b. Here, a 4-fold excess of this modifier all reacts within the space of 10 minutes. In contrast to 2,2'-dithiodipyridine, the effect of 4,4'-dithiodipyridine is more typical of that observed with cytoplasmic aldehyde dehydrogenase (Figure 3.2). This modifier, like 2,2'-dithiodipyridine, also reacts with the mitochondrial enzyme (Figure 3.3a) but unlike 2,2'-dithiodipyridine, activates approximately 3-fold.

One final point that should be mentioned here is the nature of the absorbance traces obtained in assays of mitochondrial aldehyde dehydrogenase. Allanson & Dickinson (1984) found such assays to exhibit early hysteresis characteristics before a final steady state rate is reached. These authors presented evidence to suggest that this is due to dissociation of the enzyme tetramer into dimers which are inherently more reactive; i.e. a doubling of the effective enzyme concentration. Similar hysteresis behaviour was also observed in this study. The activities of all assays were obtained, therefore, after 5-10 minutes after the initiation of the enzyme-catalysed reaction. Traces were usually linear by this stage.

3.3.2 Displacement of 4-thiopyridone and 2-thiopyridone

It has been reported that 4,4'-dithiodipyridine and 2,2'-dithiodipyridine react with cytoplasmic aldehyde dehydrogenase in a biphasic process (Kitson & Loomes, 1985a,b). Thus initial modification in the presence of NAD^+ results in a labelled thiopyridyl enzyme derivative which is activated. However, when such a reaction mixture is subsequently passed down Bio-Gel (this removes small molecules present in the assay) a time dependent release of 2-thiopyridone and 4-thiopyridone occurs. It is of interest, therefore, to see if this behaviour is observed with the mitochondrial isozyme. Figure 3.4 shows that this is indeed the case for 4,4'-dithiodipyridine. As the 4-thiopyridone label is displaced from the enzyme a concerted loss in activity occurs, and after 27 hours the rate is only 40 % of the control. In contrast, when 2-thiopyridone is displaced from this enzyme (Figure 3.5) no decline in activity is seen at all. Interestingly, these assays are still strongly activated by 4,4'-dithiodipyridine.

Figure 3.1 - Methyl 5-(1-methyltetrazolyl) disulfide (●) and methyl diethylthiocarbamyl disulfide (□) were added to enzyme (0.083 μM) in the presence of NAD^+ (1 mM) at 25 °C. The reaction was then initiated by the addition of acetaldehyde (1 mM). An alternative mixing order was also carried out where methyl 5-(1-methyltetrazolyl) disulfide was premixed with enzyme in the absence of NAD^+ (○).

Figure 3.1 Inhibitory Effect of Various Disulfiram-Like Analogues on Mitochondrial Aldehyde Dehydrogenase

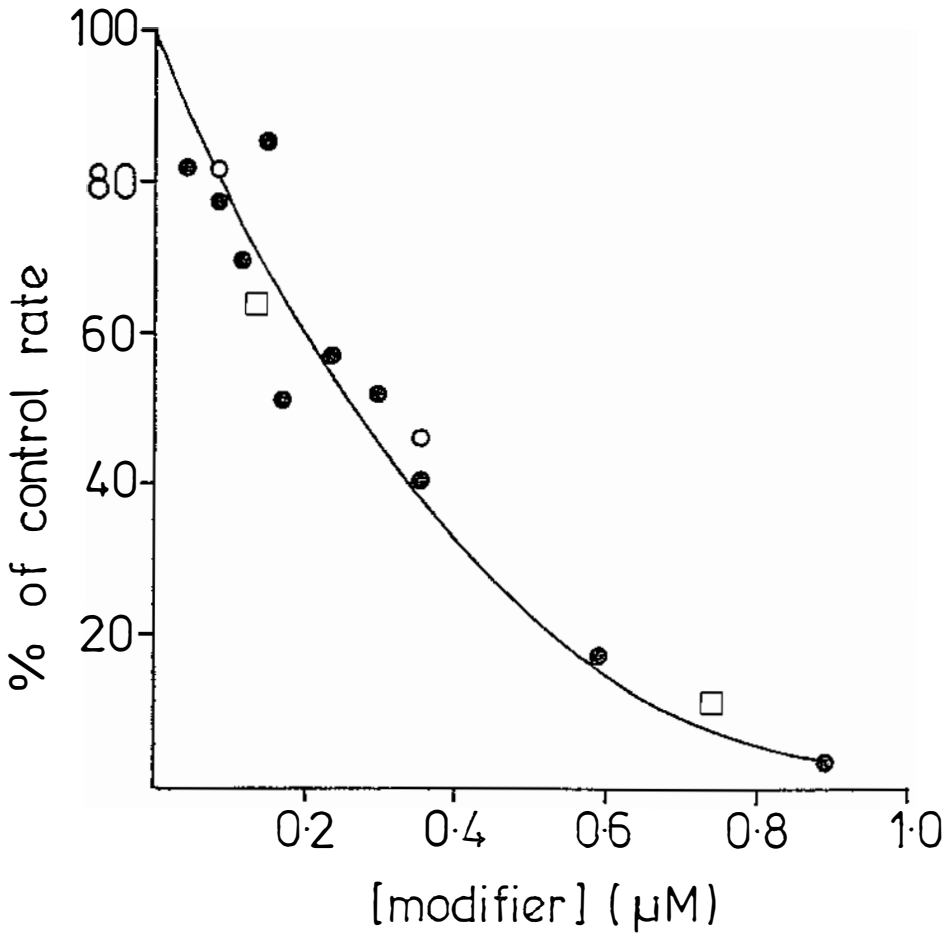
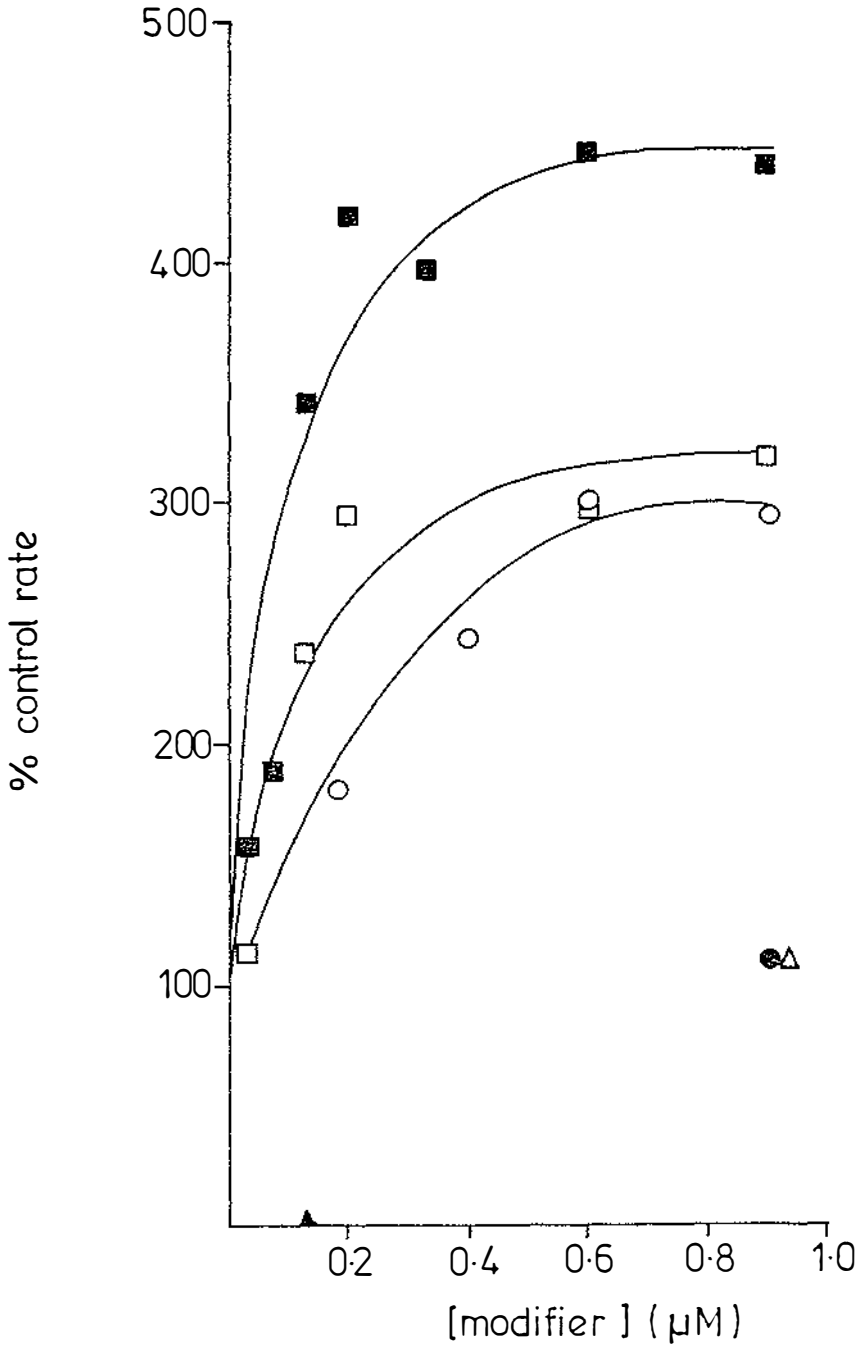


Figure 3.2 - Enzyme (0.083 μM) in the presence of NAD^+ (1 mM) was mixed with either 5,5'-dithiobis(1-methyltetrazole) (■), 4,4'-dithiodipyridine (○), 2,2'-dithiodipyridine (●), and disulfiram (Δ). Alternatively, 5,5'-dithiobis(1-methyltetrazole) was premixed with native enzyme and NAD^+ added later (□). In one experiment, methyl 5-(1-methyltetrazolyl) disulfide (0.9 μM) was added to an ongoing assay previously activated by its symmetrical disulfide (▲).

Figure 3.2 Effect of Various Thiol Modifiers on the Dehydrogenase Activity of Mitochondrial Aldehyde Dehydrogenase



4,4'-Dithiodipyridine (3.4 μM) was added to enzyme (0.80 μM) in the presence of NAD^+ (1 mM) at 25 ^oC. The production of 4-thiopyridone was monitored at 324 nm (Figure 3.3a). The same experiment was performed with 2,2'-dithiodipyridine (Figure 3.3b) except the concentrations of enzyme and modifier were 2 μM and 8.2 μM respectively. The production of 2-thiopyridone was monitored at 342 nm.

Figure 3.3 Reaction of 4,4'-Dithiodipyridine and 2,2'-Dithiodipyridine with Mitochondrial Aldehyde Dehydrogenase

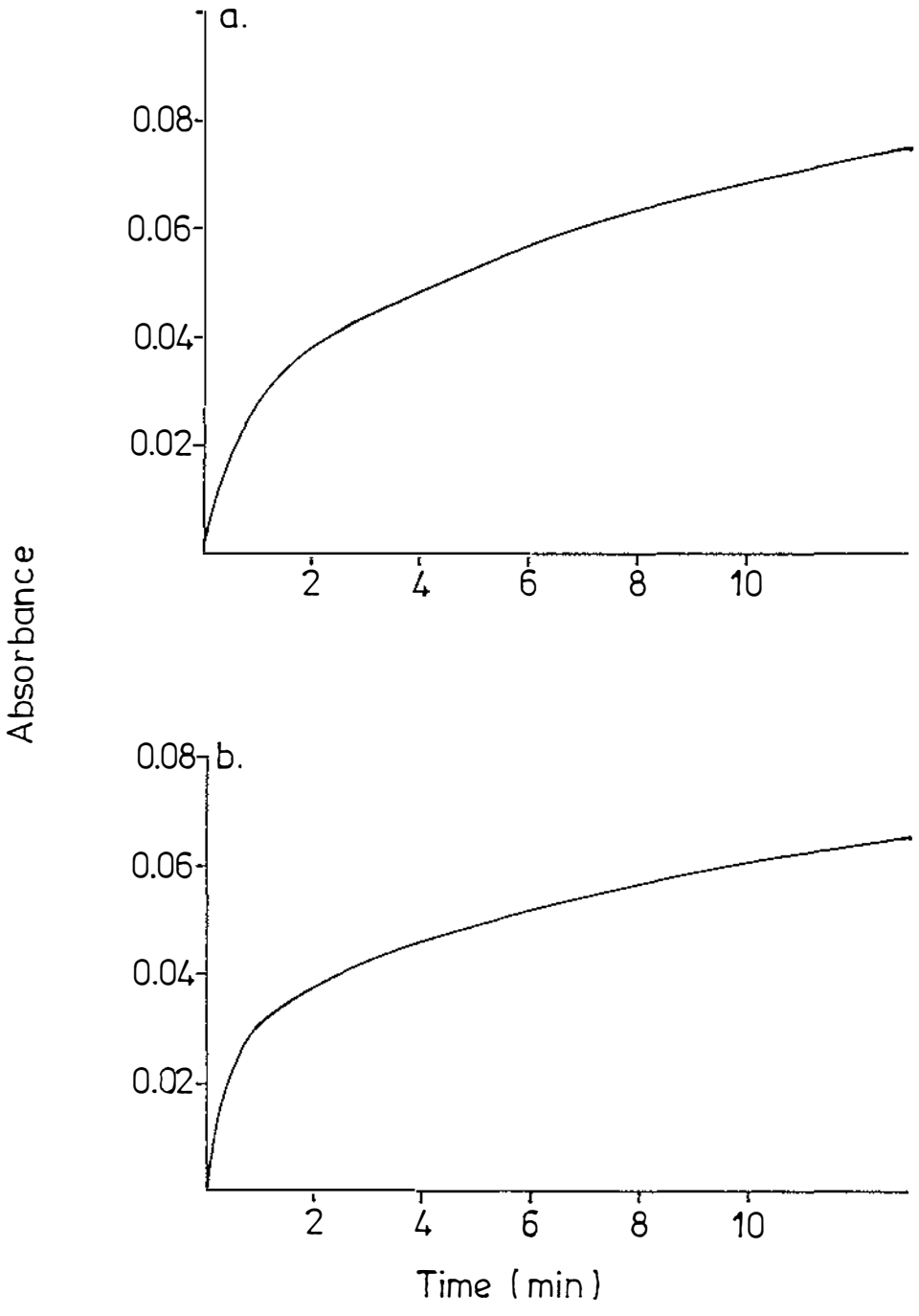


Figure 3.4 - Duplicate reaction mixtures containing 4,4'-dithiodipyridine (126 μM), enzyme (14 μM), and NAD^+ (1.6 mM) were passed down Bio-Gel P-6 columns equilibrated with 50 mM sodium phosphate, pH 7.4, at room temperature. At specified times the absorbance of each mixture was measured at 324 nm (●,○). Aliquots were also taken and assayed for dehydrogenase activity (■,□).

Figure 3.4 Displacement of 4-Thiopyridone from the Mitochondrial Enzyme

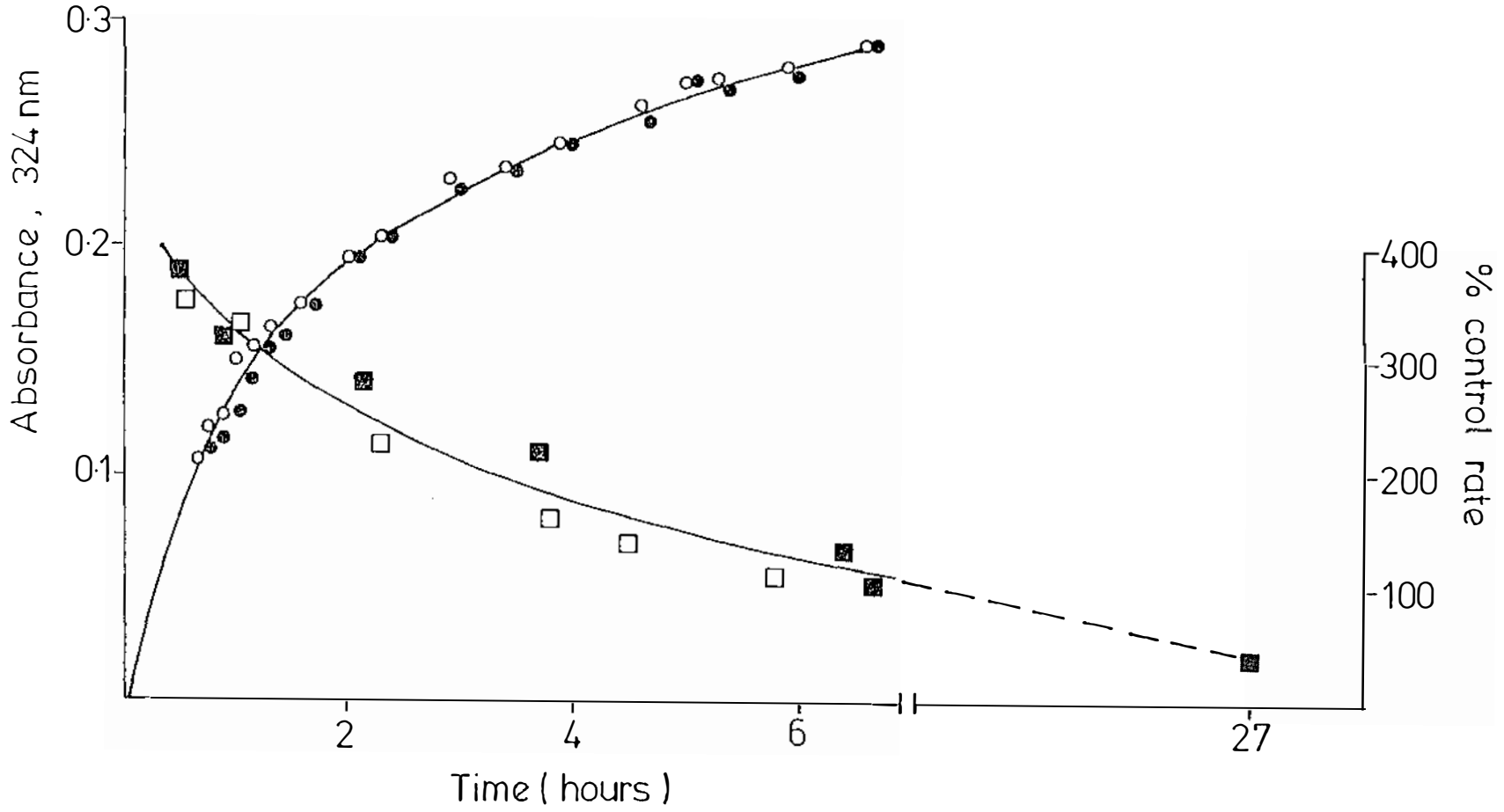
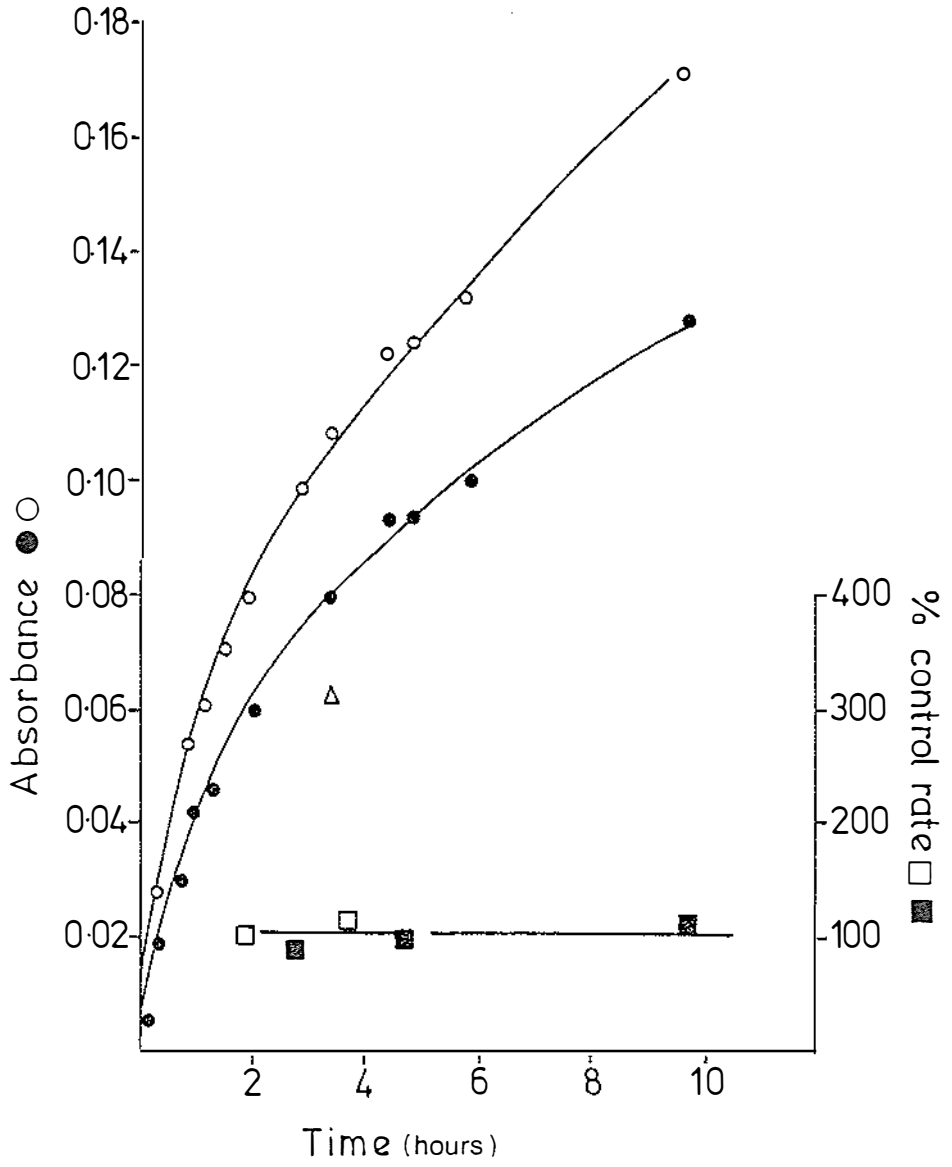


Figure 3.5 - Duplicate reaction mixtures containing enzyme (19 μM), NAD^+ (1 mM), and 2,2'-dithiodipyridine (140 μM) were passed down Bio-Gel P-6 columns equilibrated with 50 mM sodium phosphate, pH 7.4, at room temperature. The release of 2-thiopyridone from the labelled enzyme in each reaction mixture was monitored at 342 nm (●,○). Aliquots were also taken for dehydrogenase activity (□,■). In one case, 4,4'-dithiodipyridine (a 9-fold molar excess over the enzyme tetramer concentration) was added to an ongoing assay (Δ).

Figure 3.5 Displacement of 2-Thiopyridone from the Mitochondrial Enzyme



3.4 Discussion

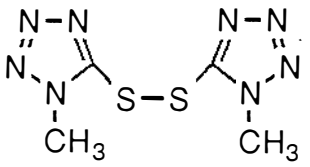
3.4.1 The nature of the disulfiram-sensitive groups

It has been commonly assumed in the literature that the active site nucleophile in the dehydrogenase reaction is a cysteine (Tu & Weiner, 1988a; Weiner *et al.*, 1985). Although as yet unproven, the prime candidate for this role has traditionally been thought to be the thiol modified by disulfiram (Weiner, 1979). This assumption is based on the known catalytic mechanism of glyceraldehyde-3-phosphate dehydrogenase which contains an active site cysteine (Fersht, 1977) and the susceptibility of the cytoplasmic enzyme to disulfiram (Agnew *et al.*, 1981).

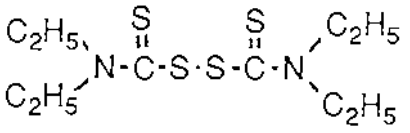
The concept of a disulfiram-sensitive catalytic thiol, however, is questionable in light of the findings with mitochondrial aldehyde dehydrogenase, namely: disulfiram has only a minimal effect on activity *in vitro*; and 5,5'-dithiobis(1-methyltetrazole) activates (Figure 3.2). This latter modifier is somewhat similar in structure to disulfiram (see later) and is a potent inhibitor of cytoplasmic aldehyde dehydrogenase activity (Kitson, 1986a).

There are two possible explanations for the general behaviour of disulfiram and 5,5'-dithiobis(1-methyltetrazole). Firstly, both react with the same thiol in both proteins. In this case the group cannot be catalytically essential in the manner proposed, for if it were, modification by these reagents would always result in complete inhibition. Secondly, disulfiram and 5,5'-dithiobis(1-methyltetrazole) react differentially with aldehyde dehydrogenase. With the cytoplasmic enzyme both react with the same thiol (which may be catalytic) while for mitochondrial aldehyde dehydrogenase they react elsewhere. For 5,5'-dithiobis(1-methyl-tetrazole) this results in activation.

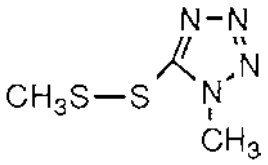
To distinguish these possibilities consider the following argument. Methyl diethylthiocarbamyl disulfide, 5,5'-dithiobis(1-methyltetrazole), and methyl 5-(1-methyltetrazolyl) disulfide can all be considered to be structural analogues of disulfiram (below)



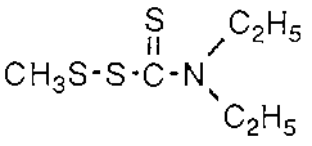
5,5'-dithiobis(1-methyltetrazole)



disulfiram



methyl 5-(1-methyltetrazolyl) disulfide



methyl diethylthiocarbamyl disulfide

The key evidence to note is that although all these modifiers potently inactivate cytoplasmic aldehyde dehydrogenase, only the mixed disulfides effectively inhibit the mitochondrial enzyme (Figure 3.1). As mentioned previously, disulfiram has only a slow inactivating effect on activity while 5,5'-dithiobis(1-methyltetrazole) is a good activator (Figure 3.2). The simplest explanation for these findings is that the mixed disulfides modify the same enzyme groups in both isozymes. On the other hand, because the symmetrical disulfides are bigger, access to these groups is hindered for mitochondrial aldehyde dehydrogenase. Consequently, there is greater scope for reaction elsewhere. For 5,5'-dithiobis(1-methyltetrazole) this scenario is confirmed by findings which show that the activated assays are still inactivated by its mixed disulfide, methyl 5-(1-methyltetrazolyl) disulfide (Figure 3.2).

Recently, several reports have emerged in the literature which question the presumed catalytic role of the disulfiram-sensitive thiol in mitochondrial aldehyde dehydrogenase. Sanny & Weiner (1987) argue along these lines on the basis that modification with disulfiram leaves the dehydrogenase active site concentration relatively unaffected during the 75 % activity loss in the steady state. Only with an excess of disulfiram is the size of the NADH burst

amplitude[1] reduced. However, these findings conflict with those of Kitson (1982a, 1987) who found both to be reduced simultaneously for sheep liver cytoplasmic aldehyde dehydrogenase.

This disparity is easily accommodated in light of the foregoing discussion if it is accepted that disulfiram reacts with the catalytic thiol in the cytoplasmic enzyme, but with a different group in mitochondrial aldehyde dehydrogenase. According to our argument the rationale for this is as follows.

It is well known that the rate of reaction between disulfiram and mitochondrial aldehyde dehydrogenase is very slow. Indeed, MacKerell *et al.* (1985) found that the second order rate constant for disulfiram is approximately 20,000 times slower than its mixed disulfide analogue methyl diethylthiocarbamyl disulfide. Consequently, Sanny & Weiner (1987) had to use a relatively high concentration of disulfiram to effect a reaction in a convenient time frame (up to 100 μM). Because the rate of reaction with disulfiram at its usual site (which may be catalytic) is so slow, other normally unreactive thiols can effectively compete. Consequently, disulfiram reacts with these other non-essential groups, and in doing so indirectly lowers the activity. When these have all been modified disulfiram has no choice but to modify its normal target thiol; the burst size then correspondingly drops. For the cytoplasmic enzyme this does not occur as the rate of reaction between disulfiram and its usual site is extremely fast (within the time of mixing; Kitson, 1982a). Consequently, side reactions are virtually non-existent and a strong correlation between the reduction of both the burst size and steady state rate is observed (Kitson, 1987).

There are several interesting pieces of evidence which support the above argument. Firstly, it has been shown for the cytoplasmic enzyme that saturating concentrations of 4-nitrophenyl acetate partially protect the enzyme against inhibition from disulfiram. The reason for this lies in the fact that 4-nitrophenyl acetate is an actual esterase substrate for this enzyme. The most obvious explanation is that because acyl hydrolysis is rate-limiting, the intermediate E^{acetyl} occurs predominantly in the steady state. As the active site is now

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1 - the burst in NADH is proportional to the active site concentration.

occupied throughout this period, disulfiram cannot react at its usual position, and instead reacts elsewhere without seriously affecting activity (Kitson, 1982a).

The link between this and the argument put forward for the mitochondrial enzyme is that the E^{acetyl} intermediate can be compared with the mitochondrial enzyme with regard to the reaction with disulfiram. The nett effect in both is the same: access of this modifier to its usual site is hindered[2]. Therefore, if (as has been shown) disulfiram can react elsewhere with cytoplasmic aldehyde dehydrogenase then it should also be possible for the mitochondrial isozyme as well.

Secondly, Sanny & Weiner (1987) found that the attached disulfiram label is quite stable. This contrasts with the cytoplasmic enzyme in which the attached label is displaced quite rapidly by an adjacent enzymic thiol (Vallari & Pietruszko, 1982). More intensive studies have shown that disulfiram specifically reacts with one of a closely positioned pair of thiols, called "A". When labelled, the diethyldithiocarbamyl moiety itself comes under attack from the other thiol, "B", and is subsequently displaced leaving behind an inactive enzyme disulfide (Kitson & Loomes, 1985a,b). The modifier 4,4'-dithiodipyridine also reacts with cytoplasmic aldehyde dehydrogenase but this time reacts with thiol B. Instead of inhibition, an activated enzyme intermediate is formed (Kitson, 1982b). Nevertheless, the eventual result is the same as disulfiram because thiol A now liberates 4-thiopyridone to again give the inactive enzyme disulfide (Kitson & Loomes, 1985a,b).

In the present study the behaviour of 4,4'-dithiodipyridine toward mitochondrial aldehyde dehydrogenase is very similar to that observed with the cytoplasmic enzyme (Figure 3.4). From this it can be inferred that 4,4'-dithiodipyridine is still reacting with thiol B, and that thiol A still has the correct stereochemistry to displace the 4-thiopyridyl label. On these grounds, if disulfiram is modifying thiol A (as it does with the cytoplasmic enzyme) then there is no reason why it could not be subsequently displaced by thiol B. That the disulfiram label is

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2 - this rests on the assumption that the dehydrogenase and esterase active sites are the same. This area is discussed in more detail in Chapters 4 and 5.

stable (Sanny & Weiner, 1987) suggests that disulfiram does not initially react with thiol A.

Finally, it is interesting to speculate on the outcome if methyl diethylthiocarbamyl disulfide was used to modify the mitochondrial enzyme instead of disulfiram in the experiments of Sanny & Weiner (1987). The reagent reacts much faster because, presumably, it is less sterically hindered than disulfiram. Although it is still possible that methyl diethylthiocarbamyl disulfide undergoes these "side reactions", they may be negligible compared to the rate of reaction at thiol A. Consequently, a much better correlation between the incorporation of this reagent and the reduction in the amplitude of the burst may be observed.

To summarise, according to the foregoing discussion the residue initially modified by disulfiram in the mitochondrial enzyme may not be thiol A. For this reason we are still left with the possibility that thiol A is essential for activity. One finding traditionally used to argue against this, however, is the small residual activity observed with cytoplasmic aldehyde dehydrogenase in the presence of excess disulfiram (Dickinson *et al.*, 1981). Obviously, if this modifier were reacting exclusively with an essential group the limiting activity should be zero. To preserve the concept that A is essential for activity consider the following.

2,2'-Dithiodipyridine is known not only to activate the cytoplasmic enzyme 2-3 fold, but it also protects the enzyme against subsequent inhibition from disulfiram (Kitson, 1979). From modifier analogue experiments it has been established that disulfiram and 2,2'-dithiodipyridine initially react with different groups: thiols A and B respectively (Kitson & Loomes, 1985a). Consequently, any protection afforded by 2,2'-dithiodipyridine must be mediated through indirect mechanisms which hinder the access of disulfiram to A - just as it is with mitochondrial aldehyde dehydrogenase.

The residual activity with disulfiram can be explained by analogy with 2,2'-dithiodipyridine. It is possible that disulfiram is not totally specific for thiol A but reacts to a small extent with thiol B. The diethyldithiocarbamyl label at this position then protects the enzyme against further inhibition at thiol A, in much the same way as the 2-thiopyridyl group does. Consequently, there will always be a small percentage of the enzyme population which is active.

The same reasoning can also be applied to 5,5'-dithiobis(1-methyltetrazole), only this time the evidence is more direct. The activation observed with the mitochondrial enzyme implicates reaction at thiol B. Therefore, modification of this residue may also occur in the cytoplasmic enzyme, albeit to a lesser extent, and could account for the relatively high residual activity of 25 % (Kitson, 1986a).

A finding in the present research which highlights yet another cytoplasmic-mitochondrial difference is the complete indifference mitochondrial aldehyde dehydrogenase displays toward 2,2'-dithiodipyridine (Figure 3.2) - this modifier activates the cytoplasmic enzyme (Kitson, 1982b). Moreover, subsequent displacement of 2-thiopyridone apparently results in an enzyme disulfide which is just as active as native enzyme (Figure 3.5). However, it is clear that the thiol modified here is not the same as that which reacts with 4,4'-dithiodipyridine, because enzyme premodified by 2,2'-dithiodipyridine is still markedly activated by the latter (Figure 3.5). Instead, it must be reacting at a position that is not essential for activity. At the present time the nature of this group, and indeed that of the resulting disulfide which forms is currently unknown.

3.4.2 The disulfiram-ethanol reaction in vivo, and disulfiram-like reactions caused by other compounds

It was mentioned in Section 1.2 that the drinking of alcohol while under treatment with disulfiram leads to unpleasant symptoms. Known as the disulfiram-ethanol reaction these effects are primarily due to the inhibition of hepatic aldehyde dehydrogenase. This leads to the subsequent build-up of acetaldehyde in the blood.

The interaction between disulfiram and aldehyde dehydrogenase *in vivo*, however, is more complicated than a one step modification process (Scheme 3.1). In the bloodstream disulfiram is almost immediately reduced to diethyldithiocarbamate by glutathione (Cobby *et al.*, 1977). This is consistent with the observation that administration of this compound to rats results in dose response curves very similar to those obtained with disulfiram (Deitrich &

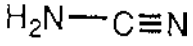
Erwin, 1971). When diethyldithiocarbamate reaches the liver it is thought to be reoxidised to disulfiram (or a derivative, see later) which in turn inhibits aldehyde dehydrogenase (Deitrich & Erwin, 1971; Kitson, 1983).

The reoxidation step to disulfiram may at first seem hard to rationalise considering the rich reducing environment present in the liver. For example, it could be envisaged that as the liver contains over a 60-fold excess of glutathione over aldehyde dehydrogenase (Kitson, 1981) that oxidation, if it occurs at all, will be negligible. However, as Kitson (1983) points out whether inactivation is observed depends on a number of factors which include the competing rate of reaction between glutathione and aldehyde dehydrogenase for any disulfiram which is formed. That disulfiram can effectively compete was clearly shown by Kitson (1981) whereby even in the presence of a 60-fold excess of glutathione, disulfiram still inactivates aldehyde dehydrogenase.

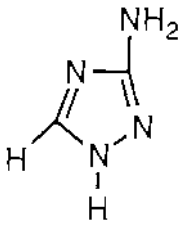
Another drug which has been used therapeutically in chronic alcoholism is calcium carbimide or cyanamide. This is usually administered in its citrated form and elicits symptoms similar to disulfiram. It also has the advantage of fewer side reactions (Kwon *et al.*, 1986; DeMaster *et al.*, 1986). Although the onset of its action is more rapid than disulfiram, the duration period is shorter (Brien *et al.*, 1978).

The actual *in vivo* metabolite of cyanamide responsible for these effects is currently unknown. Cyanamide is mostly excreted from the body as acetyl-cyanamide via an acetyl CoA dependent N-acetyltransferase (DeMaster *et al.*, 1986). However, this is not the compound responsible for the DER-like response (DeMaster *et al.*, 1983). Rather, it is known to be a bioactivation product from catalase (DeMaster *et al.*, 1984, 1985). This metabolite also has the capability of itself inhibiting catalase, in much the same way as 3-amino-1,2,4-triazole (a cyclic dimer of cyanamide). However, the two are not the same

because the latter does not inhibit aldehyde dehydrogenase *in vitro* (DeMaster *et al.*, 1984).

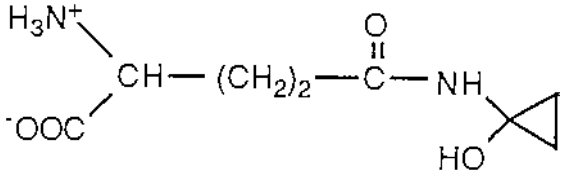


Cyanamide

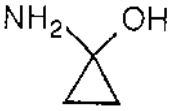


3 Amino-1,2,4-triazole

Another compound which induces DER-like symptoms is coprine (N5-(1-hydroxycyclopropyl)-L-glutamine) the active constituent in the inky cap mushroom Coprinus atramentarius (below)



Coprine



1-Aminocyclopropanol

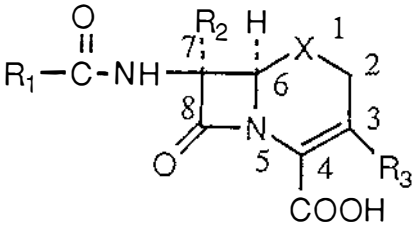
Coprine itself, however, is not the metabolite directly responsible for the inhibition of aldehyde dehydrogenase because it has no effect *in vitro* (Tottmar & Lindburg, 1977). Instead, its hydrolytic product 1-aminocyclopropanol does inactivate *in vivo* and *in vitro*. Furthermore, it has a more rapid onset than coprine when administered to rats (Tottmar & Lindburg, 1977). It is likely, therefore, that coprine is initially hydrolysed *in vivo* to glutamic acid and 1-aminocyclopropanol. The latter is in equilibrium with the hydrate of cyclopropanone and the free ketone, and it is the latter which reacts with aldehyde dehydrogenase to form a stable thiohemiketal linkage (Kitson, 1989). The resulting enzyme derivative is inhibited (Wiseman & Abeles, 1979).

In addition to the foregoing there have been various reports in the literature concerning another class of alcohol sensitising agents. It has been found that the administration of the cephalosporin antibiotics moxalactam, cefamandole, and cefoperazone all cause an adverse reaction to alcohol (Reeves & Davies, 1980;

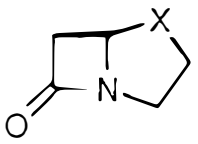
Portier, 1980). For example, Neu & Prince (1980) report the case of a young man who was being intravenously treated with moxalactam. After drinking a can of beer with his dinner he became flushed and complained of severe nausea.

Cephalosporin antibiotics are, of course, used therapeutically for their antibacterial action. Like penicillin they contain a highly reactive fused β-lactam ring which structurally mimics the substrate for a transpeptidase involved in the synthesis of the bacterial cell wall. Inhibition of this enzyme renders the bacterium incapable of crosslinking peptidoglycan strands in the final stages of cell wall synthesis (O'Callaghan & Muggleton, 1972).

The general structure of cephalosporin antibiotics differs from that of the penicillins in that they contain a six membered dihydrothiazine ring instead of a five membered thiazolidine (below)



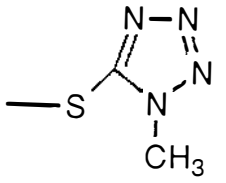
A Cephalosporin



A penicillin nucleus

The R_n (n = 1,2,3) sidechains vary widely (Gorman & Ryan, 1972) and govern factors such as the permeability of the antibiotic toward the cell wall. The core system shown above, however, is essential for antibacterial activity (O'Callaghan & Muggleton, 1972).

Interestingly, only those antibiotics where R_3 is



produce a DER-like response. For instance, in the acetaldehyde-metabolising system in rats, cefamandole, cefoperazone, and moxalactam all are effective in

elevating the blood concentration of acetaldehyde (Shimada *et al.*, 1987; Matsubara *et al.*, 1986). These three antibiotics all contain a 1-methyltetrazole-5-thiol sidechain (see Figure 3.6).

The similarity here between the above and the disulfiram-ethanol reaction led Freundt (1986a,b) to suggest these antibiotics inhibited aldehyde dehydrogenase in a competitive manner. He argued that a decrease in the apparent affinity of the enzyme for acetaldehyde would account for the DER-like symptoms. The findings of Freundt *et al.* (1985) were, however, questioned by Kitson (1986b,c) for the following reasons.

Firstly, it would be an unlikely coincidence that the only cephalosporin antibiotics which elicit a response are those which contain a 1-methyltetrazole-5-thiol sidechain (Figure 3.6; Matsubara *et al.*, 1986). Although the remote possibility exists that aldehyde dehydrogenase possesses a special binding site for this group, this is not supported by *in vitro* studies. Both 1-methyl-5-methyl-thiotetrazole and 1-methyltetrazole-5-thiol have no effect on aldehyde dehydrogenase *in vitro* (Kitson, 1986a). Secondly, the findings of Freundt *et al.* (1985) showed that a 10 mM concentration of cefamandole decreased aldehyde dehydrogenase activity by only 25 %. Kitson (1986b) claimed that such weak inhibition from such a high concentration of antibiotic would be insufficient to account for the observed effect *in vivo*. On the basis of these arguments Kitson (1984, 1986b,c) proposed instead an alternative mechanism where 1-methyl-tetrazole-5-thiol became liberated *in vivo* (Scheme 3.2).

The general mechanism of this displacement is well studied (Kaiser & Kukolja, 1972). Using an acetyl linkage instead of the 1-methyltetrazole-5-thiol in Scheme 3.2, Boyd & Lunn (1979) found the CH_2-0-Ac bond more susceptible to cleavage when the nucleophile attacked position C8. In Scheme 3.2 "Nuc" may represent β-lactamase or glutathione. The departure of a leaving group also has therapeutic advantages as well. As it is released within the cell wall it can itself be selected to have antibacterial properties (Boyd & Lunn, 1979). The 1-methyltetrazole-5-thiol which is displaced is thought to follow the same fate as diethyldithiocarbamate (Kitson, 1987b). It is oxidised in the liver to a symmetrical or mixed disulfide which subsequently inhibits aldehyde dehydrogenase (Scheme 3.2).

Throughout the foregoing the term "aldehyde dehydrogenase" has been used quite generally. However, a problem arises when a distinction is made between the cytoplasmic and mitochondrial enzyme. As outlined in Section 1.4 most acetaldehyde oxidation is thought to occur in the mitochondria. This is certainly the case for rat liver, and it is implicated for human liver (recall that Orientals who possess a mutant form of mitochondrial aldehyde dehydrogenase experience DER-like symptoms). Furthermore, studies investigating the effect on rat liver aldehyde dehydrogenase of disulfiram, β-lactam antibiotics, and 1-methyltetrazole-5-thiol (Brien *et al.*, 1985; Matsubara *et al.*, 1986) all show that mitochondrial aldehyde dehydrogenase is inhibited. This is despite the fact that disulfiram itself has little effect on this enzyme, and 5,5'-dithiobis(1-methyl-tetrazole) is an activator (Figure 3.2)

A solution to this dilemma for disulfiram was suggested by MacKerell *et al.* (1985) and involves the co-oxidation of diethyldithiocarbamate with methanethiol. The resulting mixed disulfide is a potent inhibitor of mitochondrial aldehyde dehydrogenase (Figure 3.1). Methanethiol is a normal metabolite in the body and is formed from the breakdown of methionine. The latter can normally be degraded via either a transsulfuration or transaminative pathway. In patients with liver damage, however, the transsulfuration route is diminished and most flux proceeds through the transaminative pathway. Under these conditions an elevation in the concentration of mercaptans occurs in the blood and breath (Cooper, 1983). It is interesting to note that this explanation can also apply to 1-methyltetrazole-5-thiol. The corresponding mixed disulfide methyl 5-(1-methyltetrazolyl) disulfide, which would result from co-oxidation with methanethiol, is also a potent inhibitor of mitochondrial aldehyde dehydrogenase (Figure 3.1).

In conclusion, the mechanisms by which disulfiram and β-lactam antibiotics (which contain a 1-methyltetrazole-5-thiol sidechain) elicit a DER-like response are similar. Both involve an initial reaction to liberate diethyldithiocarbamate and 1-methyltetrazole-5-thiolate respectively. It is possible that both symmetrical and mixed disulfides of these compounds are subsequently formed. However, it is the latter which affect the mitochondrial enzyme, and hence are responsible for the observed effects.

Scheme 3.1 Inactivation of Aldehyde Dehydrogenase *in vivo* by Disulfiram

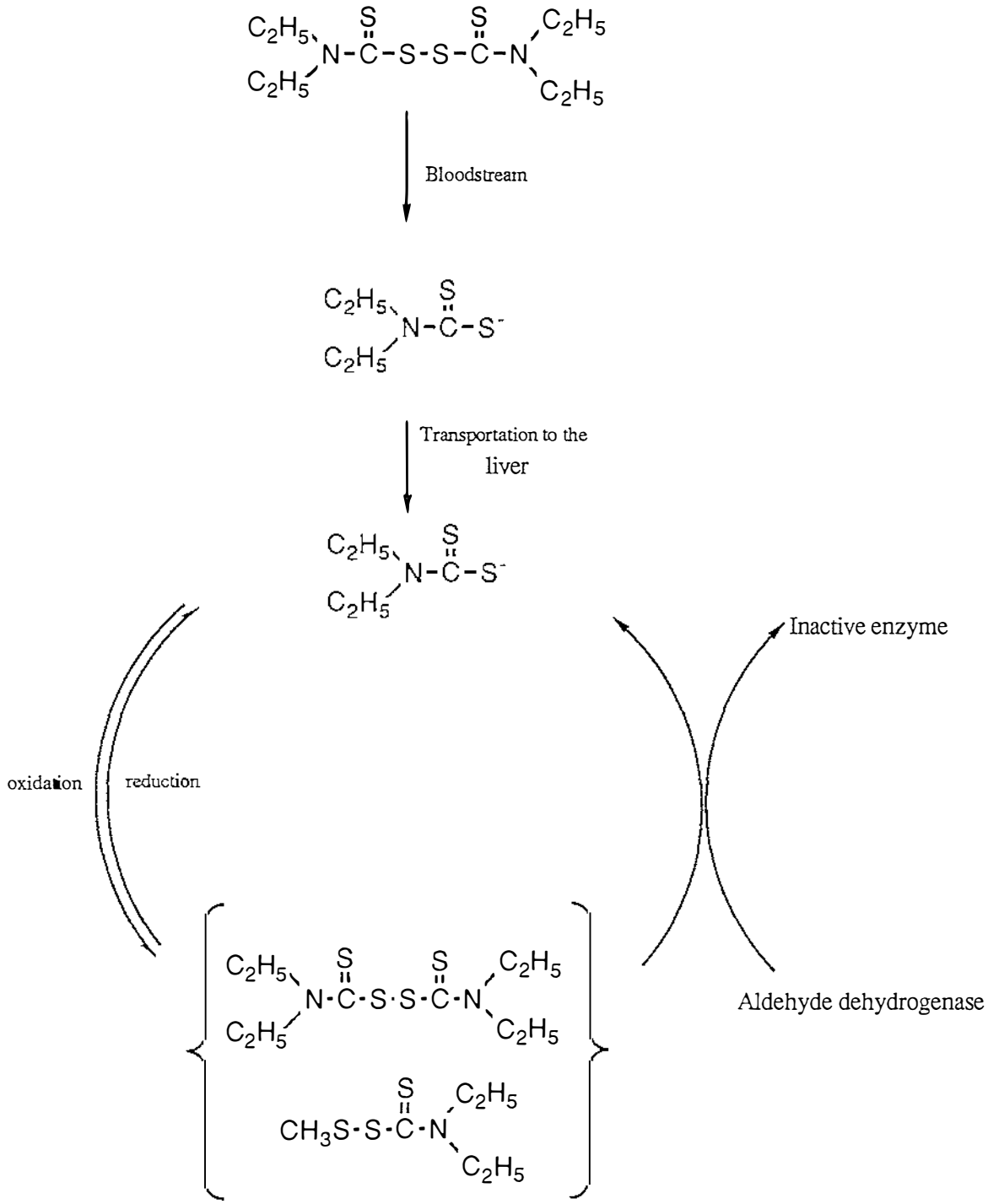
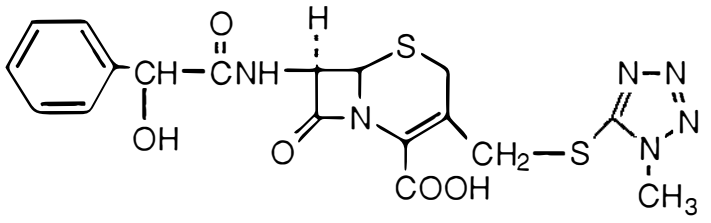
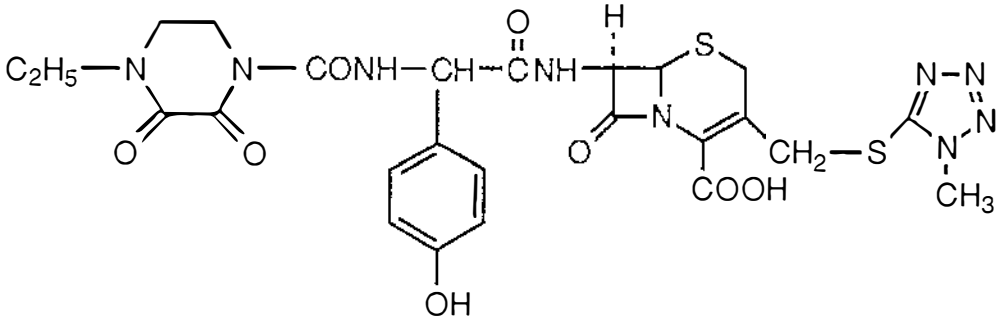


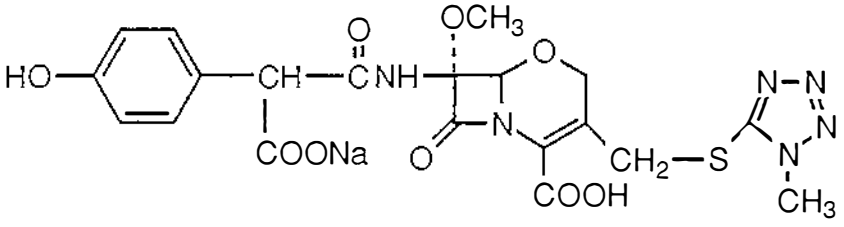
Figure 3.6 Structures of Cefamandole, Cefoperazone, and Moxalactam



CEFAMANDOLE

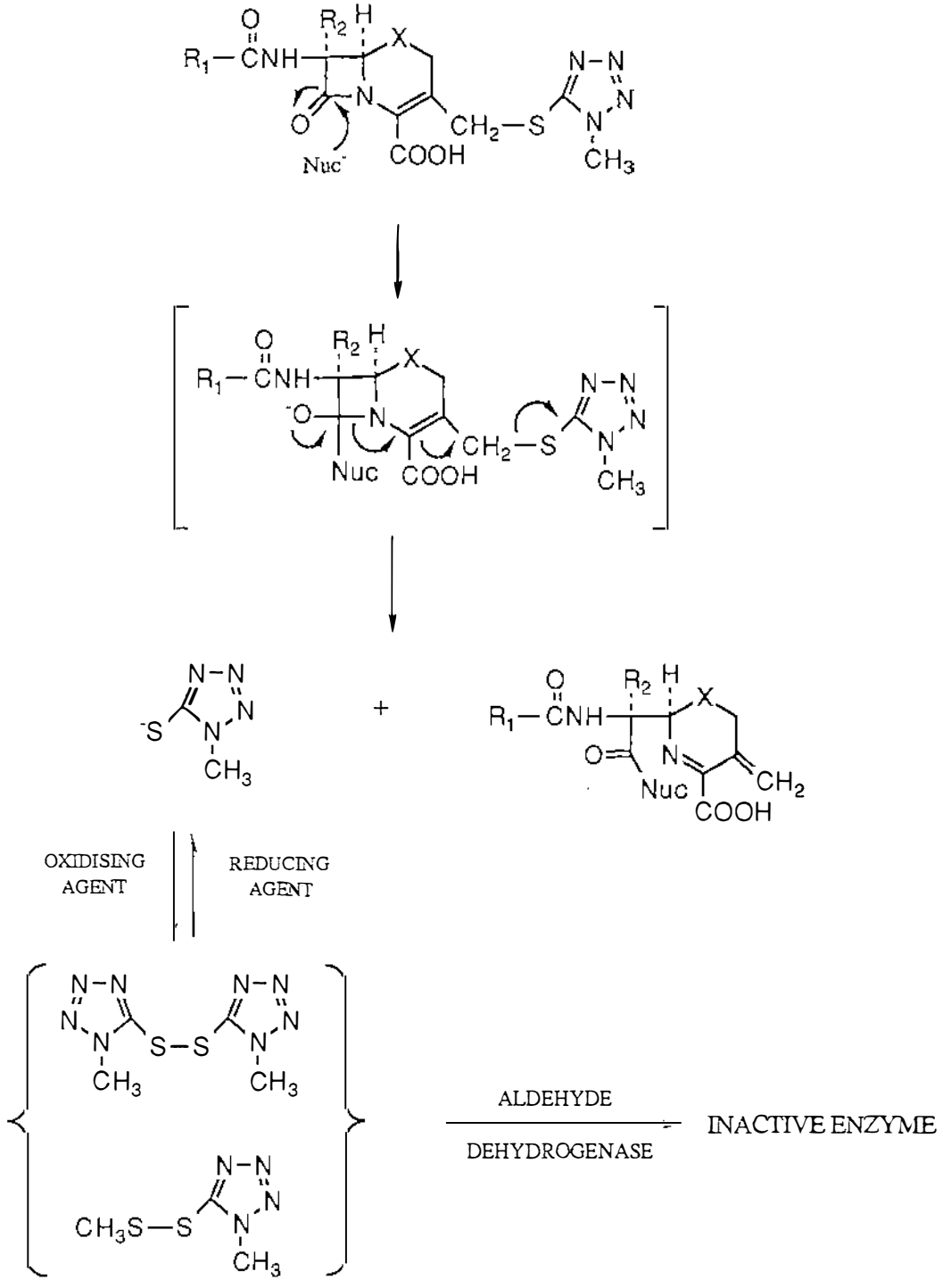


CEFOPERAZONE



MOXALACTAM

Scheme 3.2 Inactivation of Aldehyde Dehydrogenase *in vivo* by *B*- Lactam Antibiotics Containing a 1-Methyl-tetrazole-5-thiol Sidechain



CHAPTER 4

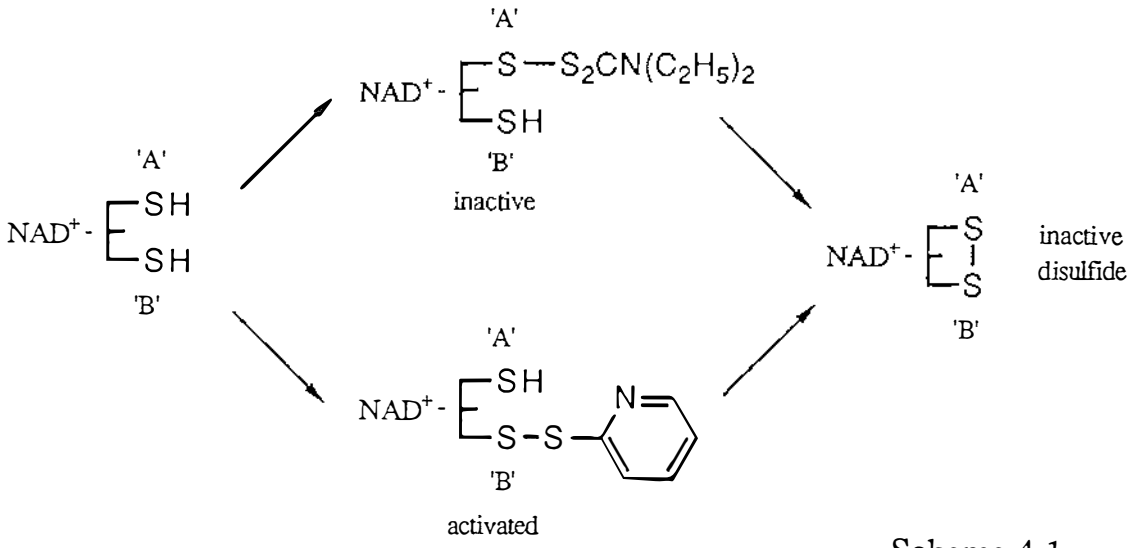
THE EFFECTS OF THIOL REAGENTS ON THE ESTERASE ACTIVITY OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE

The next two Chapters concern the one-site/two-site debate over the dehydrogenase and esterase activities of aldehyde dehydrogenase. This Chapter takes an indirect line by investigating the nature and functional properties of a pair of closely positioned enzymic thiol groups.

4.1 Introduction

In addition to its normal dehydrogenase capability aldehyde dehydrogenase also catalyses the hydrolysis of 4-nitrophenyl acetate (MacGibbon *et al.*, 1978b; Kitson, 1986d). Traditionally, it has been naturally assumed that both these activities occur at a common active site (Li, 1977; Sidhu & Blair, 1975a,b). However, other workers conclude that this esterase activity occurs at an active site completely distinct from that which catalyses the oxidation of aldehydes (Tu & Weiner, 1988b; Blackwell *et al.*, 1983; Deady *et al.*, 1985). Designated "P2" (to differentiate it from the dehydrogenase active site labelled "P1") this putative active site also has the ability to bind high concentrations of aldehyde, and is proposed to be the site modified by disulfiram (Blackwell *et al.*, 1983).

In the previous Chapter it was mentioned that aldehyde dehydrogenase possesses two closely positioned thiols, called A and B, in the tertiary structure (Kitson & Loomes, 1985a,b). Reaction with either of these groups by certain modifiers results in a labelled enzyme which is capable of forming an inactive disulfide (Scheme 4.1).



Scheme 4.1

Thus for 2,2'-dithiodipyridine labelling thiol B with a 2-thiopyridyl sidechain leads initially to an activated state, while modification of A by disulfiram leads to inhibition. In both cases, however, A and B respectively can displace the attached label to form an identical enzyme disulfide.

The functional properties of this thiol pair have a particular relevancy to the one-site and two-site models. With a common active site for both dehydrogenase and esterase activities the disulfiram-sensitive group can still be catalytically essential (see Section 3.4.1). Therefore, modification of this residue by any modifier will always lead to a concerted reduction in both activities.

In a two-site framework, however, disulfiram reacts at P2. As this alternative site is distinct from P1, thiol A (which must also reside in P2) cannot be catalytic for dehydrogenase activity. Any effect disulfiram does have on aldehyde oxidation - and indeed that of any reagent which modifies A and B - must necessarily be indirect. Consequently, there is no intrinsic requirement for a concerted reduction in both dehydrogenase and esterase activities following modification at A.

In this Chapter the effects of various thiol modifiers on the esterase activity of cytoplasmic aldehyde dehydrogenase is investigated, and compared to their corresponding behaviour with the dehydrogenase activity. It is the aim that such an analysis will provide useful information from two perspectives: firstly, a

deeper insight into the functional roles of groups A and B; and secondly, to shed more light on the one-site/two-site debate.

4.2 Experimental

To investigate the effects of various modifiers on the esterase activity of aldehyde dehydrogenase a number of different mixing orders were adopted. In some experiments the appropriate modifier was added as 15 µl of an ethanol solution on a glass nail to an assay containing enzyme in 35 mM sodium phosphate buffer, pH 7.4, at 25 ^{o}C. The total cuvette volume was 3 ml. Reaction mixtures were incubated for at least 7 minutes at 25 ^{o}C, and the enzyme-catalysed reaction initiated by the addition of 15 µl of a 4-nitrophenyl acetate stock solution in ethanol. Esterase activity was monitored by following the steady state production of 4-nitrophenoxide at 400 nm against a control containing the assay buffer. In other experiments investigating the protective effect of 4-nitrophenyl acetate the modifier was added (15 µl on a glass nail) to an ongoing assay.

The effects of NAD^{+} and NADH on esterase activity were also examined. Here, the modifier was premixed with enzyme and incubated as before. However, this time cofactor (1 mM) was added before the addition of 4-nitrophenyl acetate. Alternatively, the modifier was added to the enzyme already in the presence of cofactor, and 4-nitrophenyl acetate added later. In the experiments containing cofactor the absorbance traces are not linear but display a gradual downward curvature. Consequently, the initial rate was obtained by drawing a tangent to the curve in the early stages. In the absence of cofactor the time course is linear.

Stock solutions of 4-nitrophenyl acetate and modifiers were made up in 95 % ethanol while stocks containing cofactor were dissolved in 35 mM sodium phosphate, pH 7.4. All solutions were freshly prepared on the day of the experiment.

Disulfiram, methyl diethylthiocarbamyl disulfide, 2,2'-dithiodipyridine, 4,4'-dithiodipyridine, methyl 2-pyridyl disulfide, and methyl 4-pyridyl disulfide were obtained or prepared as described in Chapter 2.

4.3 Results

Figure 4.1 shows the inactivation profiles of disulfiram and methyl diethylthiocarbamyl disulfide on the esterase activity of aldehyde dehydrogenase. When premixed with enzyme before the addition of 4-nitrophenyl acetate both inhibit to a similar extent. Although there is some divergence in the middle part of the profiles the two nevertheless approach the same limiting value of around 10 %. However, when these modifiers are added to an ongoing assay, for disulfiram the situation is clearly different. When aldehyde dehydrogenase is in the presence of 100 μM 4-nitrophenyl acetate it is effectively protected against the otherwise inhibitory effects of disulfiram[1]. This agrees with the findings of Kitson (1982a). In contrast to disulfiram, 4-nitrophenyl acetate does not protect the enzyme against methyl diethylthiocarbamyl disulfide. Instead, addition of this modifier causes a reduction of the absorbance trace to a lower steady state over a few minutes. Although there appears to be some difference in the nature of the inactivation profile under these circumstances (Figure 4.1) the residual activity is still the same regardless of the mixing order.

The effects on ester hydrolysis of methyl 2-pyridyl disulfide and methyl 4-pyridyl disulfide are shown in Figure 4.2. The important point to note here is that in both cases inhibition occurs, although to various degrees. Furthermore, methyl 2-pyridyl disulfide and methyl 4-pyridyl disulfide have similar effects to their symmetrical parent compounds - in much the same way as disulfiram and methyl diethylthiocarbamyl disulfide do. It can be assumed that the difference in the slopes of the profiles in Figure 4.2 is real, and not due to varying extents of reaction of the modifier with enzyme. This is because experiments which monitor the production of 2- or 4-thiopyridone show the reaction to be essentially complete after a few minutes.

Figure 4.3 shows the effect of NAD^+ on the inactivation of 4-nitrophenyl acetate hydrolysis by methyl 2-pyridyl disulfide and 2,2'-dithiodipyridine. When premixed with enzyme already saturated with NAD^+, 2,2'-dithiodipyridine is still a good inhibitor. However, under these same conditions methyl 2-pyridyl

---

1 - over a period of time the absorbance trace does eventually curve down

disulfide has almost no effect, and only at high concentrations does the activity decline slightly. When methyl 2-pyridyl disulfide is premixed with enzyme and NAD^+ added later, a reduction in activity occurs in much the same way as it does in Figure 4.2. A similar trend is also seen for methyl 4-pyridyl disulfide in Figure 4.4. However, the difference in activity under these two mixing conditions is not as pronounced as that for methyl 2-pyridyl disulfide. In the presence of excess methyl 4-pyridyl disulfide both profiles converge.

Assays carried out in the presence of NAD^+ generally show a continuous downward curvature in their absorbance traces. However, it was found that by using a low concentration of 4-nitrophenyl acetate (34 μM) the amount of hysteresis was reduced. Nevertheless, the activities in these cases were obtained by drawing a tangent to the curve in the early stages of the time course. To further confirm the findings with NAD^+ the experiments with methyl 2- and methyl 4-pyridyl disulfide were repeated, but this time in the presence of NADH (Figure 4.5). Under these conditions the traces were generally more linear, and the results showed the same trend as NAD^+.

The effect of sequential addition of methyl 2-pyridyl disulfide then methyl 4-pyridyl disulfide on ester hydrolysis is shown in Figure 4.6. When enzyme was pretreated with methyl 4-pyridyl disulfide in the presence of NAD^+ (1 mM) and then assayed, a reduction in activity occurred which was identical to that obtained previously (Figure 4.4). However, when this modifier was added to enzyme preincubated with a 5-fold excess of methyl 2-pyridyl disulfide (again in the presence of NAD^+) the inactivation profile was steeper.

Figure 4.1 - Enzyme (0.13 μM) was pretreated with disulfiram (■) or with methyl diethylthiocarbamyl disulfide (●) and then assayed using 4-nitrophenyl acetate (100 μM). Alternatively, disulfiram (□) or methyl diethylthiocarbamyl disulfide (○) was added to the enzyme already in the presence of its substrate.

Figure 4.1 Effect of Disulfiram and of Methyl Diethylthiocarbamyl Disulfide on the Esterase Activity of Cytoplasmic Aldehyde Dehydrogenase with Different Orders of Mixing

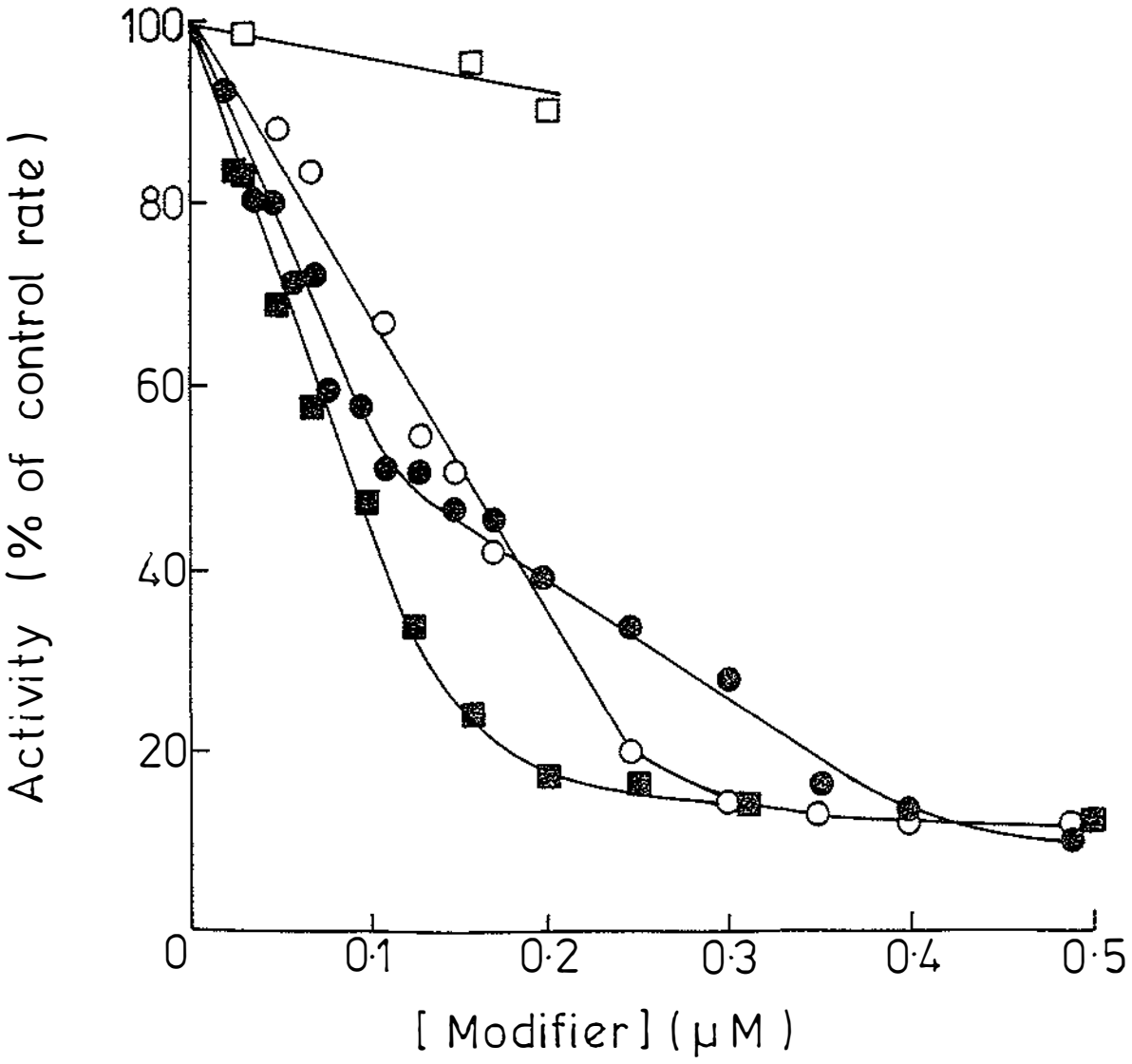


Figure 4.2 - Enzyme (0.13 μM) was pretreated with 2,2'-dithiodipyridine (□), 4,4'-dithiodipyridine (■), methyl 2-pyridyl disulfide (○) or methyl 4-pyridyl disulfide (●) and then assayed using 4-nitrophenyl acetate (100 μM).

Figure 4.2 Effect of 2,2'- and 4,4'-Dithiodipyridines and Methyl 2- and 4-Pyridyl Disulfides on the Esterase Activity of Cytoplasmic Aldehyde Dehydrogenase

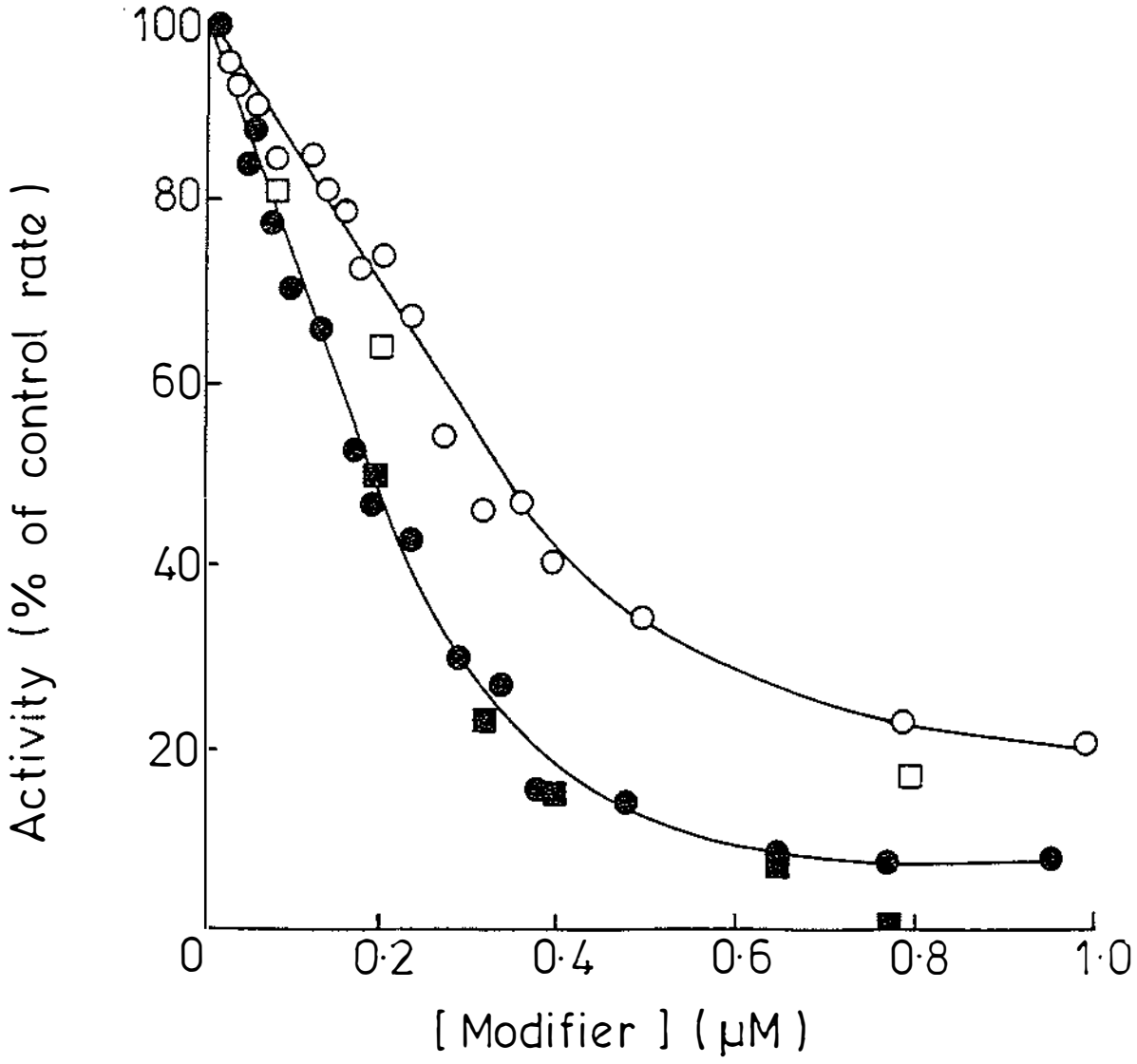


Figure 4.3 - Enzyme (0.13 μM) in the presence of NAD^+ (1 mM) was pretreated with methyl 2-pyridyl disulfide (●) or 2,2'-dithiodipyridine (■) and then assayed using 4-nitrophenyl acetate (34 μM). Alternatively, the enzyme was pretreated with methyl 2-pyridyl disulfide in the absence of NAD^+ (○).

Figure 4.3 Effect of NAD^+ on the Inactivation of Cytoplasmic Aldehyde Dehydrogenase by Methyl 2-Pyridyl Disulfide and by 2,2'-Dithiodipyridine

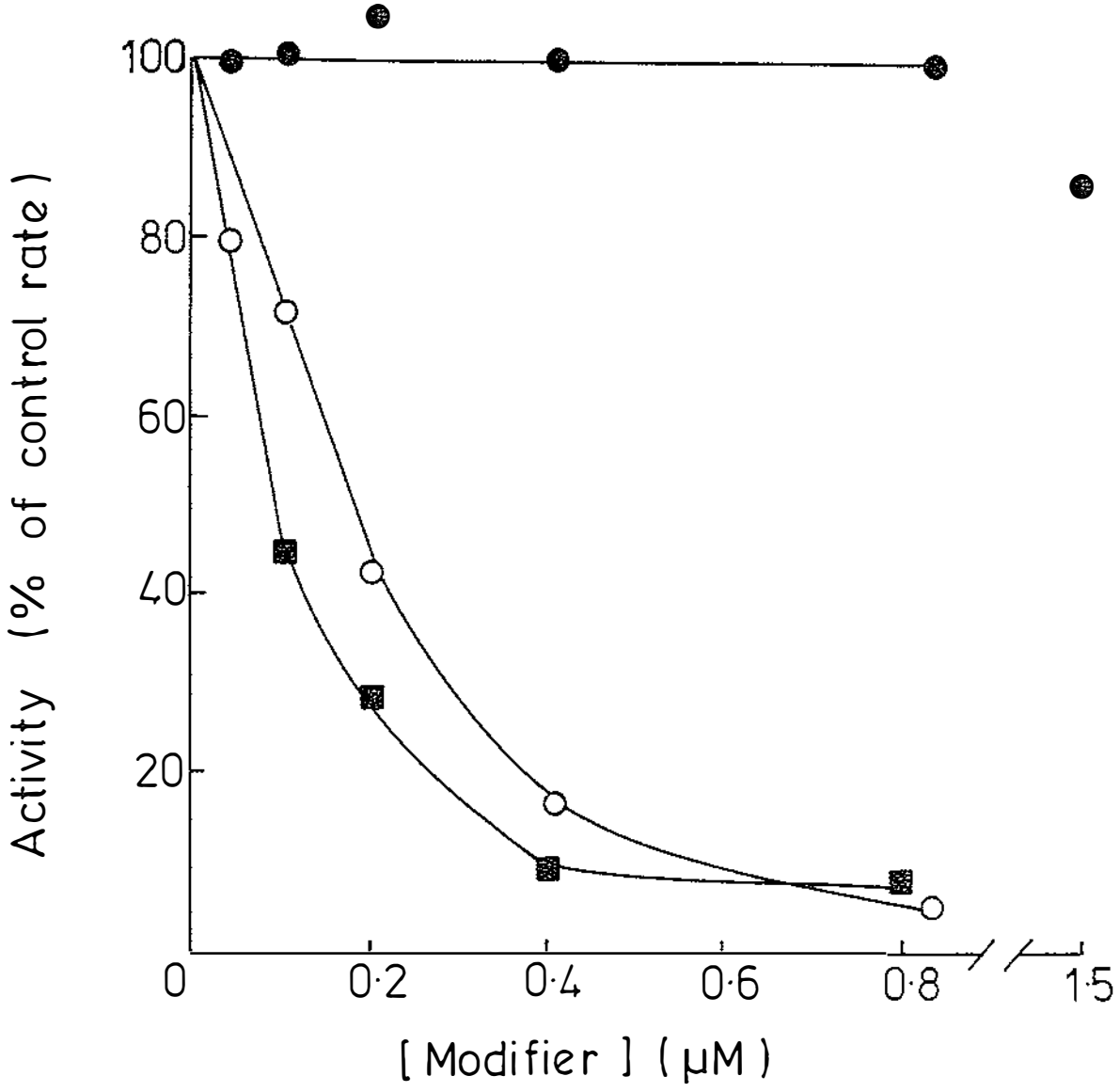


Figure 4.4 - The enzyme (0.18 μM) in the presence of NAD^+ (1 mM) was pretreated with methyl 4-pyridyl disulfide and then assayed using 4-nitrophenyl acetate (34 μM) (●). The experiment was repeated in the absence of NAD^+ (○).

Figure 4.4 Effect of NAD^+ on the Inactivation of Cytoplasmic Aldehyde Dehydrogenase by Methyl 4-Pyridyl Disulfide

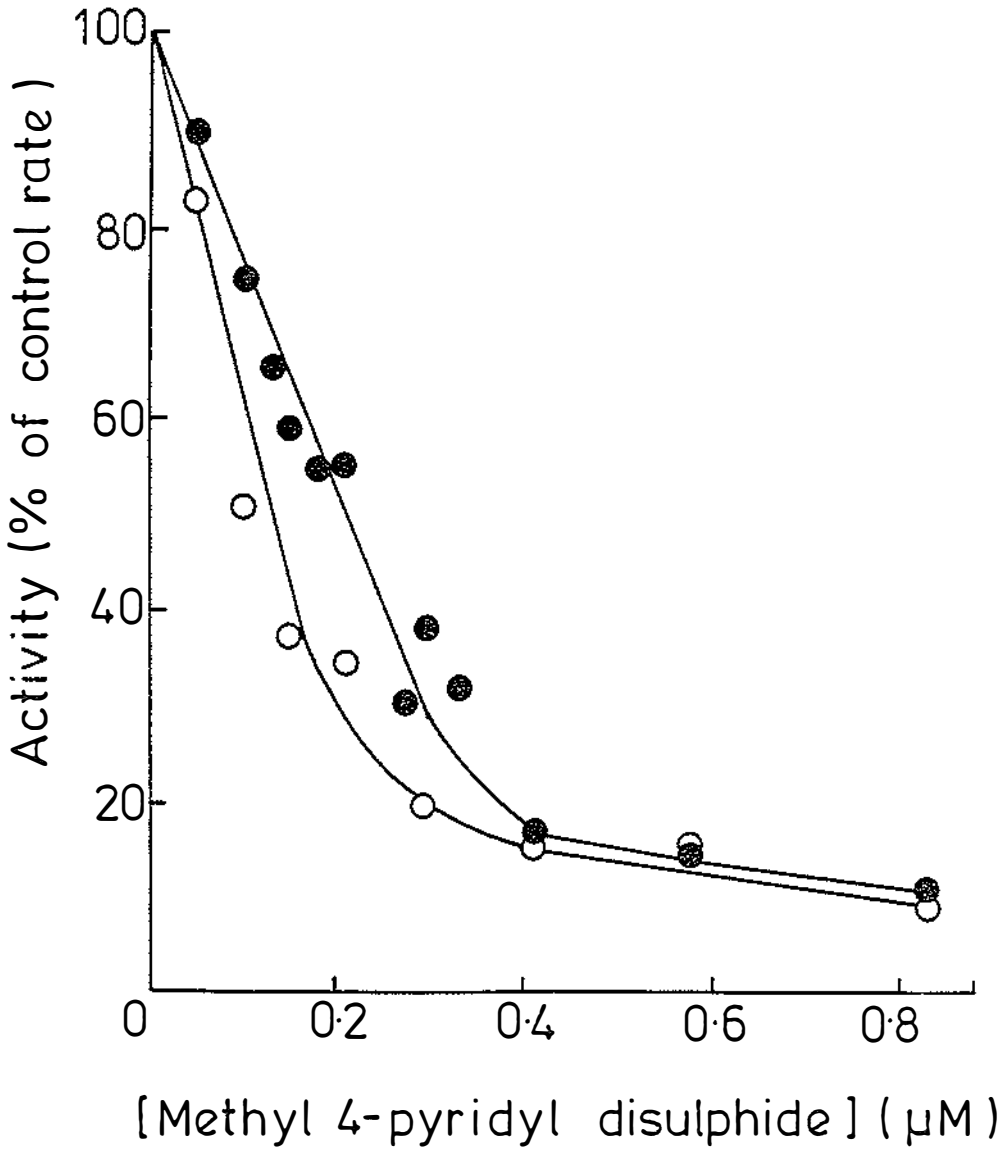


Figure 4.5 - The enzyme (0.1 μM) in the presence of NADH (40 μM) was pretreated with methyl 2-pyridyl disulfide and then assayed with 4-nitrophenyl acetate (34 μM) (●). The experiment was repeated in the absence of NADH (○). Alternatively, methyl 4-pyridyl disulfide was used, in the presence (■) and absence of (□) of NADH.

Figure 4.5 Effect of NADH on the Inactivation of Cytoplasmic Aldehyde Dehydrogenase by Methyl 2- and 4-Pyridyl Disulfides

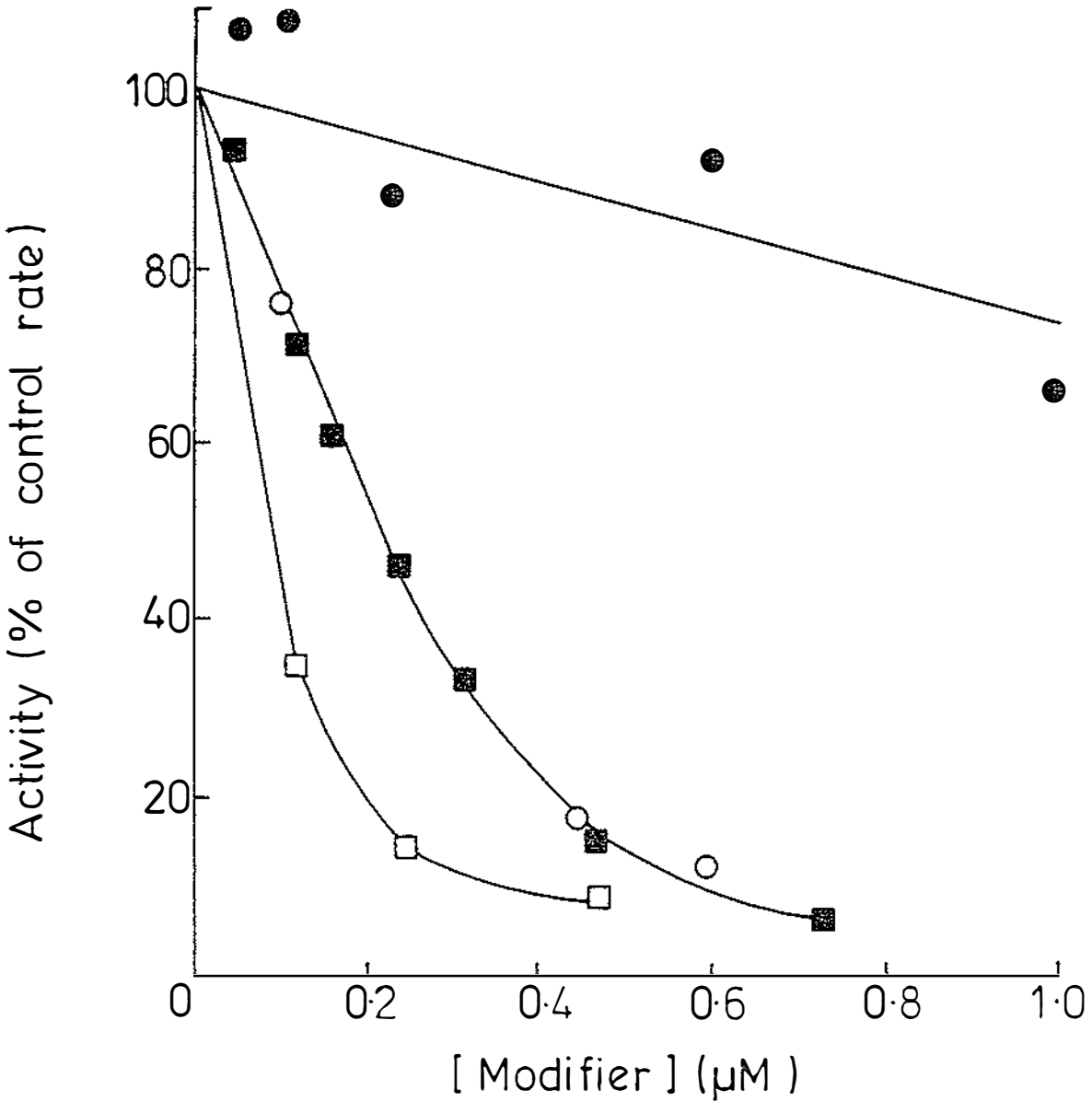
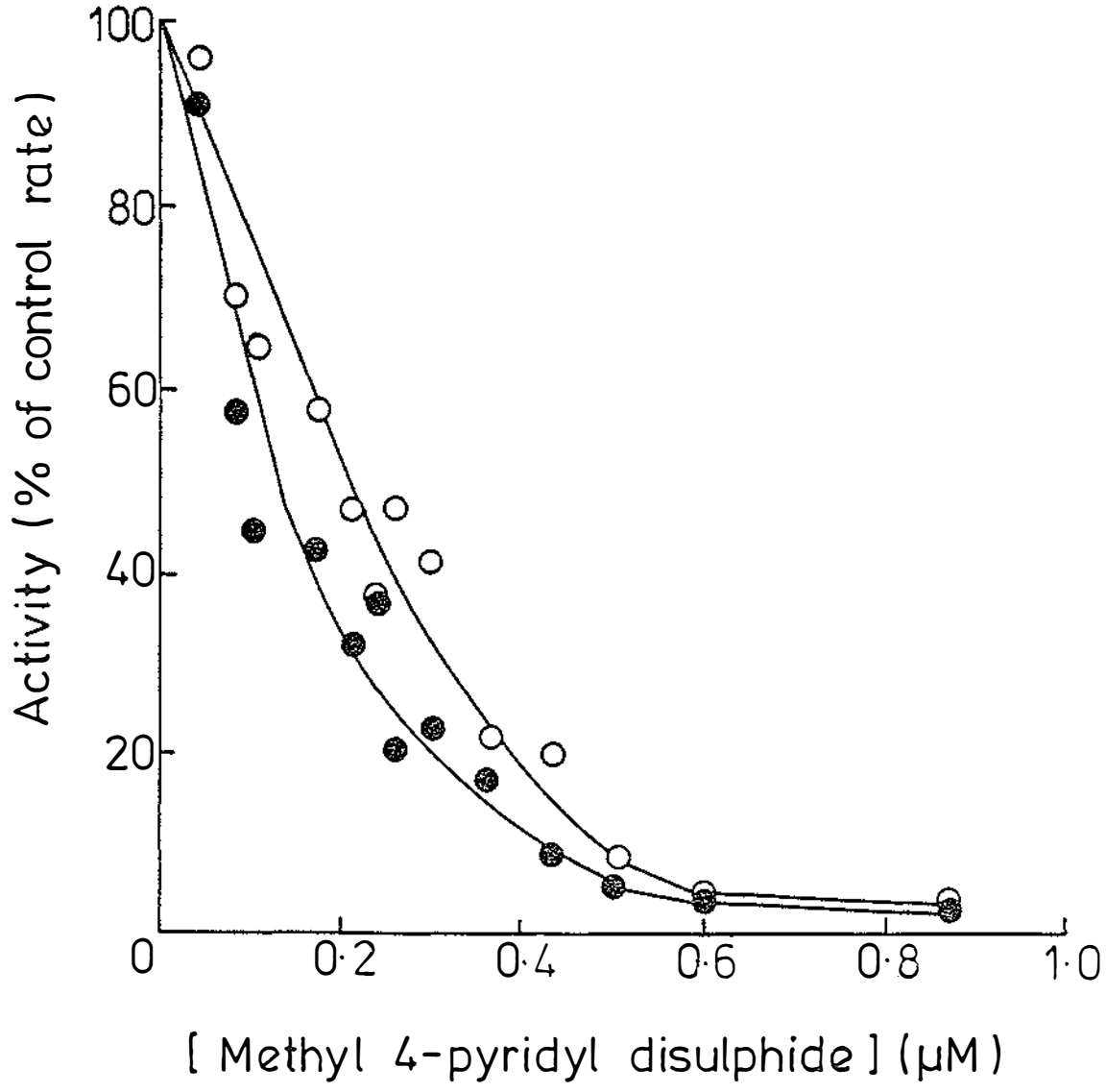


Figure 4.6 - The enzyme (0.1 μM) in the presence of NAD^+ (1 mM) was pretreated with methyl 4-pyridyl disulfide and then assayed with 4-nitrophenyl acetate (40 μM) (○). Alternatively, the enzyme in the presence of NAD^+ was treated first with methyl 2-pyridyl disulfide (0.51 μM) and then approx. 7 min later with methyl 4-pyridyl disulfide, before assaying as before (●).

Figure 4.6 Effect of Sequential Addition of Methyl 2- and 4-Pyridyl Disulfides on Cytoplasmic Aldehyde Dehydrogeanse in the Presence of NAD^+



4.4 Discussion

The results in the present study describe the effects of various modifiers on the esterase activity of aldehyde dehydrogenase under different mixing conditions. The rationale for this was to correlate these findings with those for the corresponding dehydrogenase activity (Kitson & Loomes, 1985a,b) and hence identify any patterns. From these results there is indeed a general trend. However, the rationalisation rests heavily on the assumption that these effects primarily arise from reaction with groups A and B. Because of this it will be appropriate firstly to review what is already known about these groups in terms of the existing literature.

4.4.1 The role and nature of groups A and B

It is well known that when aldehyde dehydrogenase is modified by 2,2'-dithiodipyridine in the presence of NAD^+, and assayed at 1 mM acetaldehyde, activation occurs (Kitson, 1982b). On the other hand, disulfiram is a potent inhibitor regardless of the mixing order (Kitson, 1978,1979). Under certain conditions, however, disulfiram does not inactivate. For instance, when enzyme is saturated with 4-nitrophenyl acetate this modifier has little effect at all (Figure 4.1). Kitson (1979) also found the same level of protection when aldehyde dehydrogenase is premixed with 2,2'-dithiodipyridine before the addition of disulfiram.

Protection by 2,2'-dithiodipyridine, as Kitson (1982a) points out, raises a number of interesting possibilities concerning the nature of the disulfiram-sensitive group. The simplest explanation is that 2,2'-dithiodipyridine and disulfiram react with the same group. Obviously, in this case the target thiol cannot be catalytically essential as this is incompatible with activation by 2,2'-dithiodipyridine. Alternatively, these two reagents modify different thiols and protection by 2,2'-dithiodipyridine is indirect - for example, by steric hindrance. Here, the thiol modified by disulfiram can still be catalytically essential for activity.

These two possibilities were later distinguished in studies investigating the effects of methyl 2-pyridyl disulfide, methyl 4-pyridyl disulfide, and methyl

diethylthiocarbamyl disulfide (Kitson & Loomes, 1985a,b). All these are structural analogues of 2,2'-dithiodipyridine, 4,4'-dithiodipyridine, and disulfiram respectively (see Figure 4.7). It is assumed on the basis of structural similarity that these mixed disulfides modify the same groups as their symmetrical counterparts. This is certainly the case for disulfiram and methyl diethylthiocarbamyl disulfide because both give identical inactivation profiles. For 2,2'-dithiodipyridine assays previously activated by methyl 2-pyridyl disulfide are not enhanced any further in its presence (Kitson & Loomes, 1985a).

From the foregoing it is clear that 2,2'-dithiodipyridine and disulfiram react at different groups because of the contrasting effects of their mixed disulfides. As methyl diethylthiocarbamyl disulfide inhibits and methyl 2-pyridyl disulfide activates somewhat, and because both attach a $-SCH_3$ label, different reactor groups must be involved. Call the groups modified by disulfiram and 2,2'-dithiodipyridine A and B respectively. The existence of these groups is also supported by experiments where methyl diethylthiocarbamyl disulfide inhibits assays previously activated by 2,2'-dithiodipyridine and methyl 2-pyridyl disulfide. In this case, if methyl diethylthiocarbamyl disulfide can inhibit an activated species containing a $-SCH_3$ label (from methyl 2-pyridyl disulfide) by itself attaching an identical group, then a different thiol must be involved - namely A.

Unlike its symmetrical parent, methyl 4-pyridyl disulfide is not an activator of the dehydrogenase pathway, but neither is it a potent inhibitor like methyl diethylthiocarbamyl disulfide. Assays preactivated by methyl 2-pyridyl disulfide and 2,2'-dithiodipyridine are not protected against this modifier. Furthermore, enzyme inactivated by methyl 4-pyridyl disulfide is further inhibited by methyl diethylthiocarbamyl disulfide. The simplest explanation for these results is that methyl 4-pyridyl disulfide nonspecifically reacts at A and B. In such an assay, therefore, a percentage of the enzyme population will be strongly inhibited, and a percentage mildly activated. Overall, however, mild inhibition will be observed experimentally.

If the foregoing is correct then the protection 2,2'-dithiodipyridine confers on the enzyme against disulfiram raises interesting implications about the spatial nature of A and B. It seems most likely that this is a steric phenomenon in

which the presence of a 2-thiopyridyl label at B physically prevents the access of disulfiram to thiol A. Obviously, this can only occur if A and B are closely positioned in the tertiary structure.

This conclusion is supported by a number of studies which have found aldehyde dehydrogenase capable of forming an inactive disulfide after modification by certain modifiers. Thus for 2,2'-dithiodipyridine and 4,4'-dithiodipyridine, which are proposed to modify B, these labels are subsequently displaced by a neighbouring thiol. The same occurs with disulfiram but this time removal of the diethyldithiocarbamyl label is from group A.

As Kitson & Loomes (1985a) suggest, it is very unlikely that two distinct thiol pairs exist - one containing A and the other B - both of which have the required stereochemistry to displace their respective labels. Instead, it is reasonable to assume that only groups A and B are involved in these processes. That is, 2,2'-dithiodipyridine modifies group B, initially with activation, and the label is subsequently displaced by A. Likewise, the same fate occurs with disulfiram but here the diethyldithiocarbamyl moeity is removed by B (see Scheme 4.1).

This model is supported by the finding that premodification of the enzyme with disulfiram eliminates the slower displacement reaction with 2,2'-dithiodipyridine (Kitson & Loomes, 1985a). In this case thiol A cannot displace the 2-thiopyridyl label because it is itself modified, and hence precluded from further reaction.

Support for the existence of a closely positioned thiol pair in aldehyde dehydrogenase also comes from modification experiments with 3-bromoacetylpyridinio-alkyldiphosphoadenosine (Figure 4.8). Using a butyl spacer, von Bahr-Linstrom *et al.* (1981) found that an as yet unidentified cysteine is modified. This is different to another residue, Cys-302, which is the target thiol of a similar coenzyme containing a pentyl spacer (von Bahr-Lindstrom *et al.*, 1985). This group is also specifically modified by iodoacetamide, and is implicated in the reaction with disulfiram (Hempel *et al.*, 1982a).

It is interesting to note the similarity between these findings and those obtained with alcohol dehydrogenase. Jornvall *et al.* (1975) report that the yeast and

horse liver enzymes are both modified by 5-bromoacetyl-4-methyl-imidazole dinucleotide (Figure 4.8). After digestion with chymotrypsin and subsequent fractionation, Cys-43 and Cys-174 are found to be specifically modified respectively[2]. Similar experiments have also been performed with aldehyde dehydrogenase, and show that 5-bromoacetyl-4-methyl-imidazole dinucleotide inhibits in much the same way as it does for alcohol dehydrogenase (Woenckhaus *et al.*, 1987). However, the identity of the modified group is currently unknown.

The advantage in using these brominated NAD^+ analogues is that they possess the dual capability of functioning as both hydrogen acceptor and as an irreversible modifier. Consequently, these compounds are still seen to interact at the coenzyme binding domain. Therefore, the findings for both enzymes described above imply both pairs of cysteines are spatially close to each other, and reside at a position near the coenzyme binding domain. This is especially so for aldehyde dehydrogenase in which two apparently different groups are modified by coenzyme analogues which differ only in a methylene group. In the absence of a tertiary structure the identities and exact spatial nature of A and B are still unknown. However, for alcohol dehydrogenase crystallographic data shows that Cys-46 and Cys-174 are indeed in close proximity, and are ligated to the catalytic zinc cation.

Other enzymes also capable of internal disulfide formation include chymopapain. This differs from other cysteine proteases by the presence of a non-essential thiol at the active site. With 2,2'-dithiodipyridine and 5,5-dithiobis(2-nitrobenzoate) chymopapain undergoes modification and displacement in a similar manner to aldehyde dehydrogenase (Korodi *et al.*, 1986). Glyceraldehyde-3-phosphate dehydrogenase also has two closely positioned thiol groups at the active site, one of which is Cys-149 (the catalytic nucleophile). The other is Cys-153 which is nonessential but is forced into close proximity because it occurs after one turn in a helix. Under certain conditions, for example at relatively high temperatures, both these groups are co-oxidised to an inactive disulfide (Harris & Waters, 1976).

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2 - Cys-43 corresponds to Cys-46 in the horse primary structure

4.4.2 The working model

We are now in a position to discuss in detail the findings of the present research. It is the aim of this Section to demonstrate the consistency of the present results in terms of groups A and B. The working model comprises the following two themes. Firstly, modification of A reduces both dehydrogenase and esterase activities. The more this group is modified the more inhibition is observed. Secondly, the presence of NAD^+ or NADH induces a general shift toward modification at B. As this group is proposed to have only an indirect effect on activity, activation or inhibition may occur depending on the introduced label and the assay conditions.

To demonstrate the consequences of reaction at A consider the effects the following modifiers have on the dehydrogenase activity when premixed in the *absence* of NAD^+. Under these conditions 2,2'-dithiodipyridine has relatively little effect on activity although modification by this reagent still protects the enzyme against disulfiram (Kitson, 1982a). Methyl 2-pyridyl disulfide mildly inhibits - methyl 4-pyridyl disulfide even more - and finally disulfiram and methyl diethylthiocarbamyl disulfide potently inactivate (Kitson & Loomes, 1985a,b).

Now compare the above with the corresponding behaviour on esterase activity under identical mixing conditions (Figures 4.1 and 4.2). The trend is the same; i.e. 2,2'-dithiodipyridine and methyl 2-pyridyl disulfide have least effect on activity, methyl 4-pyridyl disulfide more, while disulfiram and methyl diethylthiocarbamyl disulfide are again potent inhibitors.

We are assuming here that only groups A and B are involved with these modifiers (Section 4.4.1). The advantage in using the above mixed disulfides is that all attach a sterically identical $-SCH_3$ label. On this basis, the foregoing can easily be accommodated in terms of differing extents of reaction at A. Disulfiram and methyl diethylthiocarbamyl disulfide represent extreme cases where A is exclusively (or almost exclusively) modified, and strong inhibition is observed for both activities. However, in going from methyl diethylthiocarbamyl disulfide through to methyl 2-pyridyl disulfide the extent of reaction at A becomes less, and because of this so does the extent of inhibition.

Consider now the effects of these modifiers on the dehydrogenase and esterase activities with a different mixing order. In the presence of NAD^+ 2,2'-dithiodipyridine and methyl 2-pyridyl disulfide activate by factors of 2-3 and 1.2 respectively. Methyl 4-pyridyl disulfide still inhibits but to a lesser extent than when premixed in the absence of NAD^+. Disulfiram and methyl diethylthiocarbamyl disulfide, however, are still potent inhibitors. With the esterase activity, a similar trend toward less inhibition in the presence of cofactor is also observed for methyl 4-pyridyl disulfide and methyl 2-pyridyl disulfide. With this mixing order the former has a less pronounced inhibition profile (Figure 4.4) while the latter has almost no effect (Figure 4.3).

These findings can again be explained in terms of a general partitioning between groups A and B. In the presence of cofactor any shift towards less inhibition arises from more modification at B and less at A. For methyl 2-pyridyl disulfide reaction at B appears to be optimal under these circumstances because there is hardly any effect on ester hydrolysis. At relatively high concentrations of this modifier, however, it is possible that some reaction does occur at A. This may account for the slight reduction in activity at relatively high concentrations (Figure 4.3).

Another observation that is consistent with the above model is the steeper inhibition profile for methyl 4-pyridyl disulfide when it is added to enzyme premixed with methyl 2-pyridyl disulfide (Figure 4.6). According to our argument, in the presence of coenzyme methyl 4-pyridyl disulfide inhibits because it non-specifically reacts with A and B. Therefore, premodification of B with methyl 2-pyridyl disulfide should increase the percentage of A modified by methyl 4-pyridyl disulfide. The expected reduction in activity agrees with the findings in Figure 4.6.

Interestingly, there is one finding in the present research where a modifier has contrasting effects on the two activities, namely: under the same mixing conditions where 2,2'-dithiodipyridine activates the dehydrogenase pathway, inhibition is still observed for ester hydrolysis (Figure 4.3). It was concluded from the previous Section that 2,2'-dithiodipyridine, like methyl 2-pyridyl disulfide, is seen to exclusively modify group B. Why then are the effects of these two modifiers so different?.

In answer to this, it was mentioned previously that the advantage in using methyl 2-pyridyl disulfide, methyl 4-pyridyl disulfide, and methyl diethylthiocarbamyl disulfide is that all attach a $-SCH_3$ label. However, this is not the case with 2,2'-dithiodipyridine. The mere fact that this modifier attaches a 2-thiopyridyl sidechain introduces possible steric complications when comparisons are made with methyl 2-pyridyl disulfide. Thus although both may react with group B any differences can be rationalised in terms of the nature of the attached labels. Consider the following

Scheme 4.2

Scheme 4.2 shows a generalised steady state pathway in which substrate binds to enzyme, E, to form an intermediate species, ES. Generally speaking, there are two ways in which an attached label can affect the observed activity. It can either alter the affinity of the enzyme for its substrate, or affect the nature of k_2 which governs the breakdown of ES to products (or both). As an example, suppose that the incorporated label increases both the K_M and the size of k_2. Furthermore, assume that high concentrations of substrate can overcome this decreased affinity and still saturate the active site.

In this scenario if the substrate concentration is relatively low (that is, $k_1S << k_2$) then the limiting process will be the rate at which substrate binds. Because modified enzyme has a decreased affinity for the enzyme, inhibition will be observed experimentally. On the other hand, under conditions where substrate is saturating ($k_1S >> k_2$) ES will predominate in the steady state despite the increased K_M. As the rate determining step now depends entirely on k_2 (which is increased in the modified enzyme) activation occurs.

The foregoing is exactly what is observed for 2,2'-dithiodipyridine when premixed with enzyme in the presence of NAD^+. At low acetaldehyde

concentrations (< 30 μM) the dehydrogenase activity is inhibited. This can be interpreted as a steric phenomenon whereby the 2-thiopyridyl label physically hinders the access of acetaldehyde, in much the same way as it hinders disulfiram (Kitson, 1979). At higher acetaldehyde concentrations, however, 2,2'-dithiodipyridine is an activator. Here, acetaldehyde has overcome these indirect effects by saturation of the active site. Because the K_M no longer has any effect experimentally, activity is now largely dictated by NADH dissociation. 2,2'-Dithiodipyridine activates because it increases the rate of this process. The same reasoning can also apply to methyl 2-pyridyl disulfide except that the smaller activation observed here may simply reflect the smaller size of the $-SCH_3$ sidechain.

The model presented above can also be used to explain inhibition of esterase activity. In the present study the assay concentration of 4-nitrophenyl acetate (34 μM) is low enough to make substrate binding rate-limiting (the K_M under these conditions is 40 μM; Kitson, 1986d). Therefore, any increase in K_M by 2,2'-dithiodipyridine will always lead to inactivation. In any case, because 4-nitrophenyl acetate is larger than acetaldehyde it is possible that a 2-thiopyridyl label presents a more imposing steric threat. In fact the K_M may be elevated so much that 4-nitrophenyl acetate can never realistically saturate, and inhibition will always occur. On the other hand, a $-SCH_3$ at group B may not be large enough to significantly affect the binding of 4-nitrophenyl acetate. This would explain the lack of inhibition for methyl 2-pyridyl disulfide (Figure 4.3).

4.4.3 Relevance of the working model to the one-site/two-site debate

As mentioned in the introduction to this Chapter there is a continuing lively debate over whether the dehydrogenase and esterase activities are mediated by either one or two active sites. The two-site model was first proposed by MacGibbon *et al.* (1978b) to explain several apparent differences in these two activities. One of these is the finding that aldehyde oxidation is very sensitive to stoichiometric concentrations of disulfiram. In contrast, identical levels of this modifier have little effect on an ongoing esterase assay.

Since the first suggestion of a two-site mechanism numerous reports have emerged in the literature which present supportive kinetic evidence. For the

purposes of this discussion, however, only the salient features of the one-site/two-site model and the relevance to the working model (Section 4.4.2) will be considered. A more detailed analysis into the background kinetic evidence will be covered in the next Chapter.

Consider firstly the opposing effect of disulfiram when added to ongoing dehydrogenase and esterase assays. In a two-site model this is easily accommodated in terms of interaction at P2 by 4-nitrophenyl acetate and disulfiram. As the latter is reacting at a site positionally distinct from P1, there is no inherent reason why aldehyde should protect against its inhibitory effect. On the other hand, saturation of P2 with 4-nitrophenyl acetate protects the enzyme because disulfiram is physically denied access to its usual position.

However, this behaviour with disulfiram does not unequivically support a two-site model because it can still be preserved in terms of a one-site framework. An advocate would argue that the contrasting effects of disulfiram reflect kinetic differences in the two pathways. The rationale here is based simply on the different rate-limiting steps for dehydrogenase and esterase activities: NADH dissociation (Blackwell *et al.*, 1987) and acyl hydrolysis (MacGibbon *et al.*, 1978b) respectively. For ester hydrolysis the active site is saturated and therefore protected. For aldehyde oxidation, however, the major species in the steady state is the binary $^{*}E_{NADH}$ complex (Scheme 1.2). Disulfiram can inhibit because the same active site is now vacant.

A finding in the present study which also is equally explained by both models is the lack of protection against methyl diethylthiocarbamyl disulfide by 4-nitrophenyl acetate. Despite saturation of the active site by this ester, methyl diethylthiocarbamyl disulfide may be able to compete for its usual site more effectively than disulfiram because it is sterically smaller. Interaction may be at the point where 4-nitrophenyl acetate is bound to the enzyme but where acylation has yet to occur.

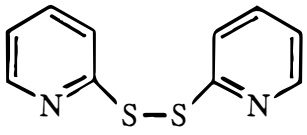
In some respects the one-site and two-site models are very similar. For example, both can keep open the possibility that "A" is essential for esterase activity. Consequently, any effects on 4-nitrophenyl acetate hydrolysis, such as the lack of protection against methyl diethylthiocarbamyl disulfide, can be explained equally from both viewpoints.

The inherent difference in the models, however, is that for the single active site model "A" may be catalytically essential for both activities. Modification of this residue will always lead to inhibition of both aldehyde oxidation and 4-nitrophenyl acetate hydrolysis. In a two-site interpretation "A" is not essential for dehydrogenase activity, and any subsequent effect must neccessarily be indirect.

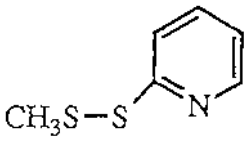
In this Chapter the present findings are consistent with a working model where modification of "A" always results in inhibition for both activities. In the light of the above discussion it is clear that this is naturally accommodated by the simpler common site viewpoint. For a two-site interpretation there is no *a priori* reason why this should be so. For example, why should methyl diethylthiocarbamyl disulfide and disulfiram be equally potent inhibitors of aldehyde oxidation when they attach sterically different labels (Kitson & Loomes, 1985a)? Rather, if inactivation is mediated by steric hindrance of the aldehyde substrate then a smaller -SCH_3 label should have less effect on activity.

In summary, the 'working model' discussed in this Chapter is an inherent requirement of the one-site model. On the other hand, to preserve the concept of two distinct sites additional constraints must be introduced which are themselves incompatible experimentally. For this reason it is concluded that a common site best explains the observed findings.

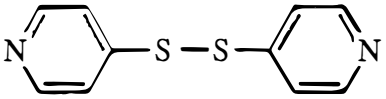
Figure 4.7 Structures of Compounds Referred to in Discussion



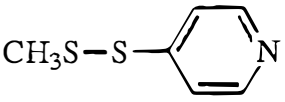
2,2'-dithiodipyridine



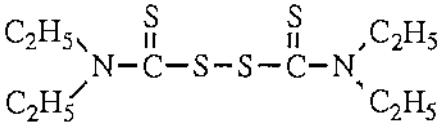
methyl 2-pyridyl disulfide



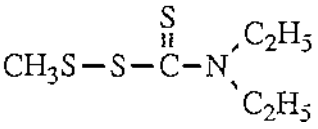
4,4'-dithiodipyridine



methyl 4-pyridyl disulfide

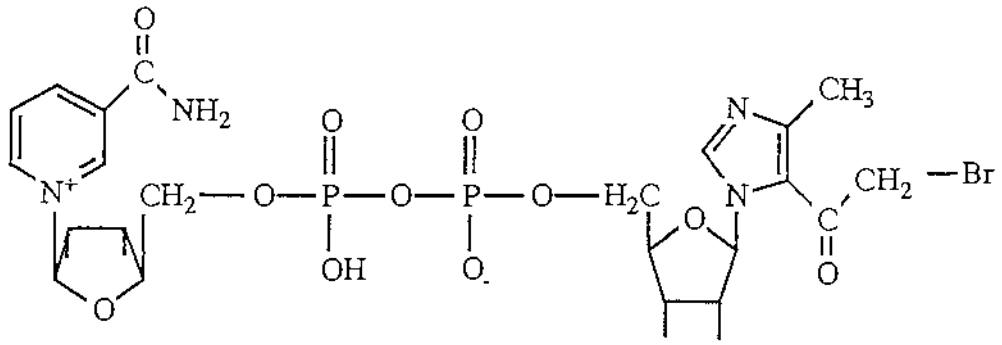


disulfiram

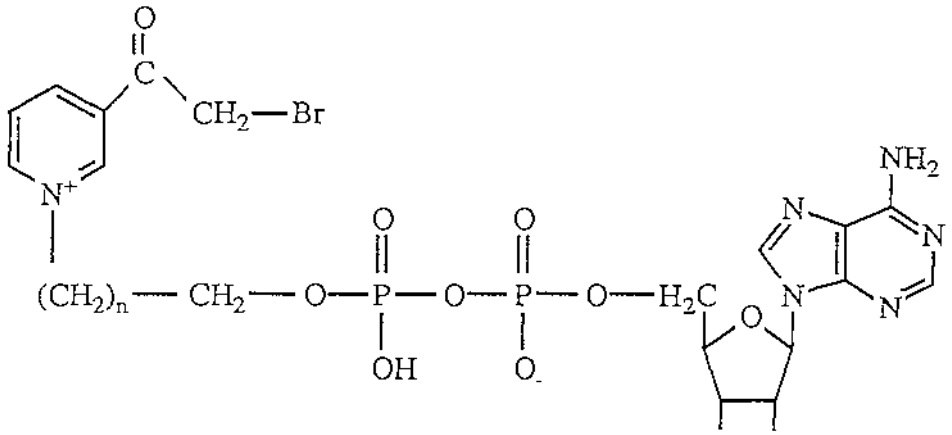


methyl diethylthiocarbamyl disulfide

Figure 4.8 Coenzyme Analogues



Nicotinamide 5-bromoacetyl-4-methylimidazole dinucleotide



3-Bromoacetylpyridinoalkyldiphosphoadenosine

CHAPTER 5

DETECTION OF ACETALDEHYDE FROM AN ESTERASE SYSTEM CONTAINING ALDEHYDE DEHYDROGENASE, 4-NITROPHENYL ACETATE, AND NADH

5.1 Introduction

In the previous Chapter it was concluded that a common active site for both dehydrogenase and esterase activities best explains the observed findings. This Chapter approaches the one-site/two-site debate from a kinetic perspective with an experiment designed to detect acetaldehyde from an esterase system containing aldehyde dehydrogenase, 4-nitrophenyl acetate, and NADH.

A common feature of both dehydrogenase and esterase activities is the formation of an acyl intermediate along the respective pathways. In a one-site model, because these activities are mediated by a common active site, the acyl intermediate formed along the esterase pathway in the presence of NADH will be identical to the acyl intermediate, $^{acyl}E_{NADH}$, from aldehyde oxidation (Scheme 1.2). Consequently, this intermediate might undergo hydride transfer to liberate acetaldehyde and NAD^+. Detection of these by-products from such an esterase system would provide convincing experimental support for the model.

That the dehydrogenase pathway is partially reversible is readily demonstrated by other acylating reagents such as acetic, propionic, or butyric anhydride (Hart & Dickinson, 1978a; Motion, 1986). In the presence of NADH the acyl species which is formed can undergo an internal reduction by NADH to form the corresponding aldehyde. Experimentally, this can be followed spectrophotometrically by monitoring the rate of NADH oxidation at 340 nm. The main problem using 4-nitrophenyl acetate, however, is that spectral analysis is very difficult because of simultaneous 4-nitrophenoxide production at 400 nm. Consequently, other methods must be devised to detect acetaldehyde.

Acetaldehyde identification was claimed by Duncan (1979) using rabbit liver aldehyde dehydrogenase. Using a procedure which involved the selective

destruction of NADH, and the extraction of unhydrolysed 4-nitrophenyl acetate and 4-nitrophenol, he was able to demonstrate the presence of NAD^+ by alkali-induced fluorescence. However, the study suffered in that acetaldehyde was not assayed directly, but only inferred from the greater amount of NAD^+ produced when yeast alcohol dehydrogenase was added to the reaction mixture. Also problematical were the spurious levels of NAD^+ in the control systems which were sometimes 25 % those of the complete reaction mixture.

The uncertainty over whether dehydrogenase substrates are produced from 4-nitrophenyl acetate was compounded by the findings of Motion (1986). Here acetaldehyde was trapped by flushing aliquots of the reaction mixture through an automated acetaldehyde detection system (Stowell *et al.*, 1978). Although acetaldehyde was detected with acetic anhydride using this method, with 4-nitrophenyl acetate none was produced within the limits of detectability (1-2 % of the initial ester concentration).

In view of these conflicting results, and the insight such information can provide, it is the aim of the present study to unequivocally determine whether acetaldehyde is produced from a reaction mixture containing aldehyde dehydrogenase, NADH, and 4-nitrophenyl [^{14}C]acetate. The strategy is to allow any [^{14}C]acetaldehyde which may be produced to distil spontaneously into a semicarbazide trap to form [^{14}C]acetaldehyde semicarbazone.

5.2 Experimental

To detect acetaldehyde a reaction mixture containing aldehyde dehydrogenase (0.80 μM), 4-nitrophenyl [^{14}C]acetate (1.1 mM), NADH (83 μM), and 35 mM sodium phosphate buffer, pH 8.0, was incubated at 30 ^{o}C for 2 hours in the outer compartment of a Conway diffusion unit (the values in brackets referring to the final concentration). The total volume was 4.5 ml. The inner compartment contained 1.0 ml of a solution of semicarbazide (0.1 M) and potassium acetate (0.18 M). The diffusion units were sealed with a greased glass plate.

In addition to the complete reaction mixture (I) the following controls were carried out in tandem, namely: (II) without NADH; (III) without enzyme; (IV) enzyme previously inactivated by disulfiram (13 μM); and (V) where 4-nitrophenyl [^{14}C]acetate was allowed to undergo complete spontaneous hydrolysis (which was confirmed spectrophotometrically) before the addition of enzyme and NADH (and semicarbazide to the inner well).

After the 2 hour incubation period 0.1 ml aliquots of the semicarbazide solutions were assayed for radioactivity and the remaining inner well contents freeze-dried. The pooled material from three of each type of reaction mixture was then extracted with 4.0 ml of ethanol, the extract concentrated to a small volume, and loaded onto a plastic backed sheet of silica gel (Merck Kiesel 60 F_{254}, 3.5 cm x 10 cm). The chromatogram was then developed with an eluting solvent of ethanol/ethyl acetate (1:1 v/v). The solvent was evaporated and the chromatogram cut into transverse sections corresponding to different R_F values. Each piece was assayed for radioactivity by scraping the silica gel into scintillation vials. In a duplicate experiment with the complete reaction mixture the silica gel corresponding to a R_F of 0.7 was extracted with ethanol. The solvent was then removed, and the residue examined by mass spectrometry.

5.3 Results

Table 5.1 shows the radioactivity trapped in reaction mixtures (I) to (V). Each value represents a separate Conway diffusion unit. In all cases the amount of incorporated radioactivity was greater in the complete reaction mixture than in any of the controls. These experiments were repeated a number of times, and although there was minor variation in the absolute levels of radioactivity, the results were always qualitatively the same.

To investigate whether the higher levels in the complete reaction mixture were due to trapped acetaldehyde, the inner well contents were analysed by t.l.c, the results of which are shown in Figure 5.1. Interestingly, all reaction mixtures exhibit a peak with a R_F value from 0.1 to 0.4, corresponding to that of authentic potassium acetate. This could arise by diffusion into the inner well by: (1) a radioactive impurity in the substrate; (2) acetic acid which is the normal hydrolysis product of 4-nitrophenyl [^{14}C]acetate; and (3) 4-nitrophenyl [^{14}C]acetate itself which is subsequently hydrolysed.

Of these possibilities, diffusion by acetic acid seems unlikely because at pH 8.0 the amount of this present in the unionised form will be negligible. The most likely reason is diffusion of the ester, and this is supported by two observations. Firstly, the freeze-dried material from the inner well was always yellow in colour, indicating the presence of 4-nitrophenoxide. Secondly, there is a correlation between the magnitude of the acetate peak in Figure 5.1 and the extent of ester hydrolysis. That is, the peak is largest when there is no catalysed hydrolysis of 4-nitrophenyl [^{14}C]acetate ((III) and (IV)); smaller when there is catalysed hydrolysis ((I) and (II)); and minimal under conditions where the ester is prehydrolysed ((V)).

The most striking feature to note from Figure 5.1, however, is the presence of a radioactive peak at a R_F of 0.6 - 0.7 for the complete reaction mixture. This is not exhibited by any of the controls, and the peak position corresponds to the R_F of authentic acetaldehyde semicarbazone. Again, repetition of these experiments always gave the same result. The identity of this peak was further confirmed by mass spectrometry where a molecular ion was observed at 101.0585 (calculated for $C_3H_7N_3O$ = 101.0589).

The amount of [^{14}C]acetaldehyde semicarbazide in Figure 5.1 indicates that at least 0.22 % of the esterase pathway can be diverted into the dehydrogenase pathway. Presumably, this represents a lower limit because not all the acetaldehyde produced may diffuse to the inner well. For instance, some may be reoxidised to acetate by the enzyme's normal dehydrogenase activity using the produced NAD^+ as a cofactor.

Table 5.1 Radioactivity Trapped in Semicarbazide Solution after Incubating 4-Nitrophenyl [^{14}C]acetate under Various Reaction Conditions

Reaction Mixture *	Radioactivity + (disintegrations min $^{-1}$)
I Complete (enzyme/NADH/ester)	490 449 481
II Control 1 (enzyme/ester)	71 90 108
III Control 2 (NADH/ester)	166 276 180
IV Control 3 (inactivated enzyme/ NADH/ester)	152 229 179
V Control 4 (pre-hydrolysed ester/ enzyme/NADH)	69 73 65

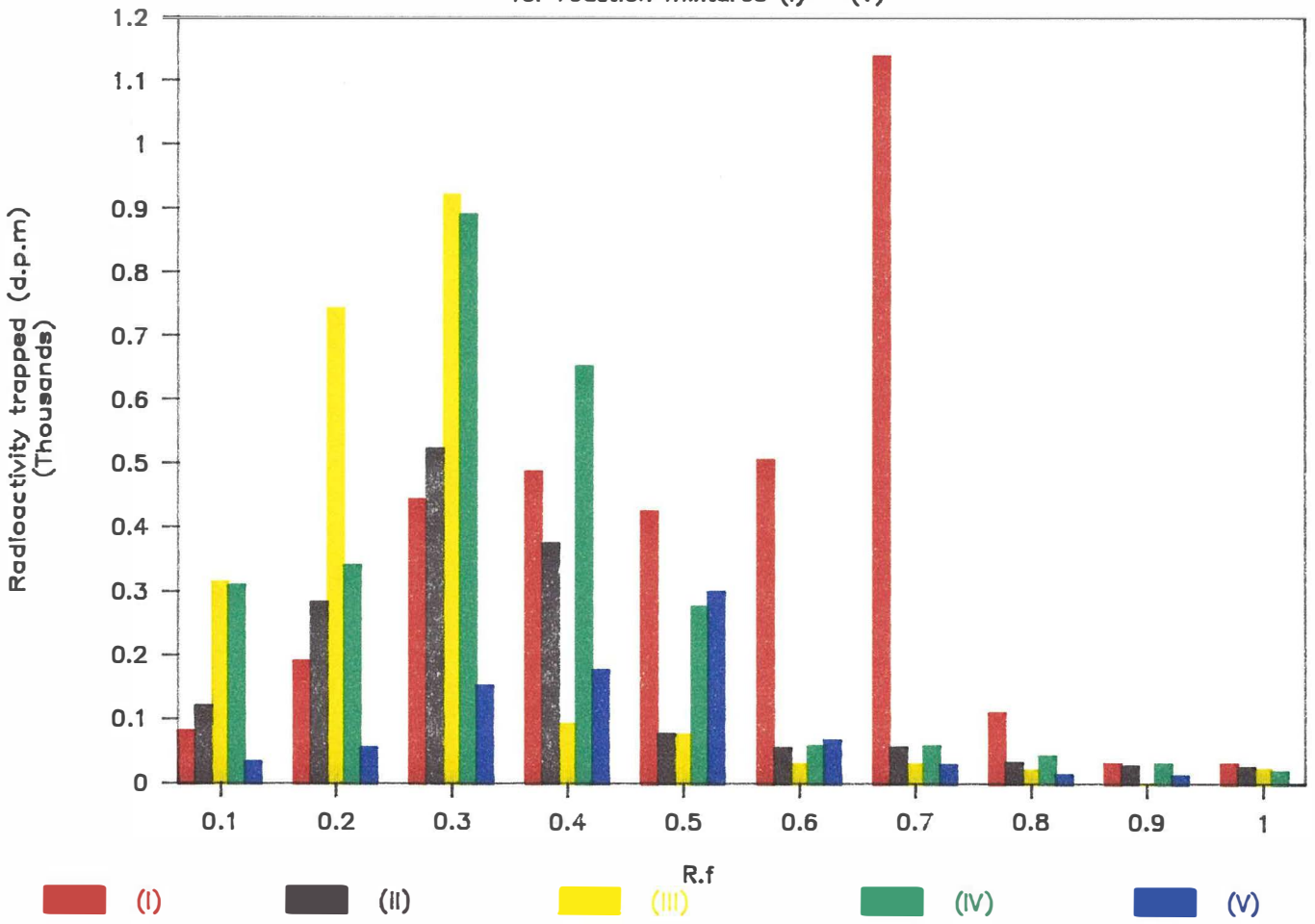
* for details see text

+ each figure refers to an individual reaction mixture

The pooled material from three of each type of reaction mixture ((I) - (V)) was extracted with 4 ml of ethanol. The extract was concentrated and loaded onto a plastic backed sheet of silica gel (Merck Kiesel 60 F_{254}, 3.5 cm x 10 cm) and the chromatogram developed with an eluting solvent of ethanol/ethyl acetate (1:1 v/v). Figure 5.1 shows the radioactivity profile after the chromatogram was cut into transverse sections corresponding to different R_F values.

Figure 5.1 T.L.C Profiles

for reaction mixtures (I) – (V)



5.4 Discussion

The one-site/two-site debate

The natural assumption that aldehyde oxidation and 4-nitrophenyl acetate hydrolysis are mediated by a common active site is supported by a number of kinetic observations. For example, both propionaldehyde and glyceraldehyde are competitive inhibitors of esterase activity in the absence of coenzyme (Feldman & Weiner, 1972b) and chloral hydrate is competitive with respect to both activities in the presence of NAD^+ (Sidhu & Blair, 1975a).

In later studies the one-site model was challenged, and a large body of kinetic evidence presented supporting the existence of two distinct sites for these activities - called P1 and P2 (Blackwell *et al.*, 1983). The two-site model proposed that aldehyde oxidation and 4-nitrophenyl acetate hydrolysis could only occur at P1 and P2 respectively. Furthermore, P2 played an additional regulatory role in the sense that it could reversibly bind high concentrations of aldehyde and glyoxylic acid, and was also the target site of disulfiram, and 2,2'-dithiodipyridine.

The two-site model in turn was criticised by Duncan (1985) who argued that the evidence supporting this viewpoint was still consistent with a common active site, particularly: (1) the partial competitive inhibition of 4-nitrophenyl acetate hydrolysis by low concentrations of propionaldehyde in the presence of NAD^+; (2) the noncompetitive inhibition of the dehydrogenase activity by glyoxylic acid; and (3) the findings relating to the phenomenon of substrate activation by aldehyde.

The underlying reasons why these findings can be interpreted in apparently two different ways will now be considered. To begin with, consider first the steady state inhibition experiments with 4-nitrophenyl acetate and propionaldehyde in the presence of NAD^+. Experimentally, inhibition of 4-nitrophenyl acetate hydrolysis by propionaldehyde is competitive but partial (Blackwell *et al.*, 1983). That is, aldehyde and ester initially compete for E_{NAD^+}, but as the aldehyde concentration becomes saturating (at approximately 100 μM) the steady state rate reaches a limit. Presumably, under these conditions the steady state concentration of E_{NAD^+} is zero.

The conclusion to draw from the foregoing is that 4-nitrophenyl acetate can be hydrolysed by a dehydrogenase intermediate other than E_{NAD^+}. The two candidates which can potentially fulfil this role are shown in Scheme 5.1. Here, propionaldehyde binds to E_{NAD^+} with a K_M of 1.1 μM, and undergoes hydride transfer to form $^{P1}E_{NADH}$. The "P1" in $^{P1}E_{NADH}$ represents the active site at which aldehyde oxidation occurs, and indicates that it is presently occupied by an acyl group. This acyl intermediate is then hydrolysed to the binary complex E_{NADH} with a first order rate constant, k_1. For convenience, it is assumed that the concentration of NAD^+ is saturating so that virtually no free enzyme exists in the steady state. Consequently, dissociation of NADH from E_{NADH} is essentially irreversible with a rate constant, k_2.

According to Scheme 5.1, because propionaldehyde and 4-nitrophenyl acetate both react with E_{NAD^+} a Lineweaver-Burk inhibition plot with 4-nitrophenyl acetate as the variable substrate will be competitive in nature. That is, propionaldehyde will increase K_{APP} but saturating ester will overcome this, and V_{MAX} will remain unchanged. To account for the partial nature at saturating propionaldehyde 4-nitrophenyl acetate must be hydrolysed by either $^{P1}E_{NADH}$ or E_{NADH}. If $k_1 < k_2$ then $^{P1}E_{NADH}$ will be the predominant species in the steady state, and the one responsible for the hydrolysis of 4-nitrophenyl acetate. As Blackwell *et al.* (1983) point out, if ester can bind and form a subsequent acyl intermediate with $^{P1}E_{NADH}$ then another active site, P2, must be involved because P1 is already occupied. The intermediate produced in this case will be $^{P1,P2}E_{NADH}$ (Scheme 5.1). This is the essence of the two-site model. Alternatively, if $k_1 > k_2$ then E_{NADH} will be the dehydrogenase intermediate which catalyses the oxidation of 4-nitrophenyl acetate. In this scenario P1 is now free, and consequently there is no reason against 4-nitrophenyl acetate reacting there. This preserves the contention that esterase activity and aldehyde oxidation are mediated by the same active site (Scheme 5.1).

Precisely the same argument applies to the finding where glyoxylic acid non-competitively inhibits the dehydrogenase pathway at low concentrations of propionaldehyde (Blackwell *et al.*, 1983; Deady *et al.*, 1985). The intercept effect can again be interpreted as glyoxylic acid reversibly binding to either $^{P1}E_{NADH}$ or E_{NADH} depending on k_1 and k_2. For the former, inhibition

occurs because $^{P1,P2}E_{NADH}$ has a decreased rate of acyl hydrolysis. For the latter it is the dissociation of NADH from $^{P1}E_{NADH}$ which is affected (Scheme 5.2)[1].

Substrate activation by aldehyde can also be rationalised from both one-site and two-site viewpoints. Generally speaking, activation can only be experimentally observed if the rate limiting step in aldehyde oxidation is increased. In a two-site framework ($k_1 < k_2$) high concentrations of aldehyde must interact with $^{P1}E_{NADH}$ at P2 (Scheme 5.3). In this case P2 acts as a modifier site which increases acyl hydrolysis to an extent where NADH dissociation becomes rate limiting (Blackwell *et al.*, 1983). On the other hand, a one-site model ($k_1 > k_2$) would propose abortive complex formation between aldehyde and E_{NADH}. Activation occurs because NADH dissociation is increased (Scheme 5.3).

Other findings which are easily accommodated by both models are the effects of high aldehyde concentrations on the binding of various reagents. For instance, the reaction rate between disulfiram and enzyme, or between 2,2'-dithiodipyridine and enzyme, is significantly slowed in the presence of 20 mM propionaldehyde (Kitson, 1985). Furthermore, high concentrations of aldehyde competitively inhibit 4-nitrophenyl acetate hydrolysis (Blackwell *et al.*, 1983) and also displace glyoxylic acid (Deady *et al.*, 1985). For the latter, substrate activation is observed . In a two-site model these interactions would be interpreted as 4-nitrophenyl acetate, glyoxylic acid, disulfiram, 2,2'-dithiodipyridine, and high aldehyde all reacting at P2. For a one-site model where E_{NADH} is involved, all still react at P1.

The foregoing clearly shows that the crucial distinction between the one-site/two-site models lies with the values of k_1 and k_2. Apart from this both are internally consistent, and both account equally well for the observed findings. According to the current literature it is generally accepted that acyl hydrolysis is much faster than NADH dissociation i.e. $k_1 > k_2$ (see Section 1.5). In the light of this it appears that E_{NADH} is the major steady state species, and hence the

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1 - Because glyoxylic acid is not a substrate for aldehyde dehydrogenase, "P2" and "P1" in $^{P1,P2}E_{NADH}$ and $^{P1}E_{NADH}$ respectively play the role of modifier sites.

one involved in the interactions discussed above. Consequently, this preserves the concept that both activities are mediated by a common active site. Strictly speaking, however, we are still left with the possibility that another site, P2, is involved in the hydrolysis of 4-nitrophenyl acetate. This is because there is no *a priori* reason why ester should react at P1 merely because it is vacant.

A convincing way to support the common involvement of P1 would be to detect acetaldehyde from a reaction mixture containing aldehyde dehydrogenase, 4-nitrophenyl acetate, and NADH. In a one-site model, and under these conditions, both dehydrogenase and esterase pathways would converge on a common $^{acyl}E_{NADH}$ intermediate (Scheme 5.4). Consequently, an equilibrium could be set up where esterase flux is diverted into the dehydrogenase pathway to liberate acetaldehyde and NAD^+. The resting assumption here is that hydride transfer can only occur if these acyl species are identical. That is, it would be unlikely that bound NADH could reduce an acyl intermediate different from that formed along the dehydrogenase pathway.

In the present research, results show that [^{14}C]acetaldehyde is indeed produced from ester hydrolysis in the presence of NADH. It could be argued that the trapped acetaldehyde arises from a reversal of the normal dehydrogenase pathway. In this case, 4-nitrophenyl acetate is still hydrolysed at P2 but reduction only occurs with the acyl intermediate formed with acetate at P1. Although acyl hydrolysis is considered essentially irreversible (Duncan & Tipton, 1971) this possibility was investigated in view of the low levels of acetaldehyde detected. However, the present findings discount this as an explanation because no acetaldehyde is produced when aldehyde dehydrogenase, NADH, and prehydrolysed ester are incubated (reaction mixture (V), Figure 5.1).

The most important point to note from the present study is the simple and unequivocal identification of acetaldehyde. This alone provides convincing evidence to support the intermediacy of a common active site for both esterase and dehydrogenase activities. To preserve a two-site model it would have to be concluded that 4-nitrophenyl acetate is still largely hydrolysed at P2, but that a very small amount of flux can now occur at P1. To support this a proponent would focus on this finding, namely: the low levels of acetaldehyde which are

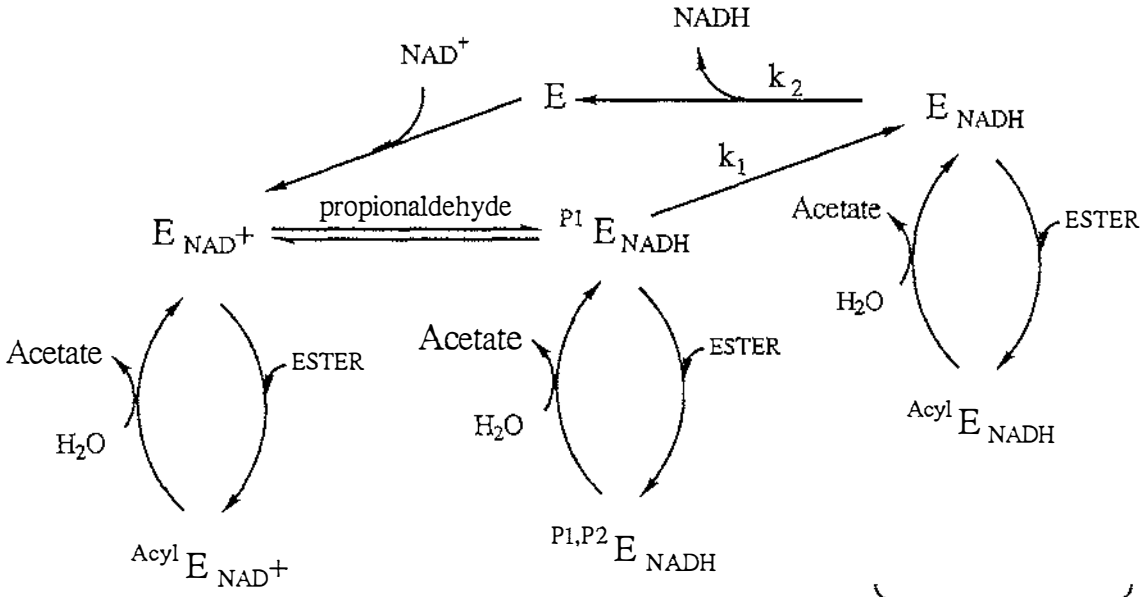
produced from the esterase pathway (Motion *et al.*, 1988) but the apparent ease with which aldehyde is formed from acetic anhydride (Hart & Dickinson, 1978a).

In terms of a one-site model this observation can be counter-argued in the following way. Consider the experimental system where 4-nitrophenyl acetate and acetic anhydride are separately added to reaction mixtures containing aldehyde dehydrogenase and NADH. Generally speaking, the absolute amounts of acetaldehyde subsequently produced in each case will depend solely on the total production of $^{acyl}E_{NADH}$ during the course of the reaction. In the presence of saturating NADH this in turn will depend only on the concentrations of 4-nitrophenyl acetate and acetic anhydride. For instance, a 2-fold increase in ester will double both the flux through $^{acyl}E_{NADH}$ and the concentration of acetaldehyde produced.

According to the literature, the initial concentrations of 4-nitrophenyl acetate and acetic anhydride used in these experiments were 320 μM (Motion *et al.*, 1988) and 16.3 mM (Hart & Dickinson, 1978a) respectively. The argument here is that NADH oxidation can be monitored spectrophotometrically with acetic anhydride only because the time course is long enough for significant buildup of NAD^+ to occur. On the other hand, with much lower starting concentrations of 4-nitrophenyl acetate the steady state is comparitively short before the reaction is over. Consequently, although the rate of acetaldehyde production may be identical to that with acetic anhydride, the absolute amount of acetaldehyde formed is only just detectable within experimental limits.

In summary, although P2 may still be involved in the hydrolysis of 4-nitrophenyl acetate there is no conclusive evidence in the foregoing discussion to support its existence. On the other hand, the detection of acetaldehyde does convincingly support the intermediacy of a common active site in both dehydrogenase and esterase activities. The one-site/two-site question will be discussed further in Chapter 8 in the light of labelling studies with *trans*-4-N,N-dimethylamino-cinnamoyl imidazole (see Chapter 7).

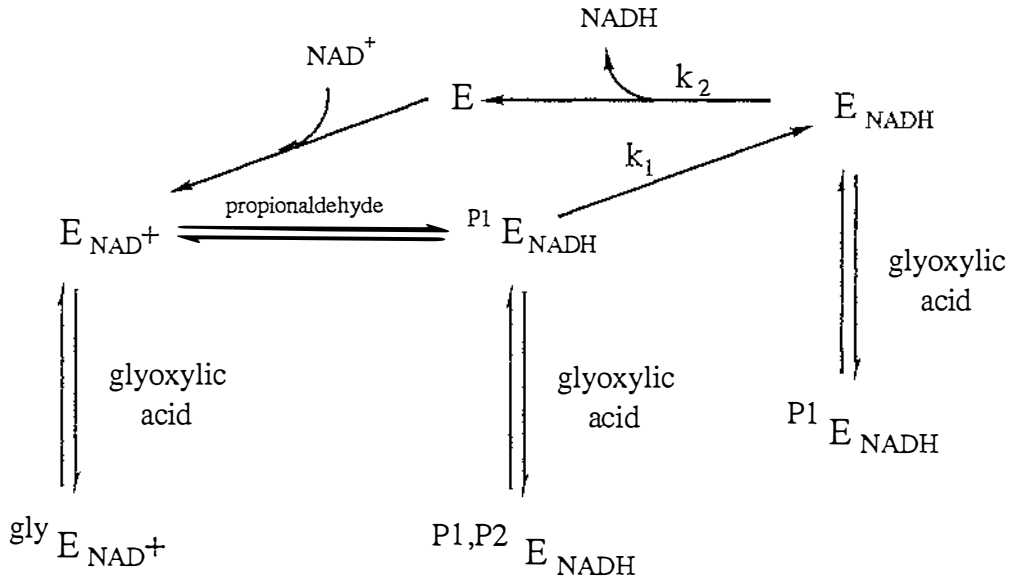
Scheme 5.1 Partial Competitive Inhibition of Esterase Activity by Propionaldehyde in the Presence of NAD^+



One site if $k_1 > k_2$
Duncan (1985)

Two site if $k_1 < k_2$
Blackwell *et al.* (1983)

Scheme 5.2 Non-Competitive Inhibition of Ester Hydrolysis by Glyoxylic Acid



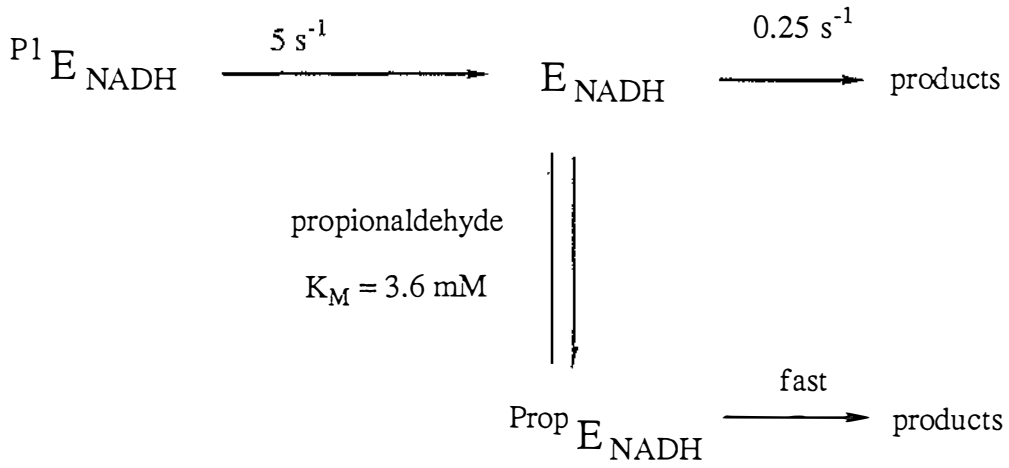
One site if $k_1 > k_2$

Duncan (1985)

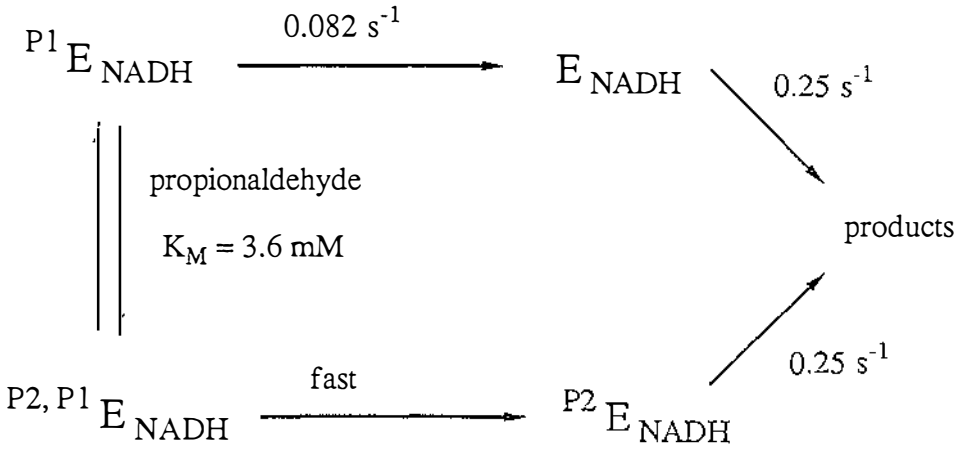
Two site if $k_1 < k_2$

Deady *et al.* (1985)

Scheme 5.3 Substrate Activation in Terms of One-Site and Two-Site Models

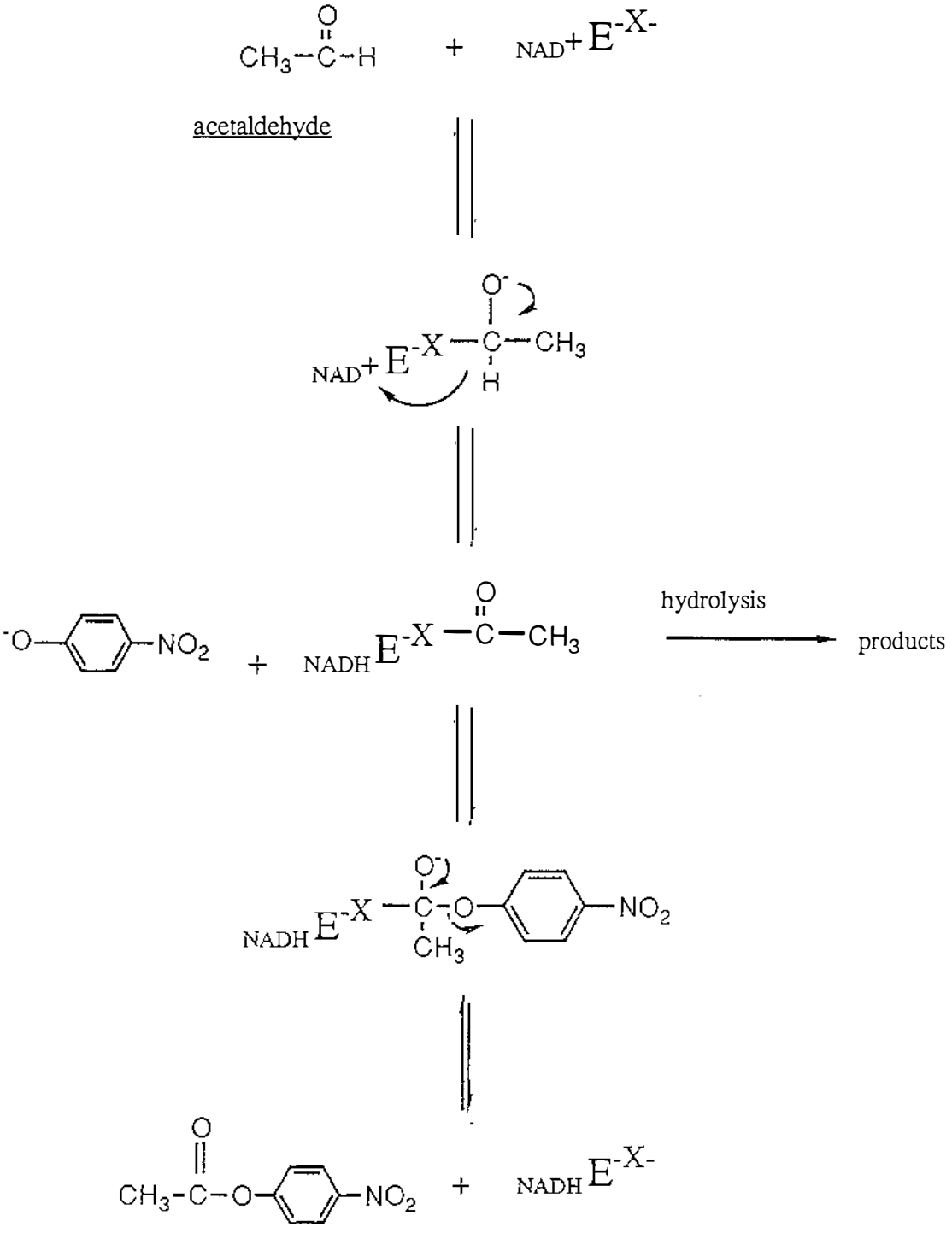


One Site Model



Two Site Model

Scheme 5.4 Convergence of the Dehydrogenase and Esterase Pathways on a Common Acyl Intermediate



CHAPTER 6

THE DISULFIRAM-SENSITIVE GROUP

6.1 Introduction

Iodoacetamide, like disulfiram, is an irreversible inactivator of dehydrogenase activity, and is thought to modify the active site on the basis of a number of supportive observations. For instance, the rate of carboxamidomethylation is enhanced in the presence of NAD^+ but slowed by aldehyde substrates and chloral hydrate (Hempel & Pietruszko, 1981). [^{14}C]Iodoacetamide specifically modifies a cysteine residue in a 35 amino acid tryptic peptide (Hempel *et al.*, 1982a) now known to correspond to Cys-302 in the complete primary structure (Hempel *et al.*, 1984).

In Chapter 4 it was mentioned that disulfiram reacts with aldehyde dehydrogenase in a biphasic manner involving initial modification of group A. In a slower process, the attached diethyldithiocarbamyl label is subsequently displaced by group B to form an inactive disulfide species (see Scheme 4.1). At the present time, Cys-302 is implicated as being group A on the basis that disulfiram blocks the incorporation of [^{14}C]iodoacetamide (Hempel *et al.*, 1982a). However, this finding suffers in that [^{14}C]iodoacetamide was not added to the reaction mixture until the disulfiram-modified enzyme was dialysed overnight by which time group B is also blocked. Furthermore, a 4-fold[1] excess of disulfiram was incubated initially with the enzyme, even though a 2-molar ratio is sufficient to inactivate almost totally (Kitson & Loomes, 1985a,b; Vallari & Pietruszko, 1982).

In the present study it is the aim to investigate the interaction between disulfiram and [^{14}C]iodoacetamide in more detail. Firstly, aldehyde dehydrogenase is labelled with [^{14}C]disulfiram in an attempt to identify the modified cysteine residue. Secondly, the incorporation of [^{14}C]iodoacetamide is monitored under

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1 - the term "-fold" used thoughout this Chapter is defined in terms of the enzyme tetramer molar concentration

various conditions where the enzyme has been premodified with various thiol modifiers.

6.2 Experimental

The gel filtration columns described in this Section were visually checked for their ability to separate large and small molecules by using blue dextran and 4-nitrophenoxide. Elution only took 3-4 minutes. The incorporation of [^{14}C]disulfiram and [^{14}C]iodoacetamide were both calculated per tetramer of enzyme.

6.2.1 Labelling with radioactive disulfiram: To 0.4 ml of enzyme stock (20 μM) in 50 mM sodium phosphate buffer, pH 7.4, at 25 ^{o}C, a molar equivalent (per enzyme tetramer) of [^{14}C]disulfiram was added (as 15 μl from a solution made up in 95 % ethanol). After a specified incubation period, enough solid urea was added to give a final concentration of 8 M, and the reaction mixture was passed down a Bio-Gel P-6 column (0.8 cm x 10 cm) equilibrated with 8 M urea. A 1.5 ml fraction was collected immediately after the void volume and a 0.1 ml aliquot sampled for radioactivity. Alternatively, no urea was added, and the reaction mixture instead passed down a Bio-Gel P-6 column (0.8 cm x 10 cm) equilibrated in 50 mM sodium phosphate buffer, pH 7.4. In other experiments, iodoacetamide (80-fold excess) or methyl diethylthiocarbamyl disulfide (100-fold excess) was added after disulfiram. On another occasion, enzyme was preincubated with NAD^+ (1 mM) and then modified with a 3-fold excess of methyl 2-pyridyl disulfide before the addition of disulfiram.

6.2.2 Incorporation of [14-C]iodoacetamide: Solutions of 1.6 ml, each containing enzyme (10 μM) and NAD^+ (1 mM) in 50 mM sodium phosphate buffer, pH 7.4, were incubated at 25 ^{o}C. To each mixture, a 2 or 4-fold excess of either disulfiram, methyl diethylthiocarbamyl disulfide, or methyl 2-pyridyl disulfide was added. A control was also included where this step was omitted. At a specified time either a 4 or 20 fold excess of [^{14}C]iodoacetamide was added (15 μl on a glass nail) to each reaction mixture. At various intervals

0.2 ml aliquots were withdrawn from each mixture, and passed down a Bio-Gel P-6 column (0.8 cm x 10 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.4. A 0.4 ml fraction was collected immediately after the void volume and assayed for radioactivity. In addition to this, the dehydrogenase activity of the control reaction mixture was monitored concurrently by the standard assay (Section 2.2.3).

6.3 Results

6.3.1 Labelling with [14-C]disulfiram: The results of the labelling experiments with [^{14}C]disulfiram are shown in Table 6.1. Each entry refers to the percentage of label incorporated calculated on the basis that all the [^{14}C]disulfiram added initially reacts with group A. In preliminary experiments [^{14}C]disulfiram was incubated with aldehyde dehydrogenase, and after 5 minutes small molecules were removed by gel filtration (reaction mixture I). In the absence of urea, 42 % of the label was incorporated - a value which is similar to that reported by Kitson (1983) under similar conditions. In its presence, however, a significant loss in bound radioactivity occurs.

In an attempt to reduce this loss, after the addition of disulfiram other unprotected enzyme thiols were modified with an excess of iodoacetamide or methyl diethylthiocarbamyl disulfide (reaction mixtures II and III respectively). Alternatively, the enzyme was premodified with methyl 2-pyridyl disulfide in the presence of 1 mM NAD^+, and disulfiram and methyl diethylthiocarbamyl disulfide added later (reaction mixture IV). For the latter experiment the enzyme precipitated on the addition of methyl diethylthiocarbamyl disulfide. Consequently, the precipitate was spun down, washed several times with water, and redissolved in 8 M urea. Radioactivity was then measured by assaying a small fraction before and after gel filtration; these values are listed in Table 6.1 as 6.5 % and 1 % respectively.

The rationale behind these experiments was that denaturation may expose other normally unreactive enzyme thiol groups which could subsequently displace the diethyldithiocarbamyl label. Consequently, the strategy was to reduce this possibility by protecting the remaining thiols with an excess of either

iodoacetamide or methyl diethylthiocarbamyl disulfide. In addition to this, it was thought that the yield in labelled material could be increased if group B (which also displaces the label) could be premodified with methyl 2-pyridyl disulfide in the presence of NAD^+. Under these conditions, reaction at B is optimised (see Chapter 4). However, findings indicate that despite these measures there is still a dramatic loss in bound radioactivity when the enzyme is denatured with 8 M urea (Table 6.1).

6.3.2 Reaction of [14-C]iodoacetamide with aldehyde dehydrogenase: Figure 6.1 shows the incorporation of a 20-fold excess of [^{14}C]iodoacetamide in the presence of 1 mM NAD^+ under various premodification conditions. The important observation to note is that a significant decrease in the rate of reaction only occurs with a 4-fold excess of either disulfiram or methyl diethylthiocarbamyl disulfide. With a 2-fold excess of the latter the data points fall on the same curve as those for the native enzyme.

To investigate the initial stages of the reaction in more detail, these experiments were repeated with a 4-fold excess of [^{14}C]iodoacetamide. The effect on the dehydrogenase activity shown in Figure 6.2 is virtually identical with the finding of Hempel & Pietruszko (1981) under similar conditions. For instance, there is a correlation between the incorporation of [^{14}C]iodoacetamide and the loss in activity; maximum inhibition occurs at a limit of approximately 1.5 molecules per enzyme tetramer. It was considered that inhibition may in part be due to a slow decline in activity of the enzyme on standing. However, a control where enzyme was incubated with 1 mM NAD^+ discounts this as a possibility because there is almost no loss in activity over a period of 24 hours (Figure 6.2).

The findings obtained when a 4-fold excess of [^{14}C]iodoacetamide is added to enzyme premodified with various thiol modifiers are shown in Figure 6.3. Interestingly, a 2-fold excess of either disulfiram, methyl diethylthiocarbamyl disulfide, or methyl 2-pyridyl disulfide only slows the rate of reaction with [^{14}C]iodoacetamide. The total amount which reacts, which is approximately 1.5 molecules per enzyme tetramer, remains unaffected.. Only with a 4-fold excess of methyl diethylthiocarbamyl disulfide do both the rate and amplitude diminish significantly. In this case, approximately 0.5 molecules of the radioactive label per tetramer is incorporated after 24 hours. Although not carried out, a 4-fold

excess of disulfiram would also be expected to fall on this latter curve on the basis of the observed similarity of these modifiers in Figure 6.1.

Table 6.1 Incorporation of [^{14}C]Disulfiram under Various Mixing Orders

ORDER OF MIXING	% INCORPORATION OF RADIOACTIVITY	
	8 M UREA	NO UREA
(I) Enzyme, [^{14}C]disulfiram (5 min)*, Bio-Gel	5.5	42
(II) Enzyme, [^{14}C]disulfiram (2 min)*, iodoacetamide (5 min)*, Bio-Gel	14	60
(III) Enzyme, [^{14}C]disulfiram (2 min)*, methyl diethylthiocarbamyl disulfide (15 min)*, Bio-Gel	2	5
(IV) Enzyme, NAD^+ (1 mM), methyl 2-pyridyl disulfide (5 min)*, [^{14}C]disulfiram (2 min)*, methyl diethylthiocarbamyl disulfide	1	6.5

* refers to the incubation time before gel filtration.

Figure 6.1 shows the incorporation of a 20-fold excess of [^{14}C]iodoacetamide at 25 ^{o}C under various premodification conditions, namely: native enzyme (○); a 2-fold excess of methyl diethylthiocarbamyl disulfide (Δ); and a 4-fold excess of disulfiram (□) and methyl diethylthiocarbamyl disulfide (●). All reaction mixtures contained 1 mM NAD^{+}.

Figure 6.1 Incorporation of a 20-Fold Excess of [^{14}C]Iodoacetamide under Various Premodification Conditions

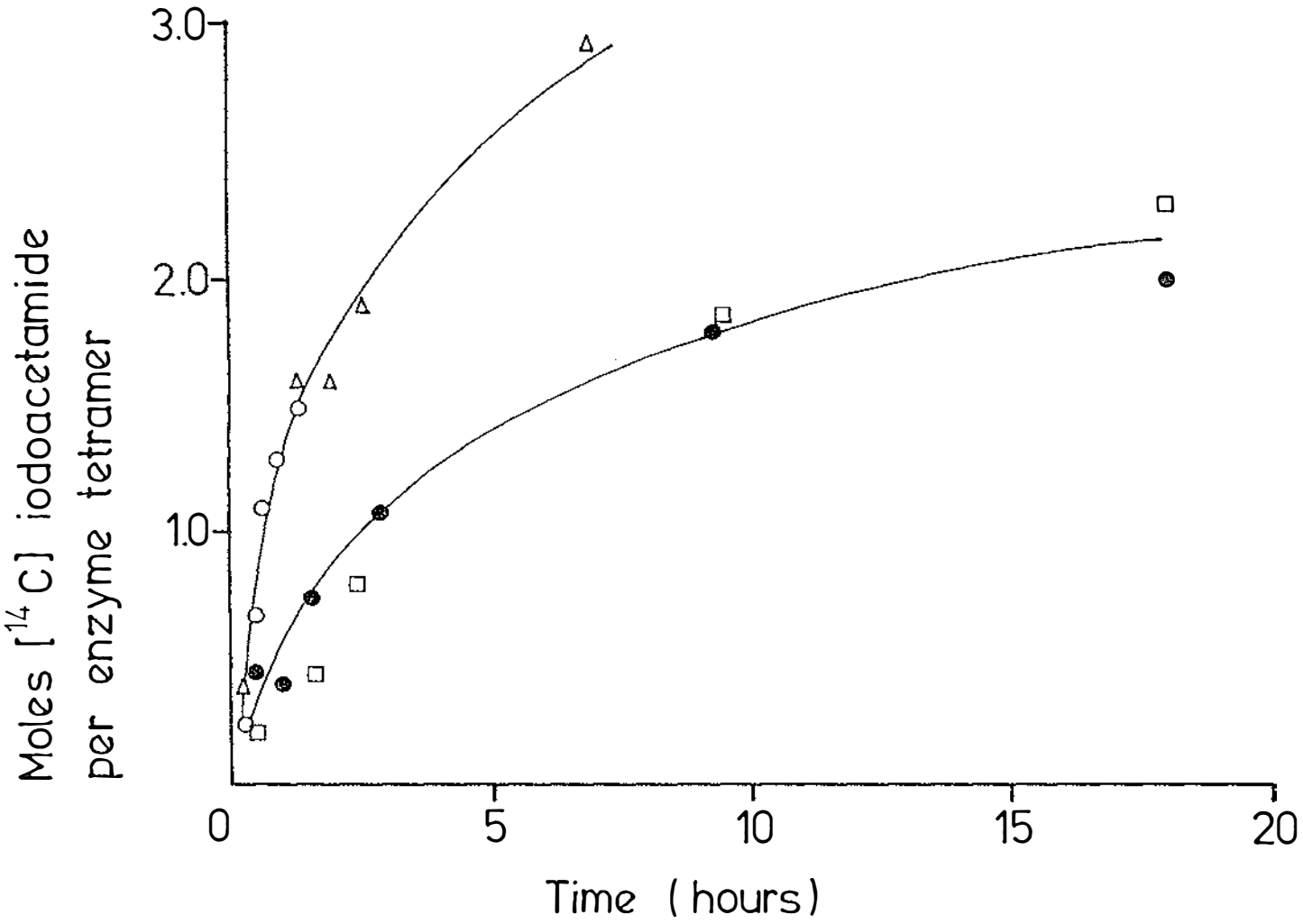


Figure 6.2 shows the inactivation of aldehyde dehydrogenase by a 4-fold excess of iodoacetamide in the presence of 1 mM NAD^+ at 25 ^oC. Incorporation of the radioactive label (○) was calculated by assaying the reaction mixture before and after gel filtration for radioactivity. In addition to this the dehydrogenase activity (●) was monitored by the standard assay (Section 2.2.3). The activity of a mixture containing 10 μM enzyme and 1 mM NAD^+ at 25 ^oC was also monitored (■).

Figure 6.2 Inactivation of Aldehyde Dehydrogenase by a 4-Fold Excess of [^{14}C]Iodoacetamide

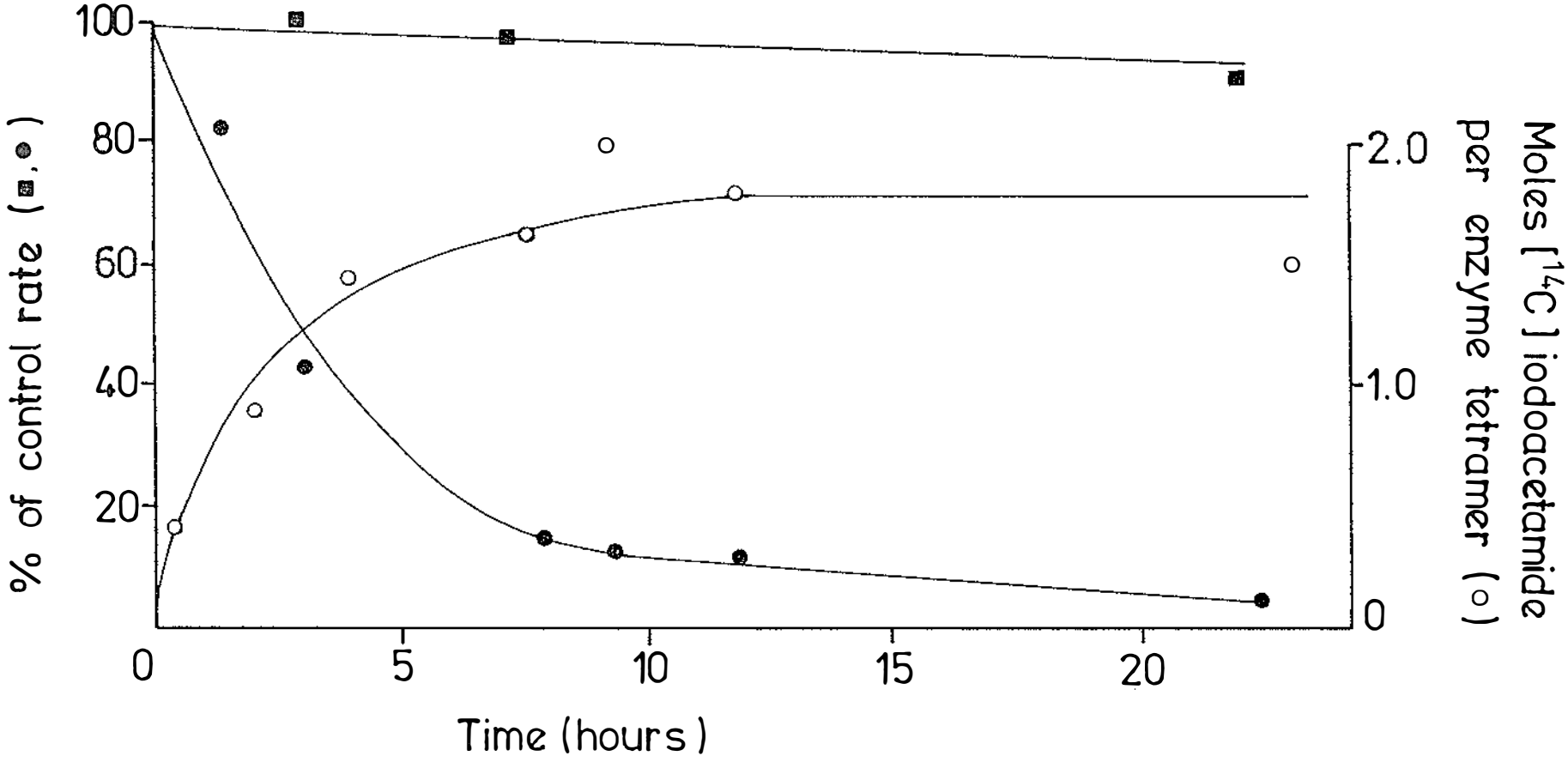
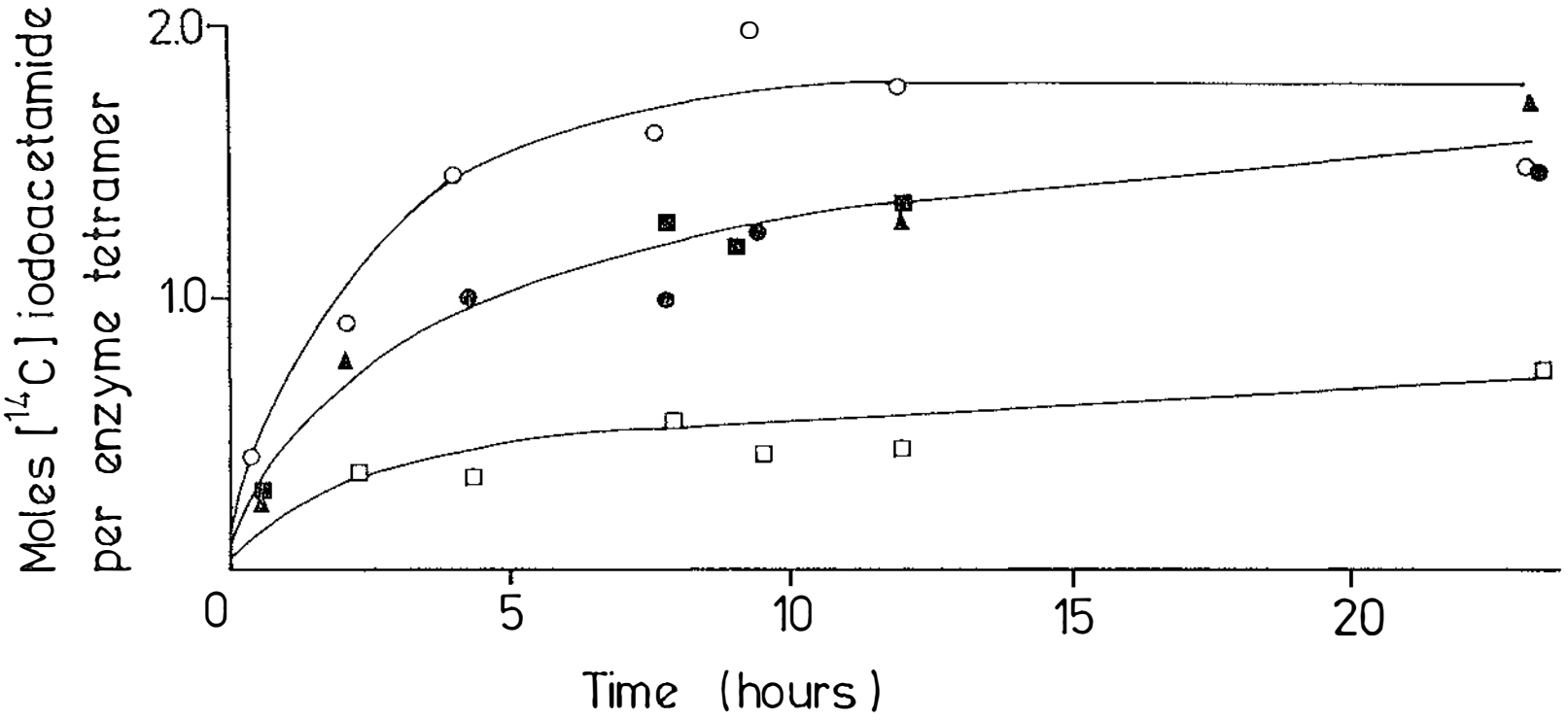


Figure 6.3 shows the incorporation of a 4-fold excess of [^{14}C]iodoacetamide at 25 ^{o}C under various premodification conditions, namely: native enzyme (○); a 2-fold excess of disulfiram (■), methyl 2-pyridyl disulfide (●), and methyl diethylthiocarbamyl disulfide (▲); and a 4-fold excess of methyl diethylthiocarbamyl disulfide (□). All reaction mixtures contained 1 mM NAD^{+}.

Figure 6.3 Incorporation of a 4-Fold Excess of [^{14}C]Iodoacetamide under Various Premodification Conditions



6.4 Discussion

Is the disulfiram-sensitive group Cys-302 ?

Attempts to identify the group modified by disulfiram have been plagued with the problem that the diethyldithiocarbamate-enzyme adduct is unstable. For instance, Vallari & Pietruszko (1982) found no bound radioactivity when aldehyde dehydrogenase was incubated with a 2-fold molar excess of disulfiram (per tetramer) for 20 hours at 25 ^{o}C. That this is due to disulfide formation was supported by the findings that the enzyme possessed fewer free thiol groups after dialysis, and full catalytic activity could be restored by the addition of 2-mercaptoethanol. Kitson (1983) subsequently showed that this displacement reaction is much slower than the initial modification of group A (Scheme 4.1).

In the present study it was the aim to label the enzyme with [^{14}C]disulfiram (at a molar stoichiometry equal to the tetramer concentration) and after a short incubation period separate unbound radioactivity by gel filtration. After denaturation, the enzyme would be digested with trypsin and the labelled peptide isolated by gel filtration and HPLC. However, findings indicate that the [^{14}C]diethyldithiocarbamyl label is too unstable to survive the denaturation step with 8 M urea. Consequently, the percentage of labelled material is too small to be carried through subsequent isolation procedures.

In the light of the inability to label aldehyde dehydrogenase with [^{14}C]disulfiram, a more indirect approach was adopted which involved investigating the interaction between [^{14}C]iodoacetamide and disulfiram. As mentioned previously, Cys-302 is implicated to be group A on the finding that disulfiram prevents the incorporation of [^{14}C]iodoacetamide (Hempel *et al.*, 1982a). In the present study, findings indicate that a 2-fold excess of either disulfiram, methyl diethylthiocarbamyl disulfide, or methyl 2-pyridyl disulfide slows the reaction rate with [^{14}C]iodoacetamide but does not affect the total amount incorporated after 24 hours. Furthermore, it is only with a 4-fold excess of disulfiram or methyl diethylthiocarbamyl disulfide that the amplitude decreases significantly (Figures 6.1 and 6.3).

These results are clearly at odds with the concept that both disulfiram and iodoacetamide react initially with group A. If this was the case then pre-

modification with a 2-fold excess of disulfiram should effectively convert group A to a disulfide species incapable of further reaction. Consequently, both the rate and amplitude of iodoacetamide incorporation will diminish concertedly (Scheme 6.1). What is found experimentally, however, is that the rate of reaction is slowed but the amplitude remains unchanged after 24 hours (Figure 6.3). This suggests that iodoacetamide does not react with A at all, but with some other group which we will call "C". In this scenario, the only effect of reaction at A or B is simply to hinder the access of [^{14}C]iodoacetamide to C (Scheme 6.2). The reduction in amplitude with a 4-fold excess of disulfiram or methyl diethylthiocarbamyl disulfide can be explained if under these conditions reaction at A is complete, and modification now occurs at group C (Scheme 6.2). This would explain the protection observed by Hempel *et al.* (1982a).

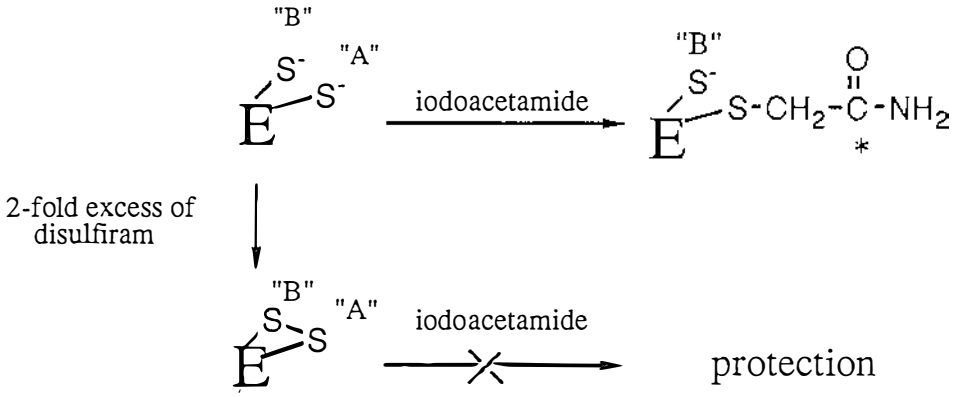
The existence of a group C which can be modified by disulfiram is supported by the finding that with more than a 2-fold excess of disulfiram, the enzyme begins to incorporate label irreversibly (Pietruszko *et al.*, 1982). The argument here is that the residues modified by a 2-fold excess of disulfiram - namely A - can possess the correct stereochemistry to be completely displaced by B. Obviously, if the additional groups modified by a 4-fold excess are also A, then displacement by B should be observed here as well. The finding that these latter modified residues are stable (Pietruszko *et al.*, 1982) suggests, therefore, that they are not A.

Interestingly, this is precisely the reasoning which led to the conclusion that disulfiram does not initially modify A in the mitochondrial enzyme (see Section 3.4.1). Here, it was argued that disulfiram preferentially reacts elsewhere because access to it normal target site, thiol A, is hindered. Modification of this other group - which we will now propose is "C" - is responsible for the assertion by Sanny & Weiner (1987) that the disulfiram-sensitive group is not catalytically essential (see Chapter 3).

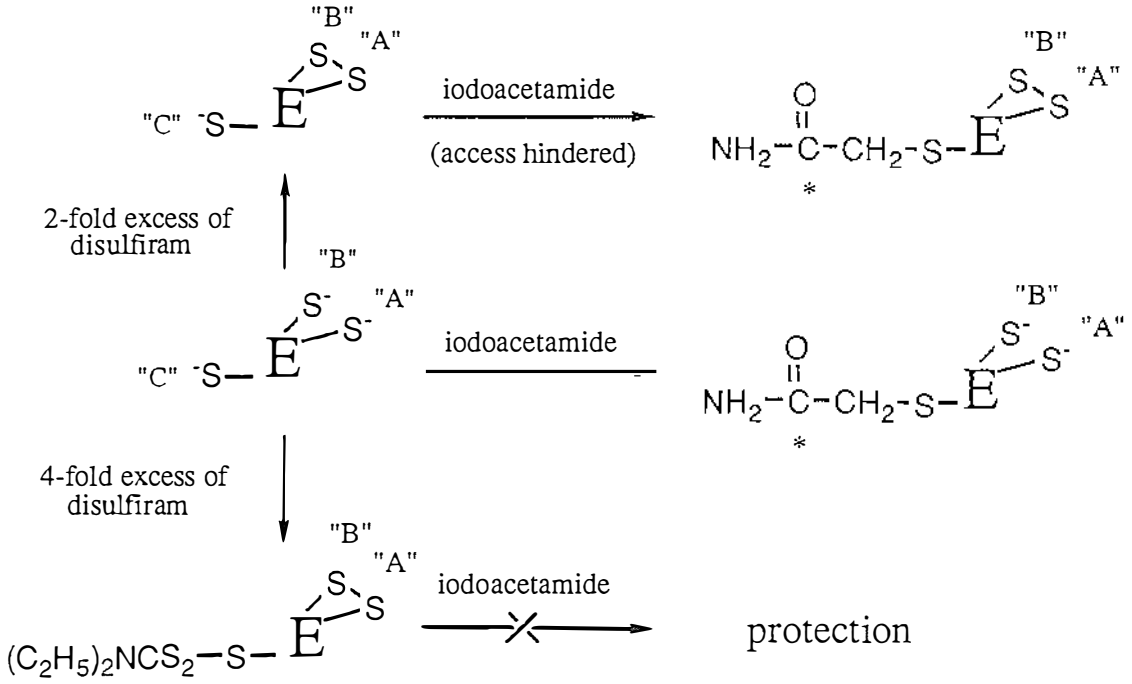
In summary, the finding of Hempel *et al.* (1982a) that disulfiram protects the enzyme against [^{14}C]iodoacetamide does not provide convincing evidence that both react with Cys-302. This is aptly demonstrated in the present study where a 2-fold excess of disulfiram - enough to convert all of group A to an internal disulfide - does not affect the extent of reaction with [^{14}C]iodoacetamide. For

this reason it is concluded that Cys-302 may be group C, and group A still remains to be identified.

Scheme 6.1



Scheme 6.2



CHAPTER 7

IDENTIFICATION OF THE ACTIVE SITE PEPTIDE CONTAINING THE CATALYTIC NUCLEOPHILE

7.1 Introduction

Although aldehyde dehydrogenase is well studied kinetically, relatively little is known about structural aspects of the active site. The tertiary structure remains to be solved, and the known primary structures have provided few clues concerning regions of functional importance (Hempel & Jornvall, 1987). In fact, the lack of sequence homology between aldehyde dehydrogenase and other enzymes has led only to tentative identifications of general features such as regions involved in coenzyme binding (Hempel & Jornvall, 1989).

One important unanswered question is the identity of the catalytic nucleophile. This is the group covalently attached to the aldehyde during oxidation, and its role is analogous to that played by Ser-195 in chymotrypsin or Cys-149 in glyceraldehyde-3-phosphate dehydrogenase. Previous studies have suggested Cys-302 as the likely candidate for this group. It resides at the active site (von Bahr-Lindstrom *et al.*, 1985) and is implicated in the reaction with disulfiram (Hempel *et al.*, 1982a).

More recently, however, a number of reports have emerged in the literature implicating other enzyme residues as possibly fulfilling this role. For instance, Pietruszko & MacKerell (1987) show that Glu-268 is specifically modified by bromoacetophenone, and conclude it is functionally important at the active site. Furthermore, Tu & Weiner (1988a) propose that Cys-49 is catalytically essential for dehydrogenase activity on the basis of their modification studies with N-ethylmaleimide.

The best way to identify the catalytic nucleophile is by isolating a true pathway intermediate using a chromophoric or radioactive substrate. For instance, these techniques have been used with success in the identification of Ser-195 in α-chymotrypsin (Noller & Bernhard, 1965) and the spectrophotometric characterisation of Cys-149 in glyceraldehyde-3-phosphate dehydrogenase

(Malhotra & Bernhard, 1968). In the present study, it is the intention to identify the catalytic nucleophile for aldehyde dehydrogenase by trapping a pathway intermediate formed during the oxidation of *trans*-4-N,N-dimethylaminocinnamaldehyde. The denatured labelled enzyme is digested, and the tryptic peptides separated by gel filtration and HPLC. Throughout these procedures, the chromophoric *trans*-4-N,N-dimethylaminocinnamoyl sidechain is used as a marker to follow the active site peptide.

7.2 Experimental

7.2.1 Acyl enzyme formation, isolation and digestion: A mixture containing 20 μM aldehyde dehydrogenase in 50 mM sodium phosphate buffer, pH 6.0, + 0.3 mM EDTA + 1 mM NAD^+ was incubated at 25 ^oC. *trans*-4-N,N-Dimethylaminocinnamaldehyde was then added (25 μl of a stock solution in acetonitrile) to give a 1 to 1.5 molar excess over the enzyme tetramer concentration. After approximately 30 seconds 70 % perchloric acid was added to precipitate the enzyme (3 % by volume) and the denatured material washed at least four times with water. The protein was redissolved in 8 M urea at a concentration of approximately 10 mg/ml. The solution was then carboxymethylated at 37 ^oC for two hours in the dark with enough iodoacetate to give a 20 % excess over the enzyme thiol concentration. After this period, the reaction mixture was dialysed against several changes of 0.1 M ammonium bicarbonate at 4 ^oC overnight. In some cases a control was included in tandem following exactly the above procedure but with the omission of NAD^+. Digestion was carried out with TPCK-trypsin (50:1 w/w) for 4 hours in 0.1 M ammonium bicarbonate at 37 ^oC.

In one experiment the effects of different trapping conditions were investigated. Here, the enzyme was precipitated with either $HClO_4$ or ethanol and washed in the usual way, or denatured by the addition of saturated urea. For the latter, the reaction mixture was immediately passed down a Bio-Gel-P6 column (0.7 cm x 12 cm) equilibrated in 8 M urea.

7.2.2 Peptide purification: After digestion, the material was freeze-dried and redissolved in 0.1 M ammonium bicarbonate at a concentration of approximately 20 mg/ml. This was then applied to a Superose-12 column (10 mm x 30 mm) equilibrated with 0.1 M ammonium bicarbonate in a loading volume not exceeding 250 µl. The column was eluted with this buffer, and the eluate monitored at 280 nm. The fraction which contained the *trans*-4-N,N-dimethyl-aminocinnamoyl chromophore (this was spectrally characterised by a peak with a λ_{MAX} at 408 nm) was loaded directly onto a Waters Associates radial-pac 8MBC181OH cartridge equilibrated with 0.1 M ammonium bicarbonate. A gradient was then run to an organic phase of acetonitrile, isopropanol, and 0.1 M ammonium bicarbonate (1:1:1 v/v) and the eluate monitored at 220 nm.

In one experiment, the major peak from HPLC which contained the *trans*-4-N,N-dimethylaminocinnamoyl chromophore was collected in two portions. Each fraction was subsequently loaded and repurified on either a Vydac Protein C_4 column (300 mm x 4.6 mm) or a RPC column, both equilibrated with 0.1 % formic acid in H_2O/CH_3CN (9:1 v/v). For both columns a gradient was run to solvent B which contained 0.1 % formic acid in H_2O/CH_3CN (1:4 v/v).

In a further experiment, prior to HPLC the coloured peak from the gel filtration column was redigested by the addition of thermolysin (approximately 18 % by weight). The reaction mixture was incubated at room temperature for approximately 10 minutes and then loaded onto the 8BC1810H column as before.

7.2.3 Differential labelling with [C14]iodoacetamide: The acyl intermediate was isolated and carboxymethylated in the usual way. After dialysis overnight in 0.1 M ammonium bicarbonate, concentrated ammonia (4 M)[1] was added, and the chromophore followed spectrally until the peak at 408 nm had decreased to 66 % of its initial value. The reaction mixture was then freeze-dried, the protein redissolved in 8 M urea (approximately 10 mg/ml) and incubated with a 4-fold

1 - The values in brackets in this Chapter refer to the final concentrations in the reaction mixtures.

excess of [^{14}C]iodoacetamide (per mole of enzyme) in the dark at 37 ^{o}C for two hours. All reagents were subsequently removed by dialysis, the denatured enzyme was digested, and the tryptic peptides were separated by gel filtration as before. Fractions were collected in 0.5 ml aliquots, a fifth of which was used for scintillation counting. A control was also included which was carried through the above procedure but where NAD^+ was omitted.

Alternatively, after carboxymethylation the denatured acyl intermediate was passed down a Bio-Gel-P6 column (0.7 cm x 12 cm) equilibrated with 8 M urea, and collected immediately after the void volume. A 7-fold excess of [^{14}C]iodo-acetamide (130 μM) was then added (the protein concentration was approximately 10 mg/ml) and the reaction mixture incubated at 37 ^{o}C for 72 hours. Reagents were dialysed out, and the protein digested as before. Fractions from the gel column were collected in 0.4 ml aliquots, and only a fifth of the labelled reaction mixture relative to the control (where NAD^+ had been omitted) was loaded.

7.2.4 Model compounds: The cysteine ester of *trans*-4-N,N-dimethylamino-cinnamic acid was prepared by adding N-acetyl-L-cysteine to a cuvette containing *trans*-4-N,N-dimethylaminocinnamoyl imidazole in water. The final concentrations of these reagents were 139 μM and 9.1 μM respectively. Formation of the cysteine ester was characterised by a shift in the λ_{MAX} from 424 nm to 398 nm. The product was not isolated.

To prepare acetyl-Ile-Gly-Ser(chromophore)-Pro-Trp-Arg-NH_2, *trans*-4-N,N-di-methylaminocinnamic acid (0.1 g) was dissolved in 5 ml tetrahydrofuran (THF) which had previously been distilled over NaH. One equivalent of triethylamine and of isobutyl chloroformate were added, and after 20 minutes the solution was filtered. The synthetic peptide acetyl-Ile-Gly-Ser-Pro-Trp-Arg-NH_2 (0.03 g in approximately 0.5 ml THF) which had been previously reacted with a 5-fold excess of NaH was added, and the solution lightly refluxed for 20 minutes. During this period small amounts of the reaction mixture were added to a cuvette containing THF, and the spectra taken. After cooling, the reaction mixture was added to excess water. The yellow precipitate which formed was isolated by filtration, and redissolved in THF. In one experiment colour which had adhered

to the filter paper was dissolved in THF and passed down a Bio-Gel P-6 column (0.8 cm x 10 cm) equilibrated with THF and water (3:2 v/v). The yellow colour eluted in approximately 4 minutes, and the fractions collected were analysed spectrophotometrically.

7.2.5 Enzyme-catalysed hydrolysis of trans-4-N,N-dimethylaminocinnamoyl imidazole: Aldehyde dehydrogenase (0.58 μM) was incubated in 50 mM sodium phosphate buffer, pH 7.4, at 30 ^{o}C. *trans*-4-N,N-Dimethylaminocinnamoyl imidazole was then added as either 15 μl or 25 μl of a solution in acetonitrile on a glass nail, and the catalysed reaction monitored by following the decrease in absorbance at 424 nm. In some experiments the enzyme was premixed with 1 mM NAD^{+} before the addition of *trans*-4-N,N-dimethylaminocinnamoyl imidazole. All assay rates were corrected for spontaneous hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole at that particular concentration. Stock solutions of *trans*-4-N,N-dimethylaminocinnamoyl imidazole were made up in acetonitrile.

To identify the catalytic nucleophile involved in the enzyme-catalysed hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole, enzyme (10 μM) was incubated in 50 mM sodium phosphate buffer, pH 7.4, at 30 ^{o}C. *trans*-4-N,N-Dimethylaminocinnamoyl imidazole (75 μM) was then added, and the reaction mixture incubated for a further 15 minutes. After this period perchloric acid was added, and the precipitate washed and redissolved in 8 M urea. Digestion and peptide separation was carried out identically to that described previously for the acyl intermediate from the dehydrogenase pathway (7.2.1 and 7.2.2).

7.3 Results

7.3.1 Peptide isolation: Figure 7.1a shows the spectrum of the denatured acyl intermediate in 8 M urea under different trapping conditions. Results show that adding saturated urea to the reaction mixture (spectrum 3) to isolate this species is less effective than when the enzyme is precipitated by either $HClO_4$ (spectrum 1) or ethanol (spectrum 2). For the latter, it is interesting to note that the enzyme requires several minutes to precipitate and needs a large excess of ethanol. Despite this, however, almost as much chromophore is trapped as when enzyme is rapidly precipitated by relatively small quantities of $HClO_4$. To demonstrate that an actual acyl intermediate was isolated, *trans*-4-N,N-dimethylamino-cinnamaldehyde was added to enzyme preincubated with NAD^+ and a control where NAD^+ had been omitted. Figure 7.1b shows the spectra after these reaction mixtures were redissolved in 8 M urea, and quite clearly the control (spectrum 2) has no associated colour whatsoever.

The first major obstacle encountered in the present study was the conditions used to reduce any disulfide linkages in the denatured protein. Normally, a 25 % molar excess of dithiothreitol (over the total thiol concentration) is added and the reaction mixture incubated in the dark at 37 ^{o}C for 2 hours (Hempel *et al.*, 1984). In preliminary experiments where this step was included, however, the chromophore diminished significantly over this time period (Figure 7.2a). Furthermore, the isolation procedure could be followed only as far as gel filtration; the chromophore was still associated with fraction 7 (see later) but only as a very slight shoulder. Consequently, dithiothreitol was omitted and the denatured protein directly carboxymethylated instead. Under these conditions there is little loss in the chromophore (Figure 7.2b).

One important observation in the present research is the progessive loss of the chromophore through spontaneous hydrolysis. This is illustrated in Figure 7.3 which shows the spectrum at various stages throughout isolation. Spectrum 3 is normalised with the others to take into account the change in volume which occurs on dialysis. Assuming that the ε_{MAX} of the chromophore is similar to that of *trans*-4-N,N-dimethylaminocinnamaldehyde (30,000 l mole^{-1} cm^{-1}) the percentage of labelled material immediately after trapping is approximately 25 % of the enzyme tetramer concentration. However, this value falls during

dialysis and digestion, and by the time the chromophore emerges from the gel column (Figure 7.4) the percentage is reduced to around 5 %. At the end of the final purification step (Figure 7.5) the value declines even further to approximately 1-2 %.

Figure 7.4 shows the gel filtration profile and associated spectra of the tryptic digest. The main advantages in using FPLC over more conventional gravity flow columns are that separation is faster and more efficient. For instance, the profile in Figure 7.4a was obtained in 50 minutes compared to the expected 1-2 days with a 2 metre long gravity flow column. The first peak in Figure 7.4a is undigested protein which elutes immediately after the void volume. This contained some colour but for clarity it is not shown in Figure 7.4b. The most important feature to note is that the chromophore is almost exclusively associated with fraction 7. The other unlabelled spectra in Figure 7.4b are representative of all the other fractions which were collected. In one of these there is a slight trace of colour which appears at the trailing edge of the largest peak in Figure 7.4a. However, because this is a partial digest it is likely that this corresponds to the same peptide, as in fraction 7, but in a less digested state.

Fraction 7 from Figure 7.4 was purified further by HPLC, the results of which are shown in Figure 7.5. Perhaps surprisingly, there is only one major peak in Figure 7.5a, indicating that fraction 7 is already quite pure. The most important finding, however, is that the chromophore is predominantly associated with the trailing edge of this peak (Figure 7.4b). When fraction 3 was sequenced the following peptide was identified (Figure 7.6)

Gln-Ala-Phe-Gln-Ile-Gly-Ser-Pro-Trp-Arg

The identity of the unlabelled peptide was also confirmed by FAB source mass spectometry (expected 1189, observed 1189). This peptide is well conserved in all the primary structures studied (Johansson *et al.*, 1988) and corresponds to T5 in the nomenclature of Hempel *et al.* (1984). The sequencing results in Figure 7.6 show that T5 is very pure, a finding also supported in Figure 7.7. Here, fractions 2 and 3 from Figure 7.5a were purified further by different columns

and solvent systems. The minor peak in Figure 7.7b was sequenced but the peptide did not correspond to any known primary sequence of aldehyde dehydrogenase.

7.3.2 Differential labelling and thermolysin redigestion: To investigate the possibility that the catalytic nucleophile is cysteine the chromophore was removed, and the denatured protein reacted with [^{14}C]iodoacetamide. Figure 7.8a shows the radioactivity profile from the gel column under conditions where the chromophore was removed by treatment with ammonia. Theoretically, if a cysteine is involved then on the basis of the observed absorbance change at 408 nm there will be a radioactive iodoacetamide incorporation of approximately 15,000 cpm. In Figure 7.8a this would correspond to a peak of approximately 3,000 cpm positioned around fraction 7 (Figure 7.4a). However, the results in Figure 7.8a show no difference in radioactivity profiles between the labelled reaction mixture and a control, and in particular, no radioactive peak around fractions 10, 11, and 12 (corresponding to fraction 7 of Figure 7.4a).

The disadvantage with the particular method described above is the time lag between chromophore removal and reaction of the putative exposed cysteine with [^{14}C]iodoacetamide. Potentially, the thiol group could be destroyed during this period. Consequently, no reaction with [^{14}C]iodoacetamide would occur anyway, and the conclusions drawn from Figure 7.8a would be misleading.

In an alternative experiment this possibility was eliminated by allowing the chromophore to hydrolyse spontaneously in the presence of [^{14}C]iodoacetamide. Presumably, if a cysteine is liberated under these circumstances it will react immediately with this reagent. Figure 7.9 shows the spectra of the chromophore initially and after 72 hours under these conditions. From the absorbance change at 412 nm[2] if the catalytic nucleophile is cysteine an incorporation of approximately 13000 cpm is expected. In Figure 7.8b this would correspond to a peak of 600 cpm positioned around fraction 15.

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2 - the λ_{MAX} of the chromophore is red shifted slightly from 408 nm in aqueous solution to 412 nm in 8 M urea

However, the results again show no difference between the labelled and control reaction mixtures, and hence support the conclusions inferred from Figure 7.8a.

A further experiment designed to distinguish between T5 and T^* (see discussion) involved redigesting fraction 7 from Figure 7.4a with thermolysin. Purification of this digest by HPLC, and associated spectra are shown in Figure 7.10. Although the profile is more complicated than that in Figure 7.5a, the findings clearly show the chromophore is specifically associated with fractions 5 and 6. All other spectra contained no colour and are omitted from Figure 7.10b for clarity. These coloured fractions were sequenced, and both were found to contain the following peptide (below)

Ile-Gly-Ser-Pro-Trp-Arg

This corresponds to a smaller segment of T5 and still contains Ser-74. The identity of the unlabelled peptide was again confirmed by FAB source mass spectrometry (expected 715, observed 715).

7.3.3 Model compounds and how they compare with the isolated chromophore:
Figure 7.11 shows the formation of the cysteine model ester. The preparation is straightforward and simply involves adding N-acetyl-L-cysteine (139 μM) to an aqueous solution containing *trans*-4-N,N-dimethylaminocinnamoyl imidazole (9.1 μM) at room temperature. The λ_{MAX} from of 398 nm is similar to the value of 404 nm reported by Dunn & Buckley (1985).

In contrast to the simplicity of the thiol ester preparation, many problems were encountered in the synthesis of the serine ester of the peptide identified as described above. For example, no reaction occurred when the peptide and *trans*-4-N,N-dimethylaminocinnamoyl imidazole were both dissolved in THF. On reflux, the spectrum of the reaction mixture simply changed to that expected for *trans*-4-N,N-dimethylaminocinnamic acid. Interestingly, purification of the peptide by HPLC from this "failed" reaction mixture (Figure 7.12a)[3] revealed

3 - The elution position of this peptide under these conditions is identical to that

the presence of strongly associated peaks at 350 nm (Figure 7.12b).

Subsequently, it was found that reaction does occur, but only with the inherently more reactive mixed anhydride derivative of *trans*-4-N,N-dimethylamino-cinnamic acid and isobutyl chloroformate under reflux in THF (Figure 7.13). Furthermore, strict precautions had to be taken to remove any traces of water. If this was not done, the spectral bands around 420 nm in Figure 7.13 simply disappeared. Perhaps the biggest obstacle in these experiments was the purification of the labelled peptide after the 20 minute reflux in Figure 7.13. Purification by HPLC using the same solvent systems as before (Figure 7.5) proved to be impossible because the peptide was now almost totally insoluble in 0.1 M ammonium bicarbonate. It was considered that this insolubility may in part be due to the fact that the synthesised peptide is protected at both N- and C-terminal ends. Consequently, the peptide was purified by adding the reaction mixture (after 20 minute reflux, Figure 7.13) to an excess of water. The bright yellow precipitate which formed was redissolved in THF.

At this stage an experiment was carried out in which a small amount of this stock solution was added to a cuvette containing water. Figure 7.14a shows the spectrum initially and again after 1 hour (the peptide it seems does have limited water solubility). A difference spectrum of these two shows a peak at around 400 nm which has presumably been lost during this period.

In an attempt to purify the peptide further, colour which had been absorbed onto the filter paper (from when the precipitate was originally isolated) was dissolved in THF. The solution was then passed down a Bio-Gel P-6 column equilibrated in a mixture of THF and water (3:2 v/v). The spectra of the early collected fractions are shown in Figure 7.14b. The important feature to note here is the association of an unsymmetrical chromophoric peak around 400 nm with a 280 nm peak which is presumably the peptide.

In the present study the properties of the cysteine model ester were compared with those of the isolated chromophore in Figure 7.5, and two major differences

of the native synthesised peptide.

were found. The first concerns the hydrolysis rates of these two compounds. Figure 7.15 shows the hydrolysis of the chromophore from Figure 7.5 in 30 mM sodium phosphate, pH 8.0, at 37 ^{o}C. Over a period of 10 hours the absorbance at 408 nm is reduced to 73 % of its initial value. The first order rate constant obtained from the inset in Figure 7.15b is 1.8 x 10^{-5} s^{-1}. Now compare the rate of hydrolysis found with the cysteine model ester under identical conditions (Figure 7.16). Here, there is no detectable loss in the chromophore over this time period. As a further comparison the rate of hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole is shown in Figure 7.17. This is by far the most rapidly hydrolysed with a first order rate constant of 1.43 x 10^{-4} s^{-1}.

The second difference concerns the λ_{MAX} of the chromophores. Figure 7.18 compares the peaks of the cysteine model ester (spectrum 1); the peak associated with fraction 7 in Figure 7.4 (spectrum 2); and *trans*-4-N,N-dimethylaminocinnamoyl imidazole (spectrum 3). All spectra were measured in aqueous solution. Although the λ_{MAX}'s of the cysteine ester and the isolated chromophore are similar there is, nevertheless, a reproducible difference of approximately 10 nm.

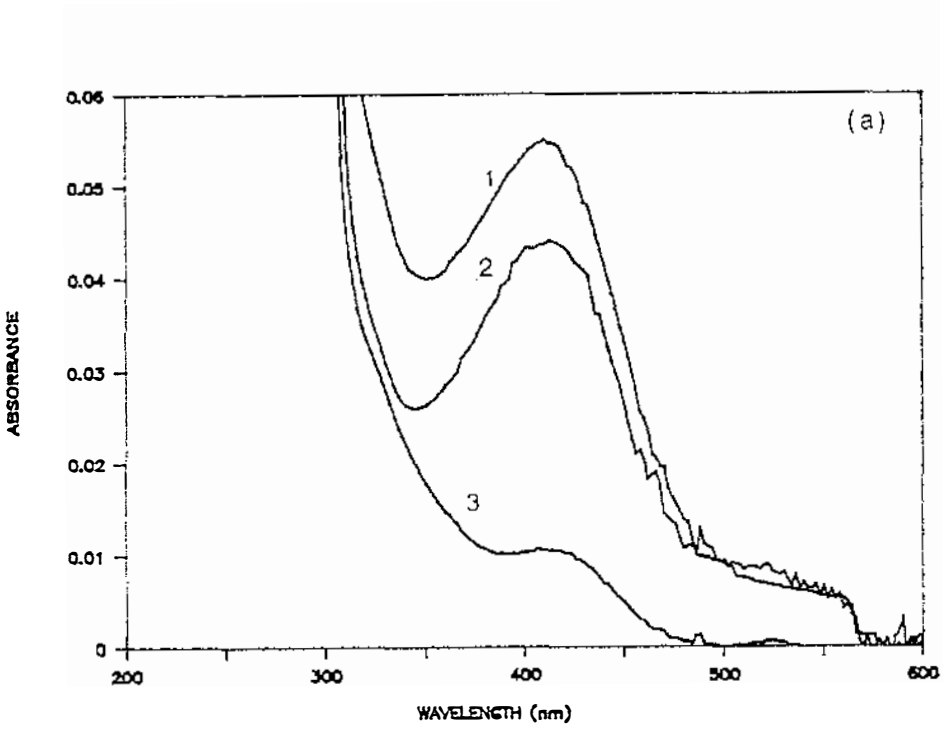
7.3.4 The enzyme-catalysed hydrolysis of trans-4-N,N-dimethylaminocinnamoyl imidazole: Figure 7.19 shows the Lineweaver-Burk plot for the enzyme-catalysed hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole. In all cases the measured assay rates were slow but linear. In the absence of NAD^+ the K_M for this substrate is 17 μM. However, in the presence of 1 mM NAD^+ both intercept and slope effects were found (Figure 7.19). Here, K_M is increased slightly to 26 μM while V_{MAX} is decreased. The latter can be interpreted as a reduction in the rate-determining step in the presence of NAD^+.

To determine whether the catalytic nucleophile for aldehyde oxidation is also involved here, the enzyme was labelled with *trans*-4-N,N-dimethylaminocinnamoyl imidazole, precipitated, and redissolved in 8 M urea. The spectrum taken at this stage (Figure 7.20a) shows the incorporation of a peak with a λ_{MAX} at approximately 412 nm. This is similar to that obtained previously in Figure 7.1 for the acyl intermediate from aldehyde oxidation. After digestion the tryptic peptides were separated by gel filtration as before, and the fractions

containing colour are shown in Figure 7.20b. The main point to note here is that these results are identical to that found previously for aldehyde oxidation (Figure 7.4). That is, the chromophore is predominantly associated with fractions 2 and 3 (Figure 7.21b) and to a lesser degree fraction 1 (Figure 7.21a). Unfortunately, the chromophore could only be followed as far as this column step because of the low levels trapped initially. For instance, further purification of fraction 3 by HPLC showed a profile identical to that in Figure 7.5, but the major peak contained no colour.

Figure 7.1a shows the spectra of the denatured acyl intermediate redissolved in 8 M urea under different trapping conditions, namely: precipitation with $HClO_4$ (spectrum 1); precipitation by the addition of ethanol (spectrum 2); and denaturation by urea (spectrum 3). Figure 7.1b compares the spectrum of a complete reaction mixture trapped with $HClO_4$ (1) and a control where NAD^+ was omitted (2).

Figure 7.1 Spectra of the Denatured Acyl Intermediate in 8 M Urea under Different Trapping Conditions



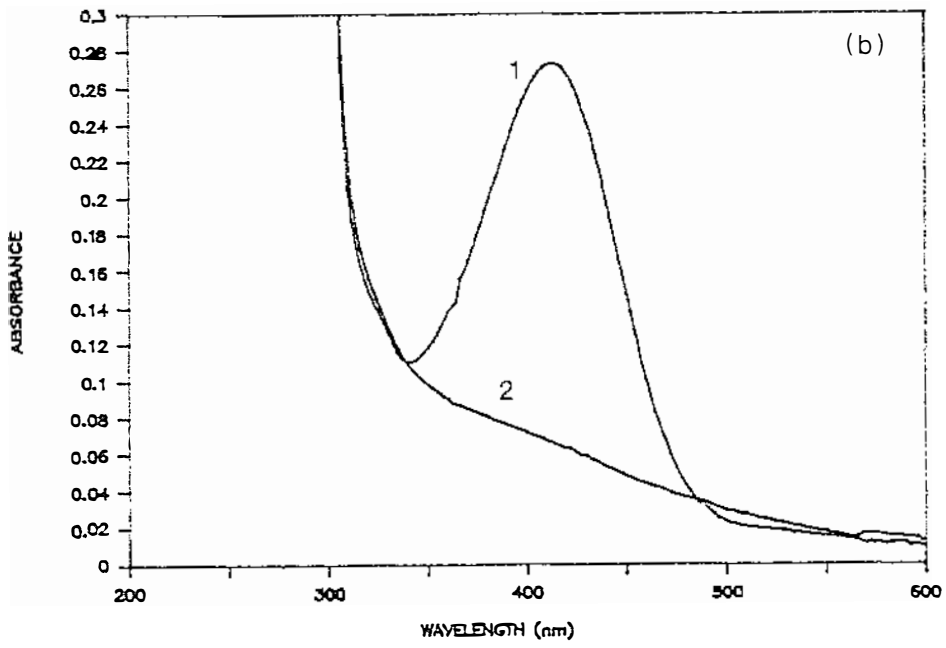


Figure 7.2a shows the spectrum of the denatured acyl intermediate (10 mg/ml) in 8 M urea at 37 ^{o}C in the presence of 5 mM dithiothreitol initially (1) and after approximately 2 hours (2). In figure 7.2b the experiment was repeated, only this time dithiothreitol was omitted from the reaction mixture. Spectra were again taken initially (1) and after 2 hours (2).

Figure 7.2 Effect of Dithiothreitol on the Chromophore Stability in 8 M Urea

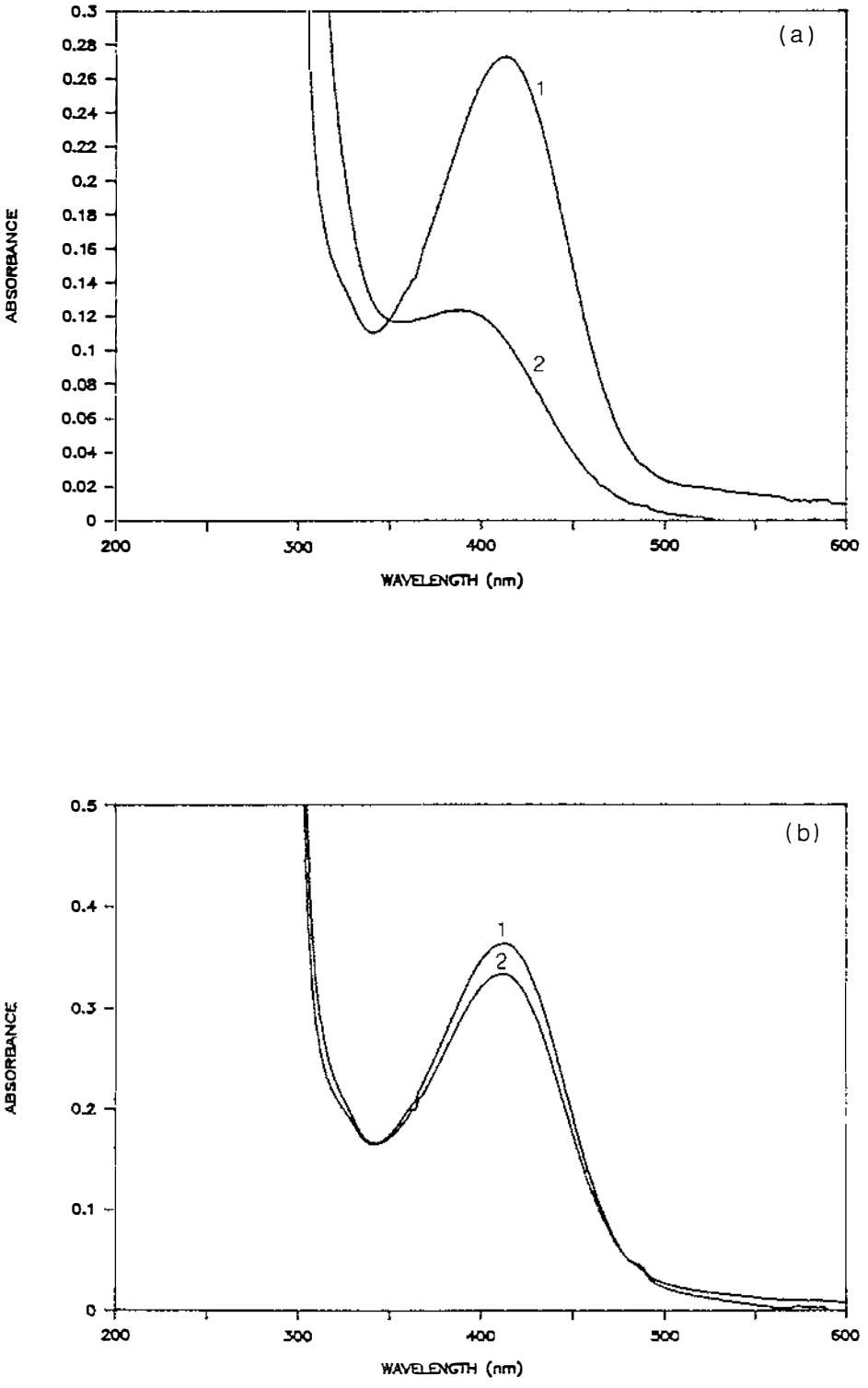
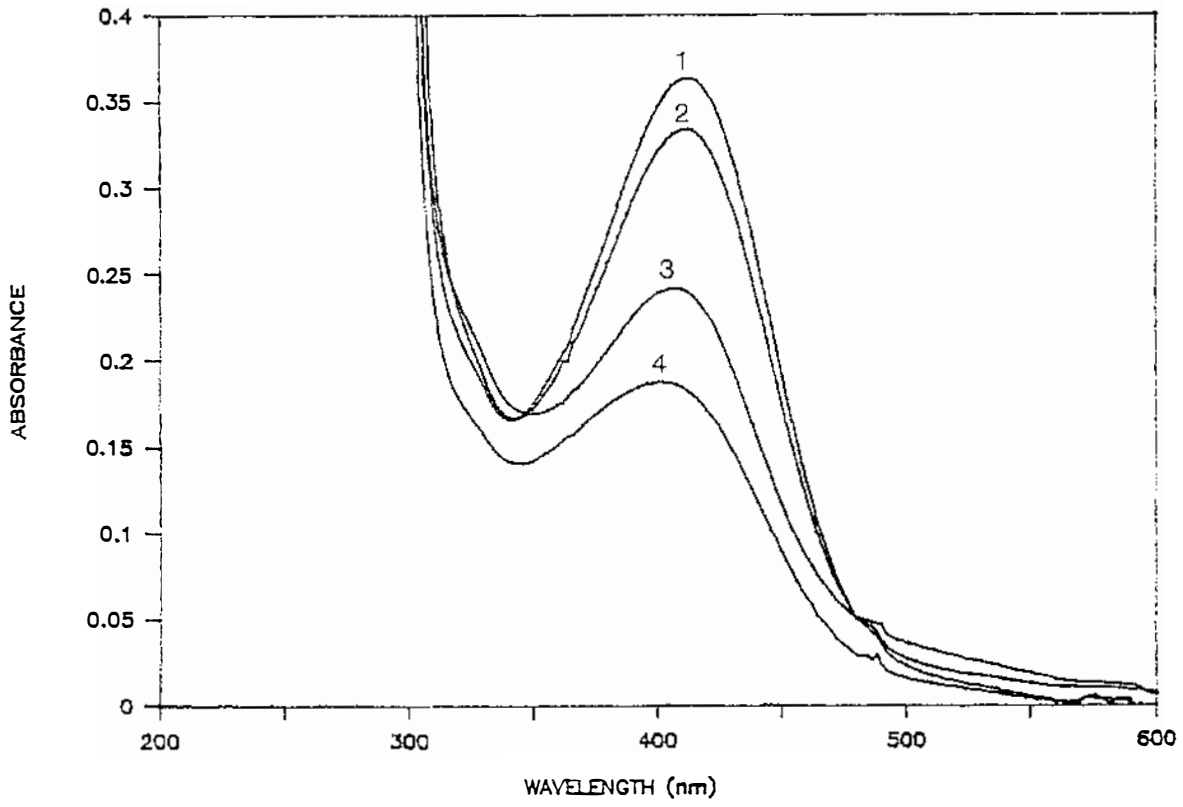


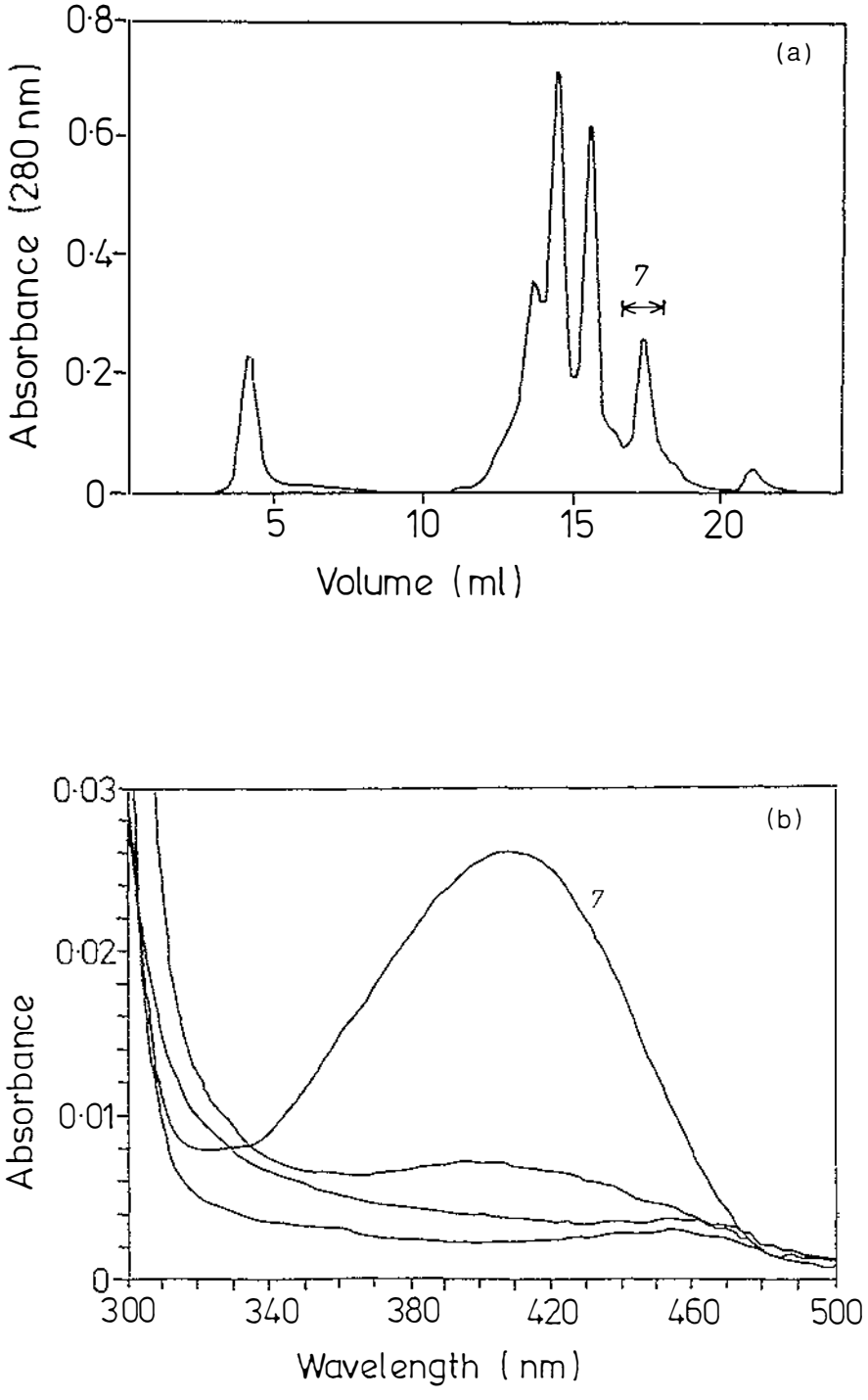
Figure 7.3 shows the progressive loss in the chromophore after: initial trapping with $HClO_4$ (spectrum 1); 2 hours in 8 M urea (spectrum 2); dialysis overnight against several changes of 0.1 M ammonium bicarbonate at 4 ^{o}C (spectrum 3); digestion with TPCK-trypsin (50:1 w/w) at 37 ^{o}C for 4 hours (spectrum 4). Spectrum 3 was normalised with the others to take into account the small change in volume which occurs on dialysis.

Figure 7.3 Loss of the Chromophore after Carboxymethylation, Dialysis, and Digestion with Trypsin



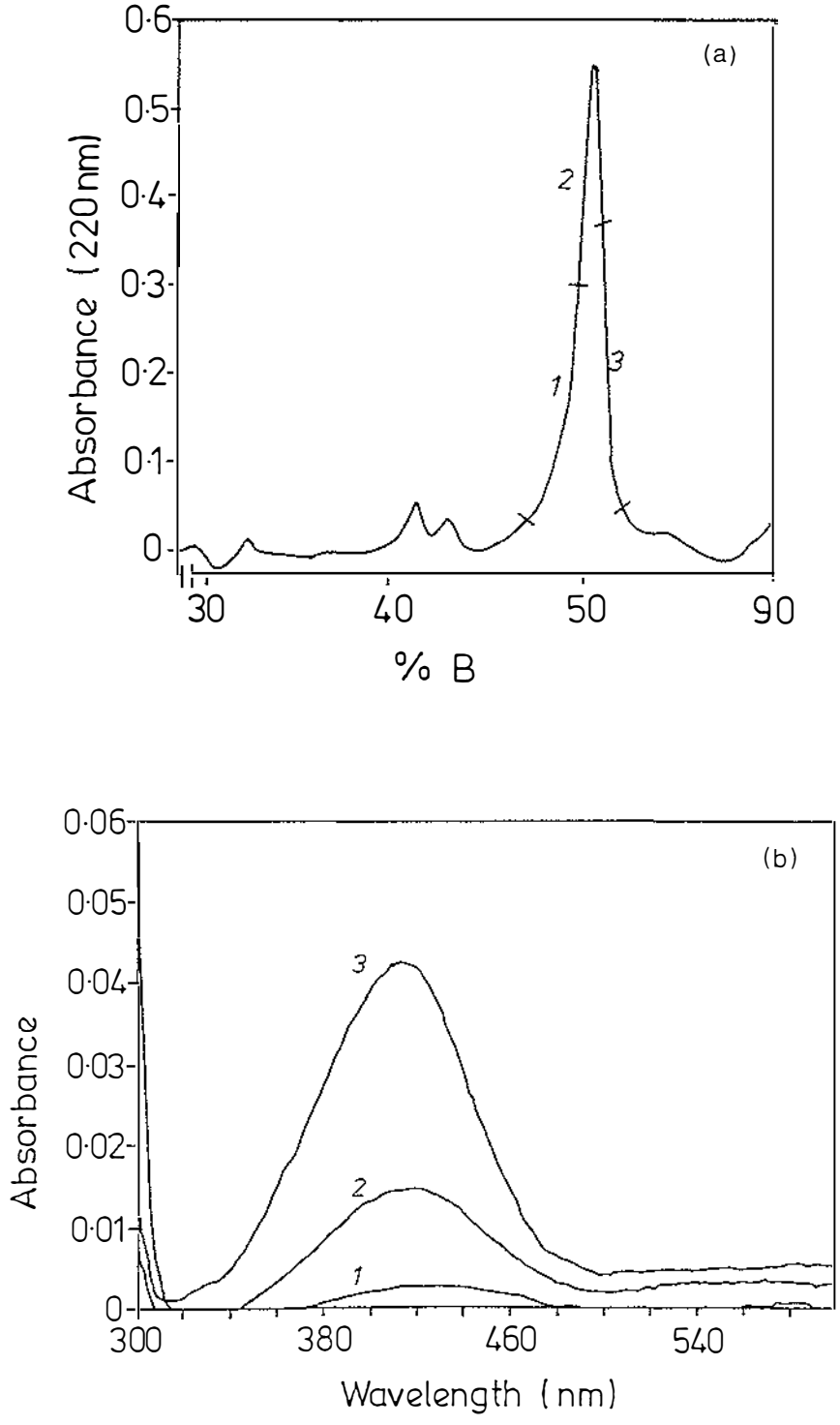
The gel filtration profile of the tryptic peptides is shown in Figure 7.4a. The digested enzyme (approximately 20 mg/ml) was loaded onto a Superose-12 column (10 mm x 300 mm) equilibrated with 0.1 M ammonium bicarbonate. The loading volume did not exceed 250 µl. The column was eluted with 0.1 M ammonium bicarbonate and the fractions monitored at 280 nm. Figure 7.4b shows the corresponding spectra of some collected fractions.

Figure 7.4 Gel Filtration of the Tryptic Digest



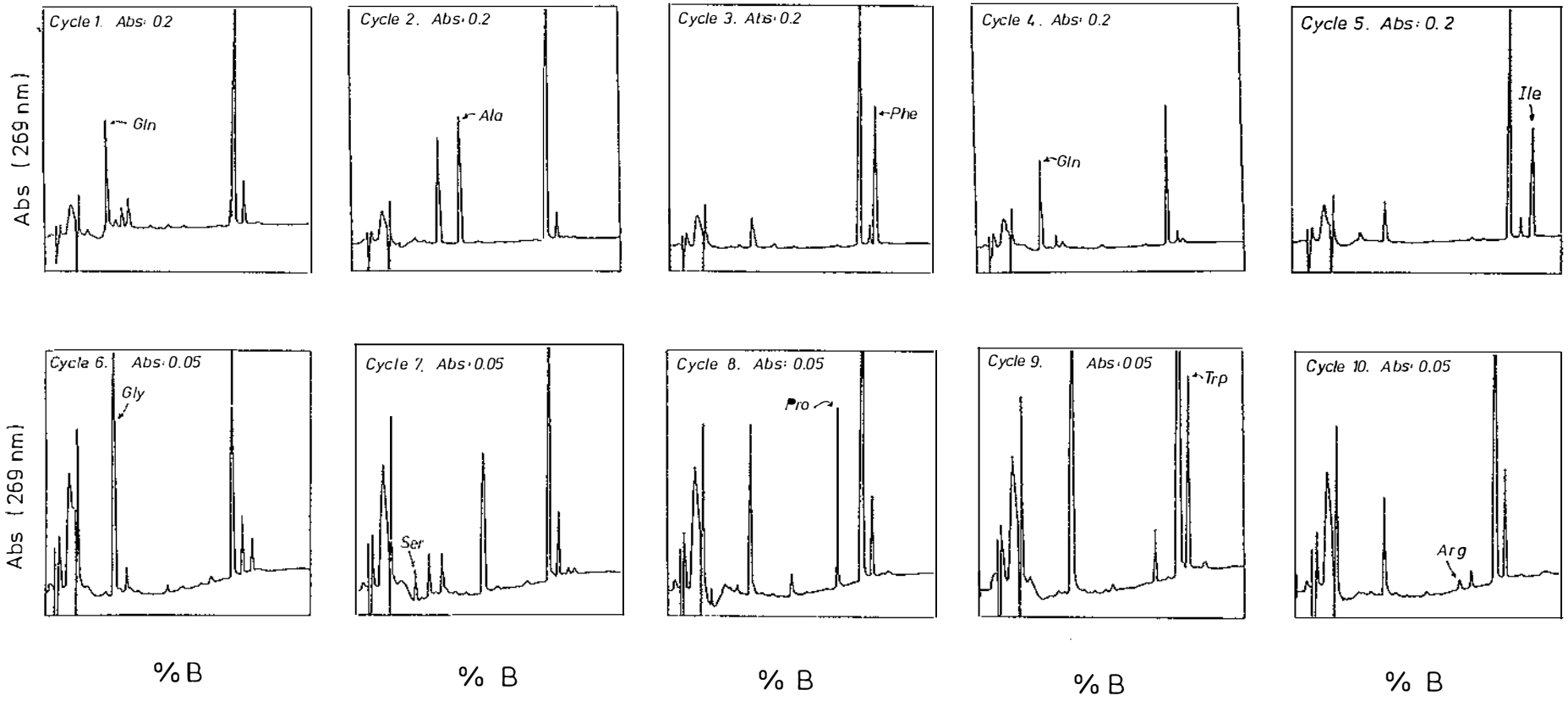
The fraction which contained colour from the gel column (fraction 7, Figure 7.4) was loaded onto a Waters Associates radial-pac 8BC1810H reverse phase column equilibrated with 0.1 M ammonium bicarbonate. A gradient was then then run to an organic solvent containing 0.1 M ammonium bicarbonate, isopropanol, and acetonitrile (1:1:1 v/v). The profile at 220 nm and the absorbance spectra of the collected fractions are shown in Figures 7.5a and 7.5b respectively.

Figure 7.5 Purification of Fraction 7 from Figure 7.4 by HPLC



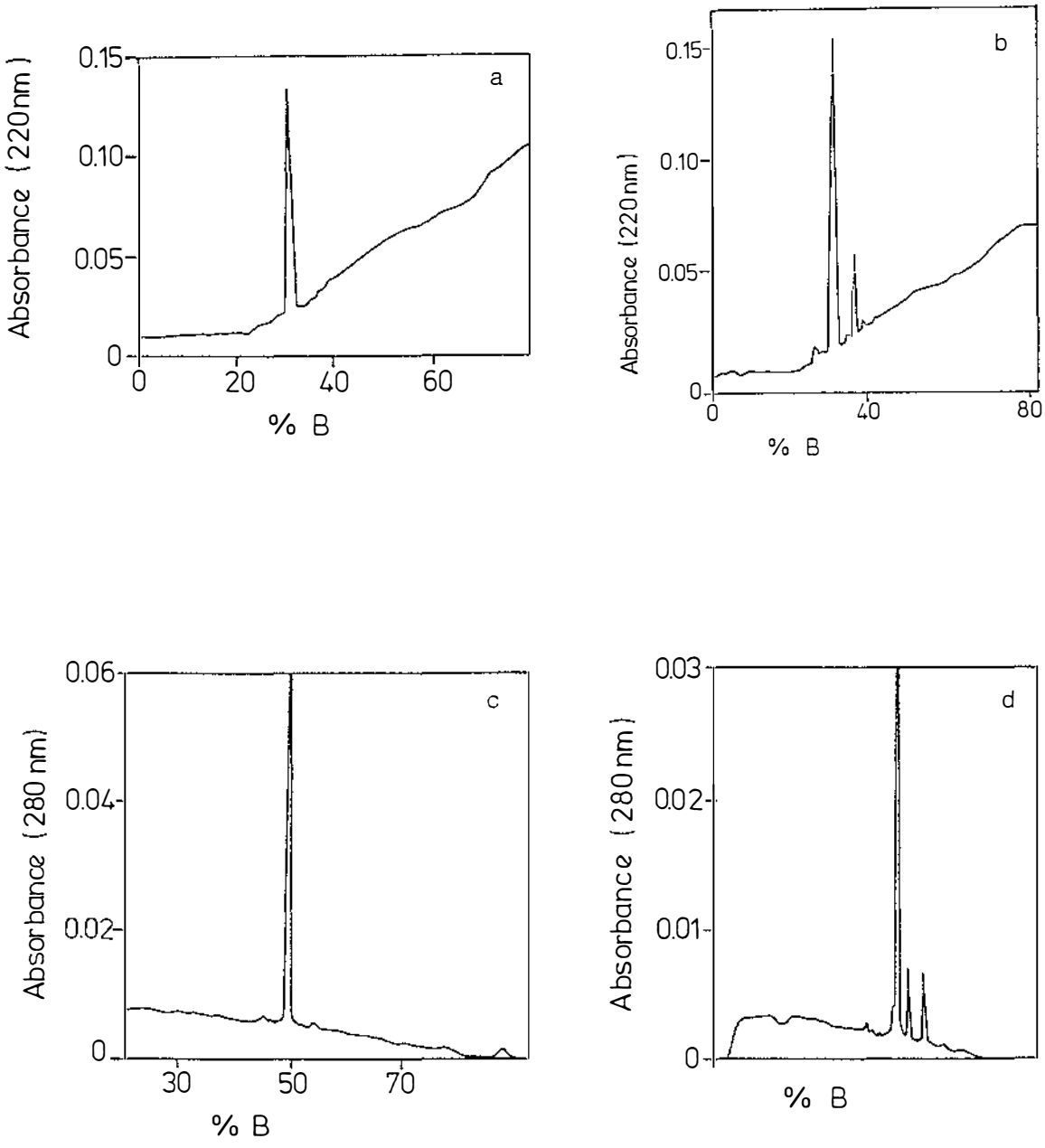
Fraction 3 from Figure 7.5 was sequenced on an Applied Biosystems 470A gas phase protein sequencer. The phenylthiohydantoin amino acid derivatives were analysed on an attached Applied Biosystems 120A PTH analyser. The first ten cycles are shown in Figure 7.6

Figure 7.6 Sequencing Results of Fraction 3 from Figure 7.5



Fraction 2 from Figure 7.5 was loaded onto either a Vydac Protein C_4 column (4.6 mm x 300 mm) or a RPC column both equilibrated with 0.1 % formic acid in H_2O/CH_3CN (9:1 v/v). In both cases a gradient was run to solvent B which contained 0.1 % formic acid in H_2O/CH_3CN (1:4 v/v). The profiles at 220 nm are shown in Figures 7.7a and 7.7c respectively. Fraction 3 from Figure 7.5 was also repurified by these columns under identical solvent conditions. The profiles from the Vydac Protein C_4 and RPC columns are shown in Figures 7.7b and 7.7d respectively.

Figure 7.7 Purification of Fractions 2 and 3 from Figure 7.5 by HPLC using Vydac and RPC Reverse Phase Columns



Aldehyde dehydrogenase was differentially labelled with [^{14}C]iodoacetamide, digested with TPCK-trypsin, and the tryptic peptides separated by gel filtration. Figure 7.8a shows the radioactivity profile of labelled and control reaction mixtures under conditions where the chromophore was removed with ammonia. Figure 7.8b is an identical experiment except that here the chromophore was allowed to spontaneously hydrolyse in the presence of [^{14}C]iodoacetamide in 8 M urea at 37 ^{o}C.

Figure 7.8 Differential Labelling of Aldehyde Dehydrogenase with [^{14}C]Iodoacetamide

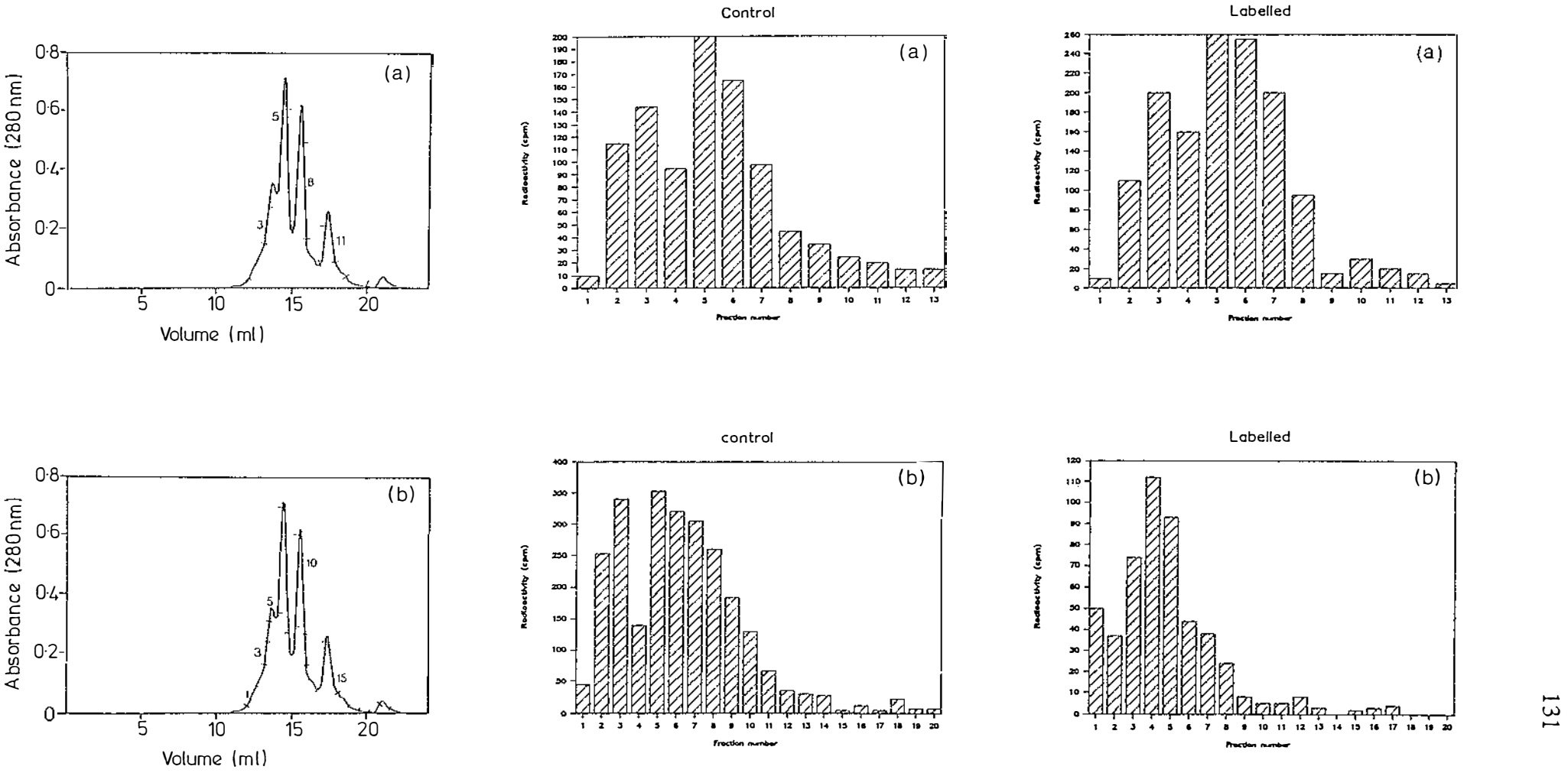
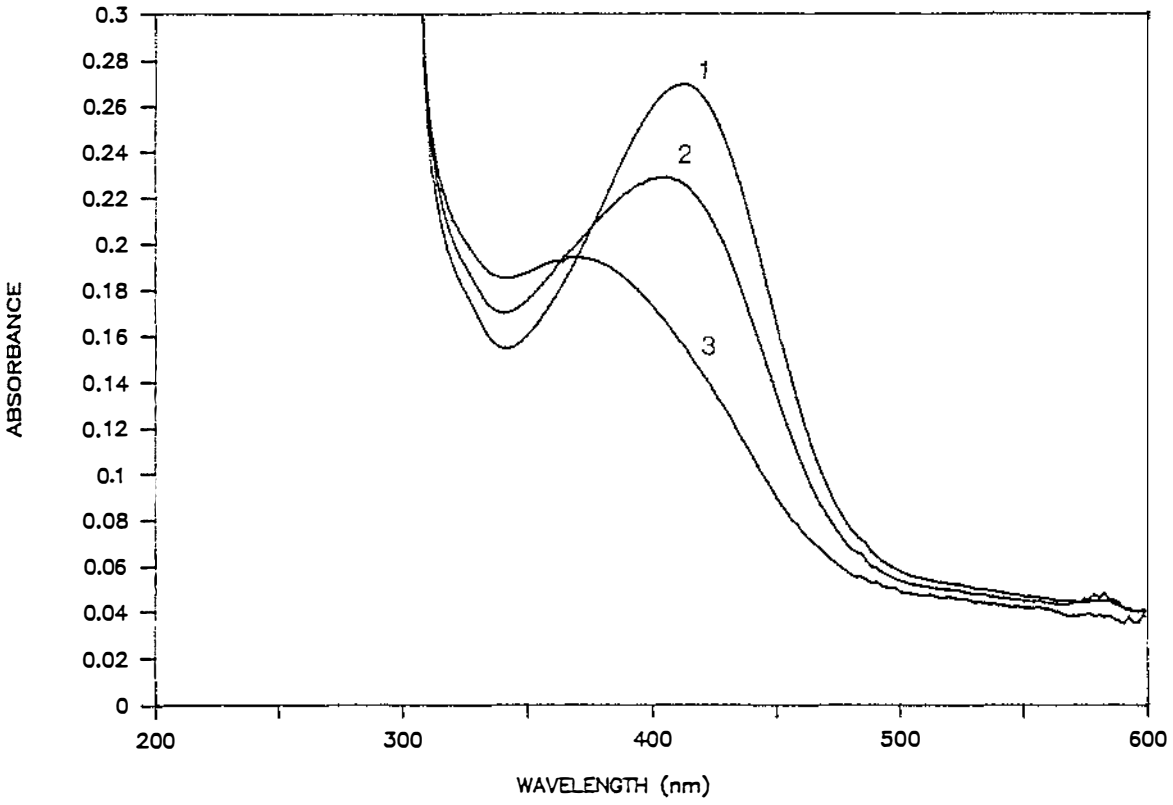


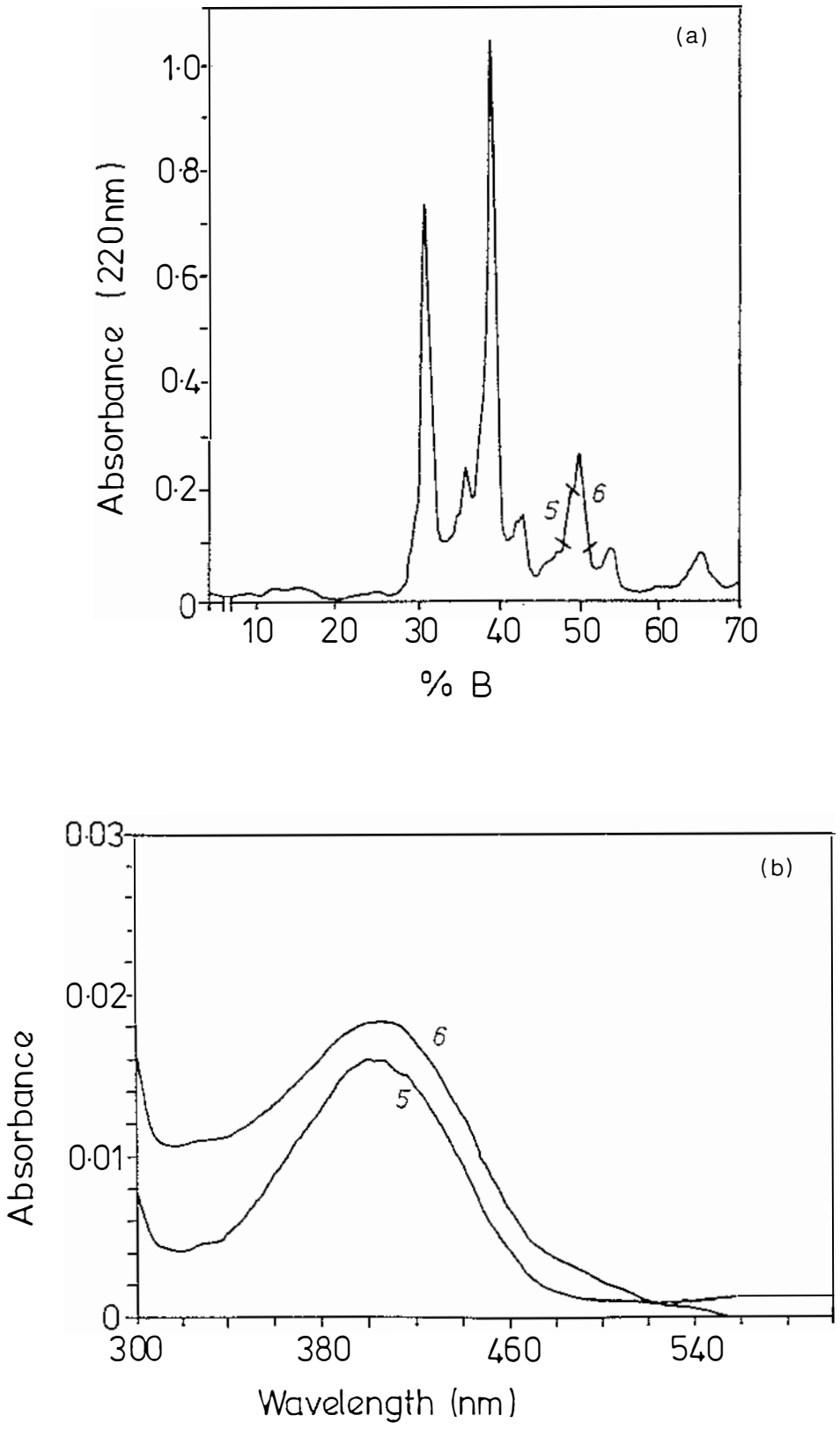
Figure 7.9 shows the spontaneous hydrolysis of the denatured acyl intermediate in 8 M urea at 37 ^{o}C in the presence of [^{14}C]iodoacetamide. Spectra were taken at: O hours (spectrum 1); 24 hours (spectrum 2); and 72 hours (spectrum 3).

Figure 7.9 Hydrolysis of the Denatured Acyl Intermediate in 8 M Urea over 72 Hours



Fraction 7 from Figure 7.4 was redigested with thermolysin (approximately 18 % by weight) for 10 minutes at room temperature. Figure 7.10a shows the profile at 220 nm when this digest was purified by HPLC under conditions identical to those of Figure 7.5. The spectra of fractions 5 and 6 are shown in Figure 7.10b.

Figure 7.10 Purification of Fraction 7 from Figure 7.4 by HPLC after Redigestion with Thermolysin



N-Acetyl-L-cysteine (139 μM) was added to an aqueous solution of *trans*-4-N,N-dimethylaminocinnamoyl imidazole (9.1 μM). In Figure 7.11, formation of the cysteine model ester is characterised by a change in λ_{MAX} from 424 nm to 398 nm.

Figure 7.11 Formation of the Cysteine Model Ester

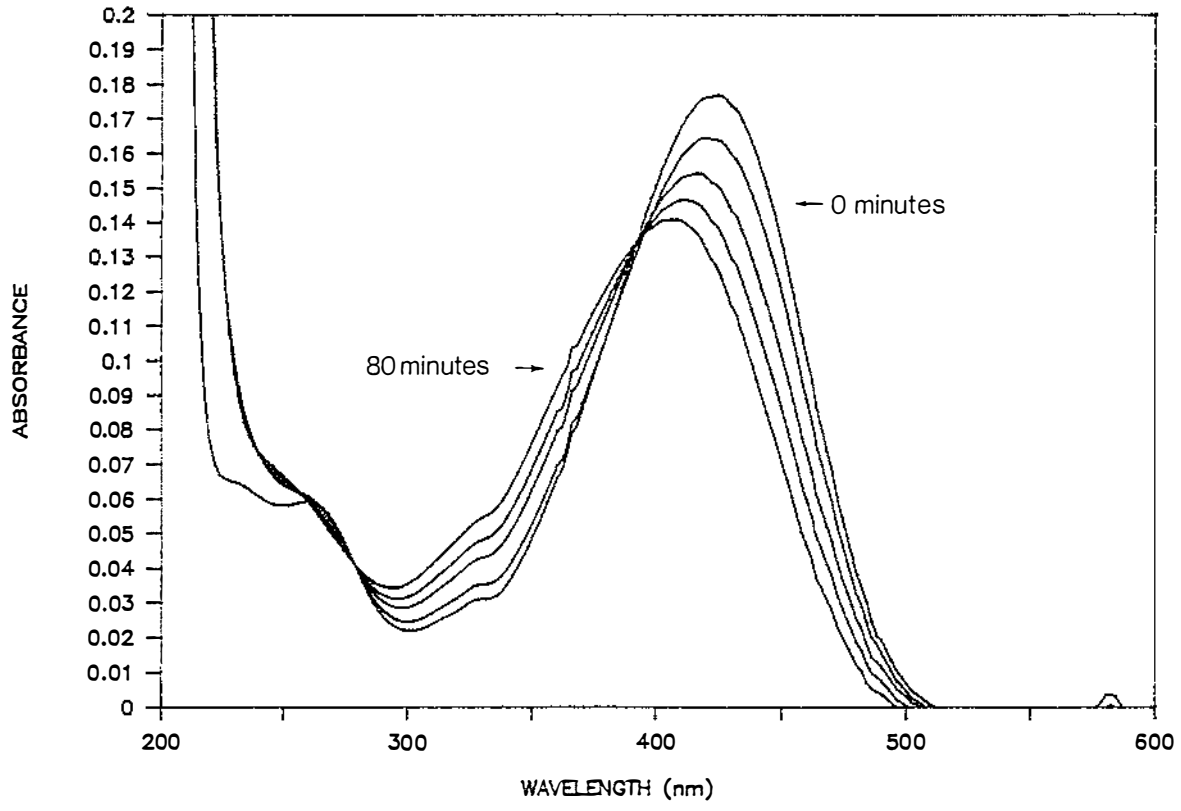


Figure 7.12a shows the purification of the synthesised peptide by HPLC from a reaction mixture where no spectral change occurred (see text). The column and solvent conditions are identical to those described previously for Figure 7.5. Figure 7.12b shows the spectra of the fractions collected in Figure 7.12a.

Figure 7.12 Purification of the Synthesised Peptide by HPLC

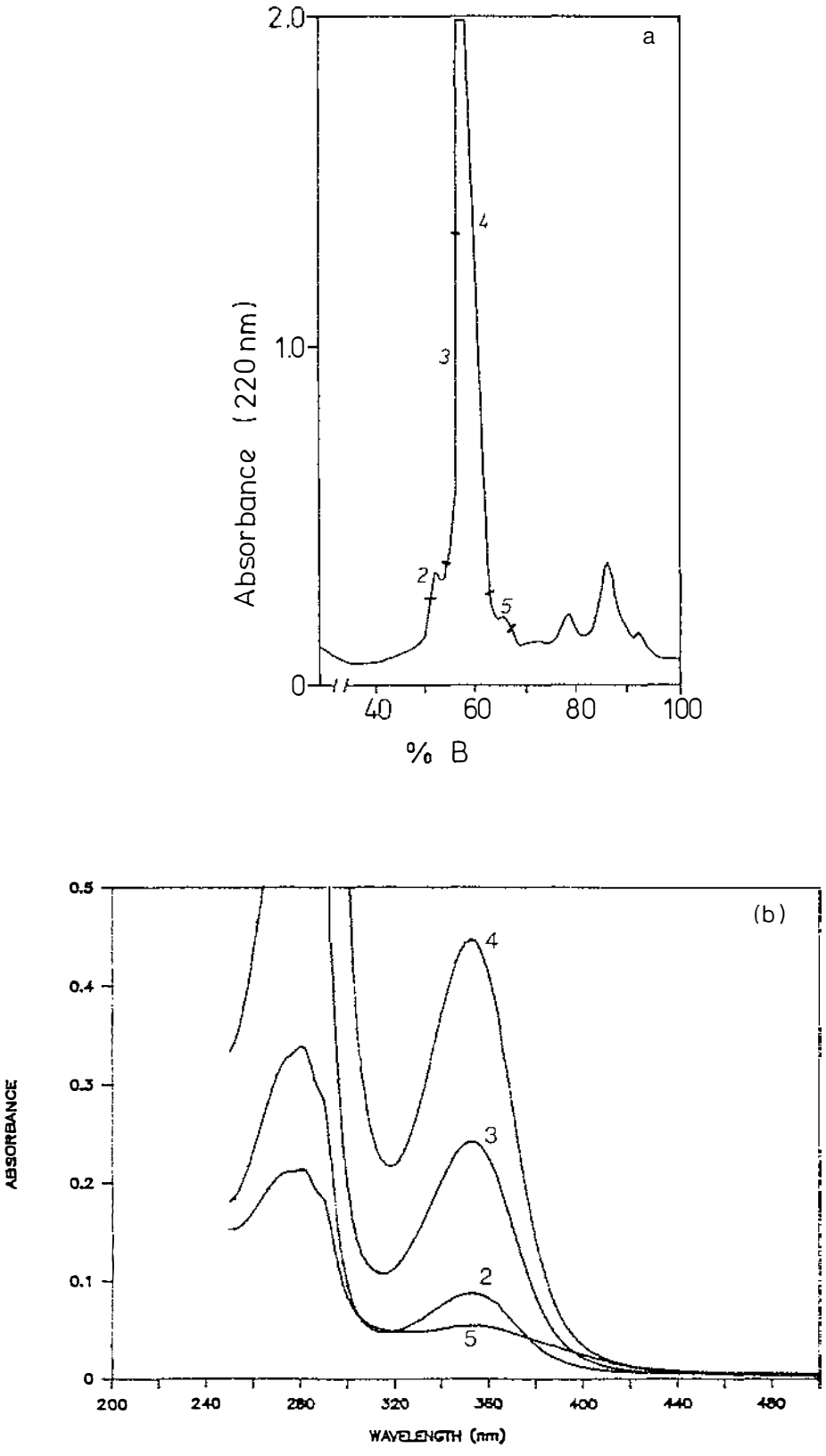
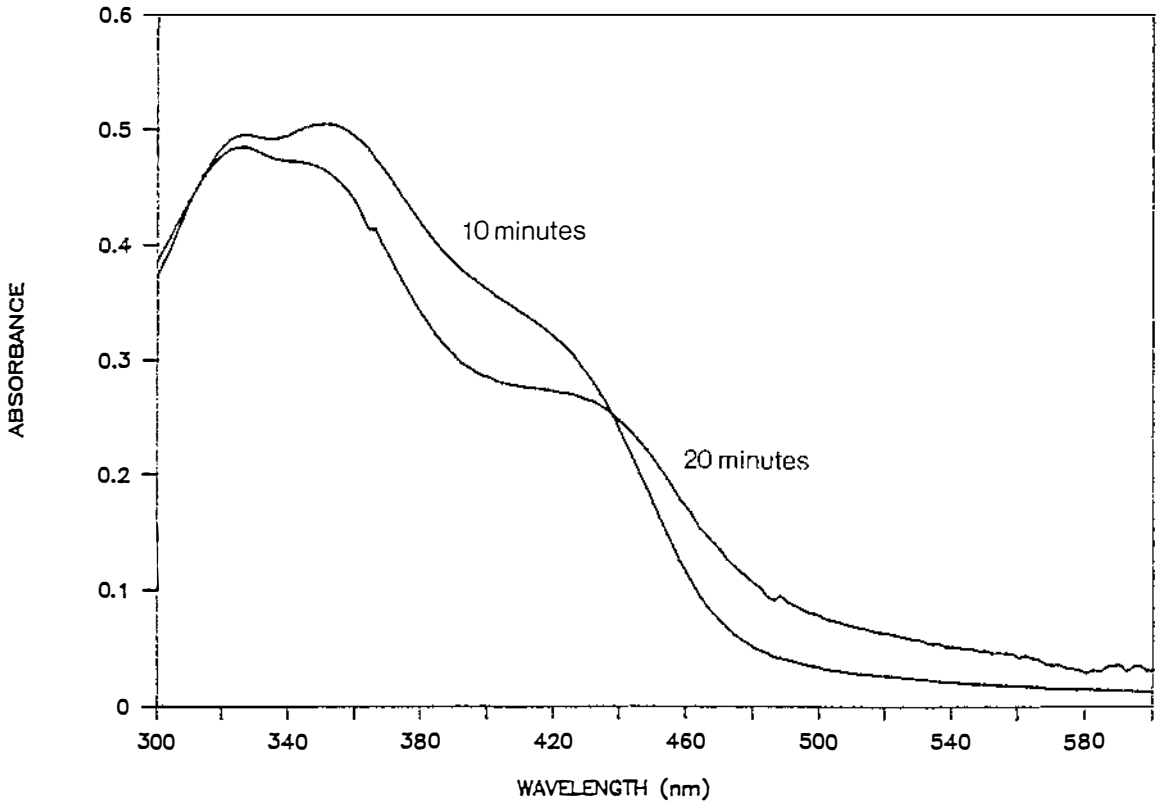


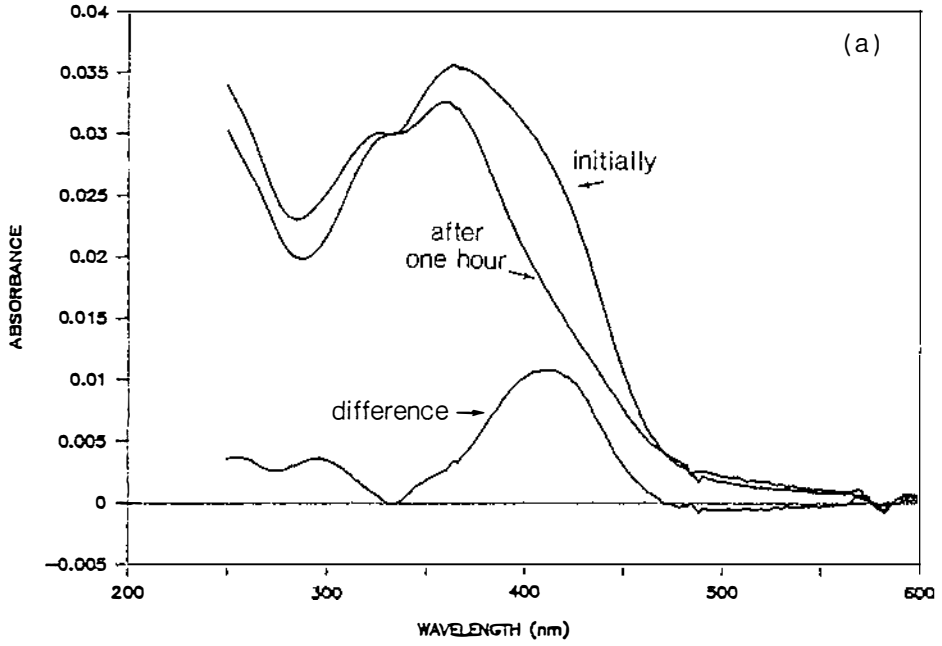
Figure 7.13 shows the spectral change when acetyl-Ile-Gly-Ser-Pro-Trp-Arg-NH_2 is refluxed with the isobutyl anhydride of *trans*-4-N,N-dimethylamino-cinnamic acid in dry THF after 10 and 20 minutes.

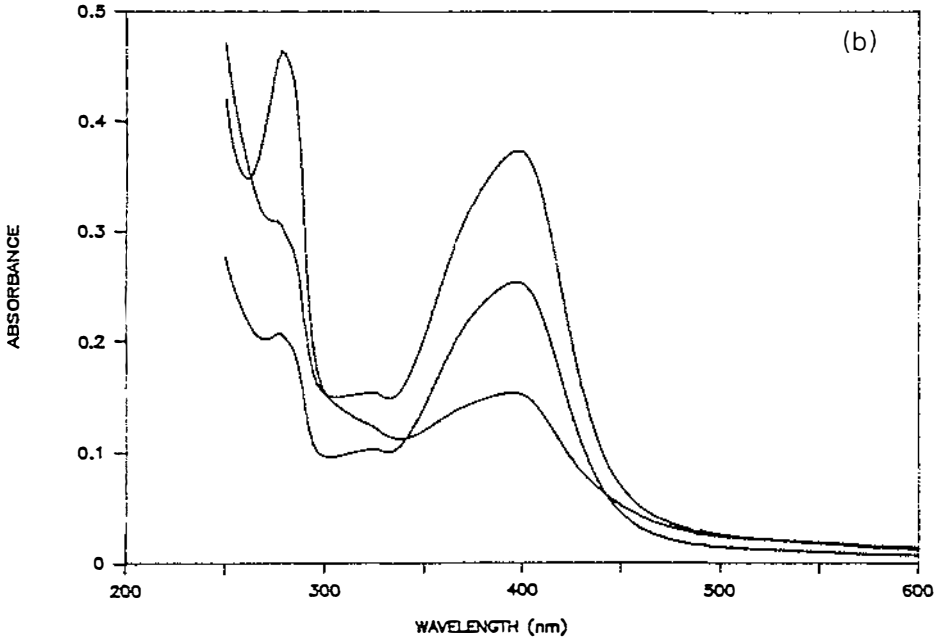
Figure 7.13 Synthesis of the Model Peptide Serine Ester



After 20 minutes reflux (Figure 7.13) the labelled peptide was precipitated by adding the reaction mixture to an excess of water. The precipitate was then redissolved in THF. A small amount of this stock solution was added to a cuvette containing water. Figure 7.14a shows the spectral change which occurs over a 1 hour period. In another experiment. some colour which had adhered to the filter paper was dissolved in THF and passed down a Bio-Gel P-6 column (0.8 cm x 10 cm) equilibrated in THF/water (3:2 v/v). The spectra of the collected fractions are shown in Figure 7.14b.

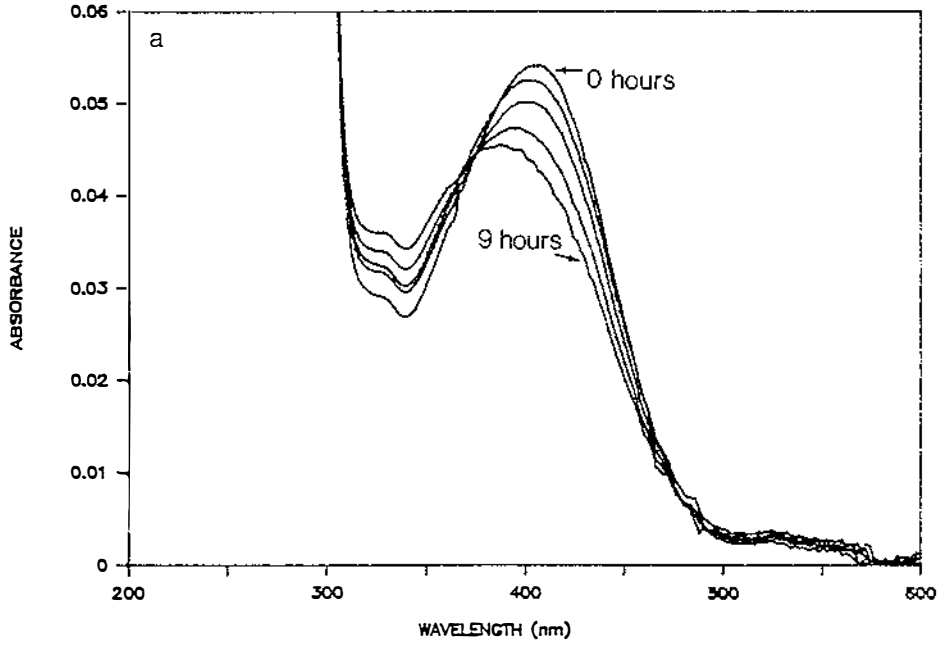
Figure 7.14 Isolation of the Model Peptide Serine Ester





Fraction 7 from the gel column (Figure 7.4) was incubated in 30 mM sodium phosphate buffer, pH 8.0, at 37 ^{o}C. Figure 7.15a shows the loss of the chromophore through spontaneous hydrolysis over time while Figure 7.15b shows the absorbance change at 408 nm - 600 nm. The inset is a first order plot and was calculated using an infinity absorbance value, A1, corresponding to the absorbance at 24 hours.

Figure 7.15 Spontaneous Hydrolysis of Fraction 7 from Figure 7.4 in 30 mM Sodium Phosphate Buffer, pH 8.0, at 37 °C



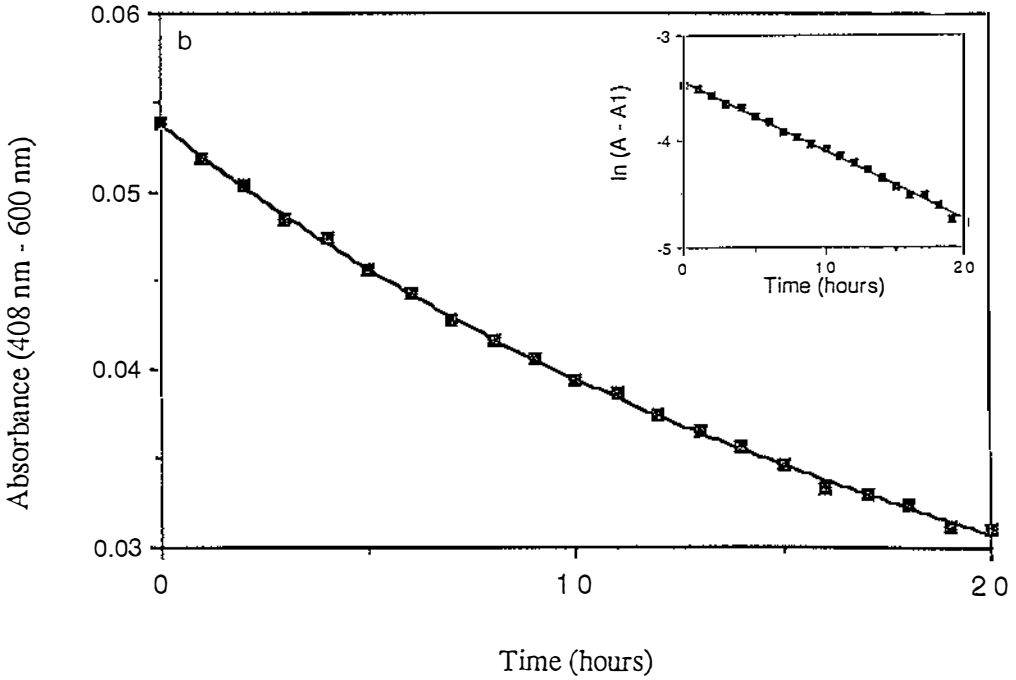
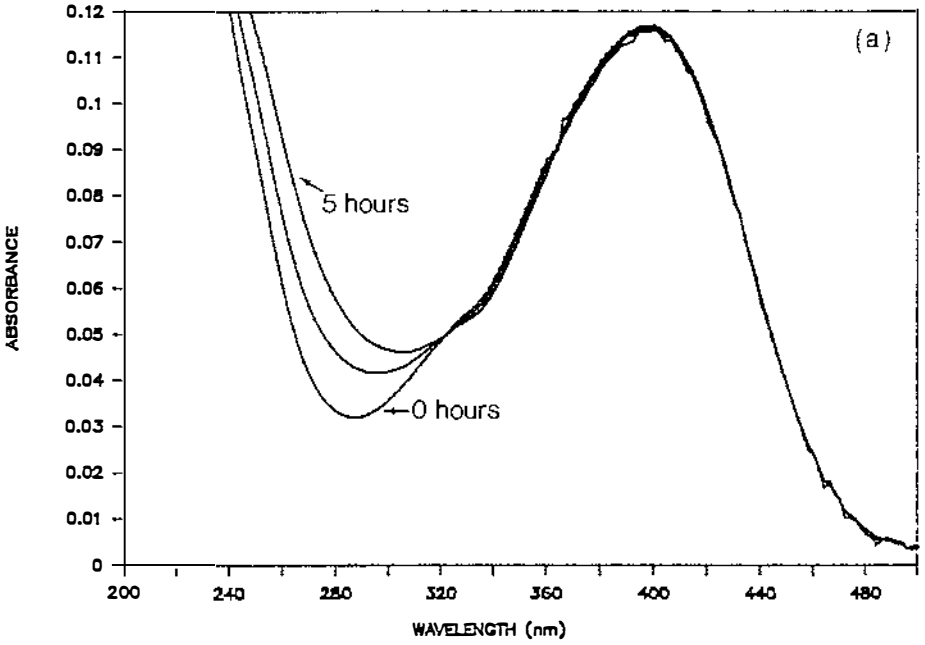


Figure 7.16a shows the spectral change of the cysteine model ester over a period of 5 hours in 30 mM sodium phosphate buffer, pH 8.0, at 37 ^{o}C. The absorbance change at 398 nm - 525 nm is shown in Figure 7.16b.

Figure 7.16 Spontaneous Hydrolysis of the Cysteine Model Ester in 30 mM Sodium Phosphate Buffer, pH 8.0, at 37 °C



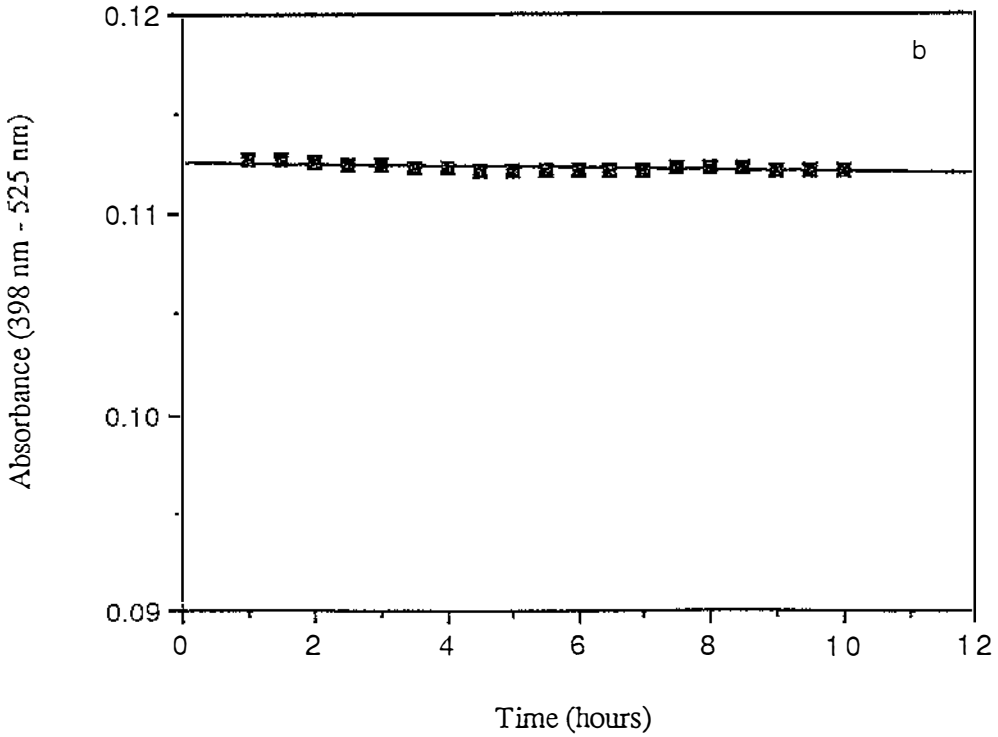
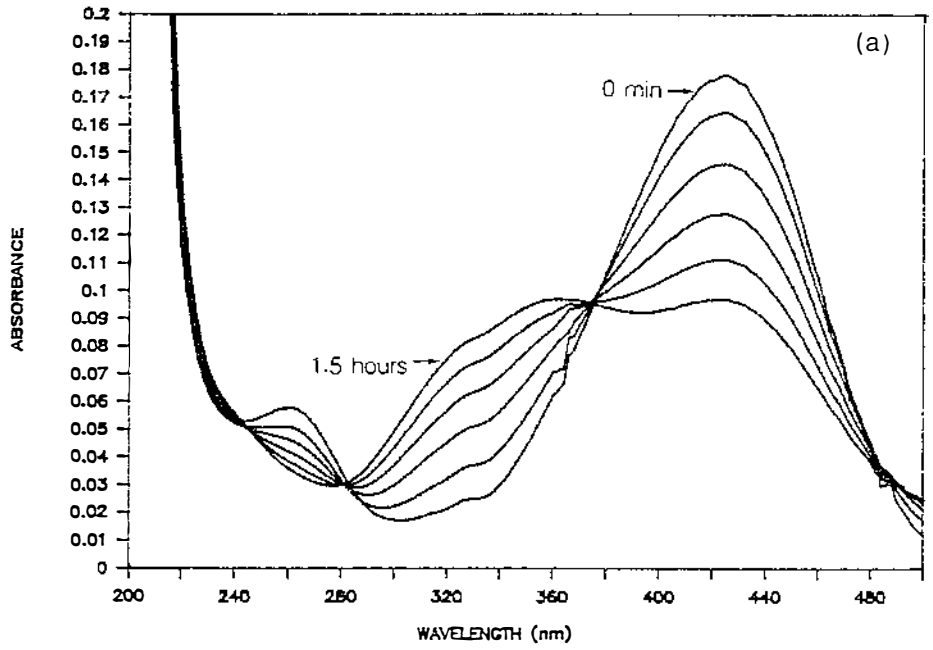


Figure 7.17a shows the spectral change of *trans*-4-N,N-dimethyl-aminocinnamoyl imidazole in 0.1 M ammonium bicarbonate at 37 ^{o}C over a period of 1.5 hours. The absorbance change at 424 nm - 565 nm is shown in Figure 7.17b, and the inset is a first order plot of these data. The infinity value in this case is zero.

Figure 7.17 Spontaneous Hydrolysis of *trans*-4-N,N-Dimethylaminocinnamoyl Imidazole in 0.1 M Ammonium Bicarbonate at 37 ^{o}C



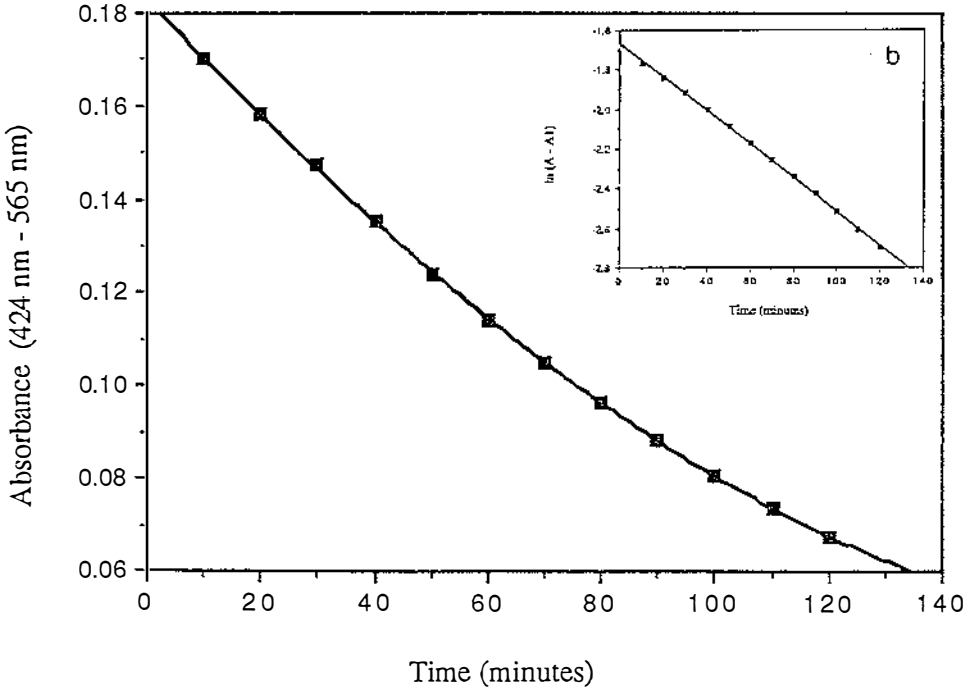


Figure 7.18 compares the spectra of the following compounds, namely: the cysteine model ester in 30 mM sodium phosphate, pH 8.0 (spectrum 1); the coloured peak from fraction 7 in Figure 7.4 in 0.1 M ammonium bicarbonate (spectrum 2); and *trans*-4-N,N-dimethylaminocinnamoyl imidazole in 50 mM sodium phosphate buffer, pH 7.4 (spectrum 3). The magnitudes of spectra 2 and 3 were normalised to spectrum 1.

Figure 7.18 Spectra of the Cysteine Model Ester, Fraction 7 from Figure 7.4, and *trans*-4-N,N-Dimethylaminocinnamoyl Imidazole

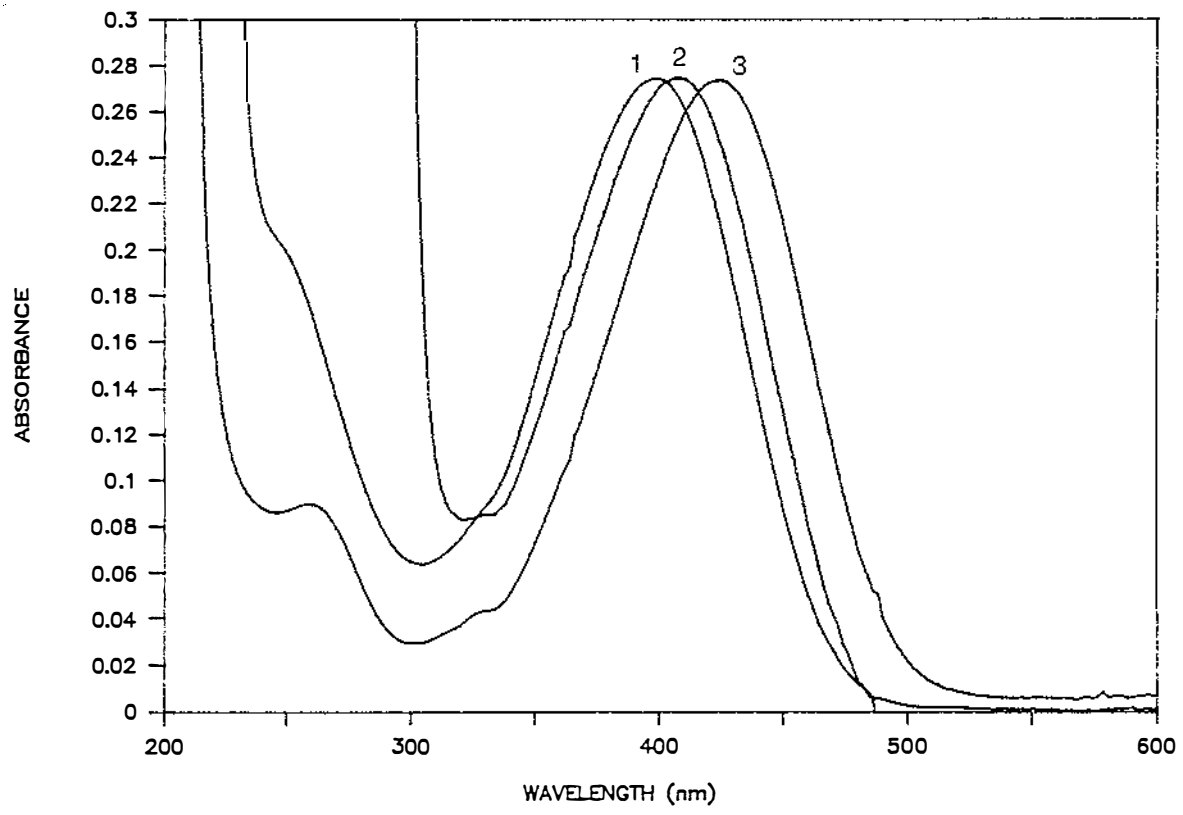
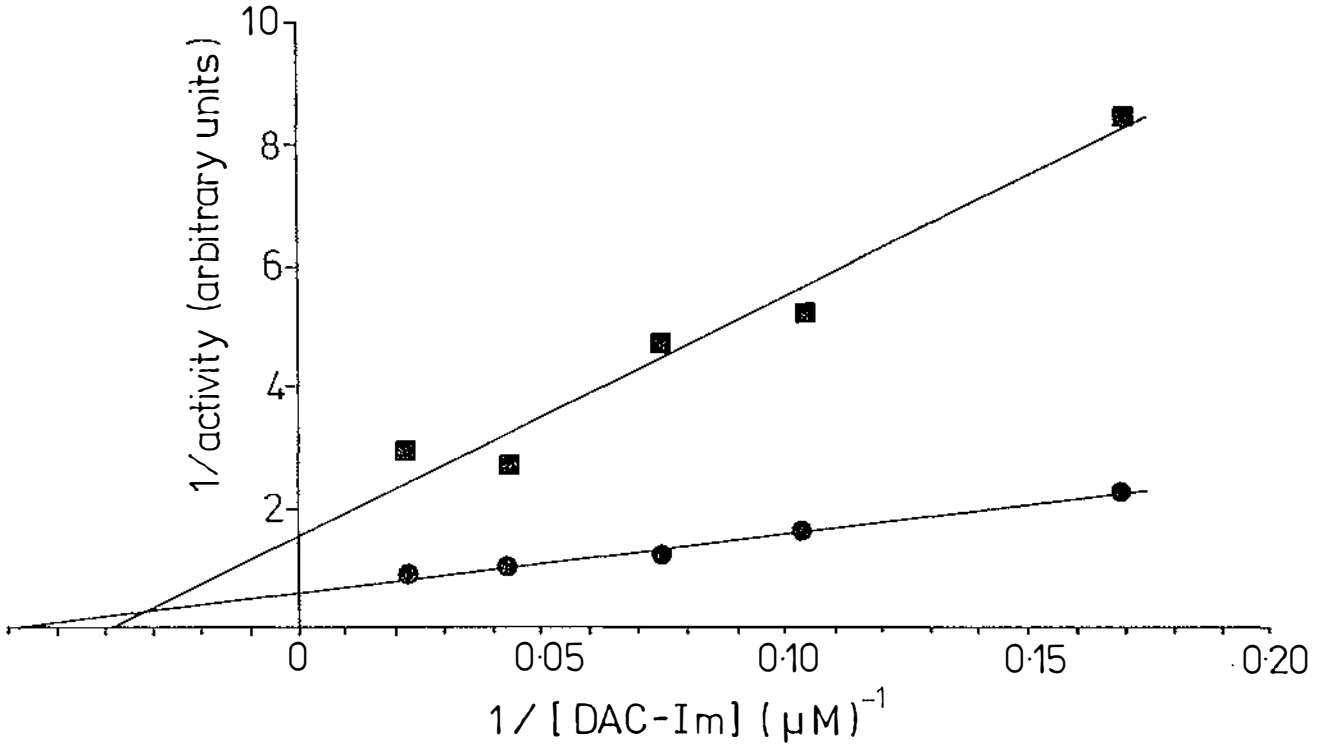


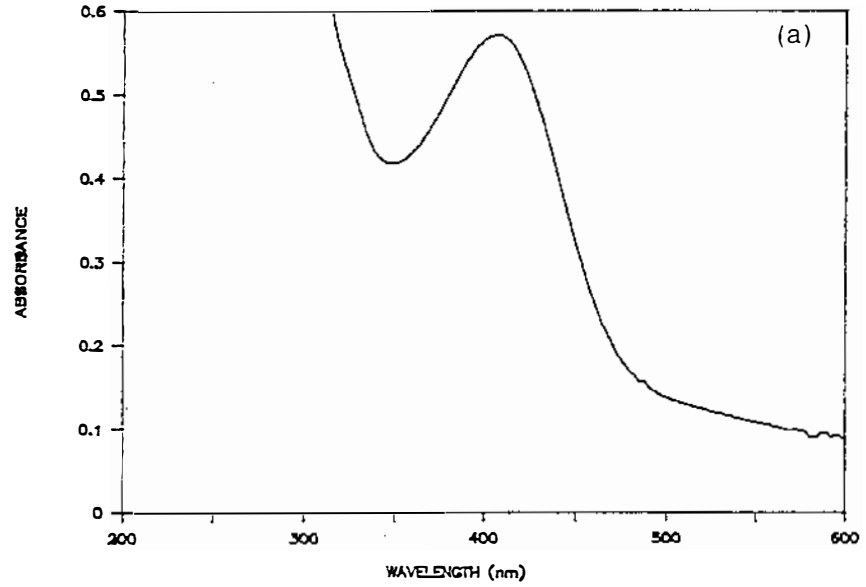
Figure 7.19 shows a Lineweaver-Burk plot of the enzyme-catalysed hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole. Assays were carried out in 50 mM sodium phosphate buffer, pH 7.4, at 30 ^{o}C, and involved adding *trans*-4-N,N-dimethylaminocinnamoyl imidazole to enzyme (0.58 μM) in the presence (■) or absence (●) of 1 mM NAD^{+} . All rates were corrected for the spontaneous hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole.

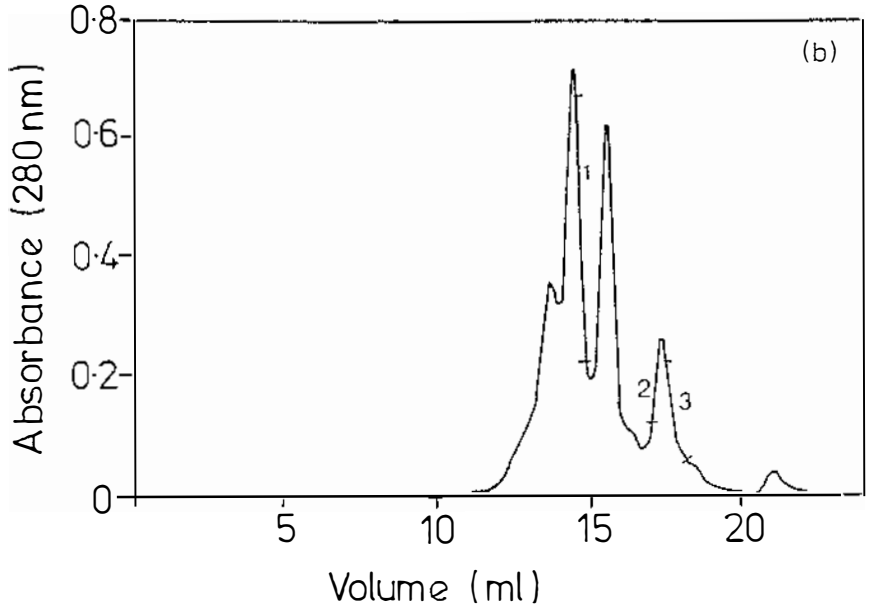
Figure 7.19 Lineweaver-Burk Plot of the Enzyme-Catalysed Hydrolysis of *trans*-4-N,N-Dimethylaminocinnamoyl Imidazole



Aldehyde dehydrogenase (10 μM) was incubated with *trans*-4-N,N-dimethyl-aminocinnamoyl imidazole (75 μM) in 50 mM sodium phosphate buffer, pH 7.4, at 30 ^{o}C for 15 minutes. The enzyme was then precipitated with $HClO_4$, and the denatured acyl species redissolved in 8 M urea. The spectrum taken at this stage is shown in Figure 7.20a. The gel filtration profile at 280 nm of the tryptic digest is shown in Figure 7.20b

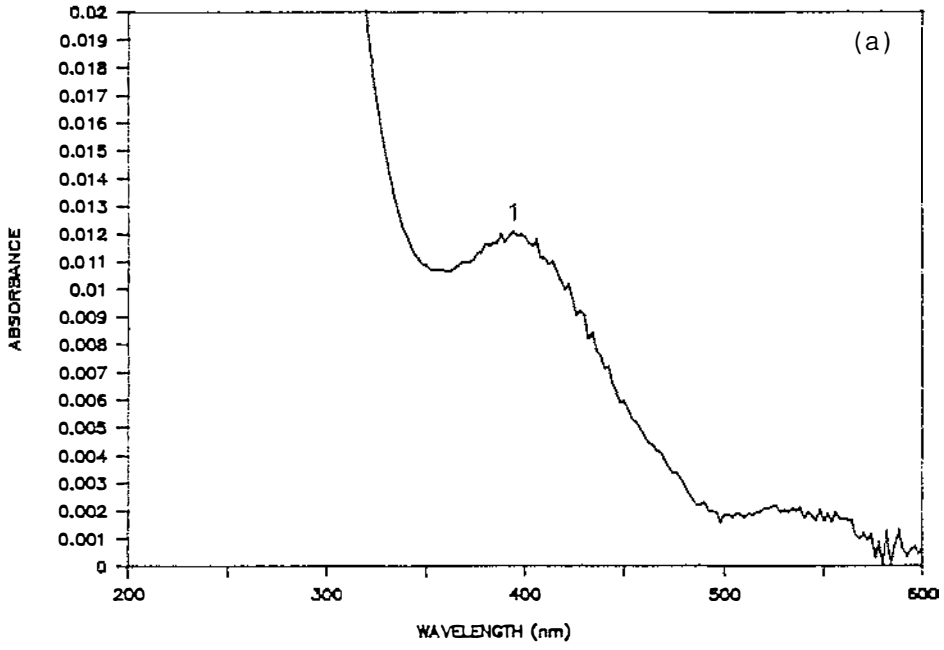
Figure 7.20 The Trapped Acyl Intermediate from the Enzyme-Catalysed Hydrolysis of *trans*-4-N,N-Dimethylaminocinnamoyl Imidazole and Subsequent Gel Filtration of the Tryptic Digest

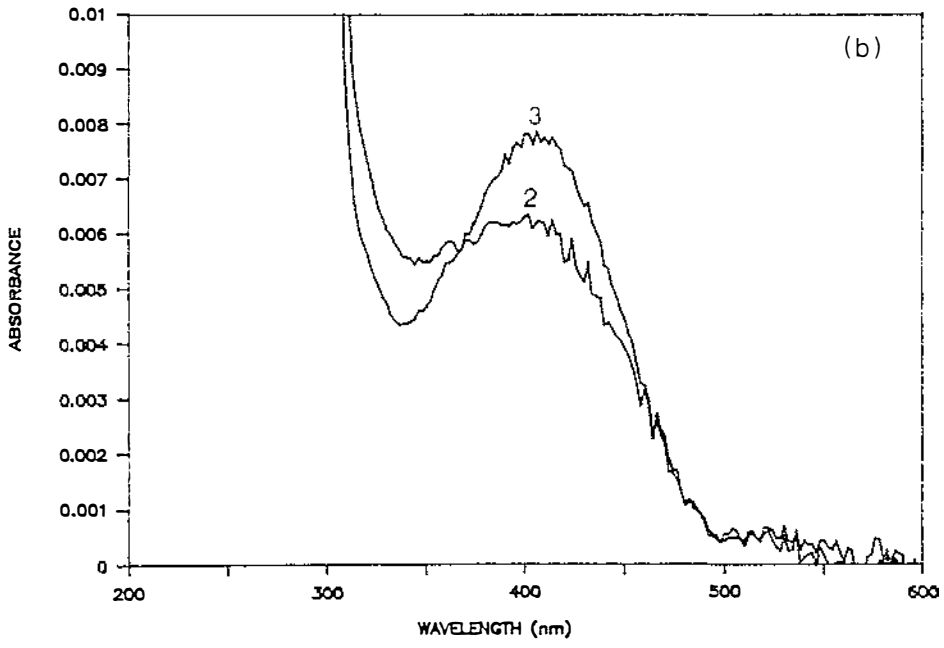




Figures 7.21a and 7.21b show the spectra of fractions 1, 2 and 3 from Figure 7.20b.

Figure 7.21 Spectra of the Collected Fractions from Figure 7.20b





7.4 Discussion

7.4.1 Groups currently thought to reside at the active site

The first identification of a group at the active site came from studies with iodoacetamide (Hempel & Pietruszko, 1981). This compound is an irreversible inhibitor of aldehyde dehydrogenase (Pietruszko *et al.*, 1982) and is proposed to react at the active site on the basis of a number of observations. For example, the rate of reaction is stimulated in the presence of NAD^+ (Pietruszko *et al.*, 1982) but decreased by aldehyde substrates and competitive inhibitors such as chloral hydrate (Hempel & Pietruszko, 1981) and cyclopropanone (Wiseman & Abeles, 1979).

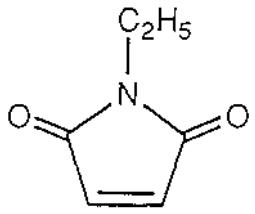
Reaction of aldehyde dehydrogenase with [^{14}C]iodoacetamide, and subsequent digestion, results in the isolation of a specifically labelled tryptic peptide which contains two contiguous cysteines (Hempel *et al.*, 1982a). The modified group is Cys-30, and corresponds to Cys-302 in the complete primary structure (Hempel *et al.*, 1984). Furthermore, Cys-302 is implicated in the reaction with disulfiram in the sense that preexposure of aldehyde dehydrogenase to a 4-fold excess of the latter dramatically reduces the reaction rate with [^{14}C]iodoacetamide (Hempel *et al.*, 1982a). That Cys-302 is at the active site is independently supported by studies which find this group specifically modified by a certain coenzyme analogue (von Bahr-Lindstrom *et al.*, 1985). Furthermore, Cys-302 may be structurally positioned close to another as yet unidentified thiol (see Section 4.4.1).

The next group identified as having possible catalytic significance was Glu-268. This is modified by bromoacetophenone, an active site directed reagent which irreversibly inactivates both dehydrogenase and esterase activities (MacKerell *et al.*, 1986). The rate of reaction is relatively fast; for example, in the absence of NAD^+, a 4-fold excess over the tetramer concentration causes a 93 % activity loss in 30 s for the human liver cytoplasmic enzyme.

MacKerrell *et al.* (1986) propose that bromoacetophenone reacts at the active site because of its structural similarity to acetophenone and benzaldehyde (Figure 7.22). This assumption is supported by the observations that chloral

hydrate protects against inactivation, and acetophenone is a competitive inhibitor of the dehydrogenase pathway. MacKerell *et al.* (1986) also performed steady state experiments with chloroacetophenone which, compared to its bromo counterpart, is a relatively slow inactivator. However, although a non-competitive pattern was found, these authors nevertheless interpreted this as an interaction between chloroacetophenone - and therefore bromoacetophenone - and the active site.

Finally, Cys-49 is proposed to be an active site residue by Tu & Weiner (1988a) on the basis of labelling studies with N-ethylmaleimide (below)



The main disadvantage these workers found with this inhibitor was that reaction with horse liver mitochondrial aldehyde dehydrogenase was relatively non-specific. For instance, dehydrogenase activity was not totally lost until a 14-fold incorporation per tetramer. Consequently, the strategy involved reacting N-ethylmaleimide with aldehyde dehydrogenase already premixed with NAD^+ and chloral hydrate. After the activity was reduced to 60 % of its initial value, small molecules were removed by gel filtration, and the modified enzyme (in the presence of NAD^+) allowed to react with radioactive N-ethylmaleimide. In this latter step activity was lost stoichiometrically with the incorporation of inhibitor.

The rationale underlying the above is that chloral hydrate, a competitive inhibitor, protects the active site. Presumably, N-ethylmaleimide can still react, but only elsewhere with non-essential groups. After gel filtration the active site is free from chloral hydrate, and hence any group now modified by N-[^{14}C]ethylmaleimide is implicated there.

In this way Tu & Weiner (1988a) have shown that Cys-49 is primarily modified, and to an extent Cys-162, depending on the reaction conditions. Qualitatively similar results were also obtained with o-nitrobenzaldehyde as a protecting

agent. This aldehyde is a relatively poor substrate and the acyl species accumulates significantly in the steady state. The effect, however, is the same as with chloral hydrate except that here protection is due to the presence of an actual pathway intermediate. Before the addition of N-[^{14}C]ethylmaleimide the aldehyde sidechain was removed by hydroxylamine (Tu & Weiner, 1988a).

7.4.2 The catalytic nucleophile

At this point it will be instructive to define clearly what is meant by a "catalytic nucleophile". For aldehyde dehydrogenase this is the group that becomes covalently attached to the aldehyde substrate, and exists in the form of an acyl intermediate immediately after the redox step of the reaction. For glyceraldehyde-3-phosphate dehydrogenase and chymotrypsin this role is fulfilled by Cys-149 (Harris & Waters, 1976) and Ser-195 (Tulinsky & Blevins, 1987) respectively.

For aldehyde dehydrogenase the catalytic nucleophile has yet to be positively identified, although Cys-302 is implicated as a likely candidate. For example, modification of this residue by iodoacetamide, or possibly disulfiram, leads to a dramatic loss in activity. However, although this is consistent with the expected properties of an essential group, strictly speaking there is no conclusive evidence to prove that Cys-302 and the catalytic nucleophile are the same. For instance, the possibility exists that inhibition by iodoacetamide arises indirectly from steric or conformational factors, and it is these which severely retard the enzyme's functional ability.

This argument applies not only to Cys-302 but quite generally to other potentially important residues such as Glu-268, Cys-49, and possibly Cys-162. Thus although all may reside at the active site (see Section 7.4.1) whether any of these is the catalytic group - or indeed if any are functionally important - is currently unknown.

The underlying reason for this uncertainty can be illustrated by reference to the labelling experiments with bromoacetophenone (MacKerell *et al.*, 1986). The justification that this inhibitor reacts at the active site rests mainly on the

observation that acetophenone is a competitive inhibitor of the dehydrogenase pathway (MacKerell *et al.*, 1986). Generally speaking, however, all that can be inferred from competitive inhibition is that the binding of inhibitor and aldehyde are mutually exclusive. While it is possible that both these react at exactly the same position, there really is no reason why different target groups should not be involved. As discussed above, in this case competition will be mediated through indirect mechanisms such as steric hindrance or conformational changes within the protein. There is no guarantee, therefore, that Glu-268 (which is modified by bromoacetophenone) is the essential nucleophile as defined previously.

A case in point is provided by similar experiments with chymotrypsin where bromoacetophenone modifies Met-192 (Lawson & Rao, 1980). As with aldehyde dehydrogenase, reaction proceeds with a concerted reduction in activity. However, although Met-192 is at the active site it is not the catalytic nucleophile, Ser-195 (Fersht, 1977). Consequently, inactivation by bromoacetophenone must necessarily be indirect.

The foregoing is not intended to imply that catalytically essential groups are never modified by affinity reagents. For instance, in chymotrypsin depending on the type of inhibitor and reaction conditions, Ser-195 can be either fractionally or exclusively modified (Lawson, 1980). As a further example, Cys-149 in glyceraldehyde-3-phosphate dehydrogenase is labelled by iodoacetic acid and 4-nitrophenyl acetate (Harris *et al.*, 1963; Perham & Harris, 1963). Rather, the foregoing is intended to demonstrate the inherent uncertainty which exists with modification experiments in general.

The best way to overcome this difficulty is to trap a true intermediate which occurs naturally along the kinetic pathway. Only then can it be absolutely certain that the labelled residue is essential for activity. For aldehyde dehydrogenase, the obvious candidate is the acyl intermediate, $^{acyl}E_{NADH}$, which is subsequently hydrolysed to the binary $^{*}E_{NADH}$ complex (Scheme 1.2). However, before isolating such an intermediate there are certain requirements which both the aldehyde substrate and assay conditions must satisfy.

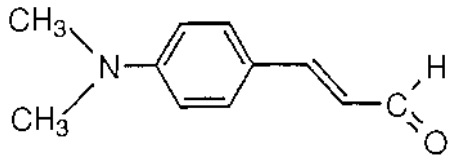
Firstly, $^{acyl}E_{NADH}$ must exist at a significant concentration in the steady state to ensure a high percentage of labelled material. Ideally, formation of this

species should be rapid, but its hydrolysis slow compared to the rate of NADH dissociation. This automatically discounts using the more conventional substrates such as acetaldehyde and propionaldehyde. Here, acyl hydrolysis is relatively fast compared to NADH release at pH 7.4 and 25 ^{o}C (5 s^{-1} versus 0.2 s^{-1}; Bennett *et al.*, 1982).

Secondly, assuming that $^{acyl}E_{NADH}$ has been successfully trapped, there must be some convenient way of monitoring the labelled active site peptide throughout subsequent isolation steps. Although one way would be to label aldehyde dehydrogenase with a radioactive substrate, it would be more convenient to use a chromophoric aldehyde instead. Preliminary studies could then be performed to confirm the formation of $^{acyl}E_{NADH}$, and to establish reaction conditions needed to optimise its survival in the steady state. After isolation and digestion the aldehyde sidechain could then be used as a chromophoric marker to detect the active site peptide.

The final consideration concerns the stability of the incorporated chromophore. In all probability the acyl linkage will be an ester - perhaps from cysteine or serine. Consequently, there is the risk that the chromophore will be hydrolysed throughout purification. This is what happens, for example, with activated esters such as 4-nitrophenyl acetate in aqueous solution (see Chapter 5). In view of this, isolation must be performed as quickly as possible using techniques which minimise any progressive loss in labelled material.

One aldehyde which satisfies these criteria is *trans*-4-N,N-dimethylamino-cinnamaldehyde (below)



This is an intense chromophore at 398 nm (ε_{MAX} = 30,000 l $mole^{-1}$ cm^{-1}) and possesses the experimental advantage in having an absorption maximum which is sensitive to changes in the external environment. For alcohol dehydrogenase, *trans*-4-N,N-dimethylaminocinnamaldehyde has been used successfully as a

probe to detect various intermediates in the kinetic mechanism (Morris *et al.*, 1980; Dunn *et al.*, 1975) and also provided the first direct evidence for the existence of a ternary complex with NAD^+ (Dahl & Dunn, 1984).

Preliminary studies with aldehyde dehydrogenase show *trans*-4-N,N-dimethylaminocinnamaldehyde is a true substrate, and forms an acyl intermediate with a λ_{MAX} at 463 nm. Figure 7.23a shows the results of a stopped flow experiment from Buckley & Dunn (1982) where *trans*-4-N,N-dimethylaminocinnamaldehyde is rapidly added to enzyme premixed with NAD^+ at pH 7.4 and 25 ^oC. Initially, the spectrum is representative of free aldehyde with only a shoulder around 400 nm (trace 4). During the time course of the reaction, however, a transient build-up at 463 nm occurs (trace 14) which then decays as the steady state phase of the reaction takes over (trace 19).

In later studies the pH dependence of *trans*-4-N,N-dimethylaminocinnamaldehyde oxidation was investigated, and the results of Dunn & Buckley (1985) are shown in Figure 7.23b. At relatively high pH's such as 9.2 and 10.5, the acyl intermediate at 463 nm forms but then decays in the same manner as in Figure 7.23a. As the pH is progressively lowered, however, the rate of acyl hydrolysis is decreased so that at pH 6.1 this process becomes rate-limiting. Consequently, the intermediate survives for several minutes. In the limit (pH 4.1 and 5.1) the association of the aldehyde is rate-limiting.

In the present study this acyl intermediate which builds up at pH 6.1 was rapidly precipitated by the addition of $HClO_4$, washed, and subsequently redissolved in 8 M urea. Figure 7.1b shows the spectrum of the denatured species at this stage, and quite clearly shows the presence of the *trans*-4-N,N-dimethylaminocinnamoyl sidechain with a λ_{MAX} around 412 nm. Perhaps the most interesting feature in Figure 7.1b, however, is the complete absence of colour in a control where NAD^+ is omitted. The conclusion to draw from this, of course, is that NAD^+ is essential for the incorporation of chromophore. This discounts the possibility of nonspecific side reactions such as Schiff base formation, and reaffirms the earlier contention that a true catalytic intermediate has been isolated.

One potential criticism is the effect of acid precipitation on the stability of the chromophore. Presumably, the possibility exists that $HClO_4$ can catalyse the

transfer of the acyl sidechain to another residue, in which case the chromophore would not be attached to the catalytic nucleophile.

In answer to this, consider the finding from the gel filtration column which shows almost all colour is associated with fraction 7 (Figure 7.4). For this to occur as a consequence of group transference during precipitation requires two unlikely assumptions, namely: (1) such a reaction goes to 100 % completion; and (2) the transfer is completely specific for a particular enzymic group. Obviously, if these criteria were not satisfied then instead of the observed specificity in Figure 7.4, colour would be spread over a number of different fractions. The complete absence of other peaks, therefore, does not support this viewpoint. In any case, it is reasonable to expect that precipitation itself would protect against such transfer reactions.

The most compelling evidence discounting any catalysed shift in the acyl sidechain are the results obtained under different trapping conditions (Figure 7.1a). In all cases the position of the chromophore is unchanged. This is despite fundamentally different denaturation conditions by ethanol and urea compared to those by $HClO_4$. Because of this, it can be concluded that acid precipitation preserves the integrity of the modified catalytic nucleophile.

In addition to the foregoing, an acyl species was trapped from the enzyme-catalysed hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole (Figure 7.20). The fundamental difference between this activity and aldehyde oxidation is that the latter acyl intermediate is formed from hydride transfer. On the other hand, *trans*-4-N,N-dimethylaminocinnamoyl imidazole reacts to form this species via a nucleophilic displacement, and hence does not inherently require the presence of coenzyme for activity.

Despite this difference, the present research strongly supports the contention that the catalytic nucleophile is involved in both these activities. This is based on the following, namely: (1) the spectrum of the denatured acyl species from *trans*-4-N,N-dimethylaminocinnamoyl imidazole catalysis (in the absence of NAD^+) is virtually identical to that isolated from the dehydrogenase pathway (Figures 7.1b and 7.20a); and (2) the chromophore is associated with T5 from the gel filtration column (Figures 7.20b and 7.21b).

7.4.3 Arguments concerning the identity of the catalytic nucleophile

The present research clearly shows that the *trans*-4-N,N-dimethylamino-cinnamoyl chromophore is associated with a ten amino acid peptide identified as T5 in the cytoplasmic primary structure (Hempel *et al.*, 1984). On chemical grounds, the only group capable of fulfilling the role of catalytic nucleophile is Ser-74.

One problem highlighted in the present findings is the gradual loss of labelled material incurred through spontaneous hydrolysis. Consequently, there is no direct evidence to support a covalent link between the chromophore and Ser-74. For instance, there is no perturbed residue from sequencing, and FAB source mass spectroscopy has confirmed only the molecular weights of unlabelled peptides.

Without this crucial information the possibility exists that the chromophore is not attached to Ser-74 at all, but to some other peptide which we will designate T^*. Call the labelled peptide T^{*dac}. Furthermore, because it has been traditionally assumed that the catalytic nucleophile is cysteine, the working hypothesis will be that T^* contains this group.

A clue to the identity of T^* is given in the gel filtration column step which separates the tryptic peptides according to molecular weight (Figure 7.4). It follows that if the acyl sidechain is attached to T^* then this peptide should also have a similar molecular weight to T5. Obviously, if the catalytic nucleophile is cysteine then this residue should be strictly conserved in all known primary structures (Johannson *et al.*, 1988). The groups which fulfil this criterion, and hence are potential candidates, are Cys-49, Cys-162, Cys-301, Cys-302, Cys-369, and Cys-455 (Hempel & Jornvall, 1989). Consequently, T^* must be one of these corresponding tryptic peptides.

Of these, the peptide of Cys-301 and Cys-302, T20, elutes in the first major peak of the profile in Figure 7.4 (von Bahr-Lindstrom *et al.*, 1985). Likewise, T13 and T14 which contain Cys-162 and Cys-49 respectively appear even earlier (supplement table 1; Hempel *et al.*, 1984). The only peptide capable of running in the same region as the chromophore in Figure 7.4 is T25, which contains Cys-

369. This has been shown to appear earlier than T5 in the human cytoplasmic enzyme (Hempel *et al.*, 1984) but later in the horse isozyme (von Bahr-Lindstrom *et al.*, 1984).

It could be argued that because the primary structure of the sheep isozyme is unknown, comparisons with other species may give misleading interpretations. That is, there is no guarantee that the tryptic peptides between species will be the same. However, while this is always a possiblity it should also be pointed out that the known primary structures of the horse and human cytoplasmic enzymes display a 91 % homology (Johansson *et al.*, 1988). It seems reasonable to assume, therefore, that if there are differences then they will be minor.

We are now in a position to discuss the possibility that the chromophore is attached to T^*, tentatively identified as T25. The first conclusion to draw from Figure 7.4 is that the concentration of T^* in fraction 7 is very small. This is based on the the observed purity of T5, namely: (1) the absence of any other major peptide peak from HPLC (Figure 7.5) and from other column techniques (Figure 7.7); and (2) the results obtained from sequencing (Figure 7.6). Because of this, an advocate of a "T^* theory" is forced to conclude that these two peptides are normally well separated by gel filtration. Consequently, the association of the chromophore with T5 must arise because T^{*dac} has elution characteristics different from T^*. A qualitatively similar argument also applies to the labelled peak from reverse phase HPLC (Figure 7.5). In this case T^{*dac} must have an almost identical hydrophobicity value to T5.

To summarise thus far, the present findings show the chromophore is associated with the peptide, T5. To preserve the concept that the chromophore is attached to another peptide, T^*, two assumptions must be made, namely: (1) T^{*dac} associates with T5 in both gel filtration and HPLC, even though these techniques separate according to fundamentally different principles; and (2) T^{*dac} is present at such low concentrations that it is undetectable within the limits of error from sequencing (Figure 7.6).

To investigate further the possibility that T^* is the true active site peptide, two important experiments performed in the present study will now be discussed. The first was to differentially label aldehyde dehydrogenase with [^{14}C]iodo-

acetamide. Here, the acyl intermediate was trapped with $HClO_4$, redissolved in 8 M urea, and carboxymethylated in the usual way. The chromophore was then removed either by ammonia or by spontaneous hydrolysis (Figure 7.9) and the denatured enzyme reacted with [^{14}C]iodoacetamide.

The strategy underlying the foregoing is very similar in principle to that of Tu & Weiner (1988a). Here, the active site was temporarily protected against reaction with N-ethylmaleimide by o-nitrobenzaldehyde (see section 7.4.1). In the present experiment, the modified catalytic nucleophile should also be protected against iodoacetate during carboxymethylation. Consequently, when small molecules such as iodoacetate are removed, subsequent hydrolysis of the acyl group will expose the catalytic nucleophile to reaction with [^{14}C]iodoacetamide.

It is at this point that the experiment differentiates whether the catalytic nucleophile is cysteine or serine. If the former, then the chromophore will be substituted by an inherently more stable [^{14}C]carboxamidomethyl sidechain. Therefore, instead of a coloured peak in fraction 7 (Figure 7.4) there will be a strongly incorporated radioactive peak. In the present study, there is no evidence to suggest the existence of such a peak by the methods used (Figure 7.8). Accordingly, these findings do not support the proposal that cysteine is the catalytic nucleophile.

The second experiment involved the redigestion of fraction 7 in Figure 7.4 with thermolysin before final purification on HPLC. The results show that the chromophore is now associated with a smaller segment of T5 which still contains Ser-74. This finding is naturally expected, of course, if Ser-74 were catalytically essential. On the other hand, there is no *a priori* reason why this should occur if T^{*dac} is involved. To preserve this viewpoint, it must firstly be assumed that the redigestion products of T^{*dac} and T5 elute together, and secondly, that the latter coincidentally happens to contain Ser-74.

Finally, support for Ser-74 being the catalytic nucleophile is provided by studies with the synthetically prepared peptide acetyl-Ile-Gly-Ser-Pro-Trp-Arg-NH_2. Under forcing conditions this couples with the mixed anhydride of *trans*-4-N,N-dimethylaminocinnamic acid and isobutyl chloroformate to give a derivative which contains an unsymmetrical peak with a λ_{MAX} around 400 nm

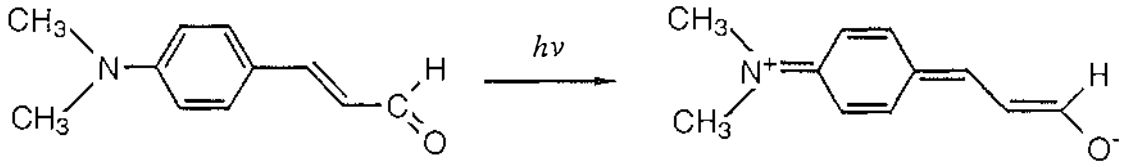
(Figure 7.14b). This peak is expected to be blue shifted slightly because of the high percentage of organic solvent present (see later). The pronounced shoulders observed here are likely to arise from competing reactions which produce peaks at 350 nm (Figure 7.12b). The latter are characteristic of amide derivatives of this chromophore (compare with 348 nm; Dunn & Buckley, 1985).

Further support is provided from the spectral difference of the synthetically labelled peptide after one hour in aqueous solution. Over this time period the spectrum of the reaction mixture changes, indicating hydrolysis of a chromophore with a peak around 400 nm (Figure 7.14a). One difficulty with this finding, however, is the observed hydrolysis rate. If it is concluded that the serine residue is modified then it is not known why this should be so high compared to that of the isolated chromophore (Figure 7.15).

7.4.4 The unusual properties of the isolated chromophore

One area yet to be considered in the present study is the reasons underlying the perturbed λ_{MAX} of the *trans*-4-N,N-dimethylaminocinnamoyl chromophore in both the native and denatured enzyme. Before discussing this, however, it will be useful first to gain some insight into the factors which affect λ_{MAX}.

The chromophoric properties of the *trans*-4-N,N-dimethylaminocinnamoyl sidechain arise from a long wavelength Π - Π^* electronic transition (below)



Generally speaking, there are at least two ways in which λ_{MAX} can be affected. First, any outside influence which stabilises the excited state relative to the ground state will lower the transition energy, and red shift the spectrum. Included in this category are "solvent affected" spectra which are shifted to longer wavelengths as the dielectric constant of the medium is increased. The

reason is that good solvents such as water can diffuse electron density on the carbonyl oxygen by hydrogen bonding, and consequently stabilise the excited state. On the other hand, organic solvents have a reduced capacity for this and generally increase the Π - Π^* transition energy. This decreases λ_{MAX}. Examples of this in the present study include the λ_{MAX} shift of *trans*-4-N,N-dimethylaminocinnamoyl imidazole from 424 nm in aqueous solution to 406 nm in acetonitrile (Figure 7.24) and the 4 nm red shift of the isolated chromophore from 408 nm (0.1 M ammonium bicarbonate) to 412 nm in 8 M urea (compare Figures 7.1b and 7.4b). For the latter, this shift is presumably due to the better hydrogen bonding capacity of urea relative to water.

For alcohol dehydrogenase, this type of mechanism can be used to explain the dramatic red shifts which occur when *trans*-4-N,N-dimethylaminocinnamaldehyde binds in the presence of coenzyme. For instance, with NADH the spectrum of the chromophore is perturbed 66 nm to 464 nm (Dunn & Hutchinson, 1973). However, it is concluded by Angelis *et al.* (1977) that solvent effects such as the interaction with a bound water molecule are too weak to account for such a big shift. Instead, this perturbation is proposed to arise from inner sphere coordination of the carbonyl group of *trans*-4-N,N-dimethylaminocinnamaldehyde with the catalytic zinc cation (Angelis *et al.*, 1977). The latter functions as a Lewis acid, and acts as an "electron sink" which pulls electron density away from the aldehyde oxygen. That *trans*-4-N,N-dimethylaminocinnamaldehyde binds in this way is confirmed by crystallographic studies (Cedergren-Zepperzauer *et al.*, 1982) and from Lewis acid model complexes (Angelis *et al.*, 1977).

Interestingly, an even bigger shift in λ_{MAX} occurs when *trans*-4-N,N-dimethylaminocinnamaldehyde forms a ternary complex with NAD^+ (Dahl & Dunn, 1984). In this case, the spectrum is shifted 97 nm to 495 nm which is 31 nm more than with NADH. It is concluded that this is due to the additional electron withdrawing properties of the nicotinamide ring of NAD^+ which is closely positioned to the aldehyde (Dahl & Dunn, 1984).

The second class of conditions which can affect λ_{MAX} includes all those factors which structurally distort the chromophore in the native protein. For example, in the catalysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole by elastase the

spectrum of the resulting acyl intermediate (which is an oxygen ester) is red shifted 32 nm compared to its denatured state (Breaux & Bender, 1976). Similar shifts ara also observed with β-arylacryloyl derivatives of subtilisin and α-chymotrypsin (Bernhard *et al.*, 1965).

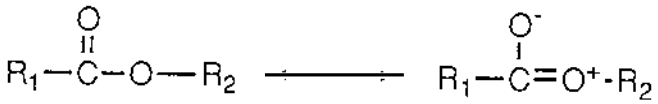
From studies with *trans*-4-N,N-dimethylaminobenzoyl imidazole and α-chymotrypsin, Argade *et al.* (1984) conclude these shifts are not due to solvent factors. Instead, λ_{MAX} is specifically affected by the native conformation of the protein. This contention is based on findings which show that the properties of the acyl intermediate approximate to those of *trans*-4-N,N-dimethylaminobenzaldehyde (Argade *et al.*, 1984). Evidently, the acyl intermediate is structurally perturbed in such a way that it becomes more "aldehyde-like". This can occur if the ester group is distorted so that there is little $\prod$-bond interaction between the carbonyl group and the alkyl oxygen (Argade *et al.*, 1984). This is supported by crystallographic studies which show that the ester group of [(3-indoleacryloyl)-Ser^{195}] acyl-α-chymotrypsin is not entirely in the s-*trans* configuration (Henderson, 1970).

For aldehyde dehydrogenase the acyl intermediate formed along the dehydrogenase pathway has a λ_{MAX} of 463 nm (Figure 7.23). Quite clearly, this represents a dramatic shift from that exhibited by either a serine or cysteine model ester (370 nm and 398 nm respectively). The exact reasons for this shift are unclear in view of what has already been discussed for alcohol dehydrogenase. From the conclusions made by Angelis *et al.* (1977) it seems unlikely that solvent effects alone could sufficiently account for this perturbed λ_{MAX}. Likewise, the out of plane bending in α-chymotrypsin which makes the ester more "aldehyde-like" could, presumably, contribute only to a small extent. This is because the observed λ_{MAX} of 463 nm far exceeds that of *trans*-4-N,N-dimethylaminocinnamaldehyde (λ_{MAX} = 398 nm). In the light of the foregoing, therefore, it is likely that both electrostatic and distortional factors contribute to the perturbed λ_{MAX}. To what extent, however, is presently unknown.

A further finding in the present study which is not not well understood is the unusual properties of the denatured acyl intermediate. Firstly, the λ_{MAX} of 408 nm is not typical of a serine-ester containing this chromophore under similar

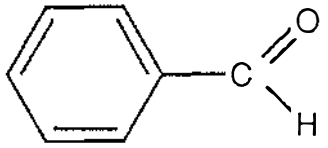
solvent conditions (compare with 370 nm; Breaux & Bender, 1976). Instead, λ_{MAX} is more typical of a cysteine model ester (Figure 7.18).

Secondly, there is a disparity between the rate of hydrolysis of the isolated peptide, the cysteine model ester, and *trans*-4-N,N-dimethylaminocinnamoyl imidazole (Figures 7.15, 7.16, and 7.17 repectively). In fact, under conditions where there is significant hydrolysis of the acyl intermediate there is no detectable loss of the cysteine model ester. Presumably, this disparity would also exist with the observed hydrolysis rate of a serine model ester. This is based on the general finding that oxygen esters are more stable than thiol esters because the alkyl oxygen has a more effective resonance contribution than sulphur (below)

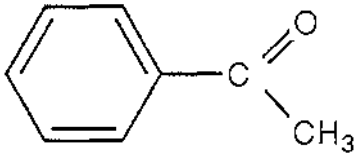


In summary, the observed properties of the isolated chromophore are not consistent with those expected of a serine model ester. On the other hand, while the hydrolysis rate of the isolated chromophore is not typical of a cysteine model ester either, the observed λ_{MAX} of 408 nm is similar. For this reason, these findings cannot discount the possibility that a cysteine residue is the catalytic nucleophile.

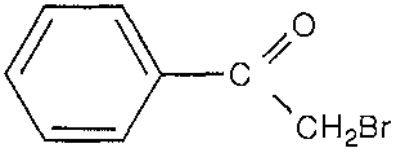
Figure 7.22 Structures of Benzaldehyde, Acetophenone and Bromoacetophenone



Benzaldehyde



Acetophenone

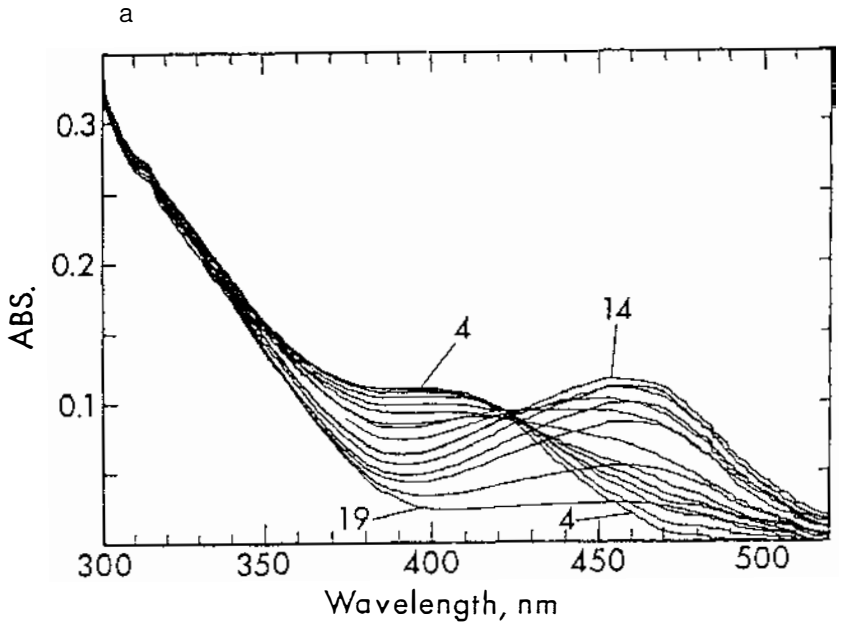


Bromoacetophenone

Figure 7.23a shows the time-resolved uv-visible spectra for the reaction of the aldehyde dehydrogenase-NAD^+ complex with *trans*-4-N,N-dimethylamino-cinnamaldehyde. The first scan in each set was initiated approximately 5 msec after flow had stopped. The repetitive scan rate was 8.605 msec/scan with additional delays introduced at longer times to space the 19 scans over 81 seconds in order to follow the fast buildup of intermediate at 460 nm and its subsequent slow decay. Little change occurred during the first three scans which are omitted from Figure 7.23a. Conditions before mixing: (Syringe 1) [Aldehyde Dehydrogenase] = 42.7 μM; [NAD^+] = 3.18 mM; (Syringe 2) [*trans*-4-N,N-Dimethylaminocinnamaldehyde] = 5.6 μM; both in 35 mM phosphate buffer (pH 7.6) at 25 ^{o}C (Buckley & Dunn, 1982).

Figure 7.23b. Single wavelength time courses for acyl-E formation and decay at 463 nm as a function of pH. The time courses are constructed from rapid-scanning data for pH values of 10.5, 9.2, 7.8, 6.1, 5.1, and 4.1 at 25 ^{o}C (Dunn & Buckley, 1985).

Figure 7.23 Stopped Flow Studies of the Enzyme-Catalysed Oxidation of *trans*-4-N,N-Dimethylaminocinnamaldehyde (Buckley & Dunn, 1982; Dunn & Buckley, 1985)



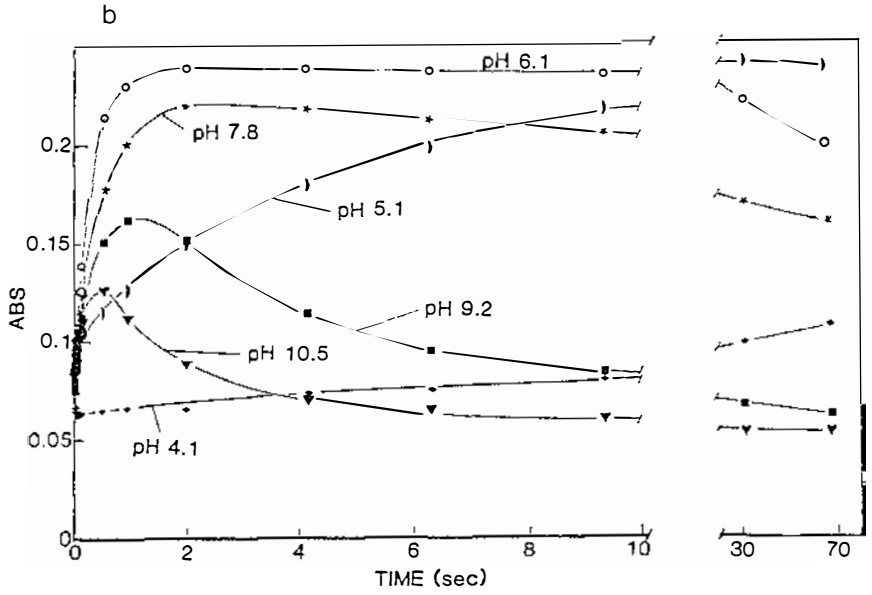
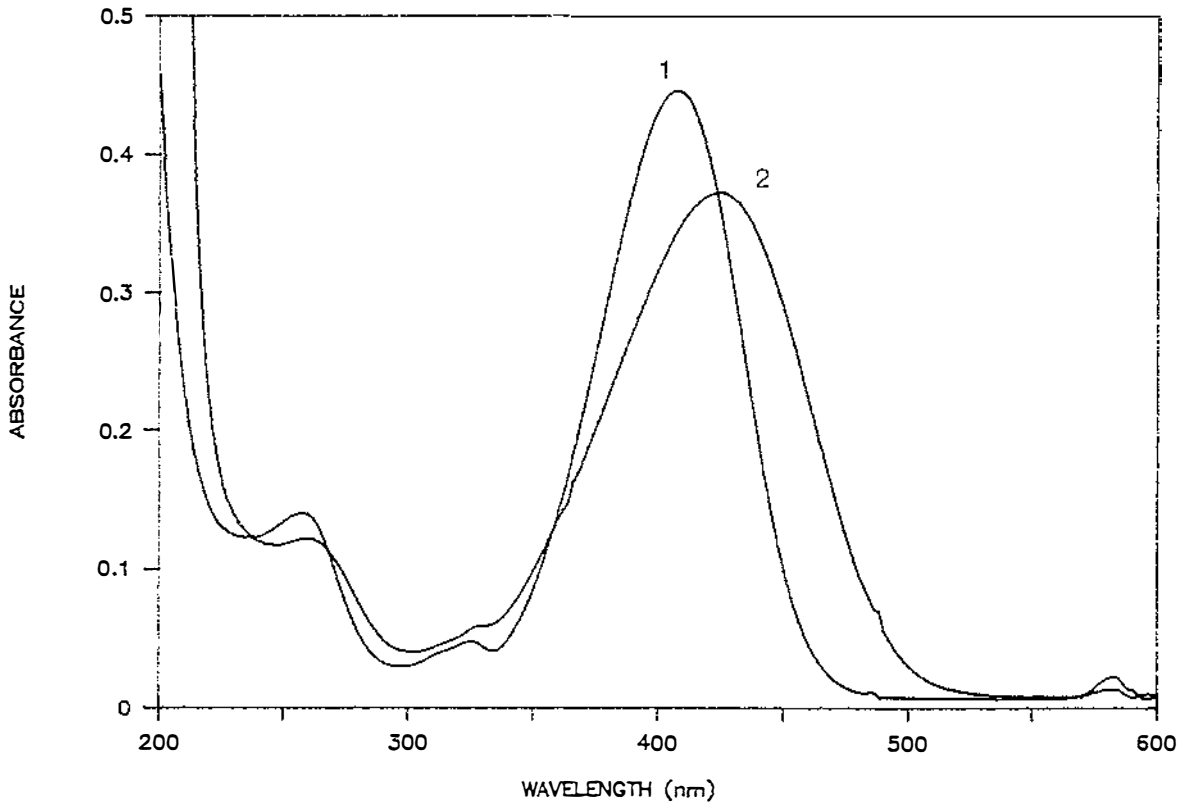


Figure 7.24 shows the spectra of *trans*-4-N,N-dimethylaminocinnamoyl imidazole (18 μM) in acetonitrile (spectrum 1) and 50 mM sodium phophate buffer, pH 7.4, + 1 % acetonitrile (spectrum 2).

Figure 7.24 Spectra of *trans*-4-N,N-Dimethylaminocinnamoyl Imidazole in Acetonitrile and Aqueous Solution



CHAPTER 8

AN OVERVIEW

In Chapters 4 and 5 it was concluded that the observed properties of group A, and the detection of acetaldehyde from an esterase system in the presence of NADH, both supported a common active site for aldehyde oxidation and 4-nitrophenyl acetate hydrolysis. The one-site/two-site question will now be discussed further in the light of recent literature, and the labelling studies from Chapter 7.

Tu and Weiner (1988b) have proposed that aldehyde dehydrogenase possesses two separate active sites on the basis of labelling studies with N-ethylmaleimide. One piece of evidence was that the incorporation of this reagent affects the dehydrogenase and esterase activities differently. Findings indicated that modification of Cys-49 inhibits the former, but the latter activity only declines when Cys-162 is modified. Tu and Weiner (1988b) interpreted this as a two-site phenomenon where Cys-49 and Cys-62 reside at what Blackwell *et al.* (1983) call P1 and P2 respectively.

Although the foregoing is consistent with a two-site model, a one-site explanation would argue in terms of kinetic differences between the respective pathways. For instance, it is quite possible that both 4-nitrophenyl acetate and aldehyde still bind normally at P1 even when Cys-49 is modified by N-ethylmaleimide. However, because processes such as hydride transfer or NADH dissociation may be specifically affected - features not shared by 4-nitrophenyl acetate hydrolysis - only aldehyde oxidation is inhibited.

Compelling evidence for a common active site is provided by the kinetic and labelling experiments with *trans*-4-N,N-dimethylaminocinnamoyl imidazole in Chapter 7. Results clearly show the hydrolysis of trans-4-N,N-dimethylaminocinnamoyl imidazole is enzyme-catalysed (Figure 7.19) and that the isolated chromophore is associated with the same peptide as that labelled by *trans*-4-N,N-dimethylaminocinnamaldehyde (Figure 7.21b). The conclusion to draw here, of course, is that the same catalytic nucleophile - and hence the same "P1" active site - can mediate two different activities. Although *trans*-4-N,N-dimethylaminocinnamoyl imidazole is not an ester like 4-nitrophenyl acetate,

there are obvious similarities between the two as substrates for aldehyde dehydrogenase. For example, both activities can proceed in the absence of coenzyme, and enzyme acylation occurs via a direct step which liberates imidazole and 4-nitrophenoxide respectively. On this basis, it seems reasonable to expect that if the same active site can catalyse both aldehyde oxidation and the hydrolysis of *trans*-4-N,N-dimethylaminocinnamoyl imidazole, then it could also catalyse the hydrolysis of 4-nitrophenyl acetate.

It could be argued that the association of the chromophore (from *trans*-4-N,N-dimethylaminocinnamoyl imidazole hydrolysis) with an earlier fraction from the gel column (Figures 7.20b and 7.21a) is due to reaction at P2. However, this view is only one of a number of possible explanations which can explain this finding. For instance, because we are dealing with a partial tryptic digest it could be that the same enzyme group is involved, but it is part of a larger incompletely digested peptide. (This conclusion was reached with *trans*-4-N,N-dimethylaminocinnamaldehyde where colour was observed in the same position, Section 7.3.1.) Alternatively, *trans*-4-N,N-dimethylamino-cinnamoyl imidazole may not be totally specific for the catalytic nucleophile but may, to a certain extent, nonspecifically react elsewhere. This was found to be the case with certain irreversible modifiers in the reaction with α-chymotrypsin (Lawson, 1980; see Section 7.4.2).

Another major focus of the present thesis has concerned the identity of the catalytic nucleophile in aldehyde oxidation. In Section 7.4.3 evidence was presented which strongly points toward Ser-74 being this group. However, the fact that the λ_{MAX} of the isolated chromphore is similar to a cysteine model ester casts doubt on the involvement of Ser-74. In the light of this, future work could aim to repeat these labelling experiments with other aldehyde dehydrogenases whose complete primary structures are known. The disadvantage in using the sheep enzyme is that T^* (see Section 7.4.3) is presumed to contain Cys-369 only by comparison with the horse and human enzyme. Although the cytoplasmic isozymes between these two latter species display a 91 % sequence homology (Johansson *et al.*, 1988) there is no absolute guarantee that the tryptic peptides of the sheep isozyme will be identical. For instance, in the present study there may be an as yet "unseen" cysteine-containing peptide which is eluting in the vicinity of T5. If this is the case then

dramatic shifts in the elution position from the gel column may be observed with other aldehyde dehydrogenases.

In conclusion, the existence of a separate P2 active site which can catalyse the hydrolysis of 4-nitrophenyl acetate and reversibly bind high concentrations of aldehyde is questionable. In the present author's view, there is no convincing experimental evidence to support a two-site model, and what evidence does exist points clearly in a one-site direction, namely: (1) the observed properties of group A (Chapter 4); (2) the detection of acetaldehyde from enzyme, 4-nitrophenyl acetate, and NADH (Chapter 5); and (3) the findings from *trans*-4-N,N-dimethylaminocinnamoyl imidazole (Chapter 7). On this basis, it is concluded that the dehydrogenase and esterase activities are mediated by a common active site. Whether the catalytic nucleophile is Ser-74 or a cysteine residue will have to be decided by future investigation.

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