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BENZYL ALCOHOL DEHYDROGENASE AND BENZALDEHYDE DEHYDROGENASE OF *ACINETOBACTER CALCOACETICUS*

BY

ROBERT WILLIAM MACKINTOSH

A thesis submitted for the degree of Doctor of Philosophy, Department of Biochemistry of the University of Glasgow, March 1987.

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ABBREVIATIONS

Abbreviations used in this thesis are those recommended by the Biochemical Society, London, except for those listed below.

BADH	Benzyl alcohol dehydrogenase
BSA	Bovine serum albumin
BZDH I	Benzaldehyde dehydrogenase I
BZDH II	Benzaldehyde dehydrogenase II
DEAE	Diethylaminoethyl
DTT	DL-Dithiothreitol
FPLC	Fast Protein Liquid Chromatography
NBRF	National Biomedical Research Foundation
NCIB	National Collection of Industrial Bacteria
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulphonyl fluoride
PMS	N-Methylphenazonium methosulphate
PQQ	Pyrroloquinoline quinone
PTH	Phenylthiohydantoin
r	Regression coefficient
SDS	Sodium dodecyl sulphate
TEMED	NNN'N'-Tetramethylethylenediamine

1. Acinetobacter calcoaceticus NCIB 8250 can grow on benzyl alcohol as sole source of carbon and energy. Previous work had shown that benzyl alcohol is oxidised to benzoate by benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II. This thesis is concerned with the purification and characterisation of these two NAD^+-dependent, soluble enzymes.

2. A quick, reliable procedure was developed for purifying both enzymes from a single batch of bacteria. The purification procedure involved disruption in the French pressure cell, ion-exchange chromatography on DEAE-Sephacel, affinity chromatography on Blue Sepharose CL-6B and Matrex gel Red A, followed by gel filtration through a Fast Protein Liquid Chromatography Superose 12 column. The enzymes co-purified as far as the Blue Sepharose CL-6B step, and were separated on the Matrex gel Red A column. Both enzymes were isolated in good yield (e.g. 0.7 mg of benzyl alcohol dehydrogenase and 1.8 mg of benzaldehyde dehydrogenase II from 9 g wet weight of bacteria) and were homogeneous as judged by both denaturing and non-denaturing gel electrophoresis. The enzymes could be stored so that they both retained more than 90% of their original activity over a three month period.

3. Dithiothreitol and $MgCl_2$ were required to stabilise both enzyme activities. $MgCl_2$ was particularly important during dialysis; it also aided binding to the triazine affinity columns.

4. The enzymes are tetramers as judged by comparison of their subunit (benzyl alcohol dehydrogenase: 39 700; benzaldehyde dehydrogenase II: 55 000) and native (benzyl alcohol dehydrogenase: 155 000; benzaldehyde dehydrogenase II: 223 000) relative molecular masses estimated by sodium dodecylsulphate gel electrophoresis and gel filtration respectively.

5. The optimum pH for enzyme activity was 9.2 for benzyl alcohol dehydrogenase and 9.5 for benzaldehyde dehydrogenase II. The pI values were determined by chromatofocusing of the enzymes on a Fast Protein Liquid Chromatography Mono P column and were 5.02 for benzyl alcohol dehydrogenase and 4.59 for benzaldehyde dehydrogenase II.

6. The absorption spectra of the two enzymes showed no evidence that they contain any cofactors such as cytochrome, flavin, or pyrroloquinoline quinone.

7. The assay procedures used to measure benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II activities involved monitoring the reduction of NAD^+ at 340 nm.

The activity of benzyl alcohol dehydrogenase is reversible, oxidising alcohol with NAD^+ and also reducing aldehyde with NADH. The equilibrium constant, K'_{eq}, for the oxidation of benzyl alcohol to benzaldehyde was calculated to be 3.08×10^{-11}M and thus reduction of benzaldehyde is favoured.

In order to obtain linear initial rates of alcohol-oxidising activity with purified benzyl alcohol dehydrogenase a modification of the assay procedure was developed in which

hydrazine was included in the reaction mixture in order to trap the aldehyde products as hydrazones. The conversion of acid to aldehyde is thermodynamically unfavourable and benzaldehyde dehydrogenase II did not appear to reduce benzoate to benzaldehyde.

8. The K_m and V_{max} values of both enzymes were determined for a range of alcohols and aldehydes and k_{cat} values were calculated. Benzyl alcohol was the most effective alcohol substrate for benzyl alcohol dehydrogenase, with a K_m of 17.3 μM and V_{max} of 231 units.(mg protein)$^{-1}$. Perillyl alcohol was the second most effective substrate, and was the only non-aromatic alcohol oxidised. The other alcohol substrates of benzyl alcohol dehydrogenase were aromatic alcohols, with <u>para</u>-substituted derivatives of benzyl alcohol being better substrates than other derivatives. Coniferyl alcohol and cinnamyl alcohol, which are known intermediates of lignin biodegradation and biosynthesis, were substrates. The reverse reaction of benzyl alcohol dehydrogenase has a V'_{max} of 366 units (mg protein)$^{-1}$ and a K'_m of 4.0 μM for benzaldehyde.

Benzaldehyde was the most effective substrate of benzaldehyde dehydrogenase II with a K'_m of 0.68 μM and V'_{max} of 76 units.(mg protein)$^{-1}$. Benzaldehydes with a single small substituent group in the <u>meta</u> or <u>para</u> position were better substrates than any other benzaldehyde derivative. Benzaldehyde dehydrogenase II could also oxidise the aliphatic aldehydes hexan-1-al and octan-1-al, albeit poorly.

9. Benzaldehyde dehydrogenase II but not benzyl alcohol dehydrogenase exhibited esterase activity with 4-nitrophenyl acetate as substrate.

10. Both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were inhibited by the sulphydryl reagents iodoacetate, iodoacetamide, 4-chloromercuribenzoate and N-ethylmaleimide. Benzyl alcohol or benzaldehyde respectively protected against these inhibitions. NAD^+ gave some protection but less than the aromatic substrates.

11. Neither benzyl alcohol dehydrogenase nor benzaldehyde dehydrogenase II was inhibited by the metal chelating agents EDTA, 2,2'-bipyridyl, pyrazole and 2-phenanthroline.

12. Neither enzyme is inhibited by a range of plausible metabolic inhibitors such as mandelate, phenylglyoxylate, benzoate, succinate, acetyl-CoA, ATP and ADP.

13. Benzaldehyde dehydrogenase II was sensitive to inhibition by several aromatic aldehydes. In particular, ortho-substituted benzaldehydes such as 2-bromo-, 2-chloro-, and 2-fluorobenzaldehydes were potent inhibitors of the enzyme.

Benzaldehyde dehydrogenase II was substrate-inhibited by benzaldehyde when the assay concentration exceeded approximately 10 μM in the reaction mixture.

14. The N-terminal amino acid sequences were determined for both enzymes. Benzaldehyde dehydrogenase II has a short sequence at the N-terminal which could be usefully converted into an oligonucleotide gene probe. No homologies were

identified between the N-terminal amino acid sequences of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II or between them and an archived data bank of amino acid sequences.

15. The final chapters include discussion of the possible role of sulphydryl groups at the enzymes' active sites; whether or not a metal is involved in the mechanism of benzyl alcohol dehydrogenase; how aromatic aldehydes might inhibit benzaldehyde dehydrogenase II; further comparisons to be made between the physical properties of the enzymes and other alcohol and aldehyde dehydrogenases; possible future lines of research.

CHAPTER 1

INTRODUCTION

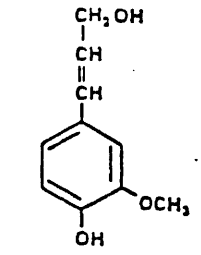
1.1 Aromatic compounds in the environment

Prokaryotes, fungi and higher plants produce an astounding array of aromatic benzenoid compounds; simple monocyclics and also more complex molecules such as flavonoids and alkaloids. In addition, many of the synthetic chemicals used as pesticides, detergents and plastics are modified aromatics. These man-made compounds are often halogenated and some are polycyclic; many are phytotoxic and carcinogenic.

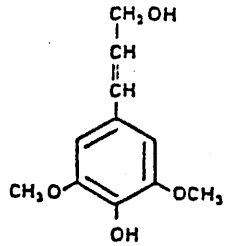
If they are soluble, aromatic compounds may be metabolised by microorganisms in soil and water. However, breakdown, especially of the man-made compounds, is often blocked if the chemical structure contains substituents which cannot react with the active sites of the inducible enzymes that are available (Hutzinger & Veerkamp, 1981).

The bulk of aromatic carbon is found not in low molecular mass compounds but in polymers, especially lignin (Higuchi, 1980). Lignin is an amorphous branched macromolecule (Figure 1.1). Its non-enzymic synthesis from monomeric aromatic alcohols such as coumaryl, coniferyl (VIII; see Appendix), and sinapyl alcohols results in the formation of carbon-carbon and ether linkages which are not easily broken either by chemical reagents or by enzymes (Dagley, 1975). This means that lignin biodegradation is a rate-limiting step in the Carbon cycle and great quantities of lignin or modified lignin accumulate. The industrial exploitation of pulping wastes to make useful by-products is limited because of this "lignin breakdown barrier" (Crawford, 1981).

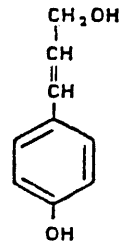
Figure 1.1 Monomeric aromatic alcohols involved in lignin biosynthesis (from Higuchi, 1980).



Coniferyl alcohol

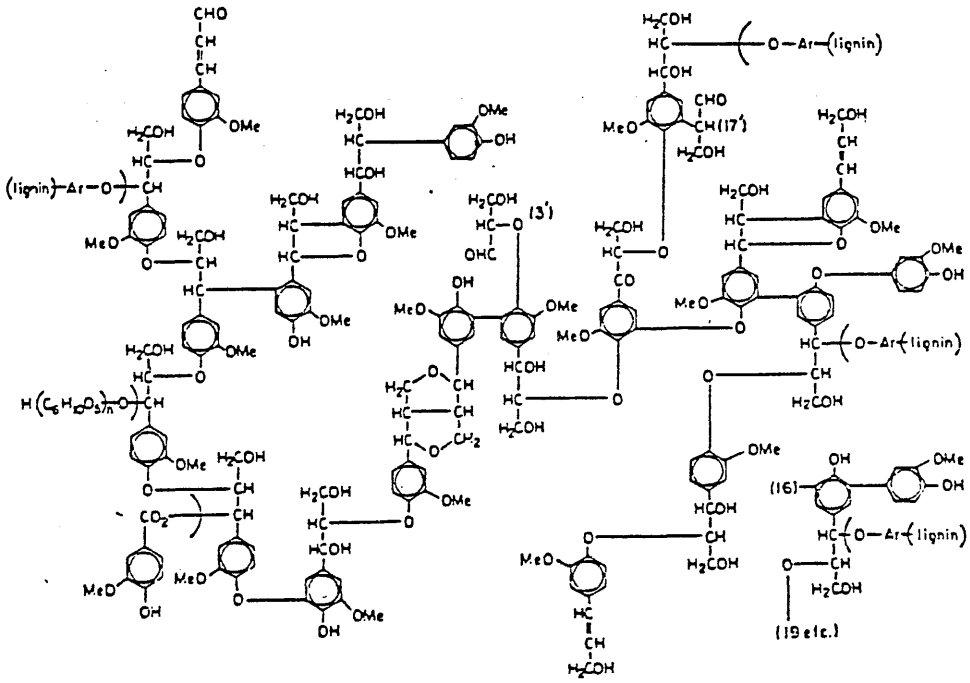


Sinapyl alcohol



3-Coumaryl alcohol

Representation of the molecular structure of spruce lignin (from Higuchi, 1980)



It appears to be primarily the white-rot fungi which are responsible for the initial attack on lignin and much of its subsequent degradation (Cain, 1980). A ligninase has been purified from the basidiomycete Phanerochaete chrysosporium (Tien & Kirk, 1984). This extracellular enzyme is a haemo-protein which requires H_2O_2 as a co-substrate for the oxidation of lignin and related substrates.

Bacteria are chiefly involved in degrading the smaller aromatic molecules such as coniferyl alcohol (VIII), 4-hydroxy-3-methoxybenzaldehyde (vanillin), 4-hydroxybenzoic acid, etc. which arise after fungal dissimilation of lignin (Cain, 1980). Inter-relationships are thought to exist amongst fungi and bacteria, the bacteria supply vitamins to the fungi and in turn the fungi provide aromatics on which the bacteria can grow (Crawford, 1981).

1.2 Pathways of aromatic catabolism in microorganisms

The enormous variety of aromatic compounds dissimilated by different microorganisms has aroused interest not only in the catabolic pathways and their regulation, but also in the individual enzymes of those pathways.

The metabolism of aromatic compounds has been studied in a variety of microorganisms. For example, Gram-negatives such as Alcaligenes spp., Azotobacter spp., and Flavobacterium spp. (Gibson, 1977), Gram-positives such as Bacillus spp. (Crawford & Olson, 1979), actinomycetes such as Nocardia spp. (Rann & Cain, 1973) and Rhodococcus spp. (Rast et al., 1980) as well as filamentous fungi and yeasts such as Aspergillus spp.,

Penicillium spp., Neurospora spp. and Rhodotorula spp. (Cain, 1980; Durham, 1984) have all been shown to grow on a range of aromatics. The most extensively investigated microorganisms with regard to their metabolism of aromatics are probably the soil microorganisms Pseudomonas spp. and Acinetobacter calcoaceticus which are able to grow on an extensive range of aromatic carbon sources (Stanier et al., 1966; Baumann et al., 1968). They utilize these compounds because of the ability to induce many convergent catabolic pathways (Hegeman, 1966a,b,c; Kennedy & Fewson, 1968a;Rosenberg, 1971). The elucidation of the metabolic pathways for degradation of aromatic compounds has been an enormous task and is still continuing (Gibson, 1984).

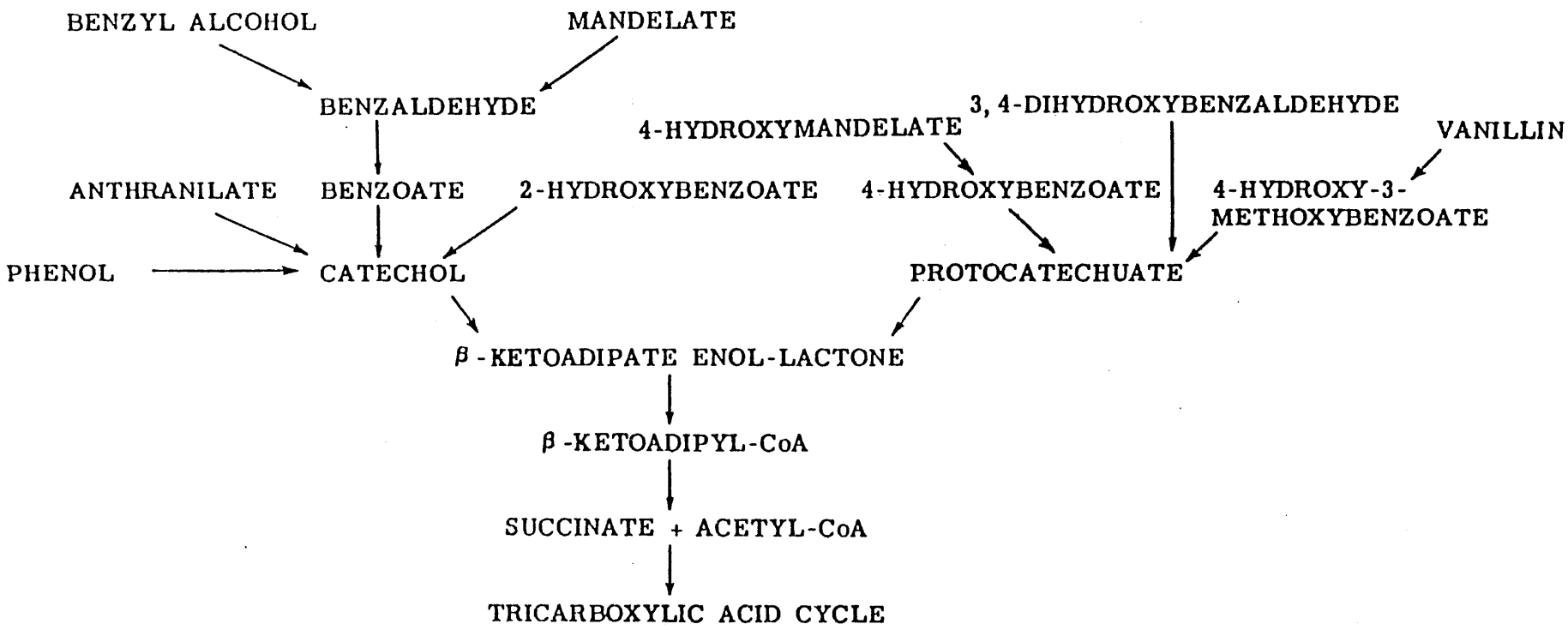
As most studies on control of metabolic pathways up to the 1960s had centred on induction and repression of enzymes concerned with carbohydrate metabolism (e.g. the lac operon; Jacob & Monod, 1961; Pastan & Perlman, 1969) and the repression and inhibition of divergent branch-chained pathways of amino acid biosynthesis (Cohen, 1965; Datta, 1969) little was known of the control systems operating in convergent, inducible, catabolic pathways. There was then an active period in which the regulation of expression of these convergent pathways was investigated in organisms such as Pseudomonas putida and Acinetobacter calcoaceticus (e.g. Stanier & Ornston, 1973) and this aspect is continuing with detailed genetic studies (e.g. Knackmuss, 1981; Williams, 1981; Harayama et al.,1986;Shanley et al., 1986). More recently, interest has turned to the detailed enzymology of the individual pathways and this thesis is concerned with an examination of the enzymes responsible for the oxidative catabolism of benzyl alcohol to benzoate in A. calcoaceticus NCIB 8250.

1.3 Acinetobacter calcoaceticus

Until recently the genus Acinetobacter had been divided into two species: A. calcoaceticus and A. lwoffii, however, Bovet & Grimont (1986) propose three new species, A. baumanii, A. johnsonii and A. juni. Acinetobacter strains have a wide distribution in Nature although their main habitats appear to be soil and freshwater (Baumann et al., 1968). It has been estimated that acinetobacters represent at least 0.001% of the total aerobic bacterial population (Juni, 1981). Many strains of A. calcoaceticus can grow on a large number of carbon sources (Baumann et al., 1968). Apart from its wide nutritional versatility and frequent occurrence, an increasing number of physiological and biochemical studies are being done with A. calcoaceticus because of its potential economic and clinical importance. Certain strains of A. calcoaceticus produce an emulsifier which is being used in the oil industry to increase the fluidity and energy output of crude oils (Gutnick & Rosenberg, 1977). Clinically, A. calcoaceticus strains have been shown to be opportunistic pathogens and so typing of strains and control of A. calcoaceticus infections in hospitals is being investigated (Joly-Guillon et al., 1984).

The bacterial strain NCIB 8250 has been classified as a strain of A. calcoaceticus (Véron, 1966; Fewson, 1967a,b; Baumann et al., 1968). This organism is unable to metabolise carbohydrates but it can utilise a wide range of organic compounds, including many aromatic compounds, as sole sources of carbon and energy (Fewson, 1967a). Figure 1.2 illustrates the general pattern of catabolism in A. calcoaceticus NCIB

Figure 1.2 The pattern of catabolism in Acinetobacter calcoaceticus NCIB 8250.



8250. Previous work with A. calcoaceticus in Glasgow has involved an investigation of the regulation of mandelate and benzyl alcohol metabolism. Unless otherwise stated all work and discussion in this thesis concerning A. calcoaceticus refers to strain NCIB 8250.

1.4 The benzyl alcohol catabolic pathway in A. calcoaceticus

The metabolism of benzyl alcohol in A. calcoaceticus can be considered in two parts: (a) manipulation of the side chain leading to the formation of catechol, and (b) ring-cleavage of catechol and the subsequent metabolism of the resulting aliphatic compounds into and through the tricarboxylic acid cycle (Figure 1.3). The regulation of the metabolism of benzoate and other aromatic acids through the so-called ortho ring-cleavage pathway has been extensively investigated in both Pseudomonas putida and A. calcoaceticus BD413 (Stanier & Ornston, 1973; Ornston & Yeh, 1982) (Figure 1.4).

Ornston and his co-workers have collated N-terminal amino acid sequence data from nearly all of the enzymes involved in the ortho ring-cleavage pathway from both A. calcoaceticus BD413 and P. putida (Yeh & Ornston, 1980; Ornston & Yeh, 1982). It may well be that the present enzymes have been derived from putative common ancestors by gene duplications and other re-arrangements. However, this is now impossible to trace given present limited information because the relationships are old and obscured by subsequent mutations, gaps and insertions (Yeh & Ornston, 1980). Rather, the patterns of homologies found in the present-day enzyme molecules reveal another

Figure 1.3 The two parts to benzyl alcohol catabolism in A. calcoaceticus.

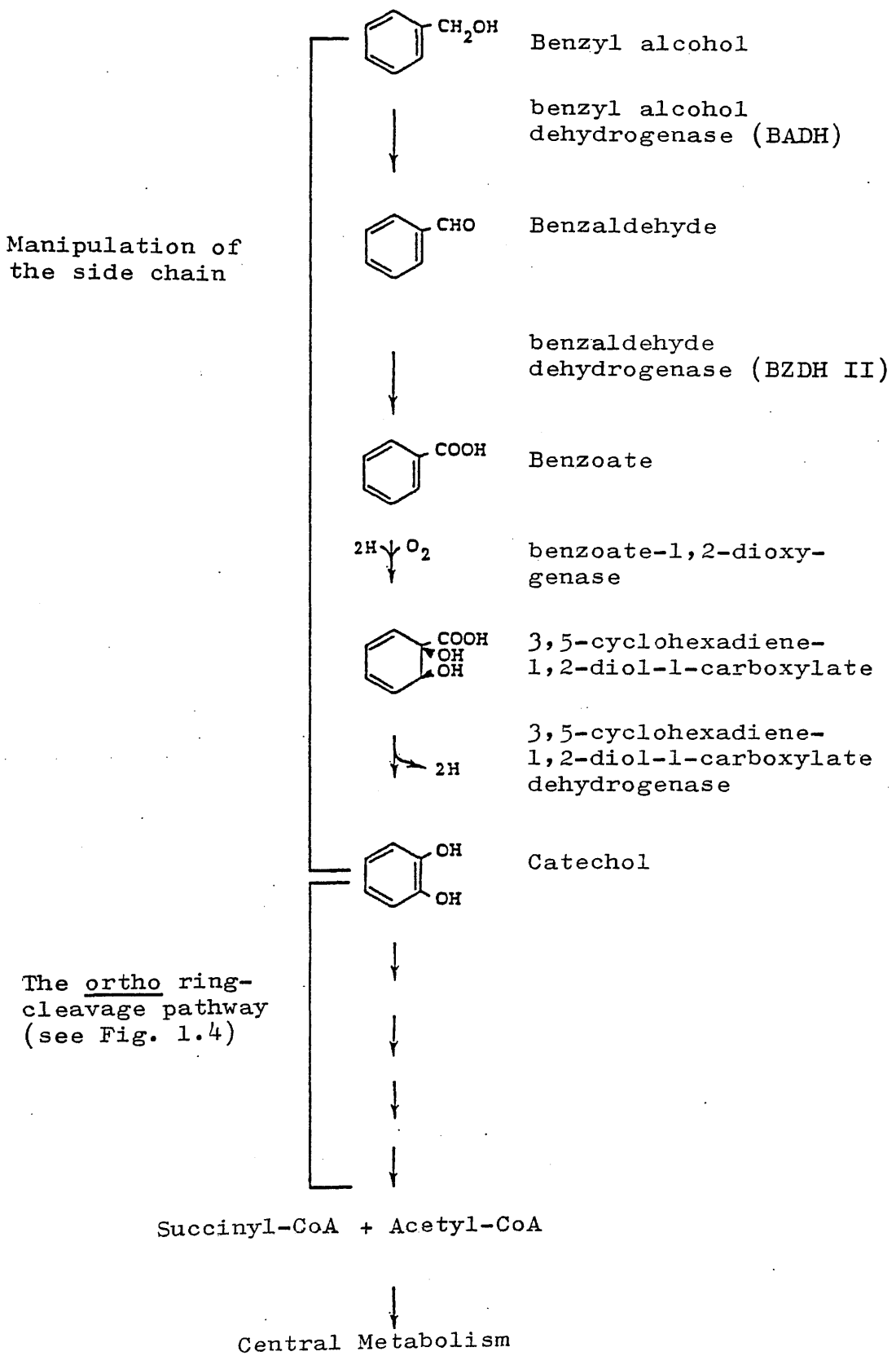
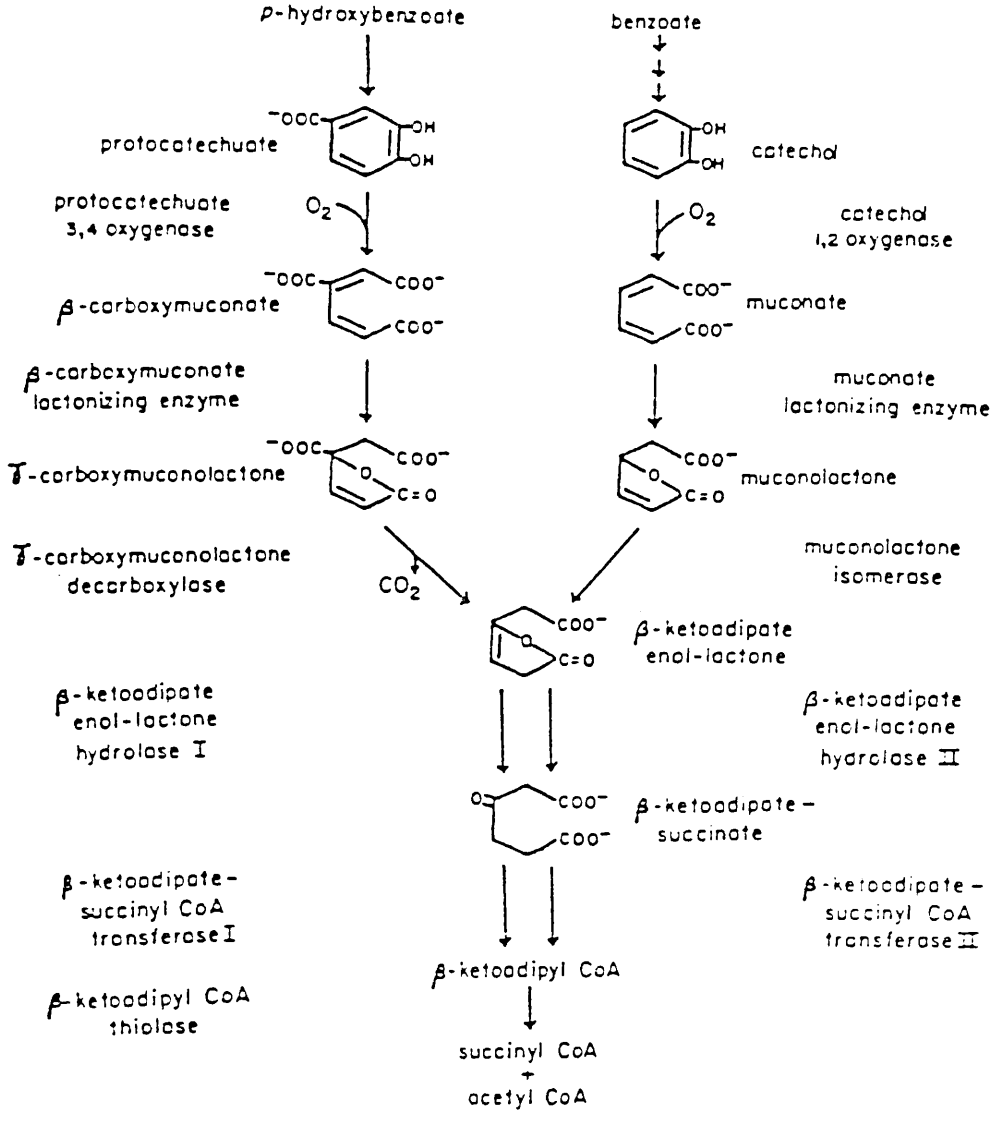


Figure 1.4 The ortho ring-cleavage pathway in A. calcoaceticus (from Patel et al., 1975).



evolutionary perspective; that there must have been complex transfers of segments of sequence among the structural genes of this pathway during their co-evolution. For example, although isofunctional enzymes such as the enol-lactone hydrolases from both A. calcoaceticus and Pseudomonas putida do show some homology with each other, even though they appear to have diverged widely, they also show some homology with the mucono-lactone isomerases from A. calcoaceticus and P. putida when gaps are inserted in the sequences (Yeh et al., 1980). However, the enol-lactone hydrolases' sequences do not share most of the sequences that they have in common with the isomerases, suggesting that the shared sequences were substituted into the hydrolase genes as they diverged from each other (McCorkle et al., 1980; Yeh et al., 1980). Immunological data show that there is conservation of sets of closely related antigenic determinants among isofunctional enzymes expressed by members of a single species (Patel & Ornston, 1976). However, immunological techniques have failed to show evolutionary relationships among isofunctional enzymes of the ortho ring-cleavage pathway from members of different genera (Patel & Ornston, 1976).

This thesis is concerned with the enzymes involved in the manipulation of the side chain of benzyl alcohol leading to the formation of benzoate, namely benzyl alcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase II (BZDH II) (Figure 1.3). The evidence for this dehydrogenative pathway of benzyl alcohol metabolism in A. calcoaceticus is as follows:

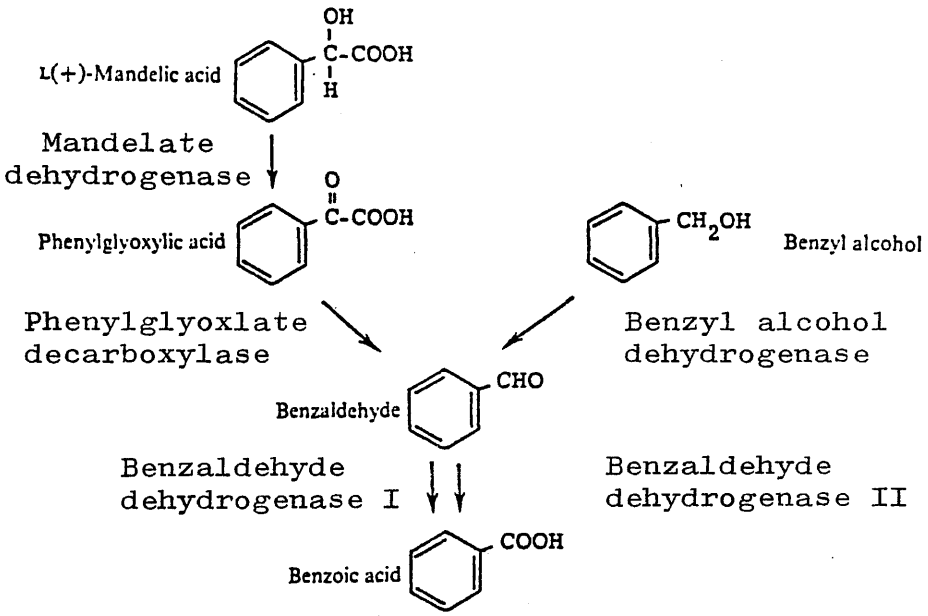
(a) the expected patterns of enzyme induction and simultaneous adaption have been observed (Kennedy & Fewson, 1966; 1968a,b), (b) mutant strains lacking the enzymes BADH or BZDH II, or both, fail to grow on benzyl alcohol (Livingstone et al., 1972), (c) both benzyl alcohol and benzaldehyde co-ordinately induce BADH and BZDH II (Livingstone et al., 1972), and (d) benzoate and catechol are formed from benzyl alcohol (Beggs et al., 1976).

The pathway of benzyl alcohol metabolism in A. calcoaceticus converges at the level of benzaldehyde with the inducible pathway of mandelate metabolism which has its own isofunctional benzaldehyde dehydrogenase (BZDH I) as shown in Figure 1.5 (Kennedy & Fewson, 1966). The two benzaldehyde dehydrogenases have quite different properties; for instance BZDH I (but not BZDH II) requires K^+ ions for maximal activity and in crude extracts at high pH BZDH I is much more heat-stable than BZDH II (Livingstone et al., 1972).

1.5 Regulation of expression of BADH and BZDH II in A. calcoaceticus

Experiments in which differential rates of enzyme synthesis were measured under a variety of conditions of induction and repression showed that BZDH II, but not BZDH I, was synthesised co-ordinately with BADH (Livingstone et al., 1972). The same experiments also showed that BZDH I and BZDH II were synthesised independently. These observations led to speculation as to whether the activities of BADH and BZDH II were located in one bifunctional enzyme or in two independent mono-functional ones (Livingstone et al., 1972). The precedent for a bifunctional

Figure 1.5 The converging benzyl alcohol and mandelate catabolic pathways in A. calcoaceticus (from Beggs & Fewson, 1974).



enzyme having both alcohol and aldehyde oxidising abilities is histidinol dehydrogenase from Salmonella typhimurium (Loper,1968).

Further investigations into the coregulation of BADH and BZDH II showed that when benzyl alcohol is used by A.calcoaceticus as sole carbon source, it supports a faster growth rate and gives a higher molar growth yield than does mandelate (Beggs & Fewson, 1974; Beggs et al., 1976). Surprisingly, however when mandelate is added to bacteria growing on benzyl alcohol or when non-induced bacteria are inoculated into medium containing both mandelate and benzyl alcohol the enzymes specific for benzyl alcohol metabolism, BADH and BZDH II, are repressed and mandelate is used in preference to benzyl alcohol (Beggs & Fewson, 1974; Beggs et al., 1976). Beggs & Fewson (1974) suggested that there were two components to the repression of BADH and BZDH II. One component occurs before benzoate, and is associated with the enzymes specific for mandelate metabolism. The enzyme phenylglyoxylate decarboxylase (see Figure 1.5) was implicated as being involved in regulation of benzyl alcohol metabolism because there was a lack of repression of BADH activity in mutants lacking phenylglyoxylate decarboxylase, but not in other blocked mutants. Also there was a positive correlation between the specific activity of phenylglyoxylate decarboxylase and the degree of repression of BADH. (As BADH and BZDH II are co-ordinately regulated (Livingstone et al., 1972) it was assumed that BZDH II was likewise affected).

It seems most likely that the expression of phenylglyoxylate decarboxylase and concomitant repression of BADH and BZDH II are controlled by some common effector. That the phenylglyoxylate decarboxylase molecule itself effects repression of BADH and BZDH II is less probable.

The second component associated with the repression of benzyl alcohol metabolism is a form of catabolite repression. Beggs & Fewson (1977) showed that addition of succinate to medium containing A. calcoaceticus pre-induced with benzyl alcohol, caused repression of BADH activity. Succinate did not, however, inhibit the activity of BADH in cell-free extracts. This suggested that the repression by succinate was a result of reduced enzyme levels rather than inhibition of enzyme activities.

Repression of benzyl alcohol metabolism by phenylglyoxylate decarboxylase leads to decreased growth rates so it is difficult to see any selective advantage. A possible explanation suggested by Beggs et al. (1976) was that the natural substrates for the converging mandelate and benzyl alcohol pathways are likely to be substituted derivatives (e.g. from lignin biodegradation). In which case, it might be advantageous for the metabolism of substrates following the mandelate pathway to override that of substrates following the benzyl alcohol pathway. Alternatively, the regulation might be an accidental consequence of the regulatory processes associated with these pathways, or it might arise from the evolution of the pathways e.g. if the enzymes were originally recruited from other pathways (Jencon, 1976).

1.6 Enzymes involved in the metabolism of benzyl alcohol and related compounds in other microorganisms

A review (MacKintosh & Fewson, 1987) of microbial aromatic alcohol and aldehyde dehydrogenases is enclosed in this thesis. The review discusses the properties of microbial alcohol and aldehyde dehydrogenases in general but with specific reference to enzymes which oxidise aromatic substrates. The topics it covers are: the enzymes roles in catabolism and anabolism, intracellular location, electron acceptors, substrate specificity, isoenzymes, and genetic location and regulation. The review goes on to compare the properties of the bacterial dehydrogenases with those of the well documented characteristics of the horse liver and yeast alcohol and aldehyde dehydrogenases.

Many microorganisms possess pathways for the metabolism of benzyl alcohol and related compounds that involve benzyl alcohol and benzaldehyde dehydrogenases. Suhara *et al.* (1969) isolated an NAD^+-linked benzyl alcohol dehydrogenase from *Pseudomonas putida* T-2 grown on toluene, and Stachow *et al.* (1967) purified an $NADP^+$-linked benzaldehyde dehydrogenase from *P. putida* grown on benzaldehyde. A 3-hydroxybenzyl alcohol dehydrogenase which is involved in the biosynthesis of the antibiotic patulin (Figure 1.6) has been purified from the fungus *Penicillium urticae* (Forrester & Gaucher, 1972; Scott *et al.*, 1986). *Rhodococcus erythropolis* possesses a coniferyl alcohol dehydrogenases which enables it to metabolise aromatics derived from lignin degradation (Jaeger *et al.*, 1981). Perillyl alcohol (XIV), a cyclohex-1-ene compound, derived

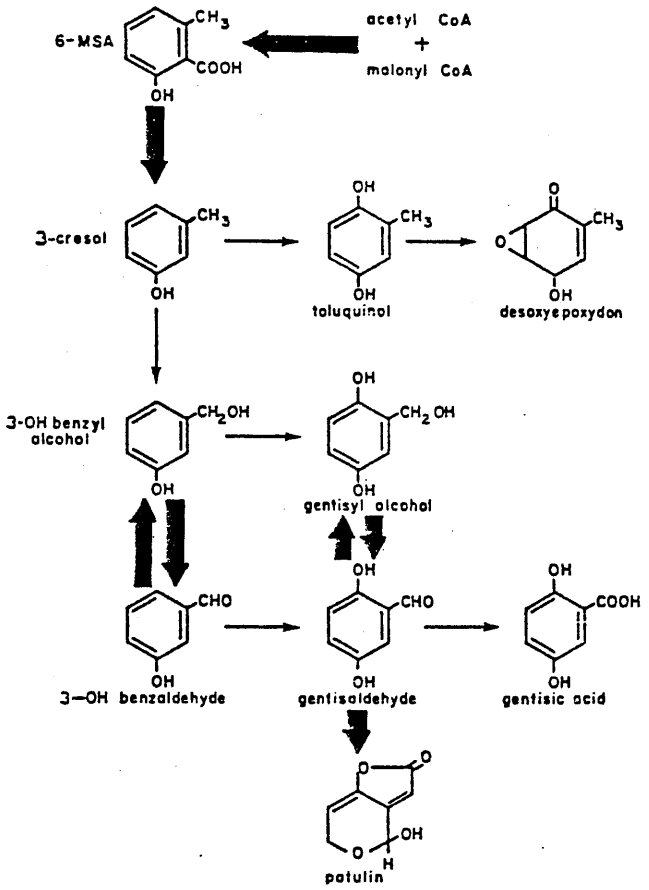
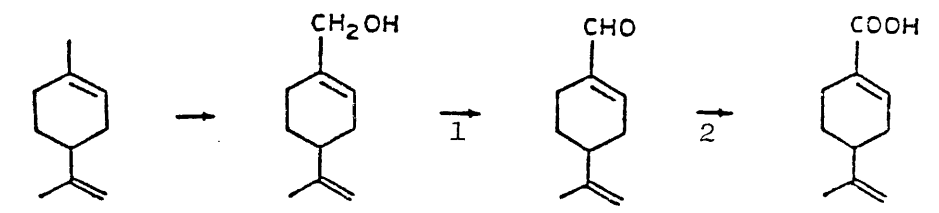


Figure 1.6 Biosynthesis of patulin. Heavy arrows indicate the steps for which the enzymes are known (Scott & Beadling, 1974; from which this figure is taken).

Figure 1.7 Catabolism of limolene to perillic acid (from Ballal et al., 1966).

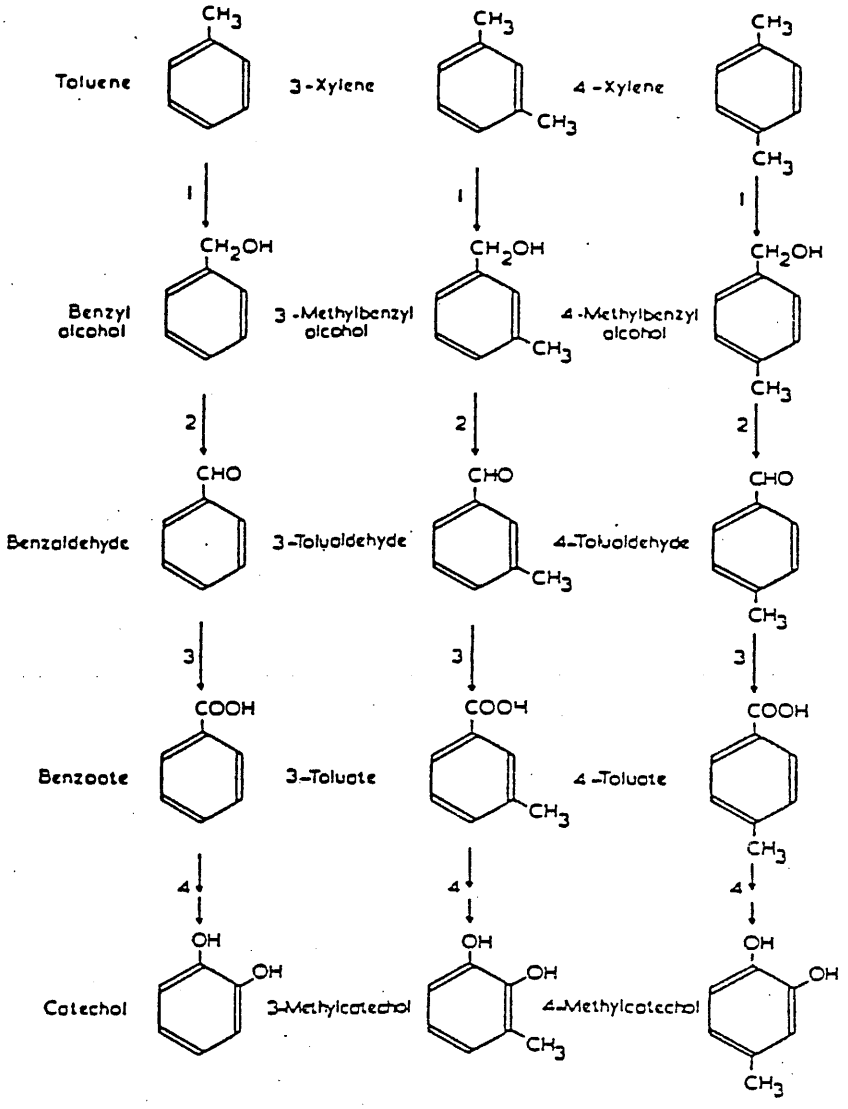


1. perillyl alcohol dehydrogenase
2. perillaldehyde dehydrogenase

from the terpene limolene, although not an aromatic, is oxidised in Pseudomonas sp. to perillic acid (XIII) by a perillyl alcohol dehydrogenase and perillaldehyde dehydrogenase (Figure 1.7); and these enzymes also oxidise aromatic compounds such as benzyl alcohol and benzaldehyde (Ballal et al., 1966, 1967).

The metabolism of benzyl alcohol in Pseudomonas putida has been extensively studied, in part because the enzymes involved are both chromosomally and plasmid encoded. P. putida mt-2 which contains a plasmid, designated TOL, can grow on toluene, 3-xylene, and 4-xylene as well as benzyl alcohol (Williams & Murray, 1974). The hydrocarbons are converted to the corresponding aromatic alcohols by the enzyme xylene oxygenase (Figure 1.8) which is encoded on the TOL plasmid. The aromatic alcohols can then be further metabolised to the corresponding benzoates by TOL-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase. Recently, it has been shown that xylene oxygenase can also oxidise benzyl alcohol to benzaldehyde; it is thought that this activity is an accidental result of the enzyme's relaxed substrate specificity (Harayama et al., 1986). Strains of P. putida which lack the TOL plasmid may still metabolise benzyl alcohol to benzoate as they possess chromosomally-encoded benzyl alcohol and benzaldehyde dehydrogenases. Collins & Hegeman (1984) investigated a wild-type strain of P. putida which, because it has very low benzyl alcohol and benzaldehyde dehydrogenase activities, and grows very slowly

Figure 1.8 Catabolism of toluene, 3-xylene and 4-xylene to the corresponding catechol by the enzymes encoded on the TOL plasmid (Worsey & Williams, 1975).



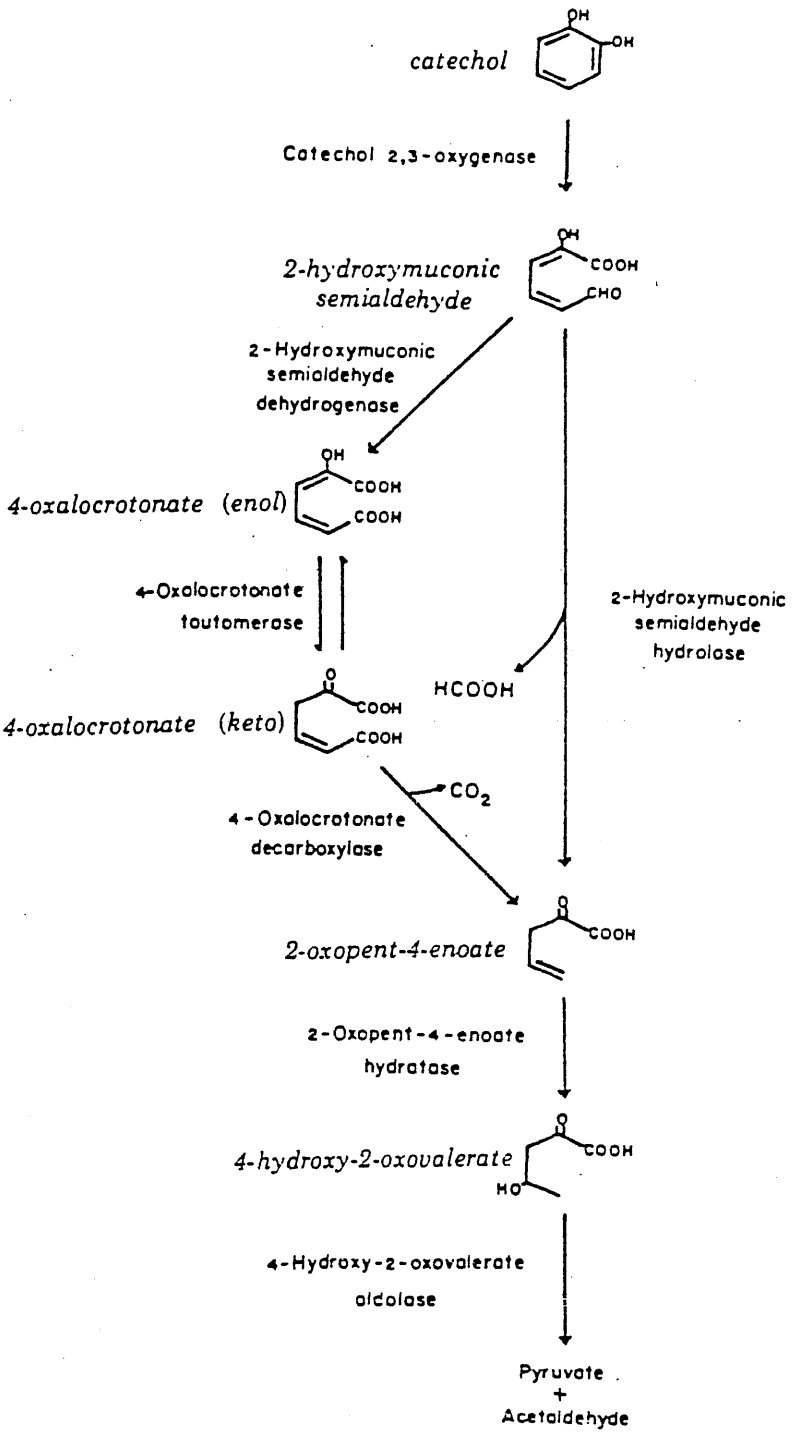
1. xylene oxygenase
2. benzyl alcohol dehydrogenase
3. benzaldehyde dehydrogenase
4. "benzoate oxidase"

on benzyl alcohol, is postulated to lack the TOL-plasmid. (This strain was not a derivative of the TOL-containing P.putida mt-2). After studying the enzymes in crude extracts, Collins & Hegeman (1984) postulated that the chromosomally-encoded pathway evolved from the enzymes for catabolism of short, linear chain alcohols, which were sufficiently non-specific to permit oxidation of benzyl alcohol and benzaldehyde.

Benzoate may be further metabolised in P.putida mt-2 by one of two pathways. The ortho pathway of ring-cleavage is chromosomally-encoded and is the same ring-cleavage pathway as found in A. calcoaceticus (Stanier & Ornston, 1973). Alternatively, benzoate may be metabolised by the meta pathway which is encoded on the TOL plasmid (Williams & Murray, 1974) (Figure 1.9). Aromatic acids derived from 3- and 4-xylenes are metabolised only via the meta pathway; this may be a result of the specificity constraints of the ortho pathway enzymes (Worsey & Williams, 1975).

Limited information is available concerning the regulation of the chromosomally-encoded pathway of benzyl alcohol metabolism in P.putida (Collins & Hegeman, 1984). The regulation of the TOL-encoded pathways has, however, been investigated in detail. Worsey et al. (1978) studied induction of the degradative TOL pathway enzymes in P. putida mt-2 and proposed a model for its regulation in which the hydrocarbons, aromatic alcohols and aromatic aldehydes induce not only the enzymes converting the hydrocarbons to their respective aromatic acids but also the meta ring-cleavage enzymes. Aromatic acids on the other hand induce only the ring-cleavage enzymes.

Figure 1.9 The meta ring-cleavage pathway which is encoded on the TOL plasmid (Williams & Murray, 1974).



1.7 Aims and scope of this thesis

The genetic regulation and organisation of the converging inducible pathways for the catabolism of a large number of aromatic compounds have been well studied, as has the enzymology of many of the ring-cleavage enzymes. However, the enzymology of the dehydrogenation steps of the pathways for metabolism of aromatic alcohols is limited to preliminary characterisation of a few dehydrogenases which are involved in a relatively diverse range of metabolic functions (MacKintosh & Fewson, 1987). The chief aim of this project was therefore to characterise BADH and BZDH II from *A. calcoaceticus*. The first objective was to design a purification procedure for the two enzymes. Purification would in turn allow investigation of the physical and chemical properties of the enzymes. For example, knowledge of the substrate specificities of the enzymes and the effect of inhibitors on their activities should give information relating to the active sites of the enzymes. Analysis of the substrate specificities might also help identify the enzymes' natural substrates. Determination of the *N*-terminal amino acid sequences might indicate any evolutionary relationships, and would also aid future gene cloning. Comparison could also be made between BADH and BZDH II and other bacterial aromatic alcohol and aldehyde dehydrogenases. It would also be possible to compare BADH and BZDH II with the alcohol and alcohol and aldehyde dehydrogenases from horse liver and yeast, for which much detailed information is available relating to structure, function, and evolution.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

All reagents used were the best grade commercially available and with the exception of those listed below were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

2.1.1 Chemicals

2-Bromobenzaldehyde, 2-chlorobenzaldehyde, coniferyl alcohol, trans-1,2-cyclohexanediol, 2-fluorobenzaldehyde, 3-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2-furanmethanol, 4-hydroxy-3-methoxybenzyl alcohol, pentafluorobenzaldehyde, perillic acid, perillyl alcohol, 3-pyridinecarboxylic acid, 4-pyridinecarboxylic acid, 2-thiophenecarboxaldehyde, and 2-thiophenecarboxylic acid were from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.

Dithiothreitol, Tris (base), NAD^+ (free acid), NADH (disodium salt), $NADP^+$ (disodium salt), and NADPH (disodium salt) were from Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K.

3-Pyridinecarboxaldehyde, 4-pyridinecarboxaldehyde, and 2-thiophenemethanol were from R.N. Emanuel Ltd., Alperton, Middx., U.K.

Histidinol and phenylglyoxylate were from Fluorochem, Glossop, Derbyshire, U.K.

2-Bromobenzyl alcohol, cis-1,2-cyclohexanediol, 2,5-dihydroxybenzaldehyde, hexahydrobenzyl alcohol, 3-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, 3-methoxybenzoic acid, and 3-methoxybenzyl alcohol were from K and K Laboratories, Hollywood, U.S.A.

4-Fluorobenzyl alcohol, 3-methoxybenzaldehyde, 4-nitrophenylacetate, 3-pyridinemethanol, 4-pyridinemethanol, and sorbitol were from Koch Light Laboratories, Colnbrook, Berks, U.K.

Acetyl-CoA (lithium salt), ADP (disodium salt), ATP (disodium salt), benzamidine-hydrochloride, Ches, decan-1-ol, N-ethylmaleimide, glycerol-3-phosphate (disodium salt), DL-isocitrate (trisodium salt), Mes, 2-phenanthroline, PMSF, and Tween 20 were from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Bovine serum albumin (BSA) was from Armour Pharmaceutical Corp., Eastbourne, U.K.

Ammonium sulphate (for media preparation) was from Formachem, Strathaven, Scotland.

Allyl alcohol was from Fisons, Loughborough, Leics., U.K.

Cooked meat medium (synthetic, CM440), nutrient agar No.1 (CM3) and nutrient broth (CM1) were from Oxoid Ltd., Basingstoke, Hants, U.K.

Hexan-1-al, octan-1-al, and propionaldehyde were from Riedel-de Haen, Seelze, Hanover, F.D.R.

L-Glutamic acid-hydrochloride was from T.J. Sas and Sons Ltd., London, U.K.

Benzyl alcohol (b.p. 204.7°C) and benzaldehyde (b.p. 179°C) were distilled under nitrogen and stored at 4°C.

2.1.2 Chromatography media

Dyematrex screening kit, Matrex gel Red A and Matrex gel Green A were from Amicon Ltd., Stonehouse, Glocs, U.K.

Blue Sepharose CL-6B, DEAE-Sephacel, FPLC chromatofocusing (Mono P), ion-exchange (Mono Q) and gel filtration (Superose 6 and 12) pre-packed columns, and Sephacryl S-300 superfine were from Pharmacia (Gt. Britain) Ltd., Milton Keynes, Bucks, U.K.

Ultrogel AcA34 was from LKB Instruments Ltd., South Croydon, U.K.

2.1.3 Proteins and enzymes

The following proteins were obtained from the Boehringer Corporation:

Combithek calibration kit for gel filtration chromatography containing aldolase (EC 4.1.2.13) from rabbit muscle, BSA, catalase (EC 1.11.1.6) from beef liver, chymotrypsinogen A from bovine pancreas, cytochrome c from horse heart, ferritin and ovalbumin.

Calibration kit for SDS-PAGE containing BSA, carbonic anhydrase (EC 4.2.1.1) from bovine erythrocyte, α-lactalbumin from bovine milk, ovalbumin, phosphorylase b (EC 2.4.1.1) from rabbit muscle, and trypsin inhibitor from soya bean.

The following were obtained from the Sigma Chemical Co. Ltd.: alcohol dehydrogenase (EC 1.1.1.1) from horse liver, aldehyde dehydrogenase (EC 1.2.1.5) from bakers' yeast, carbonic anhydrase (EC 4.2.1.1) from bovine erythrocyte, and fumarase (EC 4.2.1.2) from pig heart.

2.1.4 Miscellaneous materials

Centricon-30 microconcentrators were from Amicon Ltd., Stonehouse, Surrey, U.K.

Visking tubing was from Scientific Instruments Ltd., London, U.K.

Polybuffer 74 was from Pharmacia (Gt. Britain) Ltd., Milton Keynes, U.K.

Oxi/Ferm tubes were from Roche Products Ltd., Welwyn Garden City, U.K.

Nitrocellulose filters were from Millipore (U.K.) Ltd., Harrow, U.K.

2.2 General methods

2.2.1 pH measurements

The pH values of most solutions were measured using a direct reading pH meter (Model 7010; E.I.L. Ltd., Cumbernauld, Scotland) connected to a combined glass electrode (224; Probion Ltd., Glenrothes, Scotland) at room temperature. The pH values of small volumes was measured with a pH meter (Model PHM84, Radiometer, Copenhagen, Denmark) fitted with a microelectrode.

2.2.2 Conductivity measurements

The conductivities of solutions were measured at $4^{o}C$ using a Radiometer conductivity meter type CDM2e (Radiometer, Copenhagen, Denmark).

2.2.3 Glassware

Routinely, glassware was washed in approximately 1% (v/v) Haemo-sol solution, and rinsed thoroughly in glass-distilled water. Glassware for protein chemistry was immersed overnight in concentrated nitric acid then rinsed extensively with glass-distilled water. All glassware was dried in an oven.

2.2.4 Dialysis

Dialysis was carried out using Visking tubing (Scientific Instruments Centre Ltd., London), which had been immersed in boiling 1% (w/v) EDTA, pH 7.0 for 15 min and then washed with distilled water. Samples (which were all approximately 50 ml or less) were dialysed against three fresh 1000 ml volumes of buffer over a 16 hour period.

2.2.5 Concentration of protein samples

Samples of 10-60 ml were concentrated by vacuum dialysis. Samples of less than 10 ml were concentrated using Centricon-30 microconcentrators (Amicon Ltd., Stonehouse, U.K.) as described in the manufacturer's instructions.

2.2.6 Protein estimations

(a) In preliminary experiments using crude extracts the protein concentration was determined by the method of C.A. Fewson (unpublished results). Extracts were diluted with distilled water to give an approximate A_{280} of 1 in a Pye Unicam SP800 spectrophotometer. Calculations of total protein (mg.(ml of diluted sample)$^{-1}$) were then made by dividing the values of A_{280} by 4.9.

(b) In later experiments, including all the characterisation studies, the protein concentration of samples was determined by the method of Bradford (1976) based on the binding of Coomassie Brilliant Blue to protein. The reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G250 in 50 ml of 95% (v/v) ethanol and 100 ml of 85% (w/v) orthophosphoric acid, and water added to give a final volume of 1000 ml. The reagent was then filtered. Either 5 ml or 1 ml volumes of the reagent were added to test tubes containing 0.1 ml of sample, with protein concentrations of 0.1 to 0.9 $mg.ml^{-1}$ or less than 0.1 $mg.ml^{-1}$ respectively. Samples were vortexed and allowed to stand for 2 min at room temperature. The A_{595} of the samples was then recorded. Standard curves of A_{595} against protein concentration were constructed using BSA covering the ranges 0.01 to 0.09 $mg.ml^{-1}$, or 0.1 to 0.9 $mg.ml^{-1}$. A standard solution of BSA was prepared assuming that a 1 $mg.ml^{-1}$ solution had an A_{280} of 0.65 (Janatova et al., 1968).

(c) During the purification of BADH and BZDH II the A_{280} was monitored using a Uvicord A_{280} monitor (LKB Instruments Ltd., South Croydon, U.K.).

2.2.7 Preparation of chromatography media

DEAE-Sephacel, Sephacryl S300 superfine, and Matrex gels Red A and Green A were all obtained pre-swollen, and were poured into columns as described in the manufacturers' instructions. They were equilibrated by extensive washing in the appropriate buffer. The triazine dye columns were also pre-washed with 6M urea tc remove bound material.

FPLC Mono P, Mono Q, and Superose 6 and 12 were supplied as pre-packed columns, and were also equilibrated by washing with the appropriate buffer.

Blue Sepharose CL-6B (freeze-dried powder) was swollen in water (200 ml per g dry powder) for 15 min. Columns of the swollen gel were then washed with 6M urea to remove both material bound to the ligands and ligand unattached to the support matrix. The columns were then thoroughly washed in the buffer.

All triazine dye affinity columns were regenerated after use by washing with 100 mM potassium phosphate buffer pH 7.5, containing 1.5M NaCl. DEAE-Sephacel was regenerated using 1.5M NaCl only.

2.2.8 Lyophilisation

Samples were frozen in a suitable vessel (to allow for sample "bumping") by placing them into methanol/dry ice. The top of the vessel was covered with nescofilm punctured with a needle. Frozen samples were placed in a dessicator connected to a Flexi-dry (FTS Systems Inc., Stone Ridge, U.S.A.) and vacuum was maintained by a high vacuum pump (Javac PTY Ltd., Farnham, England).

2.3 Bacterial growth, harvesting and breakage

2.3.1 Organism, storage and characterisation

Acinetobacter calcoaceticus NCIB 8250 was originally obtained from the National Collection of Industrial Bacteria (NCIB, Torry Research Station, Aberdeen, Scotland).

The organism was maintained in Oxoid cooked-meat (CM440) nutrient agar (CM3) and nutrient broth (CM1) media, stored at 4°C. Subcultures were made into fresh Oxoid media at 6 to 12 monthly intervals. Samples of these subcultures were streaked onto Oxoid nutrient agar plates and the characteristics of individual colonies checked, using Oxi/Ferm tubes (Roche Products Ltd., Welwyn Garden City, U.K.) and an oxidase test (Cowan & Steel, 1965).

2.3.2 Preparation of media

(a) Defined media

Two types of "basal media" were used: 2g of KH_2PO_4 + 1g of $(NH_4)_2SO_4$ per litre (referred to as Z-1), and 4g of KH_2PO_4 + 2g of $(NH_4)_2SO_4$ per litre (referred to as Z-2). The pH values of both media were adjusted to 7.0 with NaOH before sterilisation. "Salts media" were prepared by the aseptic addition of 20 ml of 2% (w/v) $MgSO_4 \cdot 7H_2O$ per litre of sterile basal medium. To overcome problems of heat-lability, benzyl alcohol solutions were adjusted to pH 7.0 and sterilised by filtration (GSWP047 00, 0.22 µm; Millipore Ltd., Harrow, U.K.), and then added to the sterile medium to give the appropriate concentration.

(b) Complex medium

Complex medium contained the following constituents ($g.l^{-1}$): Oxoid nutrient broth, 26; L-glutamic acid-HCl, 0.91; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.04. The pH value of the medium was adjusted to 7.0 with NaOH before sterilisation. For every 10 litres of medium 50 ml of sterile 1% (v/v)

poly(propylene glycol) 2025 was added as antifoam (Allison et al., 1985). Benzyl alcohol solutions were adjusted to pH 7.0, filter sterilised, and added to the sterile medium to give the appropriate concentration.

(c) Nutrient broth

Nutrient broth ($13 g.l^{-1}$; CM1) was dispensed in 50 ml volumes into 250 ml conical flasks, sterilised, and then used for growing inocula.

(d) Sterilisation

Media were sterilised, before the addition of benzyl alcohol, by autoclaving at 109°C for the appropriate time (C.A. Fewson, unpublished results), and the efficiency of sterilisation was verified by using a Browne's tube (Albert Brown Ltd., Leicester, U.K.). Glass pipettes for inoculations were sterilised by sealing them in Kraft paper and heating them at 160°C in an oven for 1.75 h and the sterilization again verified by using a Browne's tube.

2.3.3 Optical density measurements

Optical densities of bacterial suspensions were estimated by measuring the OD_{500} relative to the appropriate medium in 1 cm light-path cuvettes using a Unicam SP800 spectrophotometer (Pye Unicam Instruments Ltd., Cambridge, U.K.) connected to a Servoscribe chart recorder (Smiths Industries Ltd., Wembley, Middlesex, U.K.). Samples were diluted with the appropriate medium so that the observed OD_{500} was less than 0.5, which corresponded to the linear portion of a standard curve of observed OD_{500} against actual OD_{500}.

2.3.4 Large scale growth and harvesting procedures

(a) Nutrient broth inocula

Inocula used for growing the bacteria in complex media were prepared immediately before use. Conical flasks (250 ml) containing 50 ml of nutrient broth were inoculated (0.1%, v/v) with the nutrient broth stock culture and grown, with shaking, at 30°C for 24 h.

(b) Inoculation of complex medium containing 20 mM benzyl alcohol

Nutrient broth inocula (50 ml) were aseptically transferred into 400 ml of complex medium containing 20 mM benzyl alcohol in a 2l conical flask and grown, with shaking, at 30°C for 24 h. The culture (450 ml) was then aseptically transferred into fresh complex medium containing 20 mM benzyl alcohol (final volume 10 litres) in a fermenter (Braun Biostat V; F.T. Scientific Instruments, Tewkesbury, Glos., U.K.). The fermenter was operated at 30°C using a thermostatically controlled water bath (Grant Instruments Ltd., Cambridge, U.K.) to pump water through cooling coils. The aeration rate was 4 litres of sterile air.min^{-1} and the medium was stirred at setting 2.5 (approx. 350 rev.min^{-1}).

(c) "Draw-and-fill" procedure

After 16 h of growth in the fermenter 9 litres of culture was removed for harvesting. More complex medium (9 litres) was aseptically transferred into the fermenter from a 20 litre reservoir tank. This procedure was repeated after a further 9 h. The cells were then grown for 16 hours, 9 litres of medium was removed and the remaining 2 litres in the reservoir

was aseptically transferred into the fermenter. After a further 4 h of growth the fermenter was emptied.

(d) Harvesting of bacteria

Cultures were harvested by centrifugation in 750 ml polypropylene bottles (maximum volume 500 ml, 69650, MSE Ltd., London, U.K.) at 6 000g_{av} for 20 min at 4°C in an MSE Minstral 6L centrifuge. Each pellet was resuspended in about 30 ml ice-cold sterile Z-1 basal medium and recentrifuged in 250 ml polypropylene bottles (59407; MSE Ltd.) at 12 000g_{av} for 30 min at 4°C in an MSE Highspeed 18 centrifuge. The supernatants were decanted and the bacterial pellets were stored in these bottles at -20°C until required.

2.3.5 Disruption of bacteria

(a) Ultrasonic disruption

Pelleted cells were resuspended in 100 mM potassium phosphate buffer pH 7.5 to give an approximate OD_{500} of 30.

The bacterial suspension (6.5 ml) was placed in a chilled 2 dram vial within a brass holder (Holms & Bennett, 1971) surrounded by an ice-water slurry. The suspension was disrupted with the 13 mm probe of the Dawe Soniprobe (type 1130A, Dawe Instruments Ltd., London, U.K.). The sample was sonicated for four 30s periods alternating with three cooling periods of 30s; the current was 3.2A. The homogenate was centrifuged at 15 000g_{av} for 30 min at 4°C in an MSE Highspeed 18 centrifuge to remove whole cells and debris, and the supernatant kept on ice until required for assays.

(b) French pressure cell disruption

Pelleted cells were resuspended in 4 volumes of the appropriate buffer (final volume was usually 45 ml) and disrupted by 4 passages through the French pressure cell (4-3398A; American Instruments Company, Silver Spring, Maryland, U.S.A.) at a pressure of 98 MPa (14 300 $lb.in^{-2}$) (Allison et al., 1985). The homogenate was centrifuged at either 15 000g_{av} for 30 min at 4°C in a MSE Highspeed 18 centrifuge (preliminary experiments) or 64 000g_{av} for 90 min at 4°C in a Beckman L8-M ultracentrifuge (purification procedure). The supernatant fraction was stored on ice.

2.4 Enzyme assays

2.4.1 Equipment and definition of enzyme units

Spectrophotometric assays were performed at 27°C. A Unicam SP800 spectrophotometer (Pye Unicam Instruments Ltd., Cambridge, U.K.) connected to a Servoscribe chart recorder (Smith's Industries Ltd., Wembley, Middlx., U.K.) was used for the preliminary experiments. A Unicam SP8-100 spectrophotometer with a built-in chart recorder (Pye Unicam) was used for the purification and characterisation experiments. Routinely 3 ml plastic cuvettes with a 1 cm pathlength were used, but for some studies 3 ml quartz cuvettes with a 1 cm pathlength were used instead.

Solutions were dispensed using micropipettes (Eppendorf Marburg Mikropipet; Oxford variable pipettes; Oxford Laboratories Athy, Ireland; or Finn variable pipettes, Jencons, Hemel Hempstead, U.K.). For kinetic studies enzyme, substrates,

and inhibitors were added using Hamilton glass syringes (Magnos Scientific, Sandbach, Cheshire, U.K.). Assays were mixed using plumpers (Calbiochem, San Diego, U.S.A.) or cuvettes were inverted while sealed with Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan). Blank assays were run containing all constituents except enzyme. Enzyme units are defined as μmol of substrate converted.min^{-1}, specific activities are given as units.$(mg\ of\ protein)^{-1}$. The absorption coefficient of NADH at 340 nm is $6.30 \times 10^3\ M^{-1}.cm^{-1}$ (Boehringer, 1976).

2.4.2 Assays for BADH and BZDH II activities used in preliminary experiments

BADH or BZDHII activities were assayed in certain preliminary experiments using methods described by Beggs et al. (1976) or a modified method described by Livingstone et al. (1972).

After ultrasonic or French pressure cell disruption extracts were assayed in reaction mixtures containing:

2.0 ml of 100 mM sodium pyrophosphate buffer pH 9.0 (66.7 mM assay concentration)

0.1 ml of 60 mM NAD^+ (pH adjusted to 7.0) (2 mM assay concentration)

extract (usually 0.02 to 0.1 ml)

0.1 ml of 6 mM benzyl alcohol (0.2 mM assay concentration) to initiate the reaction or

0.1 ml of 3 mM benzaldehyde (0.1 mM assay concentration) to initiate the reaction

distilled water to 3.0 ml

The rate of reduction of NAD^+ was followed at 340 nm.

2.4.3 Assays for BADH and BZDHII activities used in characterisation experiments

(a) BADH: benzyl alcohol oxidation

This assay was developed for measuring the dehydrogenase activity of purified BADH, but could also be used for measuring activities in crude and partially purified extracts. The reaction mixture contained:

2.0 ml of 150 mM Bicine/540 mM hydrazine buffer pH adjusted to 9.2 with NaOH (100 mM Bicine/360 mM hydrazine assay concentration)

0.1 ml of 60 mM NAD^+ (pH adjusted to 7.0) (2 mM assay concentration)

enzyme (usually 0.02 ml)

0.1 ml of 6 mM benzyl alcohol(0.2 mM assay concentration) to initiate the reaction

distilled water to 3.0 ml

Purified enzyme was appropriately diluted in 100 mM potassium phosphate buffer pH 7.5 and stored on ice before use. The rate of reduction of NAD^+ was followed at 340 nm.

(b) BADH: benzaldehyde reduction

This assay was developed for measuring the reductase activity of BADH. The reaction mixture contained:

2.0 ml of 150 mM Bicine/NaOH buffer, pH 8.9 (100 mM assay concentration)

0.1 ml of 7.5 mM NADH (pH adjusted to 7.0) (0.25 mM assay concentration)

enzyme (usually 0.02 ml)

0.1 ml of 3 mM benzaldehyde (0.1 mM assay concentration) to initiate the reaction

distilled water to 3.0 ml

Purified enzyme was appropriately diluted in 100 mM potassium phosphate buffer, pH 7.5 and stored on ice before use. The rate of oxidation of NADH was followed at 340 nm.

(c) BZDHII

The reaction mixture contained:

2.0 ml of 150 mM Bicine/NaOH buffer, pH 9.5 (100 mM assay concentration)

0.1 ml of 60 mM NAD^+ (adjusted to pH 7.0) (2 mM assay concentration)

enzyme (usually 0.02 ml)

0.1 ml of 0.3 mM benzaldehyde (0.01 mM assay concentration) to initiate the reaction.

distilled water to 3.0 ml

Purified enzyme was appropriately diluted in 100 mM potassium phosphate buffer, pH 7.5 and stored on ice before use. The rate of reduction of NAD^+ was followed at 340 nm.

2.4.4 Standardisation of substrate concentration

The concentrations of stock substrate and cofactor solutions were measured in the following ways:

(a) Alcohols. By using the BADH assay (Methods 2.4.3(a)) containing excess NAD^+ (2 mM) but limiting the alcohol concentration and assuming 1:1 alcohol utilization:NADH production, then the complete A_{340} corresponds to the amount

of alcohol initially added.

(b) Aldehydes. By using the BZDH II assay (Methods 2.4.3(c)) containing excess NAD^+ (2 mM) but limiting the aldehyde concentration and assuming 1:1 aldehyde utilization: NADH production, then the complete A_{340} corresponds to the amount of aldehyde initially added.

(c) NAD^+. By using the BADH assay (Methods 2.4.3(a)) or BZDHII assay (Methods 2.4.3(c)), where appropriate containing excess alcohol (0.2 mM) or aldehyde (0.1 mM) but limiting the NAD^+ concentration, the complete A_{340} corresponds to the amount of cofactor added.

(d) NADH. The A_{340} of diluted stock solution was determined.

The NADH absorption coefficient of $6.3 \times 10^3 M^{-1}.cm^{-1}$ (Boehringer, 1976) was used to convert absorbances to concentrations. All reactions were initiated with enzyme (approximately 1 $\mu g.assay^{-1}$) and, when appropriate, correction factors (Methods 2.4.8(b) were used to compensate for the absorbance of alcohols, aldehydes, or acids at 340 nm.

2.4.5 Other enzyme assays

(a) Horse liver alcohol dehydrogenase

Horse liver alcohol dehydrogenase was assayed using the BADH assay (Methods 2.4.3(a)) except that benzyl alcohol was replaced with 10 mM ethanol.

(b) Yeast aldehyde dehydrogenase

Yeast aldehyde dehydrogenase was assayed as described by Bostian & Betts (1978a) except that the pH of the assay buffer was altered to 8.4 or 7.4 as required. The reaction mixture contained:

2.0 ml of 150 mM Tris/HCl pH 8.4 or pH 7.4
(100 mM assay concentration)
0.1 ml of 15 mM NAD^+ (adjusted to pH 7.0)
(0.5 mM assay concentration)
0.1 ml of 3 mM KCl (0.1 mM assay concentration)
0.1 ml of 300 mM mercaptoethanol (10 mM assay concentration)
extract (0.01 ml)
0.1 ml of 6 mM acetaldehyde (0.2 mM assay concentration)
to initiate the reaction
distilled water to 3.0 ml

The rate of reduction of NAD^+ was followed at 340 nm.

(c) Isocitrate dehydrogenase

Isocitrate dehydrogenase was assayed as described by Holms & Bennett (1971). The reaction mixture contained:

1.5 ml of 65 mM Tris/HCl pH 7.5, 0.4 mM $MnCl_2$
(32.5 mM Tris/HCl, 0.2 mM $MnCl_2$ assay concentration)
0.1 ml of 24 mM $NADP^+$ (pH adjusted to 7.0)
(0.8 mM assay concentration)
0.1 ml of 30 mM DL-isocitrate (pH adjusted to 7.0)
(1.0 mM assay concentration) to initiate the reaction
extract (0.1 ml)
distilled water to 3.0 ml

The rate of reduction of $NADP^+$ was followed at 340 nm.

(d) NADH oxidase

NADH oxidase were assayed as described by Kennedy & Fewson (1968a). The reaction mixture contained:

0.5 ml of 100 mM sodium pyrophosphate buffer pH 7.0 (16.7 mM assay concentration)

0.1 ml of 5 mM NADH (in 50 mM sodium pyrophosphate buffer pH 7.0) (0.17 mM assay concentration)

extract (0.1 ml)

distilled water to 3.0 ml

The rate of oxidation of NADH was followed at 340 nm.

2.4.6 Esterase activity

Esterase activity was measured as described by Ting & Crabbe (1983). The reaction mixture contained:

2.0 ml of 75 mM sodium pyrophosphate buffer pH 7.4 or 8.4 (50 mM assay concentration)

0.05 ml of 30 mM 4-nitrophenyl acetate in acetone (0.5 mM assay concentration) to initiate the reaction

enzyme (usually 0.02 ml)

The enzyme was appropriately diluted in 100 mM potassium phosphate buffer pH 7.5 and stored on ice before use. The rate of appearance of 4-nitrophenol was followed at 400 nm. The absorption coefficient of 4-nitrophenol is $9.8 \times 10^3 M^{-1}.cm^{-1}$ at pH 7.4 and $18.3 \times 10^3 M^{-1}.cm^{-1}$ at pH 8.4 (Ting & Crabbe, 1983).

2.4.7 Standard deviation and linear regression analysis

Standard deviations and linear regressions were calculated on a programmable Casio fx-3500 P Scientific calculator. Averages and standard deviations are written as: the average value $\pm$ one standard deviation about the average value, and in parenthesis the number of individual experimental values

used to obtain the average and standard deviation.

Linear regression was done by use of a least means square program and the regression coefficient (r) is shown in those Figures where linear regression was used to fit the lines.

2.4.8 Analysis of initial velocities and determination of kinetic coefficients

(a) K_m and V_{max} determinations

To obtain K_m and V_{max} values for particular substrates initial velocities were measured at several non-saturating concentrations of each substrate. This information was analysed using the Enzpack computer program (Williams, 1985) designed to determine K_m and V_{max} values by the Direct-Linear method (Eisenthal and Cornish-Bowden, 1974). The Enzpack program also determines kinetic coefficients from the three linear methods: Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots. The Direct-Linear method was used to give the kinetic coefficient quoted in this thesis, however these values were checked against the coefficients derived from the linear methods. For double-reciprocal plots, lines were fitted using the K_m and V_{max} values obtained by Direct-Linear analysis.

(b) Absorption coefficients and correction factors

Some of the aromatic substrates and products of BADH and BZDHII absorb at 340 nm and so it was necessary to determine correction factors for the initial rates observed in the presence of these compounds. The absorption coefficients of these compounds were determined. Table 2.1 shows the compounds whose absorption coefficients were below $0.063 \times 10^3 M^{-1}.cm^{-1}$

(i.e. below 1% of the absorption coefficient of NADH at 340 nm) and were therefore considered negligible. The structures of certain compounds identified by Roman numerals are given in the Appendix to this thesis.

Table 2.2 gives the substrates and products of BADH and BZDHII which have absorption coefficient values of more than 1% of the value of NADH at 340 nm.

The measured changes in absorbance at 340 nm were corrected where necessary to give true estimates of the rate of formation of NADH. Correction factors were calculated in the following way:

$$\text{Correction factor} = \frac{\varepsilon_{NADH}}{(\varepsilon_{NADH} - \varepsilon_{substrate} + \varepsilon_{product})}$$

Table 2.3 lists the calculated correction factors.

As coniferaldehyde was not commercially available, its absorbance at 340 nm and hence its possible effect on the observed initial rates at 340 nm could not be determined. However, the ability of BADH to oxidise coniferyl alcohol was monitored by following, at 400 nm, the accumulation with time of coniferaldehyde which has an absorption coefficient at 400 nm (pH 9.2) of approximately 22.4 x $10^3 M^{-1}.cm^{-1}$ (Jaeger et al., 1981). The absorption coefficient of coniferyl alcohol at 400 nm (pH 9.2) was found to be only 0.04 x $10^3 M^{-1}.cm^{-1}$. The assay was as described in Methods 2.4.3(a) except that benzyl alcohol was replaced by coniferyl alcohol and hydrazine was omitted.

Table 2.1: Aromatic substrates and products with ε_{340} values of less than 1% of the ε_{340} value of NADH

(a) Using BADH assay buffer (Methods 2.4.3(a))

Benzaldehyde (I), benzyl alcohol (III), cinnamyl alcohol (VI), 3,4-dimethoxybenzyl alcohol, 2-furancarboxaldehyde (IX), 2-furanmethanol (XI), 2-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, 4-isopropylbenzaldehyde, 4-isopropylbenzyl alcohol, 2-methoxybenzaldehyde, 2-methoxybenzyl alcohol, pentafluorobenzaldehyde, pentafluorobenzyl alcohol, perillaldehyde (XII), perillyl alcohol (XIV), 2-thiophenecarboxaldehyde (XXI), 2-thiophenemethanol (XXIII).

(b) Using BZDHII assay buffer (Methods 2.4.3(c))

Benzaldehyde (I), benzoic acid (II), cinnamic acid (V), 3-fluorobenzaldehyde, 3-fluorobenzoic acid, 4-fluorobenzaldehyde, 4-fluorobenzoic acid, 2-furancarboxaldehyde (IX), 2-furancarboxylic acid (X), 4-hydroxybenzoic acid, 3-methoxybenzoic acid, 4-methoxybenzaldehyde, 4-methoxybenzoic acid, perillaldehyde (XII), perillic acid (XIII), 3-pyridinecarboxaldehyde (XV), 3-pyridinecarboxylic acid (XVI), 4-pyridinecarboxaldehyde (XVIII), 4-pyridinecarboxylic acid (XIX), 2-thiophenecarboxylic acid (XXII).

Table 2.2: Absorption coefficients of aromatic substrates and products.

(a) Using the BADH assay buffer (Methods 2.4.3(a))

Compound	$10^{-3} \times \varepsilon$ ($M^{-1}.cm^{-1}$)
Cinnamaldehyde (IV)	2.00
3,4-Dimethoxybenzaldehyde	0.16
2-Hydroxybenzaldehyde	0.70
3-Hydroxybenzaldehyde	0.61
3-Hydroxybenzyl alcohol	0.17
4-Hydroxybenzaldehyde	0.20
4-Hydroxy-3-methoxybenzaldehyde	5.60
4-Hydroxy-3-methoxybenzyl alcohol	0.07
3-Methoxybenzaldehyde	0.64
3-Methoxybenzyl alcohol	0.21
4-Methoxybenzaldehyde	1.30
4-Methoxybenzyl alcohol	0.23

(b) Using the BZDHII assay buffer (Methods 2.4.3(c))

Compound	$10^{-3} \times \varepsilon$ ($M^{-1}.cm^{-1}$)
Cinnamaldehyde (IV)	1.43
2-Hydroxybenzaldehyde	2.82
2-Hydroxybenzoic acid	0.19
3-Hydroxybenzaldehyde	1.76
3-Hydroxybenzoic acid	1.26
4-Hydroxybenzaldehyde	22.13
3-Methoxybenzaldehyde	0.55
2-Thiophenecarboxaldehyde (XXI)	0.09

Table 2.3: Initial rate correction factors

(a) Substrates of BADH

Substrates	Correction factors
Cinnamyl alcohol (VI)	0.76
3,4-Dimethoxybenzyl alcohol	0.98
2-Hydroxybenzyl alcohol	0.90
3-Hydroxybenzyl alcohol	0.93
4-Hydroxybenzyl alcohol	0.97
4-Hydroxy-3-methoxybenzyl alcohol	0.53
3-Methoxybenzyl alcohol	0.94
4-Methoxybenzyl alcohol	0.85

(b) Substrates of BZDHII

Substrates	Correction factors
Cinnamaldehyde (IV)	1.29
2-Hydroxybenzaldehyde	1.72
3-Hydroxybenzaldehyde	1.09
4-Hydroxybenzaldehyde	-0.40
3-Methoxybenzaldehyde	1.09
2-Thiophenecarboxaldehyde (XXI)	1.01

2.5 Purification of BADH and BZDHII

2.5.1 Purification buffers

All the buffers used in the purification of BADH and BZDH II were phosphate buffers. The solutions listed below were prepared by dissolving the buffer and any other additional components (i.e. DTT, $MgCl_2$,etc.) in distilled water to about 90% of the final volumes. The solutions were adjusted to the required pH value by the dropwise addition of KOH or HCl. Once at the required pH the solutions were adjusted to the final volume with distilled water. All buffers were prepared just prior to use during the purification procedure.

Buffer A: 75 mM potassium phosphate buffer pH 7.5, 2 mM DTT.

Buffer B: 110 mM potassium phosphate buffer pH 7.5, 2 mM DTT.

Buffer C: 10 mM potassium phosphate buffer pH 6.0, 2 mM DTT, 5 mM $MgCl_2$.

Buffer D: 50 mM potassium phosphate buffer pH 7.5, 2 mM DTT.

Buffer E: 100 mM potassium phosphate buffer pH 7.5, 2 mM DTT, 1 mM EDTA, 1 mM benzamidine, 1.2 mM PMSF, 40% (v/v) glycerol.

2.5.2 Purification procedure

Purification procedure steps (a) to (d) were carried out at 0 to 4°C. Step (e) was performed at room temperature.

(a) Preparation of the cell-free extracts

Cells (9g) of A calcoaceticus NCIB 8250 grown on complex medium supplemented with 20 mM benzyl alcohol and harvested as described in Methods 2.3.4, were resuspended in 36 ml of Buffer A. The cells were disrupted by 4 passages through the

French pressure cell and a cell-free extract was prepared by centrifugation (Methods 2.3.5(b)).

(b) Chromatography on DEAE-Sephacel

The extract (approximately 38 ml) was then applied to a DEAE-Sephacel column (3.8 x 2.6 cm) pre-equilibrated in Buffer A. The column was washed in Buffer A until the A_{280} of the effluent decreased to approximately 0.05. The buffer was then changed to Buffer B to elute both BADH and BZDH II. The flow rate throughout was 50 $ml.h^{-1}$. The fractions containing the enzyme activities were pooled (approximately 45 ml) and dialysed overnight against 3 x 1000 ml of Buffer C (Methods 2.2.4).

(c) Chromatography on Blue Sepharose CL-6B

The dialysed pool (approximately 50 ml) was applied to a Blue Sepharose Cl-6B column (9.5 x 2.6 cm) pre-equilibrated in Buffer C. After application the pump was stopped for 30 min and then the column was washed with Buffer C. Once the A_{280} of the effluent had returned to the base-line the washing was continued until a further 100 ml (i.e. 2 column volumes) had passed through the column. The buffer was then changed to Buffer C containing 0.12 mM NAD^+ and the buffer flow direction reversed to elute the enzymes. The flow rate throughout was 99 $ml.h^{-1}$. The fractions containing the enzyme activities were pooled (approximately 50 ml).

(d) Chromatography on Matrex gel Red A

The Blue Sepharose CL-6B elution pool (approximately 50 ml) was applied to a Matrex gel Red A column (3.8 x 2.6 cm) pre-equilibrated in Buffer C containing 0.12 mM NAD^+. After application the pump was stopped for 30 min and then the column was washed with Buffer C containing 0.12 mM NAD^+. The

The BZDH II activity was located in the wash fractions, which were pooled (approximately 50 ml). Once the effluent A_{280} had returned to the baseline the washing was continued until a further 80 ml (i.e. 4 column volumes) had passed through the column. The BADH activity was eluted by increasing the NAD^+ concentration in Buffer C to 2.75 mM, and the buffer flow direction was reversed. The flow rate throughout was 99 $ml.h^{-1}$. The fractions containing the BADH activity were pooled (approximately 30 ml). Both the BADH and BZDH II pools were concentrated overnight by vacuum dialysis. The BADH pool was further concentrated to 0.2 ml and its buffer changed to Buffer D using a Centricon-30 microconcentrator.

(e) Gel filtration on FPLC Superose 12

The concentrated BADH pool (0.2 ml) was applied to an FPLC Superose 12 gel filtration column (30 x 1 cm) (Pharmacia system) pre-equilibrated in Buffer D. The flow rate throughout was 0.3 $ml.min^{-1}$. Two peaks of A_{280} were observed, the major peak corresponded with the BADH activity. The peak fraction, containing the majority of the enzyme activity, plus the fractions immediately adjacent to the peak fraction were pooled (approximately 0.9 ml). The BADH pool and the concentrated BZDH II pool (approximately 1.0 ml), were then individually dialysed overnight against Buffer E. The dialysed pools (both approximately 0.3 ml) were then stored at $-20^{\circ}C$.

2.6 Polyacrylamide gel electrophoresis

2.6.1 SDS-PAGE (discontinuous system)

(a) Preparation of gels

SDS-PAGE was carried out using the discontinuous Tris/glycine buffer system described by Laemmli (1970).

The stock solutions for preparing gels were as follows:

Solution A: 3.0M Tris/HCl pH 8.8, 0.25% (v/v) TEMED

Solution B: 28% (w/v) acrylamide, 0.735% (w/v) N,N'-methylenebis-acrylamide (deionised solution).

Solution C: 0.1M Tris/HCl pH 6.8, 0.8% (w/v) SDS, 0.25% (v/v) TEMED.

Solution D: 20% (w/v) SDS.

The reservoir buffer was:

25 mM Tris/192 mM glycine pH 8.8, 0.1% (w/v) SDS.

Slab gels (19.0 cm x 9.5 cm x 0.15 cm) were cast and electrophoresed on our own custom-built apparatus. The gels were prepared, four at a time, from the following volumes of stock solution:

Separating gel (10% (w/v) acrylamide)

Solution A, 25 ml; Solution B, 71.5 ml; Solution D, 1 ml; distilled water, 100 ml (final volume 197.5 ml). The mixed solution was degassed before the addition of Solution D. Polymerisation was initiated by the addition of 150 mg ammonium persulphate, and the mixture poured into the casting apparatus. A thin layer of isopropanol was carefully placed on top of the gel while it set and then was washed away.

Stacking gel (6% (w/v) acrylamide)

Solution B, 17.5 ml; Solution C, 10.0 ml; distilled water, 55 ml (final volume 82.5 ml). The mixture was again degassed and polymerisation initiated with 150 mg ammonium persulphate, and the mixture was then poured on top of the separating gel. The stacking gel was allowed to polymerise around a well-forming teflon template.

(b) Sample preparation and electrophoresis conditions

Stock solutions:

(A) 143 mM Tris/HCl pH 6.8, 2.8% (w/v) SDS, 28.5% (v/v) glycerol, 0.029% (w/v) Bromophenol Blue.

(B) 1.76M DTT stored at -20°C.

Before use Solution A was mixed with Solution B in the ratio 35:4 to give the sample buffer. The sample buffer was then mixed with protein samples in the ratio 1:1 and heated at 100°C for 2 min before application to the gel. Electrophoresis was carried out at 90 mA until the dye front was approximately 0.2 cm from the bottom of the gel. The gels were cooled to approximately 4°C using a continuously flowing water chamber built into the electrophoresis apparatus.

2.6.2 Non-denaturing PAGE

(a) Preparation of gels

This was carried out in 7% (w/v) polyacrylamide gels at pH 8.9 (Davis, 1964). The following stock solutions were stored at 4°C:

Solution A: 3.0M Tris/HCl pH 8.9, 0.25% (v/v) TEMED.

Solution B: 28% (w/v) acrylamide, 0.735% (w/v) N,N'-methylenebis-acrylamide (deionised solution).

Solution C: 10 mM Tris/72 mM glycine.

7% gels were prepared by mixing 50 ml Solution A, 100 ml Solution B, and 50 ml distilled water. An equal volume of ammonium persulphate (1.4 $mg.ml^{-1}$) was prepared. Both solutions were degassed separately and then they were mixed and poured into the custom-built casting box. Slab gels (19.0 cm x 9.5 cm x 0.15 cm) were cast. The gel was allowed to polymerise around

a well-forming teflon template.

(b) Sample preparation and electrophoresis conditions

Protein samples were mixed with 5 µl of 0.05% (w/v) Bromophenol Blue and glycerol added to a final concentration of 10% (v/v). The reservoir buffer was Buffer C diluted 5-fold with distilled water plus 0.01% (v/v) mercaptoethanol. The gel was pre-electrophoresed for 30 min before samples were applied. Electrophoresis was carried out at 45 mA until the dye front was approximately 0.2 cm from the gel bottom. The gels were cooled to approximately 4°C using a continuously flowing water chamber built into the electrophoresis apparatus.

2.6.3 Staining of gels

(a) Protein staining

Gels were immersed in 0.1% (w/v) Coomassie Brilliant Blue G250, 50% (v/v) methanol, 10% (v/v) acetic acid for 1 h at 40°C and then destained in 10% (v/v) methanol, 10% (v/v) acetic acid also at 40°C.

Gels were also stained for protein by a silver method similar to that originally described by Wray et al. (1981). Gels were soaked for 2 to 3 days in 50% (v/v) methanol. The staining solution was prepared by adding 0.8g $AgNO_3$ dissolved in 4 ml distilled water to 1.4 ml 14.8M NH_4OH and 21 ml 0.36% (w/v) NaOH dropwise with stirring, distilled water was added to give a final volume of 100 ml. The gel was soaked in the staining solution for 10 min with gentle agitation and rinsed with distilled water for approximately 1 h, again with gentle agitation. The gel was then developed

by immersion in a solution consisting of 2.5 ml 1% (w/v) citric acid and 250 µl 38% formaldehyde in 500 ml distilled water. Staining was terminated by transferring the gel to 10% (w/v) methanol, 10% (v/v) acetic acid and the gel was stored in distilled water.

(b) Activity staining

After non-denaturing PAGE, gels could be stained for BADH or BZDH II activity by first soaking in 66.7 mM sodium pyrophosphate buffer pH 9.0 for 30 min at 4°C to remove the mercaptoethanol. The gels were then transferred to the staining solution (66.7 mM sodium pyrophosphate buffer pH 9.0, 2 mM NAD^+, 550 µM phenazine methosulphate, 55 µM nitroblue tetrazolium, 0.2 mM benzyl alcohol or 0.1 mM benzaldehyde) and incubated in the dark at 30°C. Enzyme activity was detectable as a purple precipitate of formazan, after approximately 10 min.

2.6.4 Gel scanning

Coomassie Blue stained gels were scanned on an LKB 2202 Ultrascan laser densitometer.

2.6.5 Standard proteins and M_r determinations using SDS-PAGE

To determine the relative M_r values of the subunits of BADH and BZDH II destained slab gels were scanned and the electrophoretic mobilities (R_f) of the proteins calculated from:

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracker dye}}$$

To calibrate the system the following standard proteins were used: phosphorylase b, 94 000 (Serry et al., 1967);

BSA, 67 000 (Castellino and Barker, 1968); ovalbumin, 43 000 (Castellino and Barker, 1968); carbonic anhydrase, 30 000 (Reynaud et al., 1971); trypsin inhibitor, 20 100 (Koide and Ikenaka, 1973); α-lactalbumin, 14 400 (Brew et al., 1967).

2.7 Protein chemistry

2.7.1 Analysis of amino acid sequence

This work was carried out in collaboration with Professor J.E. Fothergill at the Department of Biochemistry, University of Aberdeen.

(a) Dialysis

Protein samples were dialysed against 0.5% (w/v) ammonium bicarbonate, with 6 changes each of 2.5 l for 24 h at 4°C.

(b) Reduction and carboxymethylation

Carboxymethylation was carried out as described by Lumsden and Coggins (1978). Dialysed samples were lyophilised and then resuspended in 2 ml 0.1M Tris/HCl pH 8.2, 8M urea, 2 mM DTT and incubated in the dark for 1 h at room temperature under an atmosphere of N_2. Iodoacetate was added to the solution to give a concentration of 15 mM, and the solution incubated for 1 h, in the dark, under N_2. The reaction was terminated by the addition of DTT (final concentration, 30 mM). The carboxymethylated protein was dialysed against 0.5% (w/v) ammonium bicarbonate with 4 changes each of 2.5 l for 24 h at 4°C. The samples were then lyophilised.

(c) Protein sequencing

The N-terminal amino acid sequences of the carboxymethylated BADH and BZDH II were determined using a Beckman Model 890 liquid phase sequencer (Smith et al., 1982) operated by Mr B. Dunbar of Aberdeen University. The phenylthiohydantoin (PTH) amino acids were identified by chromatography on a Waters Resolve C_{18} reverse phase column with an acetate/acetonitrile pH 5.0 buffer (Russell et al., 1986) with a modified isocratic gradient.

2.8 Safety

Bacterial cultures were killed by autoclaving before disposal, and glassware washed as described in Methods 2.2.3. Any bacterial spillage was swabbed with 10% (v/v) propan-1-ol. Hydrazine was dispensed in a fume cupboard whilst wearing gloves. Care was taken not to spill any hydrazine assay buffer on to exposed skin.

All other precautions taken in the interest of safety are as described in the University of Glasgow Safety Handbook.

CHAPTER 3

PURIFICATION OF BENZYL ALCOHOL DEHYDROGENASE AND BENZALDEHYDE DEHYDROGENASE II FROM *A. CALCOACETICUS* NCIB 8250

3.1 Preliminary studies on maximising enzyme activity

These preliminary experiments were done before the development of the assay procedure for BADH involving the trapping of products with hydrazine (Methods 2.4.3(a)) and so the earlier BADH procedure (Methods 2.4.2) was used. However, in crude extracts this assay measures not only oxidation of benzyl alcohol but also oxidation of benzaldehyde by BZDH II. The contribution by BZDH II could be up to 50% of the apparent BADH activity but is probably a good deal less than this. In these preliminary studies (3.1.1 to 3.2.4) the BADH activity recorded is the observed apparent activity without any attempt at correction.

3.1.1 Cell growth and enzyme induction

There were no *A. calcoaceticus* strains constitutive for, or overexpressing, BADH or BZDH II so the optimum conditions to give the highest yields of bacteria and maximum specific activities of the enzymes had to be developed for the wild type strain NCIB 8250.

A. calcoaceticus NCIB 8250 was grown at 30°C on defined media (Z-1 and Z-2 salts media) and on a complex medium (Methods 2.3.2(a) and (b)), all supplemented with benzyl alcohol to give various final concentrations of from 5 to 50 mM. The highest yield of cells was obtained after growth on the complex medium, and the highest specific activities of both enzymes were obtained after growth on complex medium supplemented with 20 mM benzyl alcohol.

A 10-litre fermenter with a 20-litre reservoir was used to grow large amounts of cells by a "draw and fill" procedure (Methods 2.3.4). Up to 175 g (wet weight) of cells could be obtained from 30 litres of complex medium plus 20 mM benzyl alcohol. Specific activities were approximately 1.5 units. $(mg\ protein)^{-1}$ and 0.5 units. $(mg\ protein)^{-1}$ for BADH and BZDH II respectively.

3.1.2 Comparison of methods of disruption of A. calcoaceticus

Cell breakage by ultrasonication is generally convenient only when working with small volumes. Optimum conditions were therefore developed for the French pressure cell (which can hold up to 45 ml) in order to obtain the highest specific activities and recoveries of enzyme activities for BADH and BZDH II. Table 3.1 shows that four passages through the pressure cell gave most effective release of each enzyme.

3.1.3 Centrifugation of broken-cell suspensions

When homogenates of A. calcoaceticus, prepared in the French pressure cell, were centrifuged at either 15 $000g_{av}$ for 30 min at $4^{o}C$ or at 64 $000g_{av}$ for 90 min at $4^{o}C$ most of the BADH and BZDH II activities were in the supernatant fractions and the individual specific activities of each enzyme were very similar. However, centrifugation at 199 $300g_{av}$ for 3 h at $4^{o}C$ resulted in 50% loss of both BADH and BZDH II to the pellet. The distribution of enzymes in the supernatants and precipitates was comparable with the cytoplasmic marker isocitrate dehydrogenase, rather than the membrane marker NADH oxidase.

Table 3.1: Comparison of methods of disruption of A.calcoaceticus

Seven grams (wet weight) of A.calcoaceticus which had been grown on complex medium plus 20 mM benzyl alcohol (Methods 2.3.4) was resuspended in 100 mM potassium phosphate buffer pH 7.5; final volume 45 ml. A sample (7 ml) was ultrasonicated (Methods 2.3.5(a)). The remainder was passed through a French pressure cell four times (Methods 2.3.5(b)), and 5 ml samples were taken after each passage. The samples were centrifuged at 15 000g_{av} for 30 min at 4°C and the supernatants were assayed for protein (Methods 2.2.6(a)) and BADH and BZDH II activities (Methods 2.4.2).

Method of Disruption	BADH		BZDH II	
	Specific activity (units.(mg.protein)$^{-1}$)	Activity (units.(ml extract)$^{-1}$)	Specific activity (units.(mg protein)$^{-1}$)	Activity (units.(ml extract)$^{-1}$)
(a) Ultrasonication	1.79	17.4	0.84	8.2
(b) French pressure cell				
1 passage	2.08	9.0	0.78	3.3
2 passages	2.29	16.8	0.70	5.1
3 passages	2.01	16.8	0.86	7.1
4 passages	2.01	16.0	0.99	7.9

In subsequent experiments 64 000g_{av} for 90 min at 4°C was used as a compromise between sedimenting membrane and leaving the enzymes in the supernatant.

3.1.4 Effects of different buffers on the stability of enzyme activities

To achieve the maximum stability of both BADH and BZDH II activities over a time period which corresponded with that expected for any purification procedure, the effects of different buffers were examined.

Samples of an extract of A. calcoaceticus were diluted 1 to 20 in the following buffer and stored at 4°C: 100 mM sodium acetate/HCl pH 5 and 6; 100 mM potassium phosphate pH 6, 7, 7.5 and 8; 10 mM potassium phosphate pH 6 and 7.5; 100 mM Tris/HCl pH 7, 8 and 9; 100 mM sodium pyrophosphate/HCl pH 7, 8, and 9; 100 mM glycine/NaOH pH 9, 10, and 11. Samples of the diluted extracts were assayed for BADH and BZDH II activity at intervals over 5 days.

The BADH activity was, in general, more stable than the BZDH II activity; however, at least 40% of both activities was lost after only 24 h at or below pH 6 and above pH 8. Stability appeared to be independent of the buffer concentration. Between pH 7 and 8 the enzymes stored in phosphate buffer for 5 days retained more activity than in Tris buffer, i.e. approximately 80% (BADH) and 50% (BZDH II) recovery compared to 35% and 40% recovery.

Henceforth, potassium phosphate buffers at pH 7.5 were routinely used (except for binding of enzymes to triazine dye affinity columns; Section 3.2.1).

3.1.5 Effect of DTT on the stability of enzyme activities

BADH and BZDH II optimally bind to triazine dye-ligand matrices at pH 6 (Section 3.2.1). However, both enzyme activities were unstable at that pH value. The ability of DTT to stabilise the enzyme activities was examined. The results (Figure 3.1) show that 2 mM DTT partially stabilised both enzyme activities at pH 7.5 and pH 6. In further experiments 1 mM DTT was found to be less, and 5 mM DTT no more, effective than 2 mM DTT. Subsequently, 2 mM DTT was added to all buffers.

3.1.6 The effect of DTT and $MgCl_2$ on the stability of enzyme activities during dialysis

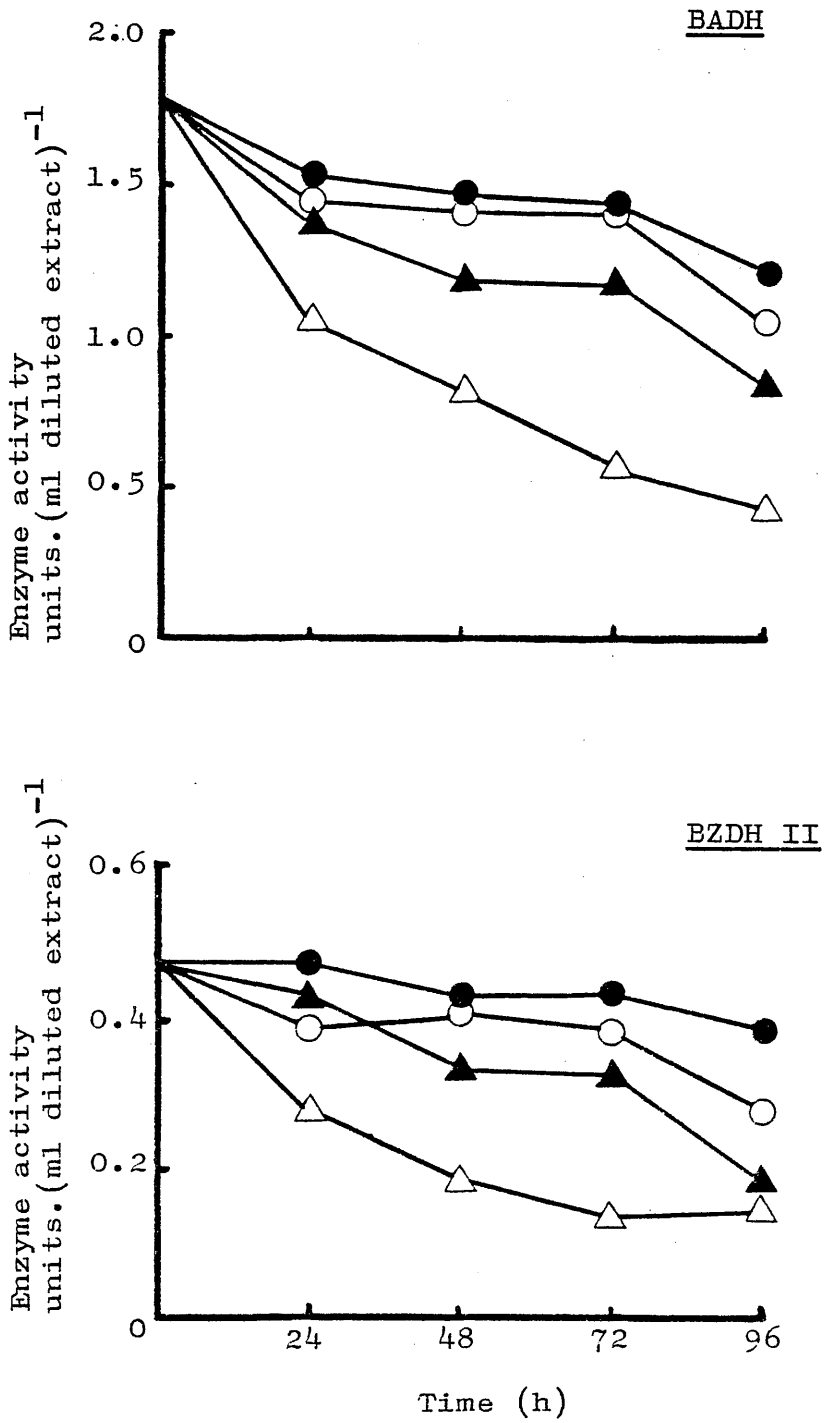
Overnight dialysis was used to change buffers between individual purification steps. However, initial experiments resulted in large losses of the activities of both BADH and BZDH II. Attempts were made to overcome this problem, and in particular to stabilise the enzyme activities during dialysis into the 10 mM potassium phosphate buffer pH 6 which was extensively used in optimizing the triazine dye-ligand chromatography procedures (Section 3.2.1).

It had been found (Section 3.1.5) that 2 mM DTT partially stabilised both BADH and BZDH II activities. Other preliminary experiments had shown that the addition of $MgCl_2$ to buffers could increase the recovery of the enzyme activities after

Figure 3.1: Effect of DTT on the stability of enzyme activities

Samples (0.1 ml) of a crude extract of A. calcoaceticus, prepared as described in Methods 2.3.5(b), were diluted 1 to 20, to approximately 0.70 mg protein.ml^{-1} in: 10 mM potassium phosphate buffer pH 7.5 (○); 10 mM potassium phosphate buffer pH 7.5, 2 mM DTT (●); 10 mM potassium phosphate buffer pH 6 (△); 10 mM potassium phosphate buffer pH 6, 2 mM DTT (▲).

Buffer pH was unaffected by addition of extract. Diluted extracts were stored at 4°C and samples assayed for BADH and BZDH II activity (Methods 2.4.2) at intervals. The initial activities are taken as those of extracts diluted 1 to 20 with 100 mM potassium phosphate buffer pH 7.5, and were for BADH 1.80 units (ml diluted extract)$^{-1}$ and for BZDH II 0.48 units.(ml diluted extract)$^{-1}$. The values plotted are averages of duplicate assays which generally agreed within 5%.


BADH
2.0
1.5
1.0
0.5
0
Enzyme activity units.(ml diluted extract)$^{-1}$
BZDH II
0.6
0.4
0.2
0
24
48
72
96
Time (h)

dialysis. The effects of both DTT and $MgCl_2$ on the recovery of BADH and BZDH II activities after dialysis were therefore tested (Table 3.2). An increase in the recoveries of either enzyme was observed only when 5 mM $MgCl_2$ was present in the buffer. In Table 3.2 the addition of 2 mM DTT to buffer containing 5 mM $MgCl_2$ caused a slight decrease in recoveries. However this was not observed in repeat experiments, and it was concluded that the presence of 2 mM DTT had little effect on the recoveries. In those repeat experiments the increase in recoveries due to the presence of 5 mM $MgCl_2$ was reproducible.

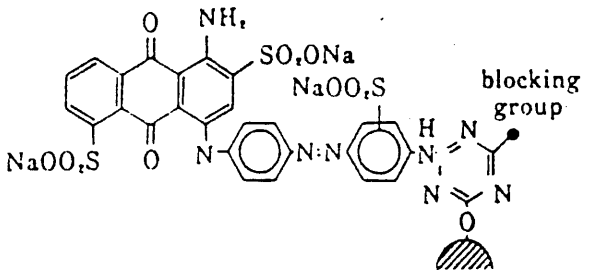
The optimum $MgCl_2$ concentration to stabilise the enzyme activities during dialysis was then determined. There was a progressive increase in the recovery of both enzyme activities as the $MgCl_2$ concentration was increased from 0 to 5 mM but there was no further increase with 10 mM $MgCl_2$. Therefore, 5 mM $MgCl_2$ was used when dialysing into 10 mM potassium phosphate buffer pH 6, 2 mM DTT.

3.2 Development of purification procedures

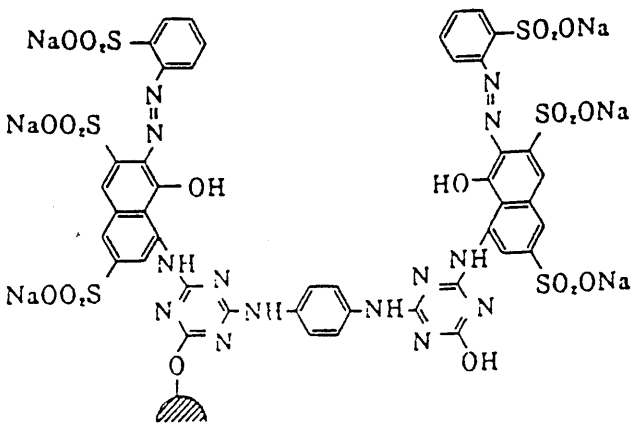
3.2.1 Triazine dye-ligand chromatography

Triazine dye-ligand chromatography is a variant of affinity chromatography in which immobilized synthetic textile dyes are used to bind proteins. It has been suggested that the interaction could be a result of the chemical structures of the dyes resembling those of the natural substrates and cofactors of the proteins (Figure 3.2) although there is debate as to the validity of this idea (Dean and Watson, 1979). Other kinds of interactions probably also play a part.

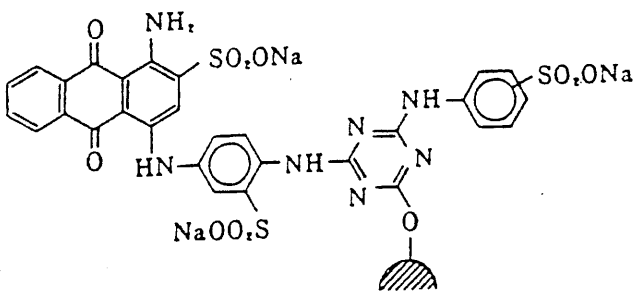
Figure 3.2 Chemical structures of some triazine dyes and NAD^+ (from Amicon, 1980).



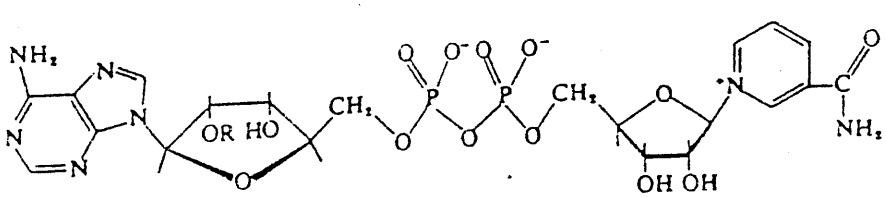
1. Procion Green H-4G



2. Procion Red HE-3B



3. Cibacron Blue F3G-A



4. NAD^+

Table 3.2: The effects of DTT and $MgCl_2$ on stabilising the enzyme activities during dialysis.

Samples (0.5 ml) of an extract of A. calcoaceticus, prepared as described in Methods 2.3.5(b), were diluted 1 to 20 to give approximately 1 mg protein.ml^{-1} in: 10 mM potassium phosphate buffer pH 6.0, containing either (a) no additions, (b) 2 mM DTT, (c) 5 mM $MgCl_2$ or (d) 2 mM DTT and 5 mM $MgCl_2$. The diluted samples were assayed for protein (Methods 2.2.6(b)) at BADH and BZDH II activity (Methods 2.4.2). The diluted extracts were then dialysed at 4^oC against 3 x 1000 ml of the buffer in which the extract had been diluted. The protein and enzyme activities were reassayed after 17 h.

Additions	BADH		BZDH II	
	Specific activity before dialysis (units.(mg protein)$^{-1}$)	Percentage specific activity remaining after dialysis	Specific activity before dialysis (units.(mg protein)$^{-1}$)	Percentage specific activity remaining after dialysis
(a) None (control)	1.08	66	0.43	52
(b) 2 mM DTT	1.28	69	0.52	50
(c) 5 mM $MgCl_2$	1.34	99	0.48	86
(d) 2 mM DTT + 5 mM $MgCl_2$	1.20	85	0.50	74

(a) Blue Sepharose CL-6B

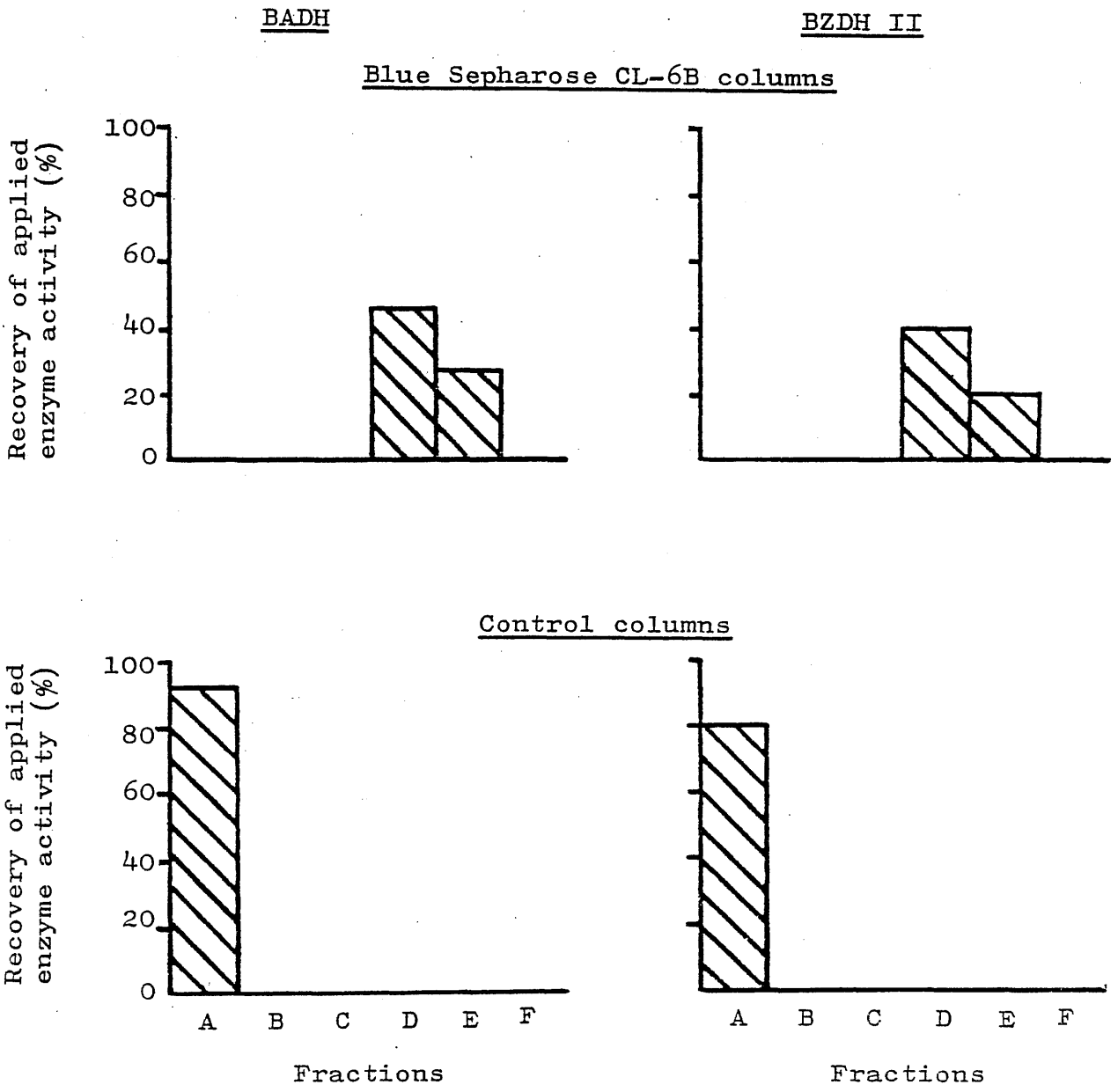
Blue Sepharose CL-6B has been widely used in the purificati of dehydrogenases (Subramanian, 1984) and experiments were therefore designed to test the potential of the material for purification of BADH and BZDH II. Several parameters, such as buffer concentration and pH, presence of metal ions and flow rate, on which the binding of proteins to dye-ligands can depend (Amicon, 1980), were investigated to define the binding conditions of BADH and BZDH II and their subsequent elution.

Preliminary experiments showed that neither BADH and BZDH II bound to Blue Sepharose CL-6B equilibrated in 100 mM potassium phosphate buffer pH 7.5. However, if the pH of the buffer was lowered to pH 6 some binding occurred, and this could be improved still further if the buffer concentration was lowered to 10 mM. The bound BADH and BZDH II could then be eluted with 10 mM potassium phosphate buffer, pH 6, containing 1 mM NAD^+; however, neither 1 mM benzyl alcohol nor 1 mM benzaldehyde could elute the enzymes. It was then shown that both enzymes began to elute from Blue Sepharose CL-6B columns with 100 µM NAD^+ (Figure 3.3) and it was also shown that this elution was unaffected by the absence of DTT from buffers.

The presence of metal ions, particularly Mg^{2+}, had been shown to increase the binding of enzymes to immobilized dye-ligands (Hughes et al., 1982). Preliminary experiments suggested that the presence of $MgCl_2$ in 10 mM potassium phosphate buffers did indeed increase the binding of BADH

Figure 3.3: Effect of NAD^+ concentration on elution of enzymes from Blue Sepharose CL-6B

An extract of A. calcoaceticus prepared as described in Methods 2.3.5(b), was dialysed into 10 mM potassium phosphate buffer pH 6, 1 mM DTT (Methods 2.2.4). Samples (0.4 ml) of the dialysed material were applied by gravity to (a) a 4 x 0.8 cm column of Blue Sepharose CL-6B and (b) a control 2.5 x 1.0 cm column of 5% cross-linked agarose. After application the flow was stopped for 30 min to aid binding. The columns were then washed under gravity with 5 ml of the following solutions in order: 10 mM potassium phosphate buffer pH 6, 1 mM DTT containing, (A) no NAD^+, (B) 1 µM NAD^+, (C) 10 µM NAD^+, (D) 100 µM NAD^+, (E) 1000 µM NAD^+, and finally (F) 100 mM potassium phosphate buffer pH 7.5, 1.5M NaCl. The BADH and BZDH II activities in the dialysed material and the wash (A) and elution fractions (B-F) of the columns were assayed as in Methods 2.4.2. The values shown are averages of duplicate assays which generally agreed within 5%.



Enzyme activity in applied material:

BADH: 1.06 units.(ml dialysed extract)$^{-1}$

BZDH II: 0.31 units.(ml dialysed extract)$^{-1}$

and BZDH II to Blue Sepharose CL-6B. Figure 3.4 shows the activities of both enzymes eluted from Blue Sepharose CL-6B columns which had been equilibrated in buffers containing different $MgCl_2$ concentrations. The highest binding capacity was observed with 5 mM $MgCl_2$ in buffers. Increasing the $MgCl_2$ concentration to 10 mM did not improve the binding of either enzyme to Blue Sepharose CL-6B. Thus 5 mM $MgCl_2$ was used to give optimal binding of both BADH and BZDH II.

The optimal conditions for binding and elution of BADH and BZDH II developed on the small analytical Blue Sepharose CL-6B columns (4 x 0.8 cm) proved successful when tested on a larger preparative column (9.5 x 2.6 cm). Gradient elution with NAD^+ on this column showed that both BADH and BZDH II could be eluted by the addition of 0.12 mM NAD^+ to the wash buffer (10 mM potassium phosphate buffer pH 6, 2 mM DTT, 5 mM $MgCl_2$).

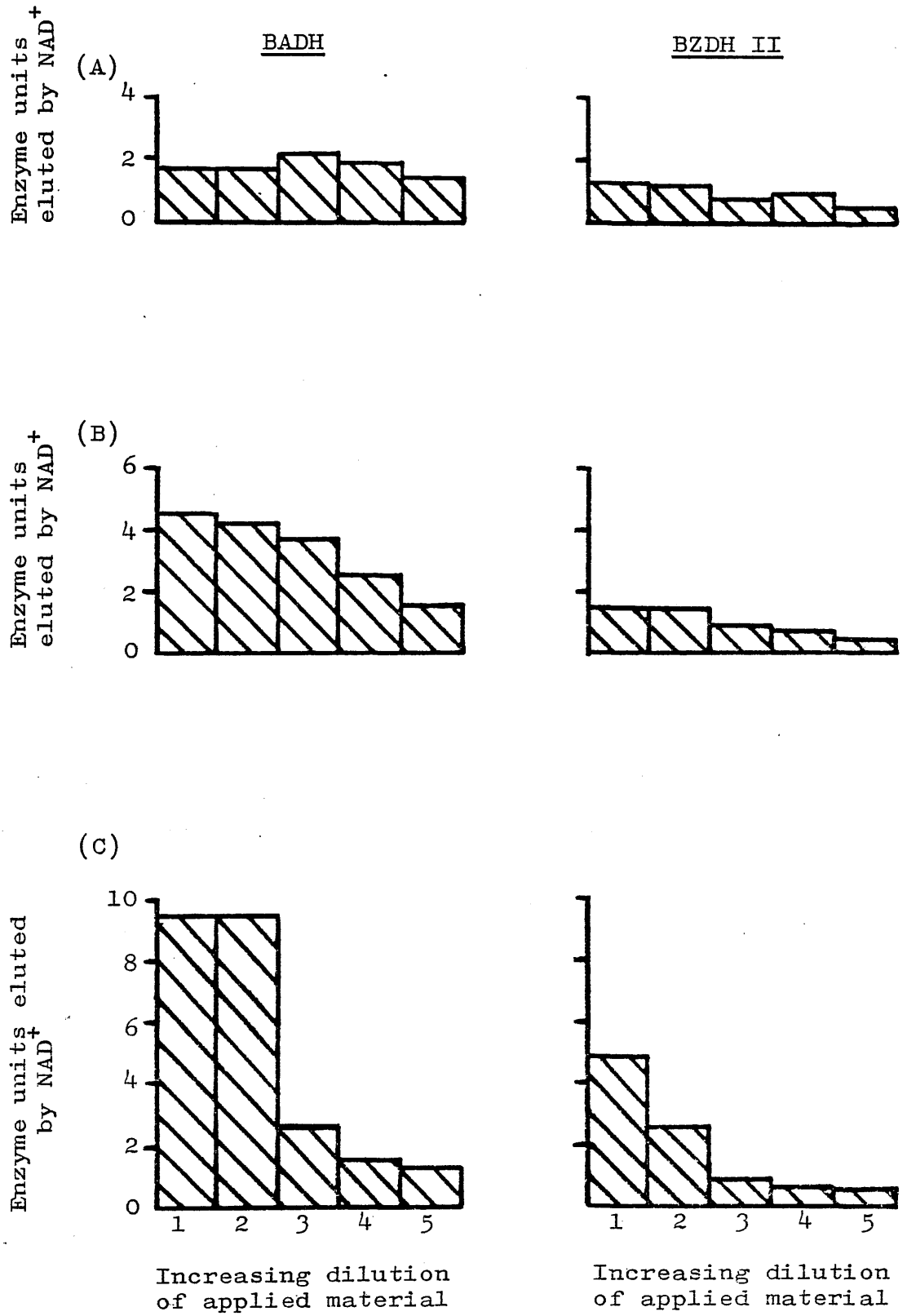
(b) Matrex gel Red A

Once optimum conditions had been developed for the interaction of BADH and BZDH II with Blue Sepharose CL-6B other dye-ligand chromatography media were examined using an Amicon Dyematrex screening kit. Neither enzyme bound to Matrex gel Blue B or Orange A, however both enzymes bound to Matrex gel Green A in 10 mM potassium phosphate buffer pH 6, 2 mM DTT, 5 mM $MgCl_2$. When $MgCl_2$ was omitted from the buffer only BADH bound to Green A.

With Matrex gel Red A, BADH bound in 10 mM potassium phosphate buffer pH 6, containing 2 mM DTT and 5 mM $MgCl_2$. Omitting the $MgCl_2$ from the buffer again appeared to reduce

Figure 3.4: Effect of $MgCl_2$ concentration on the binding of BADH and BZDH II to Blue Sepharose CL-6B.

Samples (10 ml) of ion-exchange elution pools prepared as described in Methods 2.5.2(b), were dialysed overnight (Methods 2.2.4) into one of the following solutions: 10 mM potassium phosphate buffer pH 6, 2 mM DTT containing, (A) 0 mM $MgCl_2$, (B) 1 mM $MgCl_2$ and (C) 5 mM $MgCl_2$. Each dialysed pool was then diluted, in the corresponding dialysis buffer, to give serial dilutions: (1) 1, (2) 1 to 2, (3) 1 to 4, (4) 1 to 6 and (5) 1 to 8. Then 1 ml of each diluted sample was applied to one of five 4 x 0.8 cm columns of Blue Sepharose CL-6B pre-equilibrated in the same buffer as the applied sample was diluted. After the buffer flow had been stopped for 30 min, the columns were washed twice with 5 ml of the equilibration buffer, to remove unbound excess enzyme, then with 5 ml of equilibration buffer containing 0.5 mM NAD^+ to elute BADH and BZDH II. The enzyme activities were assayed as in Methods 2.4.2. Undiluted dialysed material contained, (A) 22.2 and 6.8 units.ml^{-1}, (B) 14.5 and 4.3 units.ml^{-1} and (C) 13.0 and 5.3 units.ml^{-1} of BADH and BZDH II activities respectively. The values shown are averages of duplicate assays which usually agreed within 5%.


BADH
BZDH II
(A)
(B)
(C)
Enzyme units eluted by NAD+
Enzyme units eluted by NAD+
Enzyme units eluted by NAD+
0
2
4
6
8
10
1
2
3
4
5
Increasing dilution of applied material
Increasing dilution of applied material

the binding capacity of the column. BZDH II would not bind to Matrex gel Red A. BADH could be eluted by the addition of 2.75 mM NAD^+ to the wash buffer. It was therefore possible to apply the elution pool from a Blue Sepharose CL-6B column containing both BADH and BZDH II activities in 10 mM potassium phosphate buffer pH 6, 2 mM DTT, 5 mM $MgCl_2$, 0.12 mM NAD^+ directly on to a Matrex gel Red A column. BZDH II washed through, and bound BADH was eluted by increasing the NAD^+ concentration to 2.75 mM.

3.2.2 Development of a purification step prior to triazine dye-ligand chromatography

As the buffer system developed for the binding of BADH and BZDH II to Blue Sepharose CL-6B and Matrex gel Red A columns also resulted in the binding of many other proteins, the application of crude extracts to the dye-ligand columns resulted in a low capacity for BADH and BZDH II. An initial purification step was therefore required to produce a fraction which contained the enzymes in both a high yield and moderately purified form.

(a) Ammonium sulphate fractionation: No purification could be achieved for either enzyme using $(NH_4)_2SO_4$ fractionation procedures in 100 mM potassium phosphate buffer pH 7.5. However in 100 mM glycine/NaOH pH 9.5, a 45-55% saturation $(NH_4)_2SO_4$ fractionation purified BZDH II 7-fold with a 95% recovery of activity, but purified BADH only 4-fold with 57% recovery of activity. $(NH_4)_2SO_4$ fractionation, although useful for some preliminary work, was not used in the final purification procedure for BADH and BZDH II.

(b) Gel filtration: Extracts or $(NH_4)_2SO_4$ fractions of A.calcoaceticus were fractionated by gel filtration using AcA 34 Ultrogel. A maximal purification of only 2 to 3-fold for both BADH and BZDH II was achieved. Recovery was poor, particularly for BZDH II (80% activity being lost during the overnight filtration). Although not used as a preliminary step, gel filtration using FPLC was later used as a successful final stage purification (Section 3.2.3).

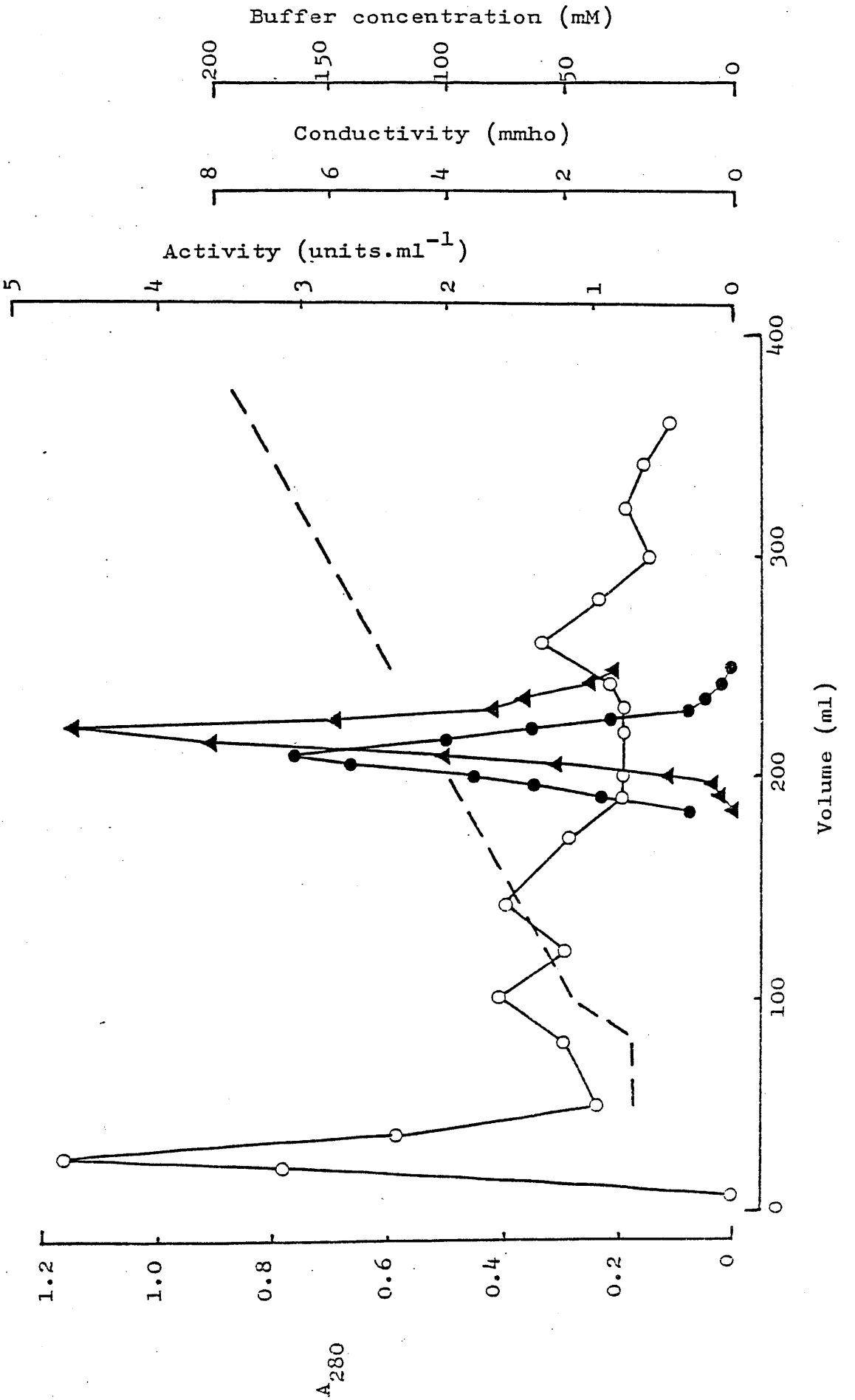
(c) Ion-exchange chromatography: Partially purified BADH and BZDH II with good recovery of activity was successfully produced by ion-exchange chromatography. Preliminary studies using 2 ml bed volume mini-columns of ion-exchange media showed that both enzymes bound to DEAE-Sephacel, but not to CM-cellulose, in 100 mM potassium phosphate buffer pH 7.5, 2 mM DTT. Elution was achieved by increasing the buffer concentration to 150 mM. Application of an extract of A.calcoaceticus to a DEAE-Sephacel column followed by elution of protein with a linear gradient of 20 to 400 mM potassium phosphate buffer, pH 7.5, 2 mM DTT (Figure 3.5) showed that both enzymes eluted between a buffer concentration of 100 mM and 120 mM. This information was used to develop the purification step described in Methods 2.5.2(b) in which a stepwise elution of the enzymes was used rather than a gradient.

3.2.3 Fast Protein Liquid Chromatography (FPLC) gel filtration

SDS-PAGE of the pool which contained BADH activity eluted from Matrex gel Red A (Section 3.2.1(b)) showed one major and one minor protein component. Several different procedures

Figure 3.5: Gradient elution of enzymes from DEAE-Sephacel ion-exchange.

An extract of A. calcoaceticus was prepared as described in Methods 2.3.5(b), except that the resuspension buffer was 20 mM potassium phosphate buffer, pH 7.5, 2 mM DTT. A sample (15 ml) was applied to a 5.6 x 2.6 cm column of DEAE-Sephacel pre-equilibrated in 20 mM potassium phosphate buffer pH 7.5, 2 mM DTT. The column was washed to remove unbound material. Once the A_{280} (○) was lowered to 0.25 a gradient of 20 mM to 400 mM potassium phosphate buffer pH 7.5, 2 mM DTT (total volume 500 ml) was started. Flow rate was 20 $ml.h^{-1}$ and 5 ml fractions were collected. BADH activity (▲) and BZDH II activity (●) were assayed as in Methods 2.4.2. The conductivity (---) of the column fractions were measured as described in Methods 2.2.2.


Buffer concentration (mM)
200
150
100
50
0
Conductivity (mmho)
8
6
4
2
0
Activity (units.ml^{-1})
5
4
3
2
1
0
400
300
200
100
0
Volume (ml)
1.2
1.0
0.8
0.6
0.4
0.2
0
A_{280}

were tested to separate the two proteins (e.g. FPLC Mono Q ion-exchange and Mono P chromatofocussing chromatography, and binding to Matrex gel Green A). However, the only purification procedure that was successful was FPLC gel filtration on Superose 6 or 12 columns. The Superose 12 column was used in the final purification procedure (Methods 2.5.2(e)).

3.2.4 Protease inhibitors

The effect of the addition of 1.2 mM PMSF (fresh solutions of which were added frequently because of its instability) and 1 mM benzamidine to the purification buffers was tested. No increase in the recovery of BADH or BZDH II activities was observed, and SDS-PAGE of samples of the pools from the individual purification steps showed no difference in the protein band patterns compared with pools obtained using buffers lacking the protease inhibitors. PMSF and benzamidine were therefore not used in the final purification buffers, but they were included in the final storage buffer (Methods 2.5.1).

3.3 Purification of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II

From the preliminary purification studies (Section 3.2) a common purification procedure was developed by which both BADH and BZDH II could be purified. The complete procedure is described in Methods 2.5 and the results are summarised in Table 3.3.

3.3.1 Ion-exchange chromatography on DEAE-Sephacel

Figure 3.6 shows a typical profile of the chromatography of an extract of A. calcoaceticus on DEAE-Sephacel. The BADH and BZDH II activities co-eluted as sharp peaks when the potassium phosphate concentration was stepped up from 75 mM to 110 mM. A 12-fold purification with approximately 80% yield of activity, of both enzyme was usually achieved in this step.

3.3.2 Dye-ligand chromatography on Blue Sepharose CL-6B

Chromatography on Blue Sepharose CL-6B is the major purification step for BADH and BZDH II. A typical chromatography profile of a dialysed ion-exchange pool on Blue Sepharose CL-6B is shown in Figure 3.7. The enzymes were co-eluted by the addition of 0.12 mM NAD^+ to the equilibration buffer. The buffer flow direction was reversed to reduce the volume in which the enzymes eluted. Both enzymes were purified a further 10-fold compared with the applied ion-exchange pool, with approximately 60% recovery of activity.

3.3.3 Dye-ligand chromatography on Matrex gel Red A

The elution pool from the Blue Sepharose CL-6B column (Section 3.3.2) was applied directly to a Matrex gel Red A column. A typical profile is shown in Figure 3.8. The BZDH II activity washed straight through the column whereas the BADH activity bound to it. There was a long wash period in order to ensure that the pool containing the BADH activity was not contaminated with BZDH II. The buffer flow direction was again reversed during enzyme elution to reduce the final pool volume.

Figure 3.6: Chromatography of an extract of A.calcoaceticus on DEAE-Sephacel.

An extract (approx. 38 ml) of A.calcoaceticus prepared as in Methods 2.3.5(b) was applied to a column of DEAE-Sephacel (3.8 x 2.6 cm) pre-equilibrated in Buffer A (defined in Methods 2.5.1). The column was washed with Buffer A to remove unbound material until the A_{280} of the effluent decreased to approximately 0.05. The buffer was then changed to Buffer B (Methods 2.5.1) (↓) to elute the enzyme activities. Flow rate was 50 $ml.h^{-1}$ and 5 ml fractions were collected. A_{280}, (○); BADH activity, (▲); BZDH II activity (●); conductivity (---).

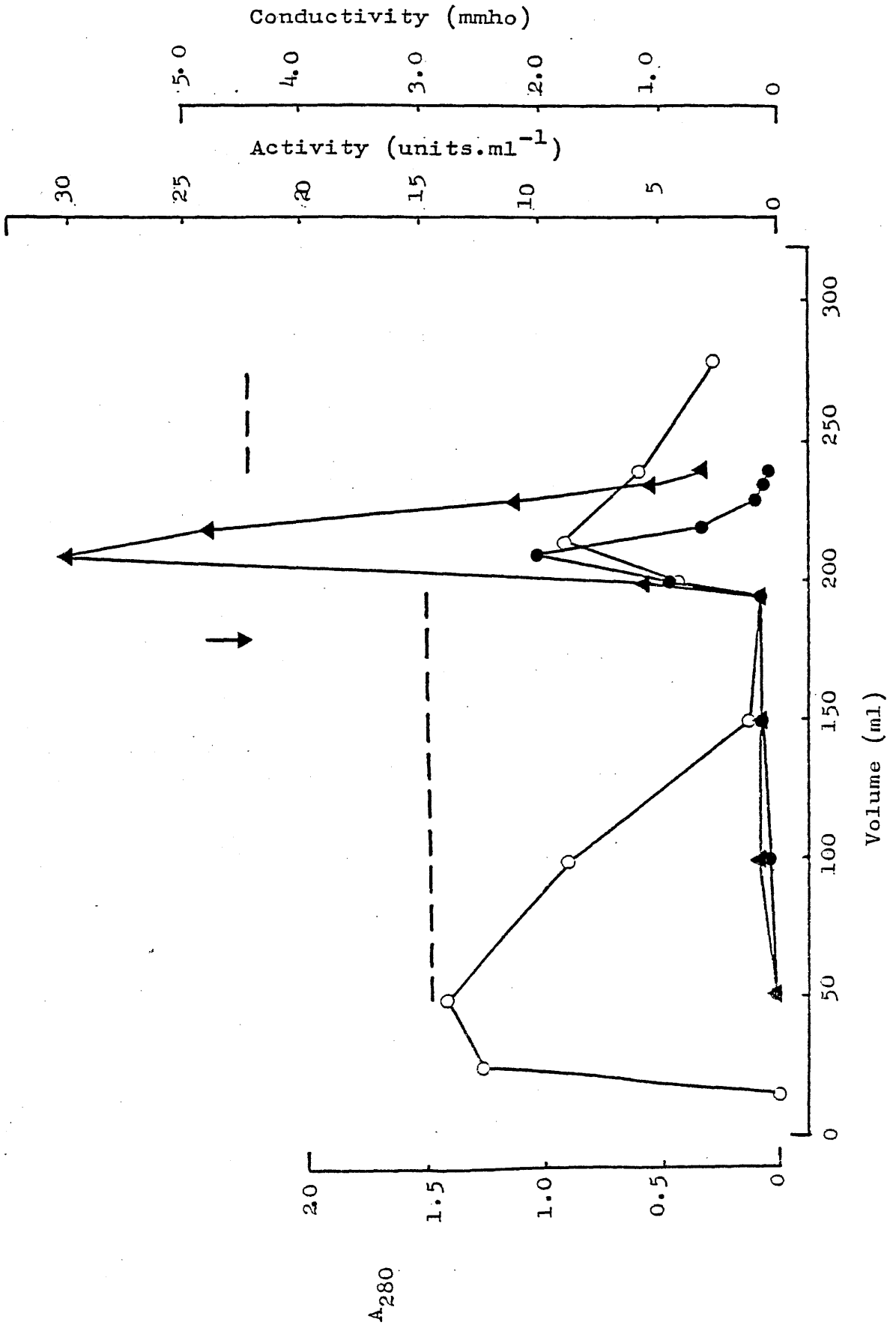

Conductivity (mmho)
5.0
4.0
3.0
2.0
1.0
0
Activity (units.ml^{-1})
30
25
20
15
10
5
0
300
250
200
150
100
50
0
Volume (ml)
2.0
1.5
1.0
0.5
0
A_{280}

Figure 3.7: Chromatography of an ion-exchange elution pool on Blue Sepharose CL-6B.

The elution pool (approx. 45 ml) from a DEAE-Sephacel column (Section 3.3.1) was dialysed overnight against 3 x 1000 ml of Buffer C (Methods 2.5.1). The dialysed pool was applied to a 9.5 x 2.6 cm column of Blue Sepharose CL-6B pre-equilibrated in Buffer C. After loading, the pump was stopped for 30 min (↓) and then the column was washed with Buffer C for 2 column volumes after the A_{280} of the effluent had returned to the baseline. The buffer was then changed to Buffer C containing 0.12 mM NAD^+, and the flow direction reversed (⇣) to elute the enzyme activities. The flow rate was 99 $ml.h^{-1}$ and 5 ml fractions were collected. A_{280}, (○); BADH activity, (▲); BZDH II activity, (●).

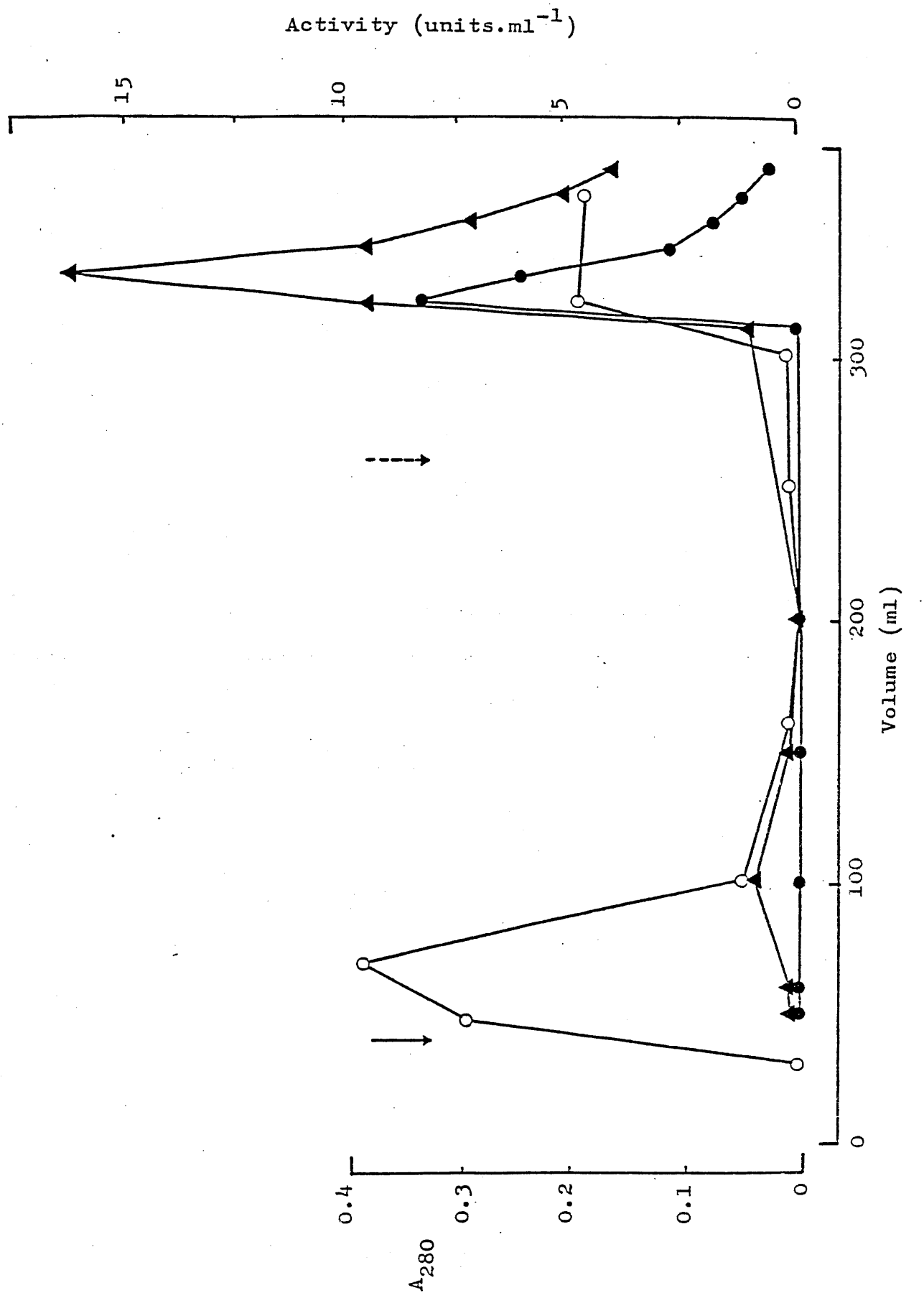
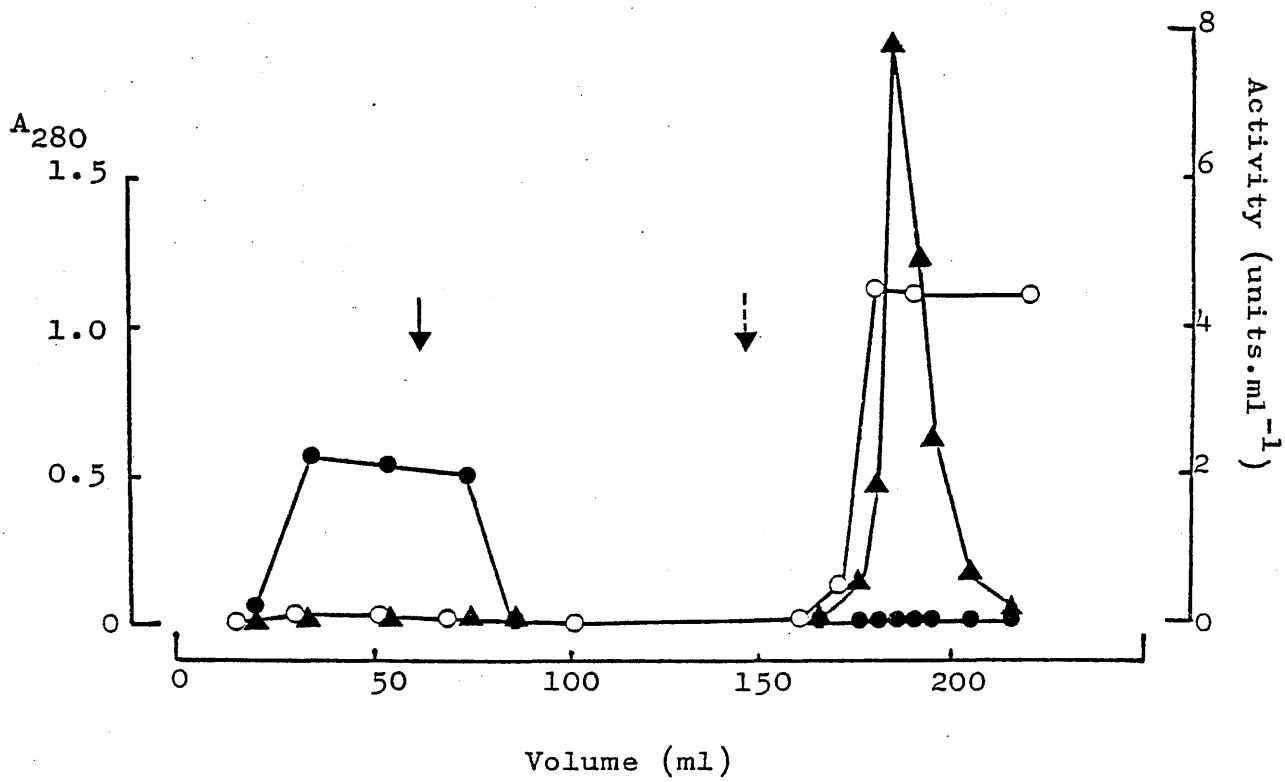

Activity (units.ml^{-1})
15
10
5
0
0.4
0.3
0.2
0.1
0
A_{280}
0
100
200
300
Volume (ml)

Figure 3.8: Chromatography of the elution pool from a Blue Sepharose CL-6B column on Matrex gel Red A.

The elution pool (approx. 48 ml) from a Blue Sepharose CL-6B column (Section 3.3.2) was applied to a column of Matrex gel Red A (3.8 x 2.6 cm) pre-equilibrated in Buffer C containing 0.12 mM NAD^+ (Methods 2.5.1). After loading, the pump was stopped for 30 min (↓), and then the column was washed with Buffer C plus 0.12 mM NAD^+, for 4 column volumes after the A_{280} of the effluent had returned to the baseline. The buffer was changed to Buffer C plus 2.75 mM NAD^+, and the flow rate reversed (⇣) to elute the enzyme activities. The flow rate was 99 $ml.h^{-1}$ and 5 ml fractions were collected. A_{280}, (○); BADH activity, (▲); BZDH II activity, (●).


A_{280}
1.5
1.0
0.5
0
0
50
100
150
200
Volume (ml)
8
6
4
2
0
Activity (units.ml^{-1})

The enzymes were purified a further 2-fold compared with the applied Blue Sepharose CL-6B elution pool, and the recovery of BZDH II activity was approximately 90% and that of BADH approximately 50%.

3.3.4 Gel filtration on Pharmacia FPLC Superose 12

Figure 3.9 shows a typical profile of the chromatography of a concentrated BADH elution pool from Matrex gel Red A (Section 3.3.3) on an FPLC Superose 12 gel filtration column. Two peaks of A_{280} were observed, the major peak corresponded with the BADH.

3.3.5 Overall purification

Details of a typical purification of *A. calcoaceticus* NCIB 8250 BADH and BZDH II are given in Table 3.3. The procedure was very reproducible and has been carried out nine times. As a result BADH was purified 168 $\pm$ 19 (7) fold over the crude extract in 13.2 $\pm$ 2.5 (7) % yield of activity. BZDH II was purified 184 $\pm$ 80 (7) fold over the crude extract in 40 $\pm$ 10 (7) % yield of activity. The purification of the enzymes, starting from cell breakage through to final dialysis (Methods 2.5) took only 60 hours.

3.3.6 Stability of the purified enzymes

The stability of the purified enzymes was tested in a variety of different buffers, at 4°C and -20°C and Buffer E (Methods 2.5.1) was finally developed. The presence of DTT was again important in stabilising the purified BADH and BZDH II, as it was with the activities in extracts of *A. calcoaceticus*.

Figure 3.9: Chromatography of a concentrated elution pool from a Matrex gel Red A column on FPLC Superose 12.

The elution pool (approx. 30 ml) from a Matrex gel Red A column (Section 3.3.3) was concentrated to 0.2 ml (Methods 2.2.5) and applied to a Superose 12 gel filtration column (30 x 1 cm) pre-equilibrated in Buffer D (Methods 2.5.1). The flow rate was 0.3 $ml.min^{-1}$ and 0.3 ml fractions were collected. A_{280}, (O); BADH activity, (▲).

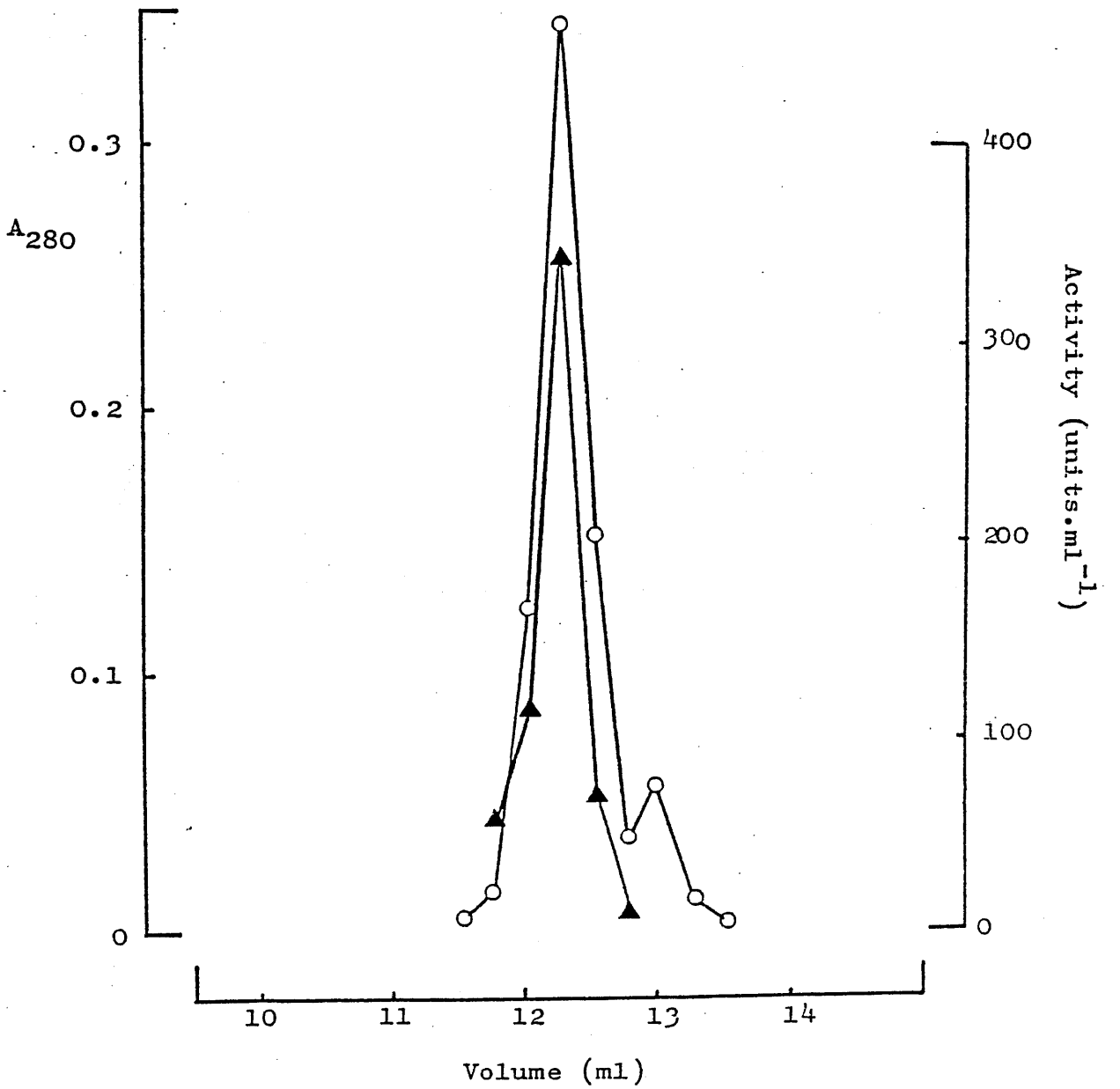

0.3
A_{280}
0.2
0.1
0
400
300
200
100
0
Activity (units.ml^{-1})
10
11
12
13
14
Volume (ml)

Table 3.3: Details of typical purification of A.calcoaceticus BADH and BZDH II.

Details of the individual purification steps are given in Methods 2.5. Protein was measured as described in Methods 2.2.6(b) and enzyme activity as described in Methods 2.4.3.

Benzyl alcohol dehydrogenase (BADH)

Step	Volume (ml)	Total activity (enzyme units)	Protein (mg)	Specific activity (units.(mg proteins)$^{-1}$)	Yield (%)	Fold Purification
Crude Extract	38	1056	704	1.8	100	1
Ion Exchange	45	938	53	17.7	89	11.8
Dialysis	50	793	50	15.9	75	10.4
Blue Sepharose Cl-6B	48	380	2.83	134	36	89
Matrex gel Red A	0.2	178	0.81	220	17	147
FPLC Superose 12	0.9	171	0.72	238	16	159
Dialysis	0.32	171	0.72	238	16	159

Benzaldehyde dehydrogenase II (BZDH II)

Step	Volume (ml)	Total activity (enzyme units)	Protein (mg)	Specific activity (units.(mg protein)$^{-1}$)	Yield (%)	Fold Purification
Crude Extract	38	388	704	0.55	100	1
Ion-Exchange	45	285	53	5.4	73	9.8
Dialysis	50	238	50	4.8	61	8.7
Blue Sepharose Cl-6B	48	190	2.83	67	49	121
Matrex gel Red A	1.2	143	1.84	78	37	142
Dialysis	0.41	143	1.80	79	37	144

Protease inhibitors were included to reduce the possibility of proteolytic degradation during long term storage. If the enzymes were simply diluted in Buffer E (Methods 2.5.1), which contained 40% (w/v) glycerol, they lost up to 90% of their activity. However when dialysed into buffer E both enzymes retained more than 90% of their original activity over a three month period at -20°C.

3.3.7 Purity

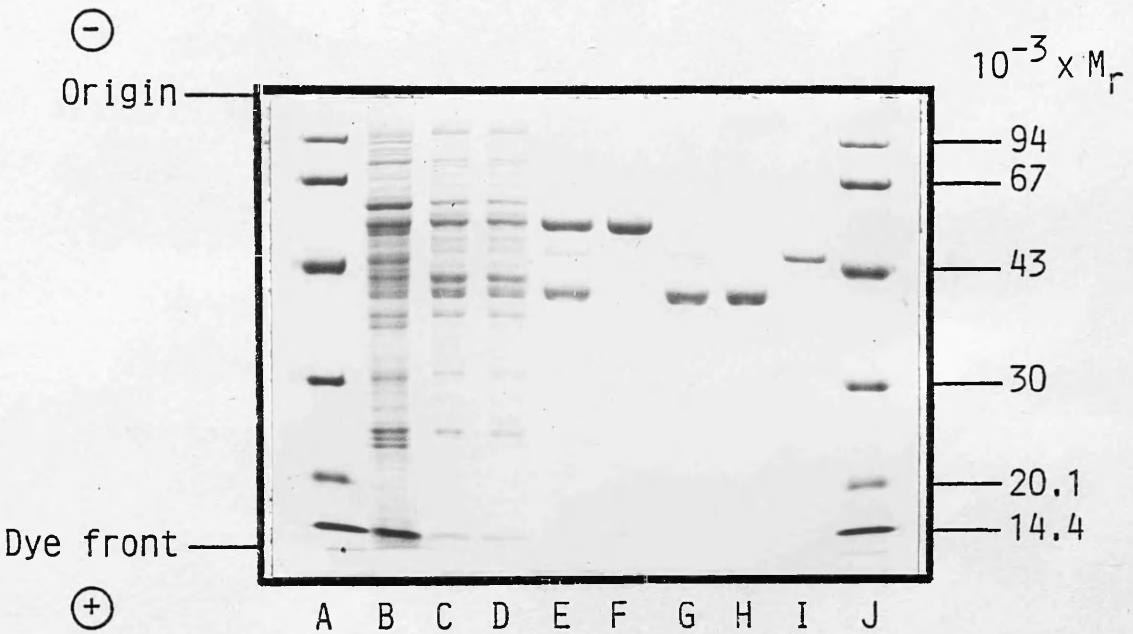
The purification of BADH and BZDH II was monitored by SDS-PAGE, and a typical gel is shown in Figure 3.10. The wash pool of the Matrex gel Red A column (Track F) contains homogenous BZDH II. The FPLC Superose 12 gel filtration pool with the BADH activity also contains homogenous enzyme (Track H). The third homogeneous protein (Track I) is the only other protein along with BADH in the Matrex gel Red A elution pool (Track G), and was separated from BADH, and purified to homogeneity, by Superose 12 gel filtration. This third protein was later identified as having glycerol-3-phosphate dehydrogenase activity (Section 3.3.8). Figure 3.11 reproduces densitometer scans of the purified BADH and BZDH II after SDS-PAGE. Gels stained with the silver procedure (Methods 2.6.3(a)) also showed no contaminant bands in the final preparations so the enzymes are sufficiently pure that it is not possible to provide an estimate of possible contaminating protein.

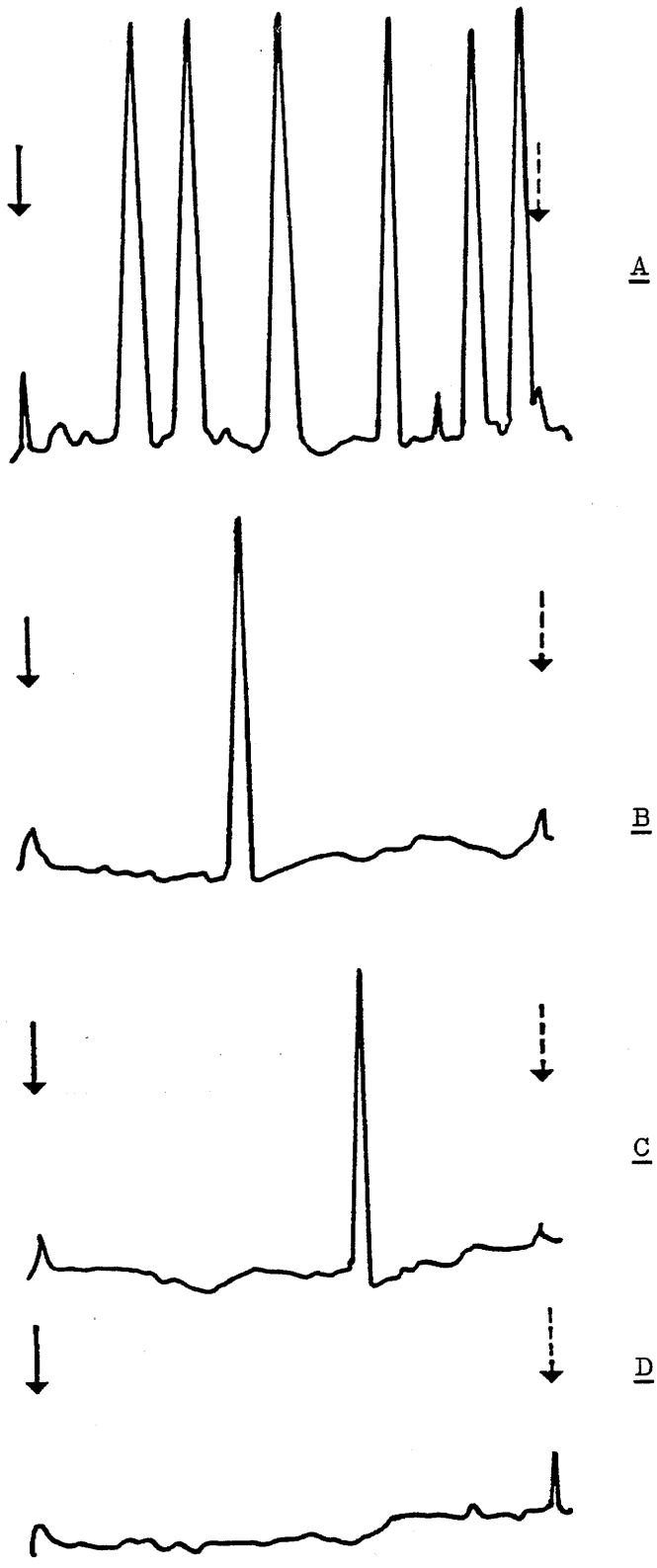
When purified BADH and BZDH II were analysed on non-denaturing 7% (w/v) polyacrylamide gels and stained for protein, a single band was observed. This band corresponded to a single band of formazan which indicated the position of the BADH

Figure 3.10: The purification BADH and BZDH II as monitored by SDS-PAGE

A 10% (w/v) polyacrylamide slab gel, containing 0.1% (w/v) SDS, monitoring the purification of BADH and BZDH II from A. calcoaceticus is shown.

Tracks: B, crude extract (20 µg protein); C, ion-exchange elution pool (10 µg protein); D, dialysed ion-exchange elution pool (10 µg protein); E, Blue-Sepharose CL-6B elution pool (2 µg protein); F, Matrex gel Red A wash pool (1 µg protein); G, Matrex gel Red A elution pool (1 µg protein); H, FPLC Superose 12 major protein peak (1 µg protein); I, FPLC Superose 12 minor protein peak (0.4 µg protein); A and J, Pharmacia low molecular mass (M_r) standard proteins (approx. 1 µg each): phosphorylase b, 94 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100; α-lactalbumin, 14 400. The gel was prepared and electrophoresed as described in Methods 2.6.1.


⊖
Origin
Dye front
⊕
10^{-3} x M_r
94
67
43
30
20.1
14.4
A B C D E F G H I J


A
B
C
D

or BZDH II activity (Figure 3.12).

3.3.8 Identification of the third homogeneous protein

The third protein that was purified along with BADH and BZDH II was found to have glycerol-3-phosphate dehydrogenase activity. The evidence for this was as follows:

(a) An increase in A_{340} was observed when enzyme and NAD^+ were mixed, without the addition of a substrate. This was attributed to the glycerol in Buffer E (Methods 2.5.1), in which the enzyme was stored, serving as a substrate for NAD^+ oxidation.

(b) Increasing the glycerol concentration in the assay from 70 to 300 mM increased the observed activity, but the highest rate was observed in the presence of 1 mM glycerol-3-phosphate.

(c) The addition of 1 mM dihydroxyacetone-3-phosphate caused reduction of NADH. Glyceraldehyde (1 mM) did not have this effect.

3.4 Discussion

Persistent early problems with loss of enzyme activities were largely overcome by the discovery that combinations of DTT and $MgCl_2$ were effective in stabilising the activities of BADH and BZDH II (Figure 3.1 and Table 3.2). Beggs and Fewson (1974) had previously observed that DTT would stabilise BADH activity in toluenised cells, although this was in part a result of the increased BZDH II activity which contributes to the apparent BADH activity measured in crude extracts (see p. 54). The mechanism by which $MgCl_2$ stabilises both of the BADH and BZDH II activities during dialysis (Table 3.2) remains unclear.

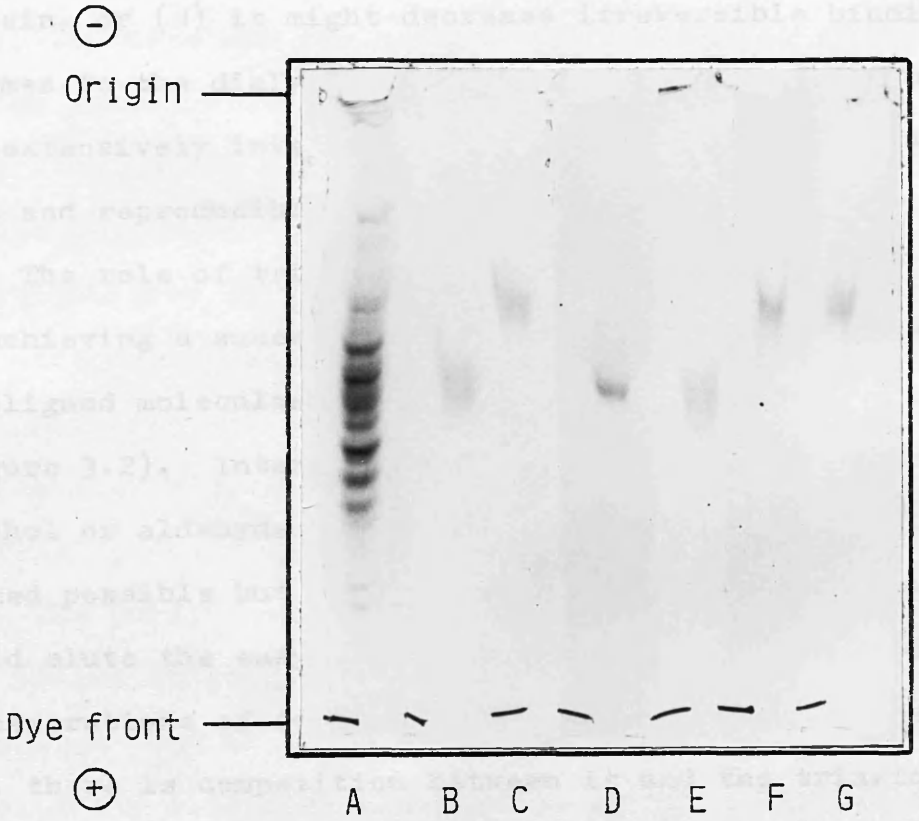

⊖
Origin
Dye front
⊕
A
B
C
D
E
F
G

Figure 3.12: Non-denaturing polyacrylamide gel and activity stain of purified A.calcoaceticus BADH and BZDH II

A 7% (w/v) polyacrylamide slab gel of purified BADH and BZDH II is shown.

Tracks: A, B and C, crude extract (20 µg protein); D and E, purified BZDH II (2 µg protein); F and G, purified BADH (2 µg protein).

Tracks A, D and F were stained for protein as described in Methods 2.6.3(a). Tracks B and E were stained for BZDH II activity and tracks C and G were stained for BADH activity (Methods 2.6.3(b)).

The gel was prepared and electrophoresed as described in Methods 2.6.2.

Several hypotheses are possible, e.g. (a) the $MgCl_2$ might stabilise the quaternary structure of the enzymes, (b) the salt might cause the enzymes to dissociate into a more active form, off-setting other activity losses, (c) it might prevent removal of any active site or structural metal atom from the protein, or (d) it might decrease irreversible binding of the enzymes to the dialysis tubing. However, the phenomenon was not extensively investigated except to establish its effectiveness and reproducibility.

The role of triazine dye affinity matrices was central to achieving a successful purification of the enzymes. The dye-ligand molecules contain several aromatic ring structures (Figure 3.2). Interaction between the ligand and the aromatic alcohol or aldehyde binding sites of BADH and BZDH II therefore seemed possible but neither benzyl alcohol nor benzaldehyde would elute the enzymes (Section 3.2.1). The elution by low concentrations of cofactor may be considered "biospecific", i.e. there is competition between it and the triazine dye-ligand for a common binding site (Subramanian, 1984).

BADH binds to both Blue Sepharose CL-6B and Matrex gel Red A but elution from the former requires a 20-times lower concentration of NAD^+ (Section 3.2.1), suggesting weaker protein-ligand interactions. BZDH II does not bind to Matrex gel Red A. The dye ligand, Procion Red HE-3B, is considerably larger than the Blue Sepharose CL-6B ligand, Cibacron Blue F3 G-A (Figure 3.2) and so the lack of binding of BZDH II to the Red A gel might be a result of steric hinderance preventing

interaction between the enzyme and specific ligand structures.

Metal ion-aided binding of proteins to triazine dye-ligands has been extensively investigated (Hughes *et al.*, 1982), and the presence of $MgCl_2$ in buffers markedly increases the binding of BADH and BZDH II to the dye matrices (Section 3.2.1). There are two models that attempt to explain protein-ligand interactions (Subramanian, 1984). Firstly, that Mg^{2+} ions bind to and neutralise the negatively charged sulphonate groups present on the ligand. This increases the ligand's hydrophobicity which in turn favours the binding of the protein. The other model suggests that the Mg^{2+} ions form the centre of a highly specific tertiary complex between the enzyme and the sulphonate, hydroxyl and azo bond regions of the ligand, thus increasing electrostatic interactions.

The purification procedure that was developed results in the isolation of two homogeneous enzymes which have either benzyl alcohol or benzaldehyde oxidising activity. The possibility raised by Livingstone *et al.* (1972) that the BADH and BZDH II activities might be part of a single bifunctional enzyme has therefore been disproved, although they co-purify through two purification steps (Section 3.3.1 and 3.3.2).

The purified enzymes are stable in the storage buffer; DTT again being the key component (Section 3.3.6). Several other bacterial aromatic alcohol and aldehyde dehydrogenases such as 3-hydroxybenzyl alcohol dehydrogenase from *Penicillium urticae* (Scott *et al.*, 1986), coniferyl alcohol dehydrogenase

from Rhodococcus erythropolis (Jaeger et al., 1981), benzaldehyde dehydrogenase from Pseudomonas putida (Stachow et al., 1967) and perillaldehyde dehydrogenase also from Pseudomonas sp. (Ballal et al., 1967) all require DTT, glutathione, or cysteine to maintain activity. A benzyl alcohol dehydrogenase purified from Pseudomonas putida T-2 grown on toluene is also unstable (Suhara et al., 1969); in this case the enzyme's instability was unaffected by the presence of glutathione but it was stabilised by the addition of acetone to buffers. BADH and BZDH II were both inhibited by acetone (results not shown).

A third protein, identified as having glycerol-3-phosphate dehydrogenase activity, was isolated along with BADH and BZDH II (Section 3.3.8). This is the first reported purification of glycerol-3-phosphate dehydrogenase from A. calcoaceticus. The regulatory properties of this enzyme will warrant study because of its role in linking glycolysis with lipid biosynthesis

CHAPTER 4

CHARACTERISATION OF PURIFIED BENZYL ALCOHOL DEHYDROGENASE AND BENZALDEHYDE DEHYDROGENASE II FROM A. CALCOACETICUS NCIB 8250

4.1 Physical and chemical characteristics of the purified enzymes

Certain physical and chemical properties of the purified BADH and BZDH II from A. calcoaceticus were measured to allow comparison of the enzymes with each other and with other bacterial and eukaryotic alcohol and aldehyde dehydrogenases.

4.1.1 Relative molecular mass values

SDS-PAGE was used to determine the subunit M_r values of the purified enzymes. A typical standard curve of electrophoretic mobility (R_f) against log M_r is shown in Figure 4.1. Measurement of the mobilities of the enzymes gave subunit M_r values of 39700 ± 400 (24) for BADH and 55000 ± 900 (33) for BZDH II. Six batches of BADH and eight batches of BZDH II were used for these determinations.

The native M_r values of BADH and BZDH II were obtained by gel filtration chromatography. A standard curve of $V_e - V_o/V_t$ against log native M_r was constructed with proteins of known native M_r (Figure 4.2). In two independent experiments for each enzyme the $V_e - V_o/V_t$ values gave average native M_r values for BADH of 155000 (from two identical values) and 223000 (216000, 229000) for BZDH II.

Comparison of the subunit and native M_r values of the enzymes suggests that both are tetrameric under the conditions used for the native M_r estimations.

4.1.2 Isoelectric point determinations

Figure 4.3 shows the profiles of A_{280} BADH and BZDH II activities after chromatofocussing on an FPLC Mono P column.

Figure 4.1. Typical standard curve of R_f against log M_r from an SDS-PAGE gel.

SDS-PAGE was performed using a 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS as described in Methods 2.6.1. The gel comprised six tracks of standard proteins (●) of known M_r values, 6 tracks of BADH (○) (from 6 batches of enzyme), and 8 tracks of BZDH II (△) (from 8 batches of enzyme). The standard proteins were:

	Protein	Subunit M_r
1	Phosphorylase b	94 000
2	BSA	67 000
3	Ovalbumin	43 000
4	Carbonic anhydrase	30 000
5	Trypsin inhibitor	20 100
6	α-Lactalbumin	14 400

After electrophoresis the gel was stained with Coomassie Blue; destained (Methods 2.6.3(a)) and scanned using an LKB densitometer (Methods 2.6.4). The R_f values of the protein bands were calculated (Methods 2.6.5). The average R_f value of each protein is plotted (with standard deviations indicated by bars, ⧳) against log M_r.

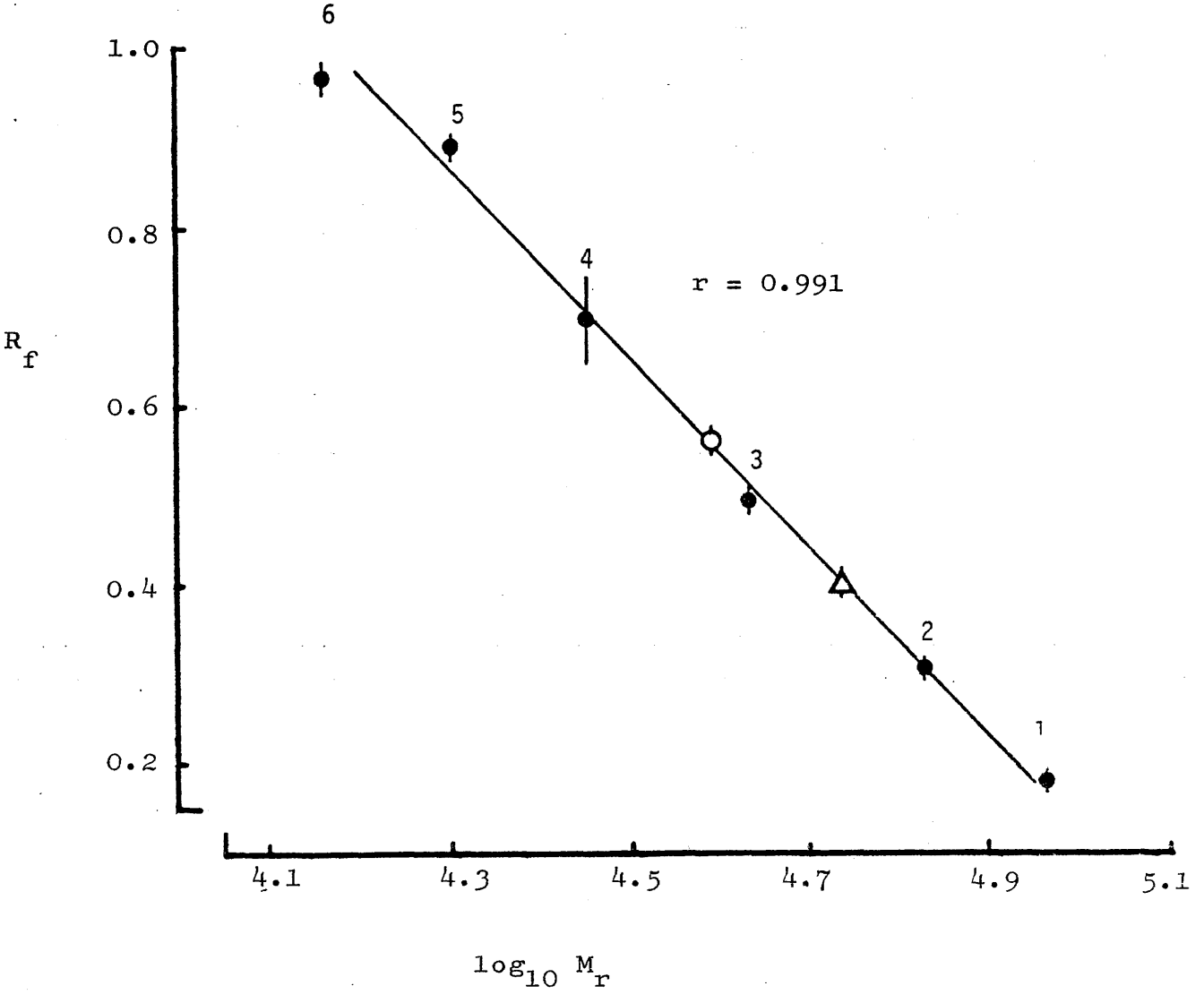

6
5
4
3
2
1
1.0
0.8
0.6
0.4
0.2
R_f
r = 0.991
4.1
4.3
4.5
4.7
4.9
5.1
$\log_{10} M_r$

Figure 4.2. Standard curve of $V_e - V_o/V_t$ against log M_r

A Sephacryl S-300 superfine column (75 x 1.6 cm) was calibrated with proteins of known molecular mass. The proteins were those of the Combithek calibration kit plus carbonic anhydrase and fumarase:

	Protein	Native M_r
1	Cytochrome c	12 500
2	Chymotrypsinogen A	25 000
3	Carbonic anhydrase	30 000
4	Ovalbumin	45 000
5	BSA	67 000
6	Aldolase	158 000
7	Fumarase	194 000
8	Catalase	240 000
9	Ferritin	450 000

The calibration proteins (●) were applied to the column three at a time and the A_{280} of the elution fractions were measured on a Pye Unicam SP8-100 spectrophotometer, to determine the V_e values. The V_e values of BADH and BZDH II were determined by measuring the eluted enzyme activities (Methods 2.4.3(a) and (c)). The buffer was 100 mM potassium phosphate pH 7.5, 2 mM DTT and the flow rate 20 ml.h^{-1}. The $V_e - V_o/V_t$ values for BADH (○, two identical values) and BZDH II (△) were obtained from two independent experiments for each enzyme.

V_e = elution volume of the protein

V_o = void volume

V_t = total bed volume

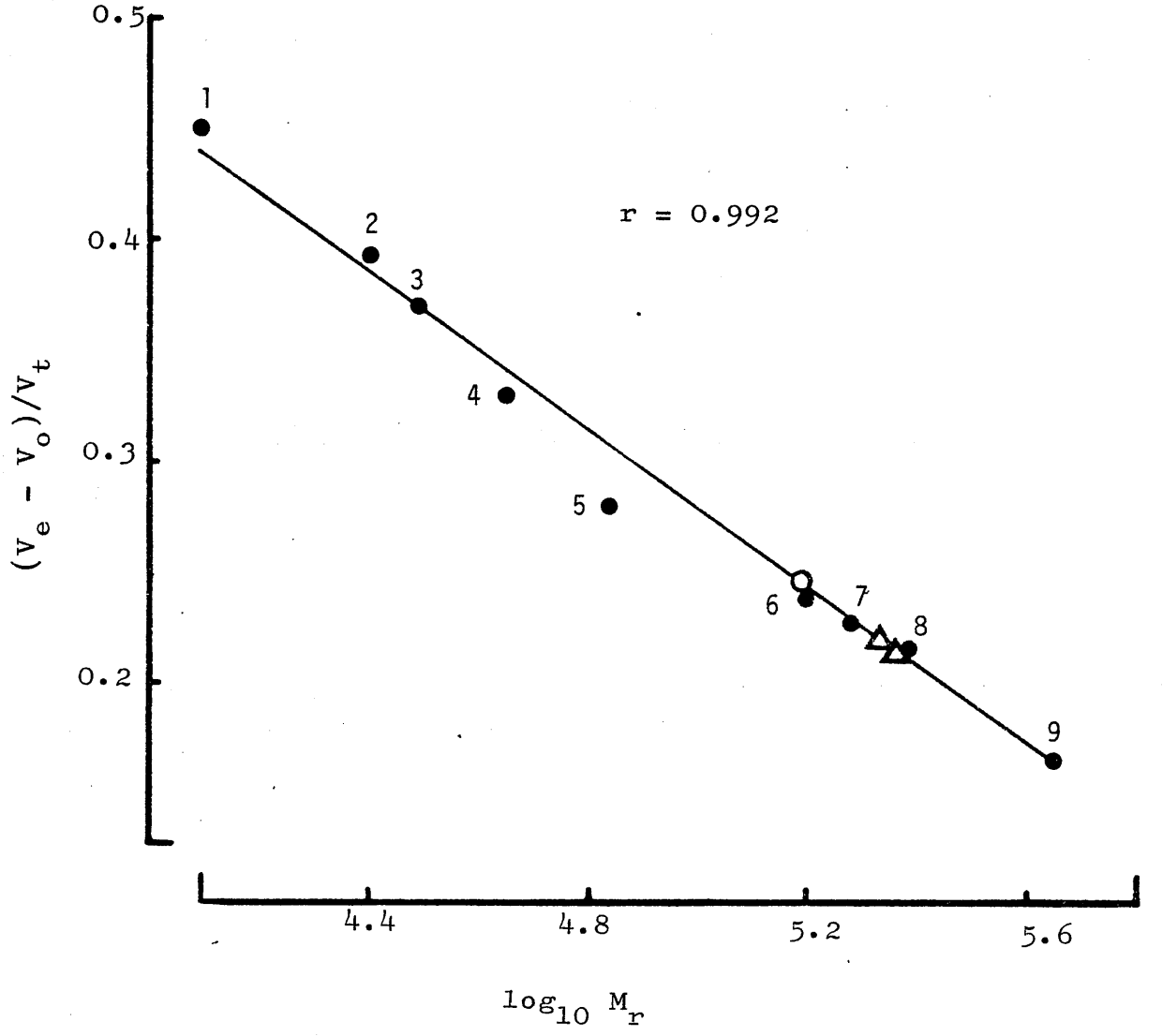
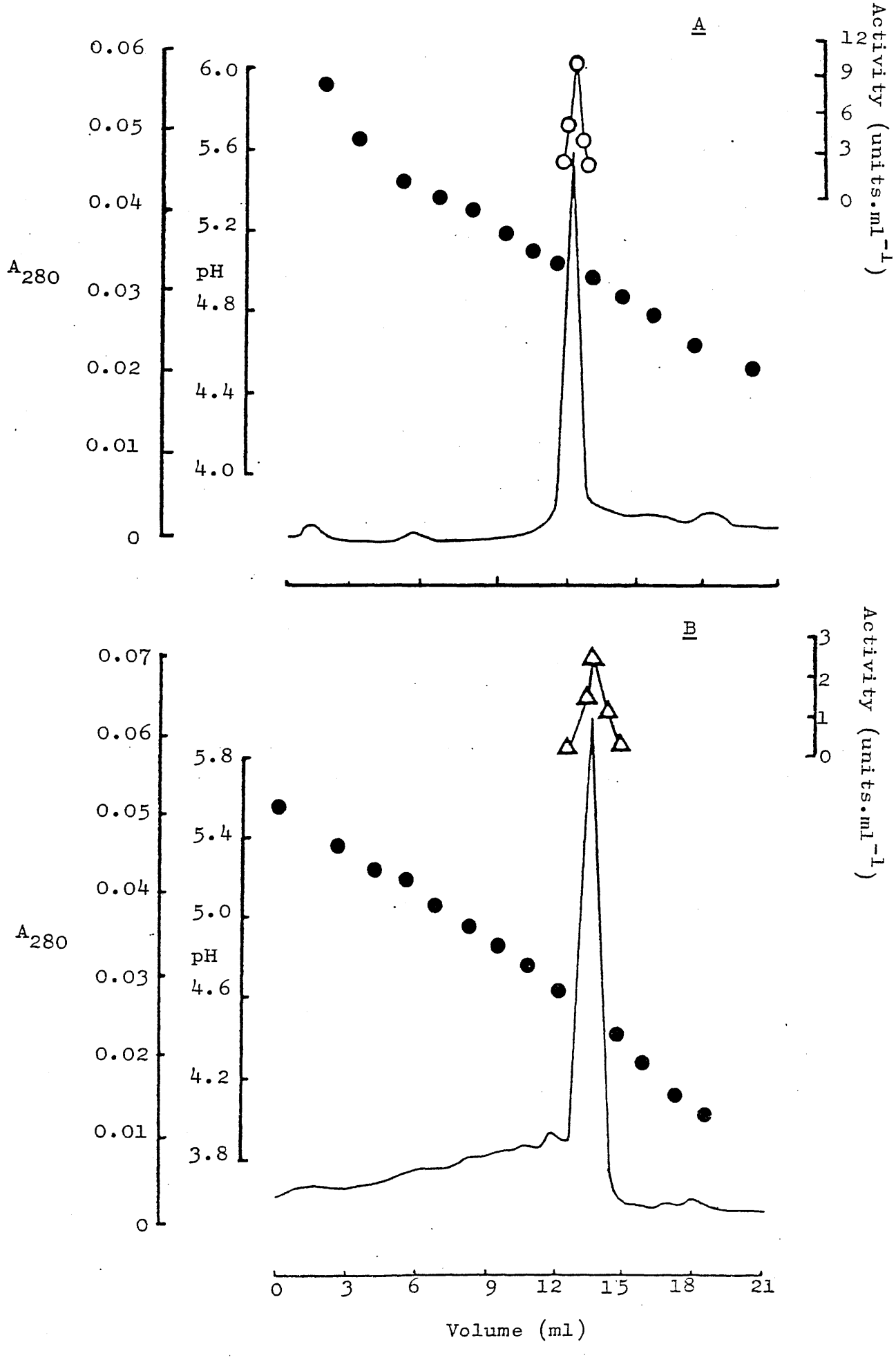

0.5
0.4
0.3
0.2
$(V_e - V_o)/V_t$
1
2
3
4
5
6
7
8
9
r = 0.992
4.4
4.8
5.2
5.6
$\log_{10} M_r$

Figure 4.3. Determination of isoelectric point.

Purified BADH and BZDH II (0.1 mg) in 20 mM piperdine/HCl buffer pH 5.7 (0.5 ml) were applied to a FPLC Mono P chromatofocussing column (20 x 0.5 cm) pre-equilibrated in the same buffer. Protein was eluted with Polybuffer PB 74 diluted 1 to 8 with water and adjusted to pH 3.6 with HCl. The flow rate was 1 $ml.min^{-1}$ and 0.75 ml fractions were collected. The fractions were assayed for enzyme activity (Methods 2.4.3). The A_{280} (——) was monitored and the pH value (●) of fractions measured (Methods 2.2.1). (A), BADH (○); (B) BZDH II (△).


A
B
A_{280}
pH
Activity (units.ml^{-1})
Volume (ml)
0.06
0.05
0.04
0.03
0.02
0.01
0
6.0
5.6
5.2
4.8
4.4
4.0
12
9
6
3
0
0.07
5.8
5.4
5.0
4.6
4.2
3.8
2
1
0
3
6
9
12
15
18
21

Both enzymes were chromatofocussed twice and the average pI value for BADH was 5.02 (5.00, 5.04) and for BZDH II 4.59 (4.52, 4.65).

4.1.3 N-terminal amino acid sequence analysis

Table 4.1 lists the N-terminal amino acid sequences of BADH and BZDH II obtained by automatic protein sequencing of the intact proteins. Residues 2 to 41 of BADH and 1 to 40 of BZDH II were determined before the build up of background made further identification impossible.

The following residues could not be assigned: for BADH, 1, 18, 20, 26, 27 and 38, and for BZDH II, 2, 9, 10, 26, 31, 34, and 38. Degradation cycle 23 of the BZDH II analysis contained four identifiable amino acids, however the recovery of three of the amino acids corresponded in total to only 22% of the recovered threonine. Residue 23 of BZDH II was thus tentatively designated as threonine. The recovery of some identifiable residues could not be determined as their elution peaks from the HPLC were too close to other background peaks to permit integration by the in-built computer program.

These preliminary sequences were further analysed using an archived data bank (NBRF) of amino acid sequences in collaboration with Drs A. Coulson and J. Collins at the Department of Molecular Biology, University of Edinburgh.

4.1.4 Absorption spectra

Figure 4.4 shows the absorption spectra of BADH and BZDH II. The absorption maxima for both the enzymes were at 214 nm and 280 nm. No other maxima were observed suggesting

Table 4.1. The N-terminal amino acid sequence of BADH and BZDH II.

Approximately 25 nmoles of BADH and 32 nmoles of BZDH II were individually applied to a Beckman 890C liquid-phase sequence and the PTH-amino acids were analysed by reversed-phase HPLC (Methods 2.7.1). The recovery values recorded refer to PTH-amino acids obtained after each degradation cycle.

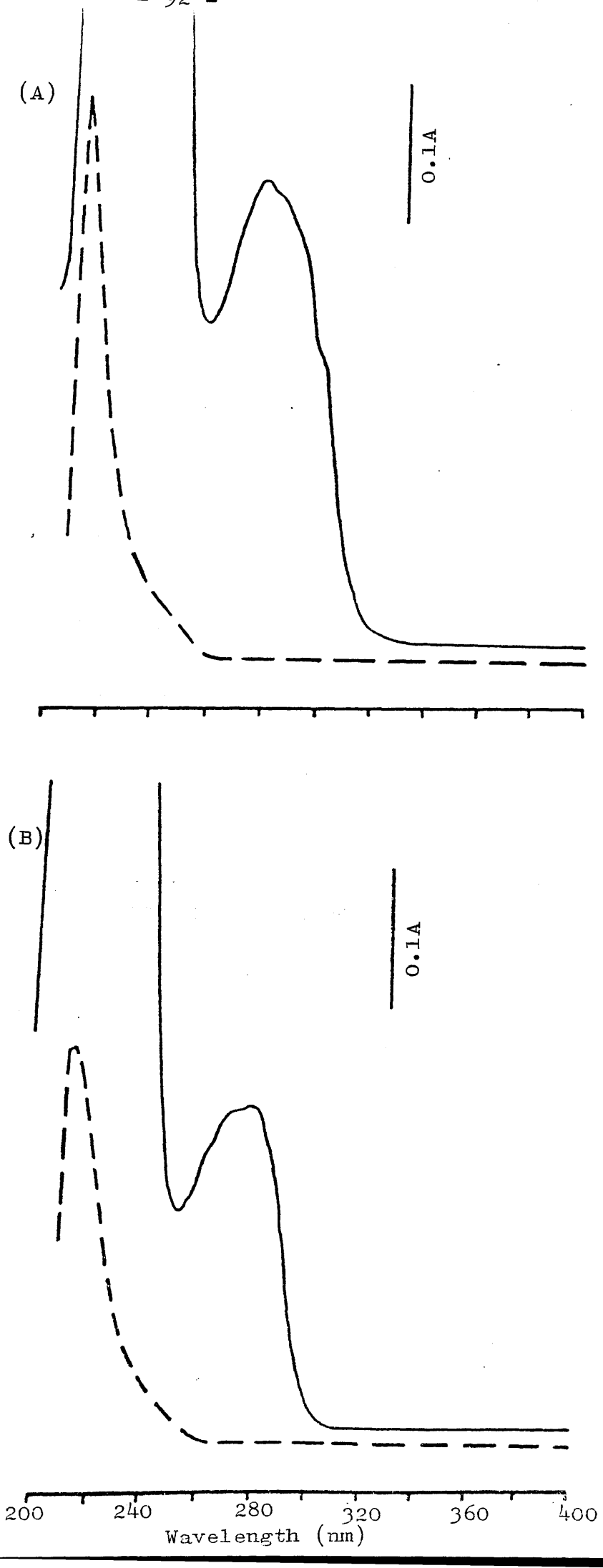
Sequence number	BADH PTH amino acid	BADH nmoles recovered	BZDH II PTH amino acid	BZDH II nmoles recovered
1	-	-	Gly + Ser	7.5 + 4.8
2	Glu	16.8	-	-
3	Leu	20.7	Phe	5.7
4	Lys	10.2	Thr	3.6
5	Asp	15.2	Lys	3.3
6	Ile	6.9	Glu	4.8
7	Ile	5.4	Leu(Ser)	-
8	Ala	14.1	Asp	16.8
9	Ala	13.5	-	-
10	Val	3.6	-	-
11	Ser	3.6	Gly	-
12	Pro	7.2	Leu	6.3
13	Cm-Cys	-	Phe	5.4
14	Lys	3.0	Asp	6.0
15	Gly	8.7	Gly	5.1
16	Ala	8.4	Ser	2.4
17	Asp	9.0	Trp	1.8
18	-	-	Gln	5.4
19	Glu	9.0	Asp	-
20	-	-	Ala	3.6
21	Gln	7.5	Gln	3.0
22	Cm-Cys	-	Asp	2.4
23	Glu	4.2	Thr	6.0
24	Lys	2.6	Tyr	2.1
25	Glu	4.5	Ser	-
26	-	-	-	-
27	Gln	4.5	Glu	1.5
28	Pro	1.8	Glu	1.8
29	Asp + Glu	4.8 + 2.7	Val	1.2
30	Gly	4.8	Ala	1.1
31	Asp	-	-	-
32	Glu	4.5	Gly	0.8
33	Val	1.4	Gly	0.9
34	Pro	1.8	-	-
35	Val	1.4	Leu	0.9
36	Lys	1.2	Gly	2.1
37	-	-	Leu	0.4
38	-	-	-	-
39	Ala	1.5	Gly	0.5
40	Thr	-	Tyr	0.6
41	Gly	1.8		

Figure 4.4. Absorption spectra of BADH and BZDH II.

A sample of each enzyme in 100 mM potassium phosphate buffer pH 7.5 was placed in a 1 ml quartz cuvette and scanned from 600 nm to 200 nm in a Pye-Unicam SP 8-100 spectrophotometer. The scan speed was 1 $nm.sec^{-1}$. There was no detectable absorption from 600 nm down to 400 nm.

(A) BZDH II at 0.3 $mg.(ml\ of\ buffer)^{-1}$ (———) and 0.01 $mg.\ (ml\ of\ buffer)^{-1}$ (---)

(B) BADH at 0.3 $mg.(ml\ of\ buffer)^{-1}$ (——) and 0.01 $mg.\ (ml\ of\ buffer)^{-1}$ (---).


(A)
0.1A
(B)
0.1A
200
240
280
320
360
400
Wavelength (nm)

that neither enzyme contained a bound cofactor or prosthetic group such as cytochrome, flavin, or PQQ.

4.2 Preliminary kinetic studies

4.2.1 Development of assay procedure for BADH

Purified BADH exhibited a non-linear increase (concave down) of A_{340} during the oxidation of benzyl alcohol to benzaldehyde with NAD^+, using the assay procedure developed by Beggs *et al.* (1976) which contained sodium pyrophosphate to buffer at pH 9.0 (Methods 2.4.2). This assay was used during the preliminary studies on the BADH activity in crude extracts and during the development of the purification procedure, and gave linear progress curves when assaying unpurified BADH.

The non-linearity observed with purified BADH could not be abolished by lowering the enzyme concentration in the assay. However, it was found that BADH could reduce benzaldehyde back to benzyl alcohol with concomitant oxidation of NADH; this reaction was considered to be responsible for causing the non-linearity of the progress curves of the forward reaction, benzyl alcohol to benzaldehyde. When assaying BADH activity in crude or partially purified extracts benzaldehyde would not accumulate, but rather be oxidised to benzoic acid by BZDH II.

The effect of benzaldehyde reduction was eliminated by the presence of hydrazine in the assay to trap the benzaldehyde product as the hydrazone derivative. The optimum concentration

of hydrazine was 0.36 M. Figure 4.5 shows the progress curves of benzyl alcohol oxidation by BADH in the presence and absence of hydrazine. Purified BADH was therefore routinely assayed in the presence of 0.1M Bicine/0.36M hydrazine buffer pH 9.2, 2 mM NAD^+, and 0.2 mM benzyl alcohol (Methods 2.4.3(a)). Bicine buffer was chosen to replace sodium pyrophosphate as it was inert (Ches buffer was inhibitory) and buffered at the pH optima of both BADH and BZDH II.

4.2.2 Assay for the reduction of benzaldehyde by BADH (the reverse reaction)

Once it had been established that BADH catalysed the reduction of benzaldehyde, a routine assay was developed in order to measure this properly. The reaction was measured in the presence of 0.1M Bicine/NaOH pH 8.9, 0.25 mM NADH, and 0.1 mM benzaldehyde, and the decrease in A_{340} followed (Methods 2.4.3(b)). Doubling or halving the benzaldehyde concentration had no effect on the observed rate; halving the NADH concentration reduced the rate by approximately 20% (the NADH concentration could not be doubled because of its absorbance at 340 nm).

4.2.3 Reproducibility of assays

Purified BADH was assayed for benzyl alcohol oxidation and benzaldehyde reduction, and purified BZDH II was assayed for benzaldehyde oxidation. Each reaction was measured a total of ten times. The standard deviation was in all cases not more than 4% of the mean (Table 4.2).

Table 4.2. Reproducibility of enzyme assays.

Purified BADH and BZDH II were assayed as described in Methods 2.4.3(a) and (c). The assay system used is identified by the assay buffer and its pH value. Each method of assay was used ten times. The enzyme concentrations were: BADH, 9.6 ng.(ml of assay)$^{-1}$ for benzyl alcohol oxidation and 4.5 ng.(ml of assay)$^{-1}$ for benzaldehyde reduction; BZDH II 10 ng.(ml of assay)$^{-1}$ for benzaldehyde oxidation.

Enzyme and reaction	Assay buffer	Specific activities units.(mg protein)$^{-1}$
BADH		
benzyl alcohol oxidation	66.7 mM sodium pyrophosphate pH 9.0	157 $\pm$ 6.2 (10)
benzyl alcohol oxidation	100 mM Bicine/360 mM hydrazine pH 9.2	216 $\pm$ 3.3 (10)
benzaldehyde reduction	100 mM Bicine/NaOH pH 8.9	355 $\pm$ 8.6 (10)
BZDH II		
benzaldehyde oxidation	66.7 mM sodium pyrophosphate pH 9.0	53 $\pm$ 2.0 (10)
benzaldehyde oxidation	100 mM Bicine/NaOH pH 9.5	70 $\pm$ 1.6 (10)

4.2.4 Dependence of enzyme activities on protein concentration

The effect of varying the enzyme concentration in the assay mixture was examined in order to ensure a linear dependency of rate and protein concentration. The amount of enzyme used in the assays was then set at a maximum of (a) 10 ng.(ml of assay)$^{-1}$ for the oxidation reaction of BADH, (b) 5 ng.(ml of assay)$^{-1}$ for the reduction reaction of BADH, and (c) 50 ng.(ml of assay)$^{-1}$ for the oxidation reaction of BZDH II.

4.2.5 pH profiles and apparent optimal pH values

The effect of assay buffer pH on the activities of BADH and BZDH II is shown in Figure 4.6. The maximum activities were estimated to be at the following pH values: benzyl alcohol oxidation by BADH, pH 9.2; benzaldehyde reduction by BADH, pH 8.9; and benzaldehyde oxidation by BZDH II, pH 9.5. These pH optima were confirmed in a repeat experiment. The presence of hydrazine in buffers had no effect on the pH optimum for benzyl alcohol oxidation, but because of its own alkalinity could only be tested at high pH. (Figure 4.6(A)). Bicine buffer at the appropriate pH value was subsequently used to assay the purified enzymes.

4.2.6 K'_{eq} and $\Delta G'_o$ determinations

BADH catalyzes both the oxidation of benzyl alcohol and the reduction of benzaldehyde. The equilibrium constant, K'_{eq}, of the reaction can be calculated from the progress curve of benzyl alcohol oxidation in the absence of hydrazine. The progress curve plateaued when the concentration of

Figure 4.5. The effect of hydrazine in the BADH assay.

Enzyme was added to the cuvette to give 9.6 $ng.ml^{-1}$ and assayed in the presence of either (a) 0.1M Bicine/ 0.36M hydrazine pH 9.2, 2 mM NAD^+, 200 µM benzyl alcohol, or (b) 0.1M Bicine/NaOH pH 9.2, 2 mM NAD^+, 200 µM benzyl alcohol, as described in Methods 2.4.3(a).

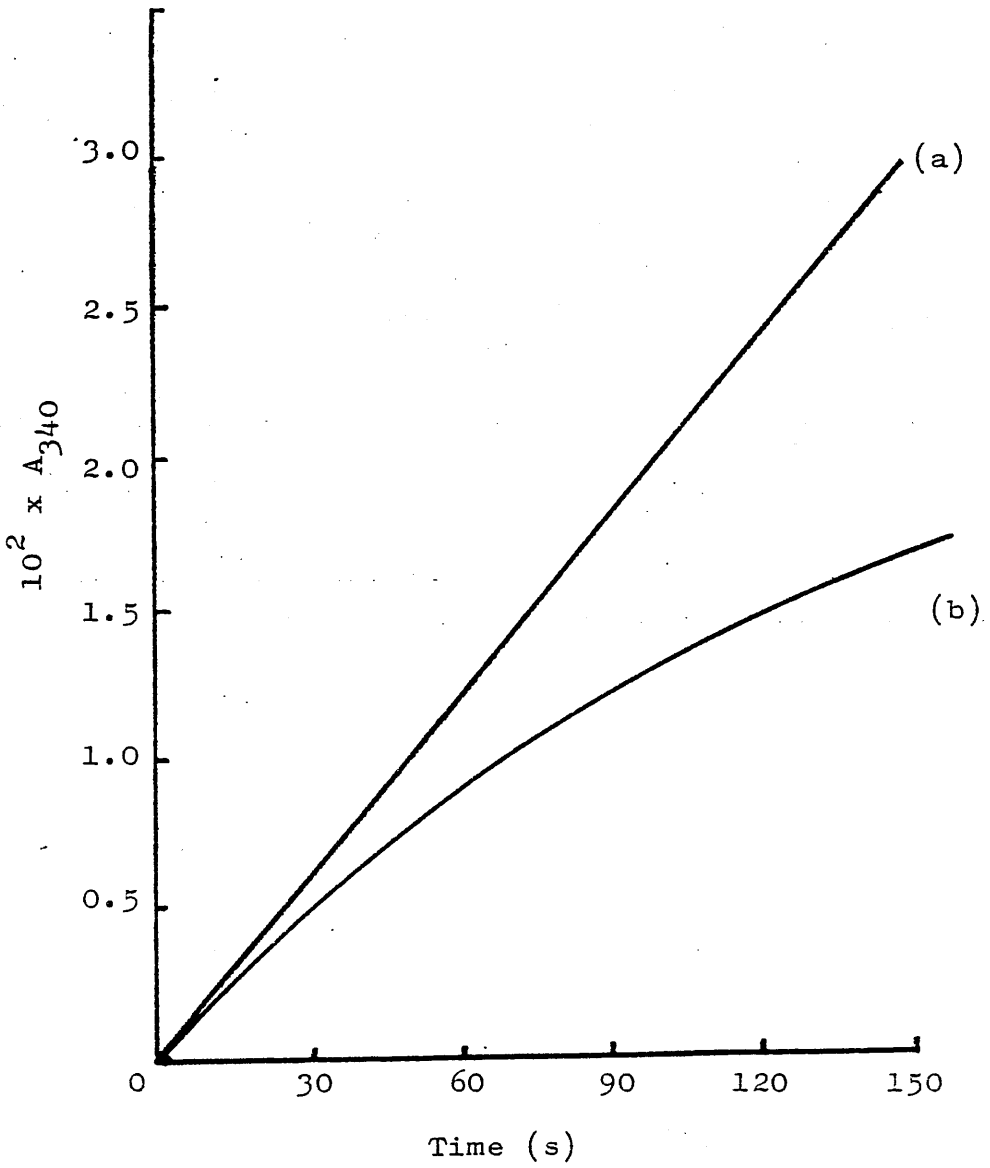
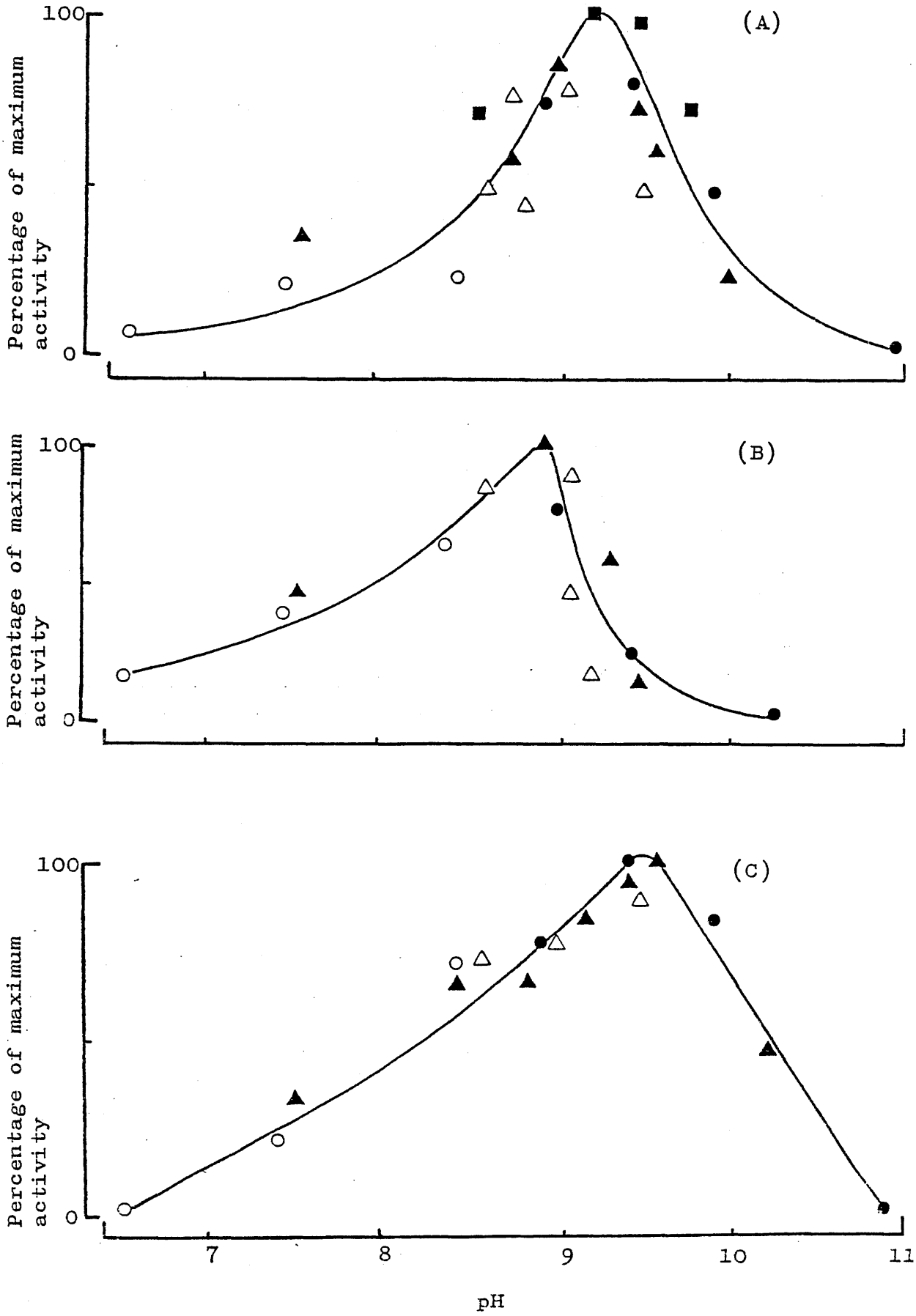

3.0
2.5
2.0
1.5
1.0
0.5
0
30
60
90
120
150
10^2 x A_{340}
Time (s)
(a)
(b)

Figure 4.6. Effect of assay buffer pH on the activities of BADH and BZDH II.

Enzyme was assayed for (A) BADH activity, benzyl alcohol oxidation (B) BADH activity, benzaldehyde reduction, and for (C) BZDH II activity, benzaldehyde oxidation, as described in Methods 2.4.3(a),(b) and (c) except that the enzymes assayed in the presence of the following buffers: 0.1M Hepes/NaOH pH 6.5 to 8.5, (○); 0.1M glycine/NaOH pH 9 to 12, (●); 0.1M phosphate buffer pH 8.5 to 10.5, (△); 0.1M Bicine/NaOH pH 7.5 to 11 (▲); and 0.1M Bicine/0.36M hydrazine buffer pH 8.5 to 10 (■). The pH values used for plotting the figures were measured in the cuvette at the end of the assay. The relative percentage activities plotted are averages of values obtained from duplicate assays which generally agreed to within 5%. The concentration of the enzymes in the assays was, (A) 10 ng.ml for BADH, (B) 5 $ng.ml^{-1}$ for BADH, and (C) 7.6 $ng.ml^{-1}$ for BZDH II.

The 100% values were for BADH 224 $units.(mg\ protein)^{-1}$ (benzyl alcohol oxidation) and 330 $units.(mg\ protein)^{-1}$ (benzaldehyde reduction); BZDH II, 83 $units.(mg\ protein)^{-1}$ (benzaldehyde oxidation).


100
0
Percentage of maximum activity
(A)
(B)
(C)
7
8
9
10
11
pH

substrates and products reached equilibrium (Figure 4.7), and assuming 1:1 stoichometry of NADH and benzaldehyde production, the K'_{eq} and $\Delta G'_{o}$ values can be calculated from the equations:

$$(a)\quad K'_{eq} = \frac{[\text{benzaldehyde}] \cdot [\text{NADH}]}{[\text{benzyl alcohol}] \cdot [\text{NAD}^+]} \times \frac{1}{\text{antilog pH}}$$

The concentrations are those at equilibrium.

$$(b)\quad \Delta G'_{o} = -RT \ln K'_{eq} \qquad R = 8.303 \text{ J.deg.}^{-1}\text{mol}^{-1}$$

$$T = 300^{o}\text{K}$$

The average K'_{eq} and $\Delta G'_{o}$ values for the oxidation of benzyl alcohol were estimated to be 3.08×10^{-11}M and $+60.3$ kJ.mol^{-1} respectively. Thus the reduction of benzaldehyde is favoured at pH 9.2. These values compare with those (K'_{eq}: 2.98×10^{-11}M, $\Delta G'_{o}$: 60.4 kJ.mol^{-1}) calculated from the redox potentials of benzyl alcohol and NAD^+ (Scharschmidt et al., 1984).

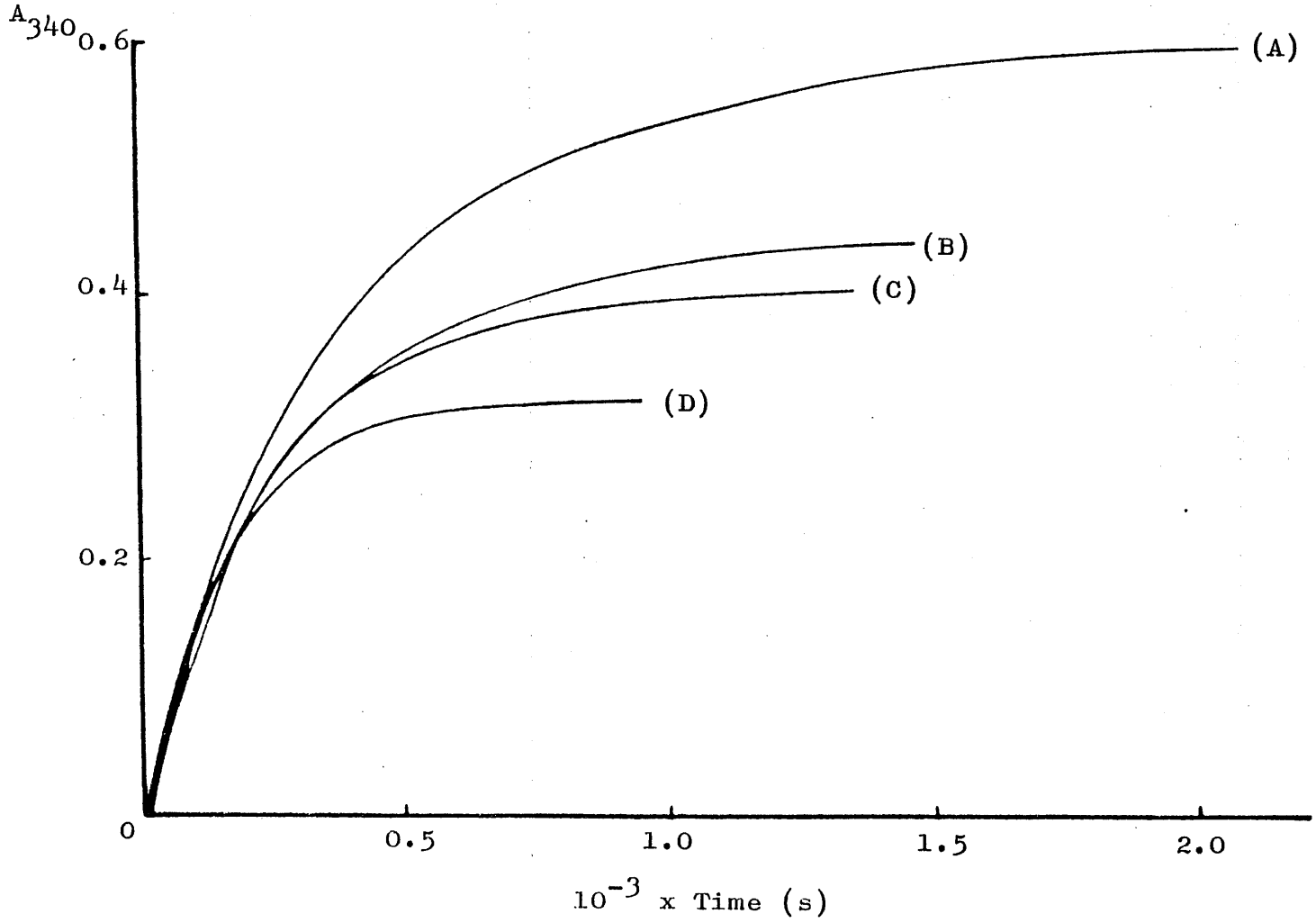
BZDH II did not reduce benzoic acid (1 mM), with NADH (0.25 mM) to benzaldehyde at a detectable rate. The oxidation of benzaldehyde appears to go to completion within 0.5% error.

4.2.7 The effect of salts on enzyme activities

BADH and BZDH II were neither activated nor inhibited when assayed (Methods 2.4.3(a) and (c)) in the presence of the following salts: 1 mM $MgCl_2$, 1 mM NaCl, 1 mM KCl, 1 mM Na_2SO_4, or 1 mM NH_4Cl.

Figure 4.7. Estimation of K'_{eq} and $\Delta G'_{o}$ values for benzyl alcohol oxidation reaction with BADH.

Assaying procedure as in Methods 2.4.3(a), except BADH assayed in presence of 0.1M Bicine/NaOH pH 9.2 and enzyme initiated reaction. Starting concentrations of benzyl alcohol and NAD^+ were, (A) 205 µM and 1.82 mM, (B) 105 µM and 1.82 mM, (C) 205 µM and 0.93 mM, and (D) 104 µM and 0.93 mM respectively. Enzyme concentration was 2.4 µg.(ml of assay)$^{-1}$). Substrate concentrations were determined as described in Methods 2.4.4.


A_{340}
0.6
0.4
0.2
0
(A)
(B)
(C)
(D)
0.5
1.0
1.5
2.0
10^{-3} x Time (s)

4.2.8 Electron acceptors

Both BADH and BZDH II were specific for NAD^+ under the assay conditions used. When NAD^+ was replaced by $NADP^+$ or the artificial electron acceptor PMS no activity (i.e. less than 0.5% of the rate with NAD^+) was observed for either enzyme. Similarly the reductase activity of BADH was specific for NADH, and NADPH was ineffective.

4.3 Steady-state kinetics

Alcohol dehydrogenases are perhaps the most extensively characterised enzymes to date, both physically and kinetically, and many of their reaction mechanisms are well studied. The properties of aldehyde dehydrogenases are known to a lesser extent. It should therefore be useful to compare the kinetic properties of BADH and BZDH II with the body of information available about other dehydrogenases. This may give clues suggestive of the chemical nature of the enzymes' mechanisms, active sites, and the effects of inhibitors, as well as possible evolutionary relationships.

The kinetic coefficients of the substrates of BADH and BZDH II were determined by the construction of primary, and where necessary secondary, plots as described by Engel (1977). Initial rates were obtained (Methods 2.4.1 and 2.4.3) and analysed as described in Methods 2.4.8(a). Three general points should be made: (a) $K'_{m.}$ and V'_{max} refer to the apparent Michaelis constant and maximum velocity obtained for one substrate at a fixed concentration of the second substrate (in these studies usually fixed cofactor concentration).

K_m and V_{max} refer to the Michaelis constant and maximum velocity for a substrate when the concentration of the other substrate has been extrapolated to infinity, by the two substrate method described by Engel (1977).

(b) Several of the alcohols and aldehydes tested as substrates of the enzymes absorbed light at 340 nm. The initial rate measurements were corrected when necessary using the appropriate absorption coefficients (Methods 2.4.8(b)).

(c) The concentration of stock solutions of substrates were standardised as described in Methods 2.4.4.

4.3.1 Kinetic coefficients of the forward and reverse reactions of BADH with benzyl alcohol and benzaldehyde

Figure 4.8 shows typical double reciprocal plots of the initial velocities of the forward reaction of BADH against benzyl alcohol concentration at different NAD^+ concentrations. The secondary plots of intercepts or slopes from the primary plots against the reciprocal of the NAD^+ concentration are also shown.

Figure 4.9 shows typical double reciprocal plots of the initial reactions of the reverse reaction of BADH against benzaldehyde or NADH concentration at a fixed concentration of the second substrate.

Table 4.3 gives the kinetic coefficients for BADH obtained from plots for benzyl alcohol, benzaldehyde, NAD^+, and NADH.

The maximum velocity of the reverse reaction was about 1.6 times that of the forward reaction, and the K'_m for benzaldehyde (4.0 μM) was over four times lower than the K_m

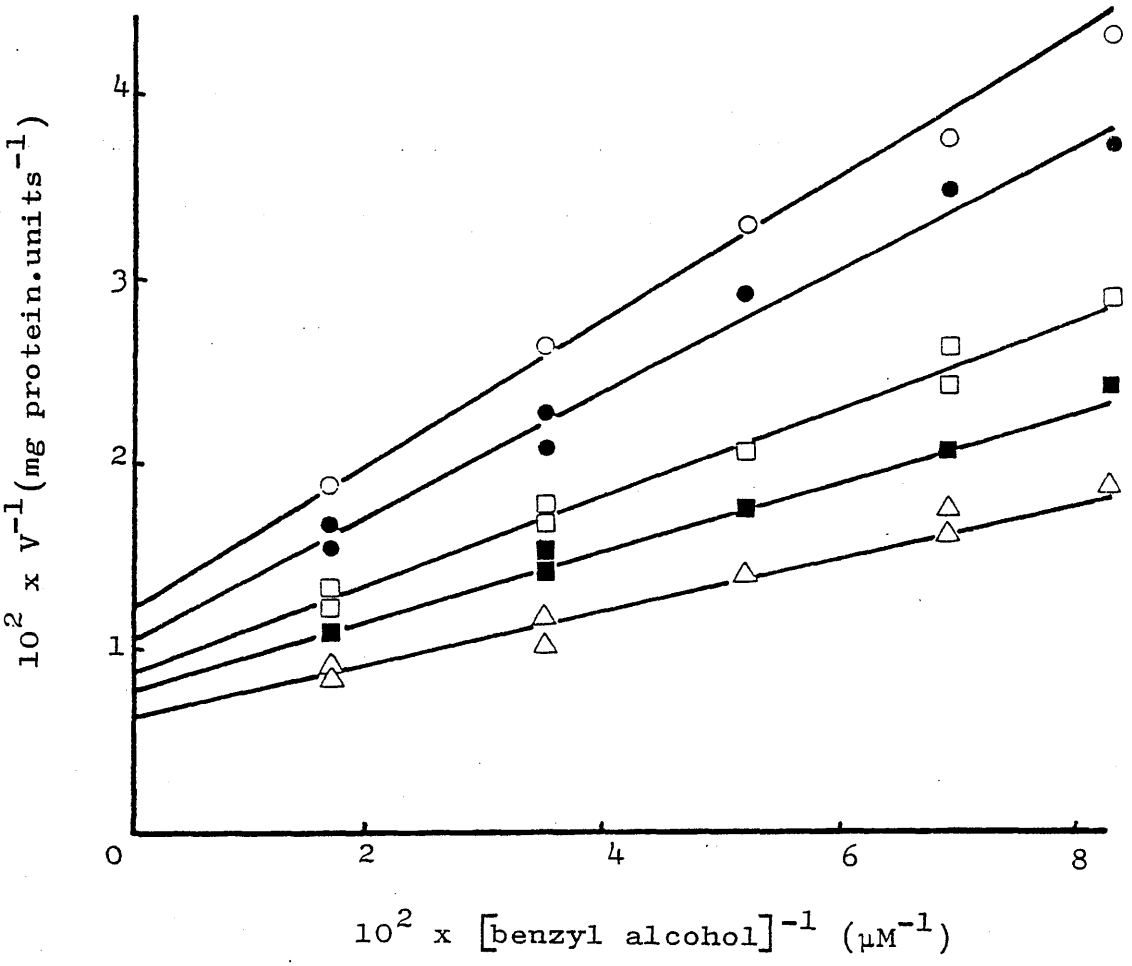
Figure 4.8. Typical plots of initial velocities of the forward reaction of BADH with benzyl alcohol and NAD^+.

(A) Double reciprocal plot of the initial velocities against benzyl alcohol concentration at a series of fixed NAD^+ concentration: 9.75 μM (○); 11.7 μM (●); 15.6 μM (□); 23.4 μM (■); 46.8 μM (△).

(B) Replot of slopes (▲), and intercepts (◇) as a function of $[NAD^+]^{-1}$.

Enzyme (9.6 ng.(ml of assay)$^{-1}$) was assayed, in the presence of hydrazine, as described in Methods 2.4.3(a), and the initial velocities analysed as described in Methods 2.4.8(a). Each determination was made in duplicate and all experimental points are shown; where a single point is given the duplicates were identical.

A


10^2 x V^{-1} (mg protein.units^{-1})
0
1
2
3
4
2
4
6
8
10^2 x [benzyl alcohol]$^{-1}$ (μM^{-1})

B

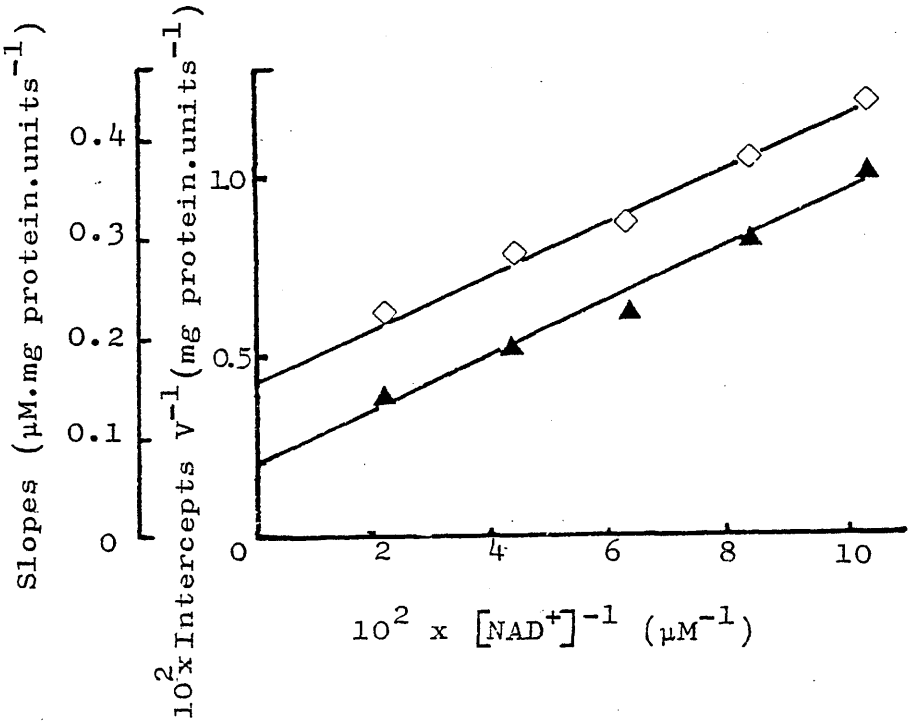
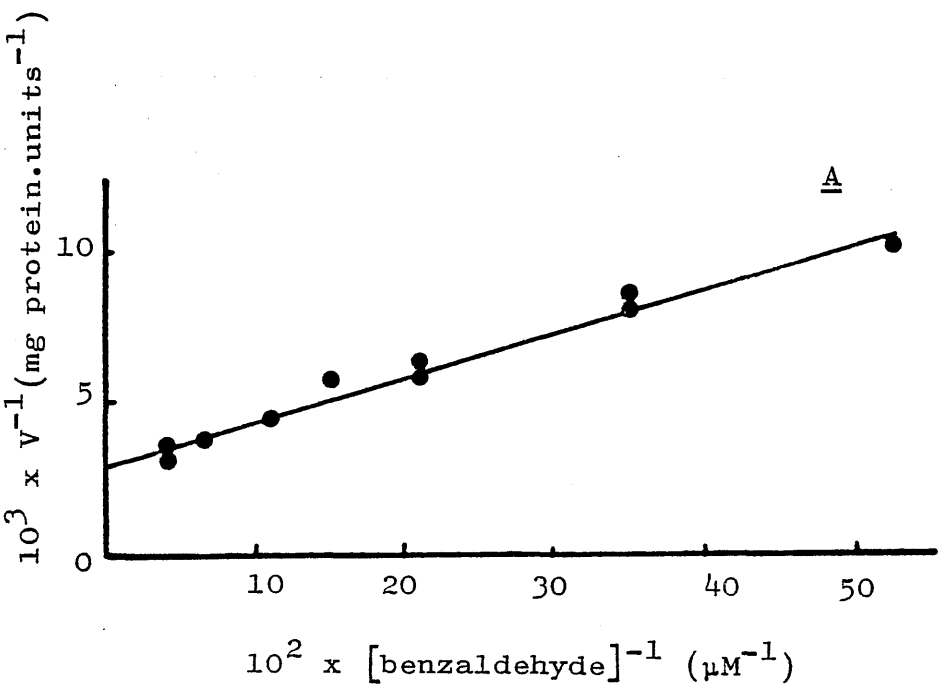

Slopes (μM.mg protein.units^{-1})
0
0.1
0.2
0.3
0.4
10^2x Intercepts V^{-1} (mg protein.units^{-1})
0
0.5
1.0
2
4
6
8
10
10^2 x [NAD^+]$^{-1}$ (μM^{-1})

Figure 4.9. Typical plots of initial velocities of the reverse reaction of BADH with benzaldehyde and NADH.

Double reciprocal plots of the initial velocities against, (A) benzaldehyde concentration at fixed 0.25 mM NADH, and (B) NADH concentration at fixed 0.1 mM benzaldehyde. Enzyme (4.5 ng.(ml of assay)$^{-1}$) was assayed as described in Methods 2.4.3(b), and the initial velocities analysed as described in Methods 2.4.8(a). All determinations were made in duplicate and all experimental points are shown; where a single point is given the duplicates were identical.


A
$10^3 \times V^{-1}$ (mg protein.units^{-1})
0
5
10
10
20
30
40
50
$10^2 \times$ [benzaldehyde]$^{-1}$ (μM^{-1})

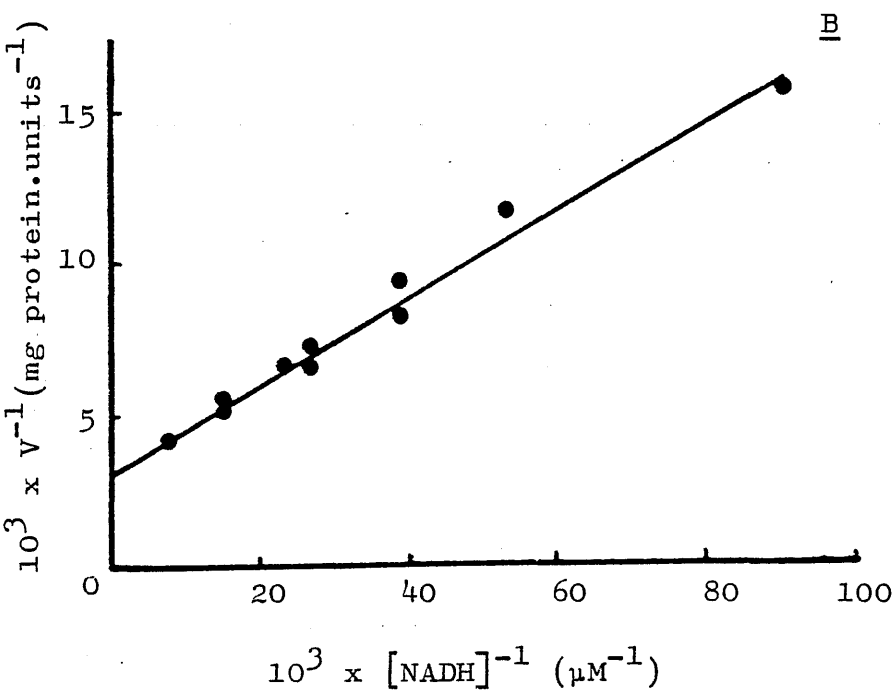

B
$10^3 \times V^{-1}$ (mg protein.units^{-1})
0
5
10
15
20
40
60
80
100
$10^3 \times$ [NADH]$^{-1}$ (μM^{-1})

Table 4.3. Kinetic coefficients of BADH with benzyl alcohol, benzaldehyde, NAD and NADH.

Reaction	Kinetic parameter	Estimated values
benzyl alcohol + NAD^+ ↓ benzaldehyde + NADH + H^+	Km (benzyl alcohol) (μM)	17.3 ± 0.65 (3)[1]
		17.5 ± 0.75 (3)[2]
	Km (NAD) (μM)	15.2 ± 2.2 (3)[1]
		19.3 ± 0.61 (3)[2]
	V_{max}(units.(mg protein)$^{-1}$)	231 ± 8.7 (3)[1]
		192 ± 21.4 (3)[2]
benzaldehyde + NADH + H^+ ↓ benzyl alcohol + NAD^+	K'_m(benzaldehyde) (μM)	4.0 (3.1, 4.9)
	K'_m(NADH) (μM)	39.8 (33.3, 46.3)
	V'_{max}(units.(mg protein)$^{-1}$)	366 ± 40 (4)

For typical plots see Figures 4.7 and 4.8. Assay and analysis procedures as described in Methods 2.4.3(a) and (b), and 2.4.8(a). Number of individual experiments are given in parentheses, except where only two experiments were done in which case the individual values are given in parentheses.

[1] assayed in the presence of hydrazine.

[2] assayed in the absence of hydrazine.

for NADH (39.8 μM) was approximately twice that of the K_m for NAD^+ (15.2 μM, hydrazine assay). The presence of hydrazine in the assays appeared to have little effect on the kinetic coefficients obtained for benzyl alcohol and NAD^+.

4.3.2 Kinetic coefficients of BZDH II with benzaldehyde

Figure 4.10 shows typical double reciprocal plots of the initial velocities of BZDH II against benzaldehyde or NAD^+ concentration at a fixed concentration of the second substrate. The K'_m obtained for benzaldehyde was 0.68 μM (0.57, 0.80) and for NAD^+, 250 μM (221, 284). The V'_{max} of the reaction was 76 ± 16 (4) units.(mg protein)$^{-1}$.

4.3.3 Substrate inhibition of BZDH II by benzaldehyde

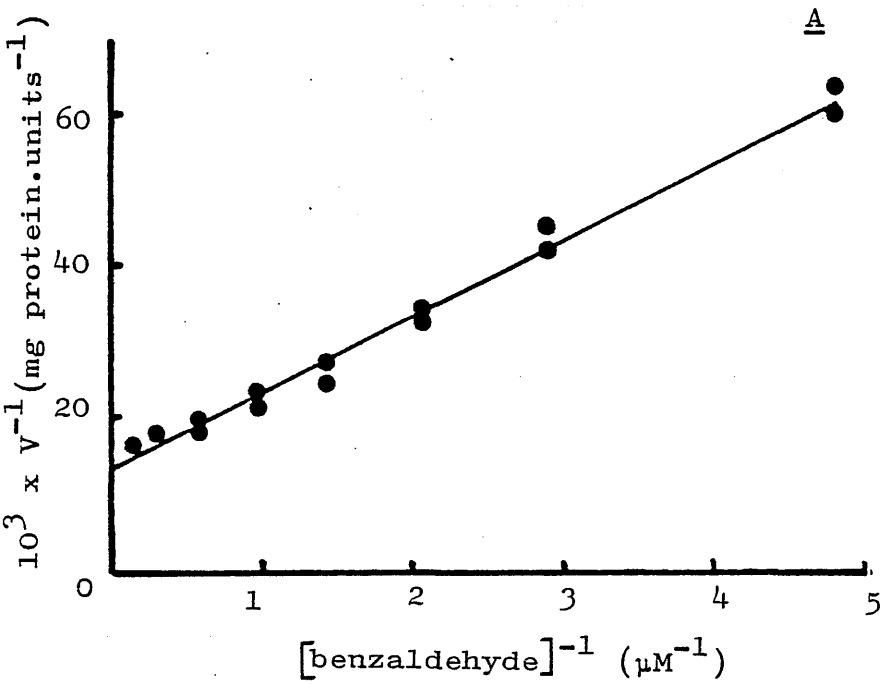
The effect of high concentrations of benzaldehyde on the initial velocities observed with BZDH II is shown in Figure 4.11. As the benzaldehyde concentration is increased above 10 μM there is a progressive inhibition of the enzyme activity at either 2 mM or 0.5 mM NAD^+ until approximately 50 μM benzaldehyde.

4.3.4 The substrate specificities of BADH and BZDH II

An extensive variety of aliphatic and aromatic alcohols and aldehydes was tested as potential substrates for BADH and BZDH II. Table 4.4 lists these compounds which were not oxidised at above 0.5% of the rate observed with benzyl alcohol or benzaldehyde in the presence of NAD^+. Table 4.5 gives the percentage activity relative to benzyl alcohol or benzaldehyde, of those alcohols and aldehydes which were substrates of the enzymes. BADH is generally specific for aromatic

Figure 4.10. Typical plots of initial velocities of the reaction of BZDH II with benzaldehyde and NAD^+.

Double reciprocal plots of the initial velocities against, (A) benzaldehyde concentration at a fixed 2 mM NAD^+, and (B) NAD^+ concentration at a fixed 0.01 mM benzaldehyde. Enzyme (10 ng.(ml of assay)$^{-1}$) was assayed as described in Methods 2.4.3(c), and the initial velocities were analysed as described in Methods 2.4.8(a). All determinations were made at least in duplicate and all experimental points are shown; where a single point is given replicates were identical. Up to four initial rate values were obtained for the lower benzaldehyde concentrations in plot A.


A
10^3 x V^{-1} (mg protein.units^{-1})
0
20
40
60
1
2
3
4
5
[benzaldehyde]$^{-1}$ (μM^{-1})

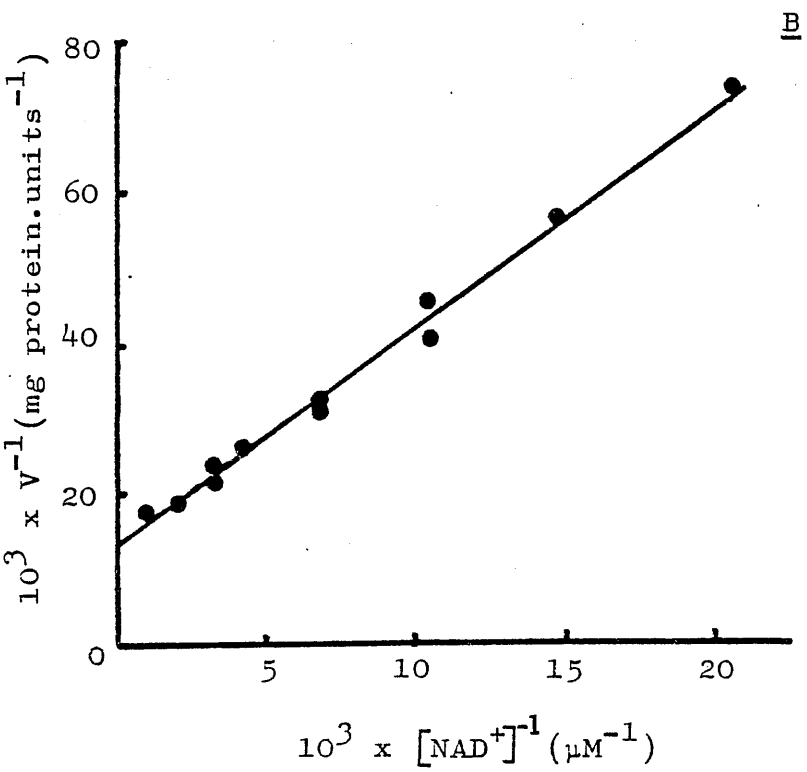
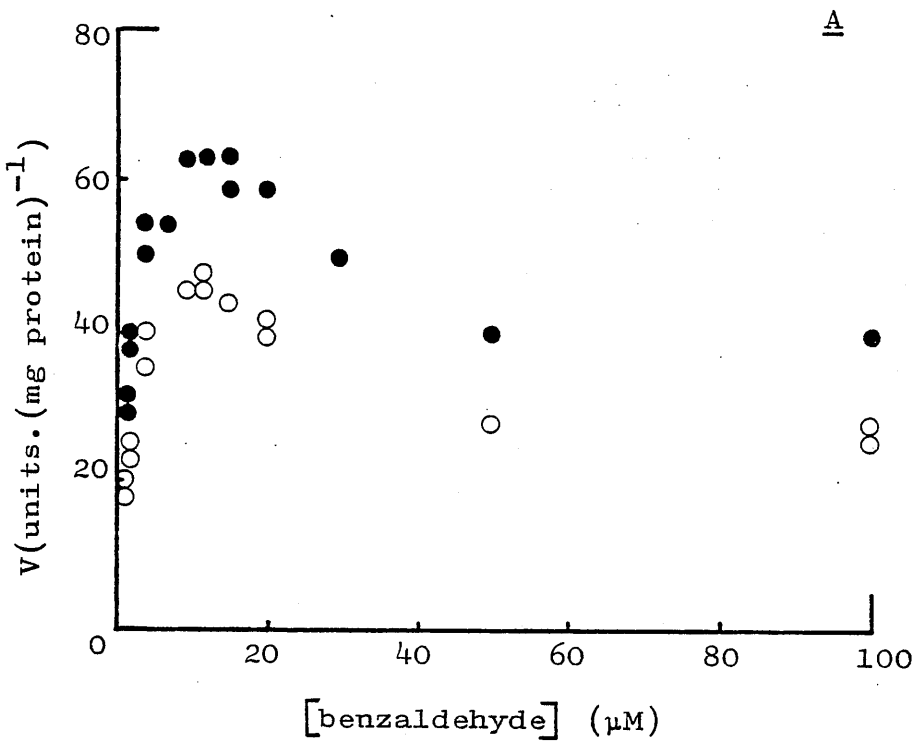

B
10^3 x V^{-1} (mg protein.units^{-1})
0
20
40
60
80
5
10
15
20
10^3 x [NAD^+]$^{-1}$ (μM^{-1})

Figure 4.11. Substrate inhibition of BZDH II by benzaldehyde.

(A) Plot of initial velocities against benzaldehyde concentration at fixed NAD^+ concentrations (a) 2 mM (●) and (b) 0.5 mM (○). Enzyme concentration was 10.1 ng. $(ml\ of\ assay)^{-1}$ was assayed as described in Methods 2.4.3(c).

(B) Double reciprocal plots of the initial velocities against benzaldehyde concentration at fixed NAD^+ concentrations 2 mM (●) and (b) 0.5 mM (○). The enzyme concentration was 10.1 ng. $(ml\ of\ assay)^{-1}$.

All determinations were made in duplicate and all experimental points are shown; where a single point is given the duplicates were identical.


A
80
60
40
20
0
V(units.(mg protein)$^{-1}$)
20
40
60
80
100
[benzaldehyde] (μM)

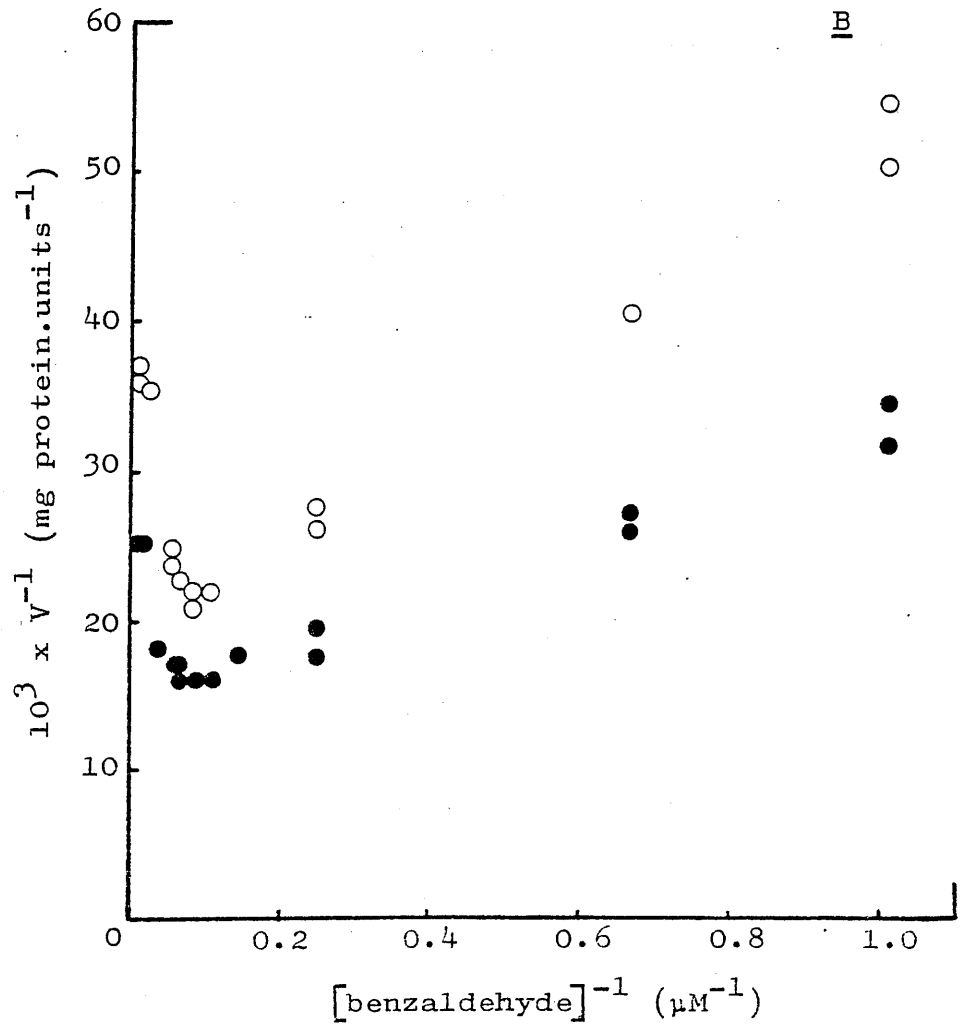

B
60
50
40
30
20
10
0
10^3 x V^{-1} (mg protein.units^{-1})
0.2
0.4
0.6
0.8
1.0
[benzaldehyde]$^{-1}$ (μM^{-1})

Table 4.4. Alcohols and aldehydes not oxidised by BADH or BZDH II.

(A) BADH (9.6 ng.(ml of assay)$^{-1}$) was assayed as described in Methods 2.4.3(a), with 0.2 mM alcohol and fixed 2 mM NAD^+.

(B) BZDH II (40.5 ng.(ml of assay)$^{-1}$) was assayed as described in Methods 2.4.3(c), with 0.1 mM and 0.01 mM aldehyde and fixed 2 mM NAD^+.

(A) Alcohols which were not oxidised by BADH

Aromatic alcohols:

2-Bromobenzyl alcohol, DL-1-phenylethanol, 2-phenylethanol.

Other alcohols:

Allyl alcohol, butan-1-ol, butan-2-ol, cis-1,2-cyclohexanediol, trans-1,2-cyclohexanediol, cyclohexanol, decan-1-ol, ethanol, heptan-1-ol, hexan-1-ol, hexahydrobenzyl alcohol, inositol, mannitol, methanol, octan-1-ol, pentan-2-ol, propan-1-ol, propan-2-ol, 3-pyridinemethanol (XVII), 4-pyridinemethanol (XX), sorbitol.

(B) Aldehydes which were not oxidised by BZDH II

Aromatic aldehydes:

2-Bromobenzaldehyde, 2-chlorobenzaldehyde, 2,6-dichlorobenzaldehyde, 2,5-dihydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 2,4-dimethoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, 2-fluorobenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, 4-isopropylbenzandehyde, 2-methoxybenzaldehyde, 2-methylbenzaldehyde, 3-methylbenzaldehyde, pentafluorobenzaldehyde, phenylacetaldehyde.

Aliphatic aldehydes:

Acetaldehyde, butyraldehyde, decylaldehyde, formaldehyde, propionaldehyde

Table 4.5. Relative activity of alcohol and aldehyde substrates with BADH and BZDH II.

BADH (9.6 ng.(ml of assay)$^{-1}$) was assayed as described in Methods 2.4.3(a), with 0.2 mM alcohol, and fixed 2 mM NAD^+. Coniferyl alcohol oxidation was assayed as described in Methods 2.4.8(b).

BZDH II (40.5 ng.(ml of assay)$^{-1}$) assayed as described in Methods 2.4.3(c), with (A) 0.1 mM and, (B) 0.01 mM aldehyde, and fixed 2 mM NAD^+. Initial velocity measurements were corrected when necessary for substrate and/or product absorbance at 340 nm. Correction factors were obtained as described in Methods 2.4.8(b). The values shown are averages calculated from duplicate assays which generally agreed within 5%. The 100% activity of BADH was 225 units.(mg protein)$^{-1}$ and 69 units.(mg protein)$^{-1}$ for BZDH II.

Substrates	BADH: Activity relative to the rate with benzyl alcohol (%)
Benzyl alcohol (III)	100
Cinnamyl alcohol (VI)	64
Coniferyl alcohol (VIII)	28
3,4-Dimethoxybenzyl alcohol	13
2-Furanmethanol (XI)	25
2-Hydroxybenzyl alcohol	40
3-Hydroxybenzyl alcohol	26
4-Hydroxybenzyl alcohol	67
4-Hydroxy-3-methoxybenzyl alcohol	39
4-Isopropylbenzyl alcohol	80
2-Methoxybenzyl alcohol	14
3-Methoxybenzyl alcohol	67
4-Methoxybenzyl alcohol	103
Pentafluorobenzyl alcohol	22
Perillyl alcohol (XIV)	80
2-Thiophenemethanol (XXIII)	50

	BZDH II: Activity relative to the rate with benzaldehyde at 10µM (%)	
	A	B
Benzaldehyde (I)	ND	100
Cinnamaldehyde (IV)	42	29
3-Fluorobenzaldehyde	31	0
4-Fluorobenzaldehyde	85	65
2-Furancarboxaldehyde (IX)	40	15
Hexan-1-al	40	12
2-Hydroxybenzaldehyde	25	0
3-Hydroxybenzaldehyde	42	25
4-Hydroxybenzaldehyde	ND	44
3-Methoxybenzaldehyde	51	42
4-Methoxybenzaldehyde	57	48
Octan-1-al	21	0
Perillaldehyde (XII)	12	0
3-Pyridinecarboxaldehyde (XV)	90	48
4-Pyridinecarboxaldehyde (XVIII)	109	54
2-Thiophenecarboxaldehyde (XXI)	73	42

ND = not determined

alcohols, whereas BZDH II has a preference for aromatic aldehydes, although it will oxidise two of the aliphatic aldehydes tested.

4.3.5 Kinetic coefficients of selected substrates of BADH and BZDH II

Table 4.6 records the kinetic coefficients of a range of alcohols and aldehydes oxidised by BADH and BZDH II. Substrate concentrations of 0.01 mM to 0.1 mM for BADH, and 1 μM to 0.2 mM for BZDH II, were used, in most cases, to give initial rates which were analysed (Methods 2.4.8(a)) to give the coefficients. The turnover number, k_{cat}, was determined from the V'_{max} values and this allowed the calculation of the so-call "specificity constant", k_{cat}/K'_m. This is an important kinetic constant in determining the specificity of competing substrates, since it combines both the rate of conversion of substrate and product with the binding of substrate to enzyme. A high k_{cat}/K'_m value suggests that there is a high degree of complementarity between the enzyme and the transition-state analogue of the particular substrate (Fersht, 1984).

Benzyl alcohol and benzaldehyde have the highest specificity constants with BADH and BZDH II respectively, and the acceptability of many of the other substrates appears to depend on the position and size of the substituent groups on the aromatic ring (See Discussion, Section 4.5).

Table 4.6. Kinetic coefficients of selected substrates of BADH and BZDH II.

(A) BADH (9.6 ng.(ml of assay)$^{-1}$ and (B) BZDH II (40 ng.(ml of assay)$^{-1}$ or 10 ng. ml^{-1} with benzaldehyde) were assayed as described in Methods 2.4.3(a) and (c), and the initial velocity measurements, corrected where necessary (Methods 2.4.8(b)), were analysed as described in Methods 2.4.8(a). The NAD^+ concentration was fixed at 2 mM, and the substrate concentrations ranged in most cases from 10 to 100 μM for the alcohols, and 1 to 200 μM for the aldehydes. All assays were done in duplicate. The k_{cat} was calculated from the equation:

$$k_{cat} = \frac{V'_{max}}{E_t} = \frac{\text{Specific activity}}{\text{concentration of active sites}}$$

The concentration of active sites (assuming one per subunit) was calculated from the subunit M_r values of 39732 for BADH, and 55002 for BZDH II (Section 4.1.1).

(A) BADH:

Substrate	K'_m (μM)	V'_{max} (units.mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K'_m ($s^{-1}.\mu M^{-1}$)
Benzyl alcohol (III)	26.5	196	131	4.94
Perillyl alcohol (XIV)	18.3	124	83	4.54
4-Methoxybenzyl alcohol	56.9	174	116	2.04
4-Hydroxybenzyl alcohol	45.5	120	80	1.76
Cinnamyl alcohol (VI)	93	212	141	1.52
4-isopropylbenzyl alcohol	111	218	144	1.30
4-Hydroxy-3-methoxybenzyl alcohol	74.5	141	94	1.26
Coniferyl alcohol (VIII)	44.3	72	48	1.08
3,4-Dimethoxybenzyl alcohol	52.6	84	56	1.06
3-Methylbenzyl alcohol	145	230	156	1.06
2-Hydroxybenzyl alcohol	90	140	93	0.66
2-Thiophenemethanol (XXIII)	111	98	65	0.59
2-Methoxybenzyl alcohol	111	76	51	0.46
2-Furanmethanol (XI)	265	105	70	0.26

(B) BZDH II:

Substrate	K'_m (μM)	V'_{max} (units.mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K'_m ($s^{-1}.\mu M^{-1}$)
Benzaldehyde (I)	0.57	78.4	72	126
4-Fluorobenzaldehyde	2.8	69.8	64	23.1
3-Fluorobenzaldehyde	3.9	44.3	41	10.3
2-Thiophenecarboxaldehyde (XXI)	5.1	56.5	52	10.2
3-Methoxybenzaldehyde	6.5	35.9	33	5.07
4-Pyridinecarboxaldehyde (XVIII)	19.9	71.6	66	3.30
4-Methoxybenzaldehyde	15.8	53.0	49	3.08
3-Pyridinecarboxaldehyde (XV)	26.1	74.8	68	2.62
Cinnamaldehyde (IV)	14.5	28.8	26	1.82
4-Hydroxybenzaldehyde	18.4	22.2	20	1.10
2-Furancarboxaldehyde (IX)	58.6	50.3	46	0.79
3-Hydroxybenzaldehyde	62.5	31.5	29	0.46
Hexan-1-al	72	31.1	29	0.39
2-Hydroxybenzaldehyde	187	44.5	41	0.22
Perillaldehyde (XII)	152	18.8	17	0.11
Octan-1-al	460	28.7	26	0.06

4.3.6 Esterase activity

Aldehyde dehydrogenases from several sources have esterase activity with 4-nitrophenyl acetate as substrate (e.g. Feldman & Weiner, 1972b). The esterase activities of BADH, BZDH II, and of yeast aldehyde dehydrogenase as a reference are recorded in Table 4.7.

BADH showed no detectable esterase activity but BZDH II was active with 4-nitrophenyl acetate. The percentage esterase activity of BZDH II is low compared with yeast aldehyde dehydrogenase, but this is a result of the high dehydrogenase activity of BZDH II. The esterase activity could not be measured above pH 9, because of the high blank rates of non-enzymic hydrolysis.

4.4 The inhibition of enzyme activity

4.4.1 The effect of sulphydryl reagents on the activities of BADH and BZDH II

Preliminary experiments showed that DTT, at concentrations of up to 500 μM did not appear to protect BADH or BZDH II against inactivation by 5 μM 4-chloromercuribenzoate or 100 μM N-ethylmaleimide. Therefore, the amount of DTT present in the incubation mixture after dilution of the stored enzyme (4 μM DTT for the BADH incubation and 0.8 μM DTT for the BZDH II incubation) would not significantly interfere with any possible inactivation in the following experiments.

Figures 4.12 and 4.13 show the effect on the BADH and BZDH II activities, with respect to time, of incubating the

Table 4.7. Esterase activity with 4-nitrophenyl acetate

The dehydrogenase activity of BADH (9.1 ng.(ml of assay)$^{-1}$), BZDH II (40.5 ng.(ml of assay)$^{-1}$), and yeast aldehyde dehydrogenase (333 ng.(ml of assay)$^{-1}$) was assayed, at fixed 2 mM NAD^+ concentration, as described in Methods 2.4.3(a) and (c) and 2.4.5(b).

The esterase activity of BADH (2.1 µg (ml of assay)$^{-1}$), BZDH II (5.1 µg.(ml of assay)$^{-1}$) and yeast aldehyde dehydrogenase (16.7 µg.(ml of assay)$^{-1}$) was assayed as described in Methods 2.4.6. The values shown are averages of duplicate assays which generally agreed within 5%.

Enzyme	pH of assay	Dehydrogenase activity (units.mg^{-1})	Esterase activity (units.mg^{-1})	Esterase activity relative to dehydrogenase activity(%)
BADH	9.5	178	ND	ND
	8.4	ND	0.5% of BZDH II activity	ND
	7.4	ND	0.5% of BZDH II activity	ND
BZDH II	9.5	72	ND	ND
	8.4	53	0.65	1.2
	7.4	30	0.12	0.4
Yeast aldehyde dehydrogenase	9.5	5.7	ND	ND
	8.4	10.0	0.33	3.3
	7.4	2.9	0.16	5.5

ND = not determined.

Figure 4.12. The effect of sulphydryl reagents on the activity of BADH.

BADH was assayed as described in Methods 2.4.3(a) except that the stored enzyme was diluted 1 to 500 in 100 mM potassium phosphate buffer pH 7.5 containing the sulphydryl reagent. The diluted enzyme was stored on ice, and 20 µl samples were assayed in duplicate at 30s, 300s, and 900s after dilution. The protein concentration in the incubation mixture was 1.44 $\mu g.ml^{-1}$ and in the assay 9.6 $ng.ml^{-1}$. The values shown are averages of duplicate assays which generally agreed to within 5%. The 100% activity was 214 units.$(mg\ protein)^{-1}$.

(A) Iodoacetate: Control (○) 0.1 µM (●) 1 µM (△)

(B) Iodoacetamide: Control (○) 1 µM (△) 10 µM (▲) 100 µM (□)

(C) 4-Chloromercuribenzoate: Control (○) 1 µM (△) 10 µM (▲) 100 µM (□)

(D) N-Ethylmaleimide: Control (○) 50 µM (■) 200 µM (▽) 500 µM (▼)

Control incubations contained no sulphydryl reagent.

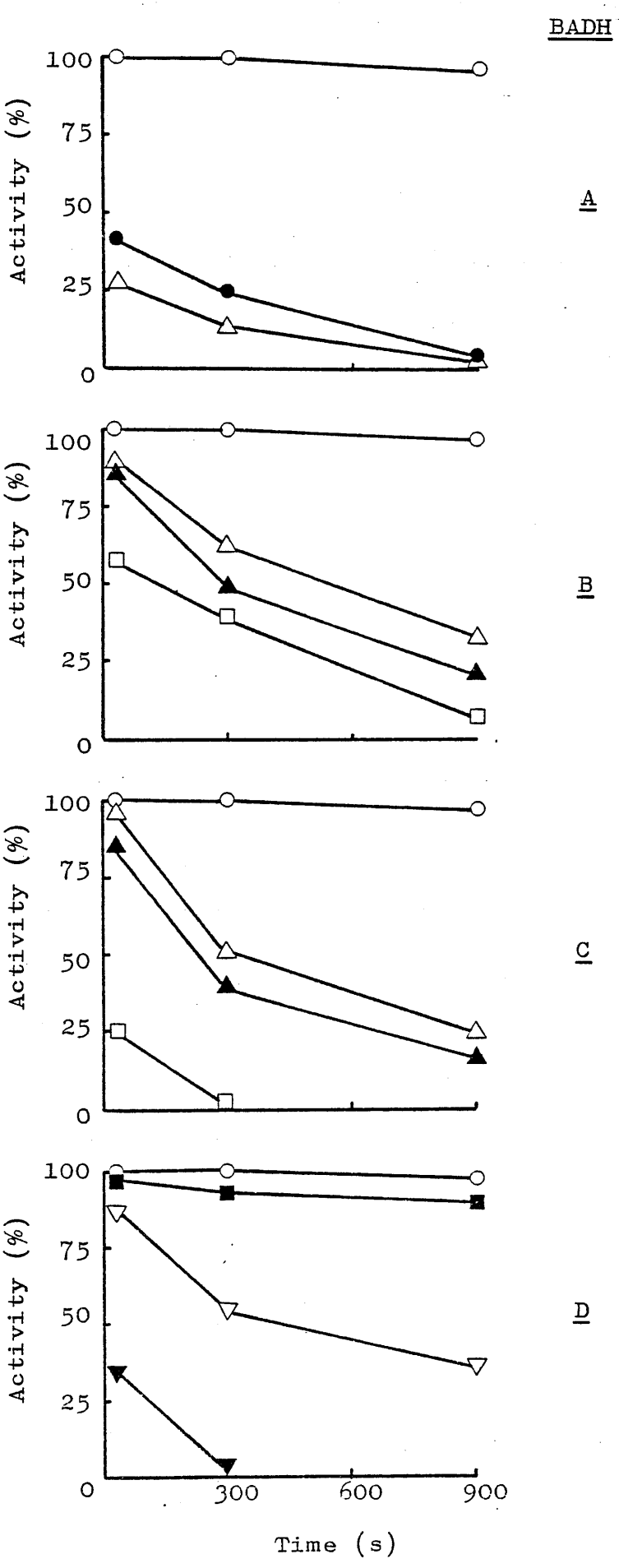

BADH
A
B
C
D
Activity (%)
100
75
50
25
0
300
600
900
Time (s)

Figure 4.13. The effect of sulphydryl reagents on the activity of BZDH II.

BZDH II was assayed as described in Methods 2.4.3(c) except that the stored enzyme was diluted 1 to 2500 in 100 mM potassium phosphate buffer pH 7.5 containing the sulphydryl reagent. The diluted enzyme was stored on ice and 20 µl samples were assayed in duplicate at 30s, 300s, 900s and 1800s after dilution. The protein concentration in the incubation mixture was 6.08 $\mu g.ml^{-1}$ and in the assay 40.5 $ng.ml^{-1}$. The values shown are averages of duplicate which generally agreed within 5%. The 100% value was 68 $units.(mg\ protein)^{-1}$.

(A) Iodoacetate: Control (○) 100 µM (□) 1 mM (◇) 10 mM (◆)

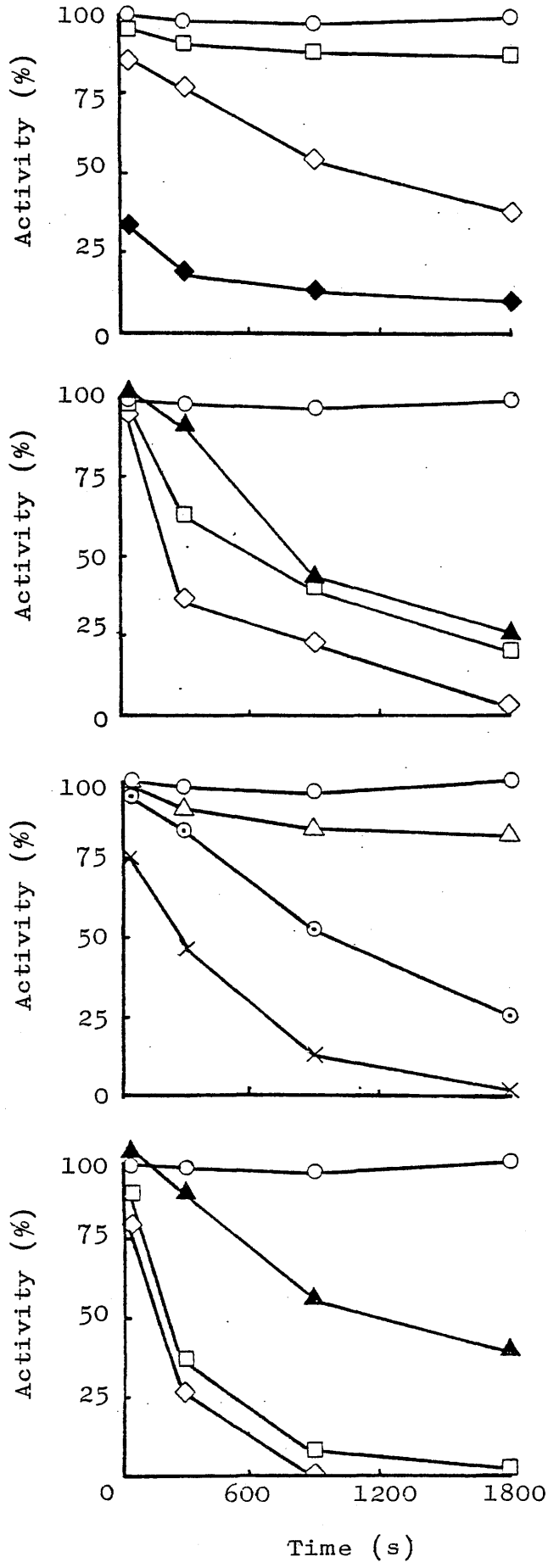
(B) Iodoacetamide: Control (○) 10 µM (▲) 100 µM (□) 1 mM (◇)

(C) 4-Chloromercuribenzoate: Control (○) 1 µM (△) 2 µM (⊙) 5 µM (X)

(D) N-Ethylmaleimide: Control (○) 10 µM (▲) 100 µM (□) 1 mM (◇)

Control incubations contained no sulphydryl reagent.

BZDH II



enzymes with the sulphydryl reagents: iodoacetate, iodoacetamide, 4-chloromercuribenzoate, N-ethylmaleimide. Table 4.8 records the estimated time required to reach 50% inhibition of the enzymes with the different concentrations of reagents. Each enzyme exhibits different sensitivities to each of the reagents. BADH is particularly sensitive to inactivation by iodoacetate (50% inhibition: < 30 sec with 0.1 μM) whereas BZDH II is most sensitive to 4-chloromercuribenzoate (50% inhibition: 266 sec with 5 μM).

4.4.2 Substrate protection against inactivation by sulphydryl reagents

If BADH or BZDH II was incubated in the presence of benzyl alcohol, benzaldehyde, or NAD^+ and 4-chloromercuribenzoate or N-ethylmaleimide, the enzymes were less inhibited by the sulphydryl reagent (Figure 4.14). The cofactor appeared less protective than the alcohol or aldehyde. These results suggest that the sulphydryl reagents interacted with a thiol group nearer the substrate binding site than the cofactor binding site.

4.4.3 The effects of metal chelators on the activities of BADH and BZDH II

Both BADH and BZDH II were assayed (Methods 2.4.3(a) and (c) in the presence of the following metal chelators: 10 mM EDTA 1 mM 2,2'-bipyridyl, 1 mM pyrazole, and 1 mM 2-phenanthroline. The assay concentrations of benzyl alcohol and benzaldehyde were 0.02 or 0.2 mM and 0.002 or 0.01 mM respectively. No inhibition

Figure 4.14. Substrate protection of BADH and BZDH II from inactivation by sulphydryl reagents.

BADH and BZDH II were diluted in 100 mM potassium phosphate buffer pH 7.5 containing sulphydryl reagents and substrates, or cofactors. Samples were assayed (Methods 2.4.3(a) and (c)) at intervals after dilution. The protein concentration of incubation mixtures were 1.2 $\mu g.ml^{-1}$ for BADH and 5.6 $\mu g.ml^{-1}$ for BZDH II, and in the assay 8.0 $ng.ml^{-1}$ for BADH and 37.3 $ng.ml^{-1}$ for BZDH II. The values shown are averages of duplicate assays which generally agreed within 5%. The 100% activities were for BADH 218 units.$(mg\ protein)^{-1}$, and for BZDH II 64 units.$(mg\ protein)^{-1}$.

BADH:

(A) Control (○), 1 μM 4-chloromercuribenzoate (△), 200 μM benzyl alcohol and 1 μM 4-chloromercuribenzoate (▲), 200 μM NAD^+ and 1 μM 4-chloromercuribenzoate (□).

(B) Control (○) 100 μM N-ethylmaleimide (▽), 200 μM benzyl alcohol and 100 μM N-ethylmaleimide (▼), 200 μM NAD^+ and 100 μM N-ethylmaleimide (■).

BZDH II:

As above except 200 μM benzyl alcohol was replaced by 10 μM benzaldehyde, and the NAD^+ concentration was 2 mM.

Control incubations contained no sulphydryl reagents, and addition of benzyl alcohol, benzaldehyde, or NAD^+ to buffers had no effect on enzyme activity.

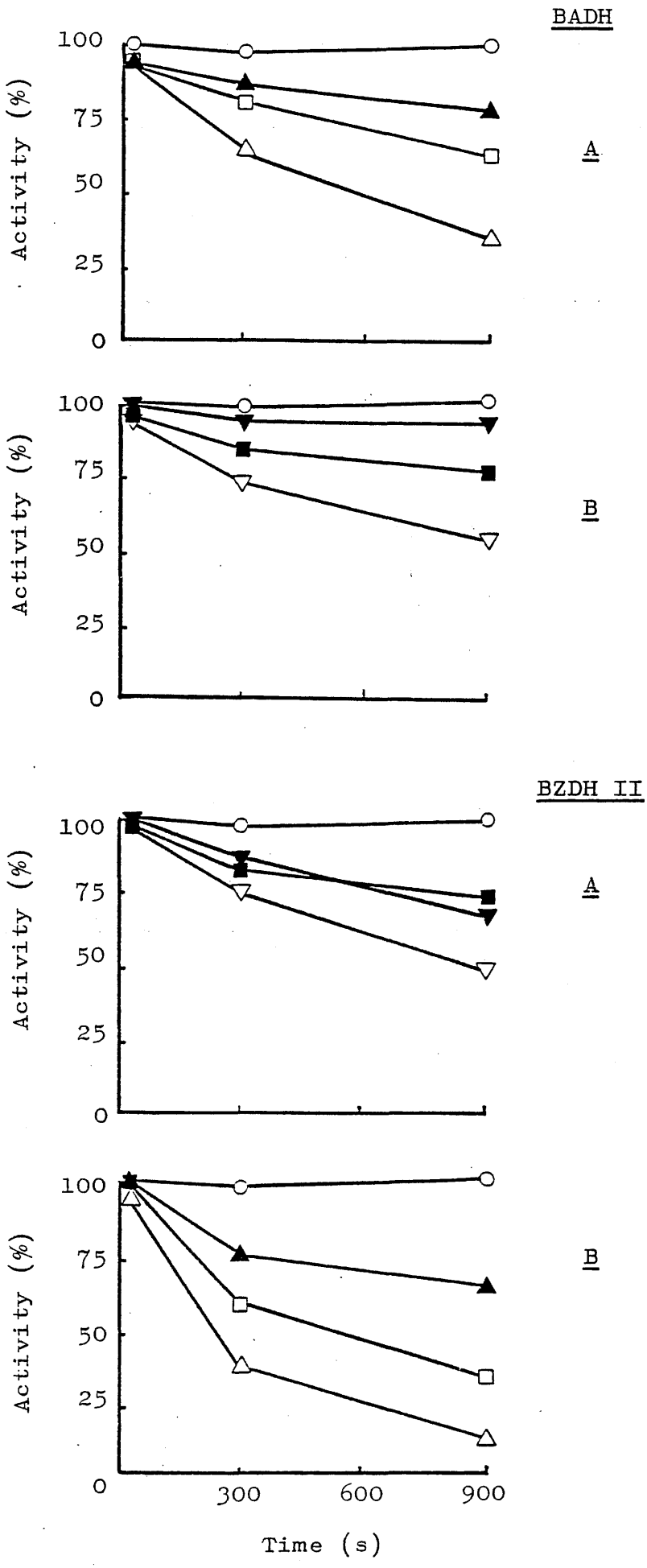

BADH
A
B
BZDH II
A
B
Activity (%)
100
75
50
25
0
300
600
900
Time (s)

Table 4.8. The time taken to reach 50% inhibition of BADH and BZDH II incubated with sulphydryl reagents.

Each enzyme was incubated with sulphydryl reagents as described in Figures 4.11 and 4.12. From the plots of percentage activity against time the 50% inhibition points were estimated for the different inhibitor concentrations.

Inhibitor	BADH		BZDH II	
	Inhibitor concentration	Time to reach 50% inhibition (s)	Inhibitor concentration	Time to reach 50% inhibition (s)
Iodoacetate	0.1 µM	30	1 mM	1170
	1 µM	30	10 mM	30
Iodoacetamide	1 µM	540	10 µM	855
	10 µM	300	100 µM	600
	100 µM	135	1 mM	210
4-Chloromercuri-benzoate	1 µM	330	2 µM	1000
	10 µM	235	5 µM	266
	100 µM	30		
N-Ethylmaleimide	200 µM	480	10 µM	1200
	500 µM	30	100 µM	215
			1 mM	160

of BADH and BZDH II was observed. However in a control experiment horse liver alcohol dehydrogenase was inhibited (by 72%) when assayed (Methods 2.4.5(a)) in the presence of 1 mM pyrazole. The activities of BADH and BZDH II in crude extracts of A. calcoaceticus were also unaffected by metal chelators.

4.4.4 Inhibition by non-substrate alcohols and aldehydes

Table 4.9 shows the effect of assaying BADH and BZDH II in the presence of certain alcohols and aldehydes which were not substrates for the enzymes. BADH was inhibited by cyclohexanol and hexahydrobenzyl alcohol, however BZDH II was inhibited by several non-substrate aldehydes; in particular 2-bromo-, 2-chloro-, and 2-fluorobenzaldehydes were potent inhibitors.

4.4.5 Possible metabolic inhibitors

The effect of selected metabolites on the activities of BADH and BZDH II was examined. The enzymes were assayed (Methods 2.4.3(a) and (c)) in the presence of 0.2 mM of the following compounds: mandelate, phenylglyoxylate, benzoate, succinate, acetyl-CoA, ATP and ADP. The assay concentrations of benzyl alcohol and benzaldehyde were 0.02 or 0.2 mM and 0.002 or 0.01 mM respectively. No inhibition of either enzyme activity was observed with any of the compounds.

Table 4.9. Inhibition by non-substrate alcohols and aldehydes.

BADH and BZDH II were assayed as described in Methods 2.4.3(a) and (c) except that certain alcohols and aldehydes were also present. The values shown are averages of duplicate assays which generally agreed within 5%. The 100% activity of BADH was 192 units.(mg protein)$^{-1}$ and for BZDH II 78 units.(mg protein)$^{-1}$. Enzyme concentration was 9.6 ng.(ml of assay)$^{-1}$ and 40.5 ng.(ml of assay)$^{-1}$ for BADH and BZDH II respectively.

BADH

Non-substrate alcohols present	Activity (%)
None	100
0.2 mM allyl alcohol	100
0.2 mM 2-bromobenzyl alcohol	100
0.2 mM cyclohexanol	80
0.2 mM hexahydrobenzyl alcohol	60
0.2 mM DL-(1)-phenylethanol	100
0.2 mM 2-phenylethanol	100
0.2 mM 3-pyridinemethanol (XVII)	100

BZDH II

Non-substrate aldehyde present	Activity (%)
None	100
0.01 mM 2-bromobenzaldehyde	18
0.1 mM 2-bromobenzaldehyde	10
0.01 mM 2-chlorobenzaldehyde	5
0.1 mM 2-chlorobenzaldehyde	0
0.1 mM cyclohexane	103
0.1 mM 2,6-dichlorobenzaldehyde	100
0.1 mM 3,4-dimethoxybenzaldehyde	90
0.01 mM 2-fluorobenzaldehyde	14
0.1 mM 2-fluorobenzaldehyde	0
0.05 mM 4-hydroxy-3-methoxybenzaldehyde	97
0.01 mM 4-isopropylbenzaldehyde	13
0.1 mM 4-isopropylbenzaldehyde	37
0.1 mM 2-methoxybenzaldehyde	100
0.1 mM 2-methoxybenzaldehyde	87
0.1 mM 3-methoxybenzaldehyde	25
0.1 mM pentafluorobenzaldehyde	56

4.5 Discussion

4.5.1 Quaternary structure

Both BADH (4 x 39 700) and BZDH II (4 x 55 000) are tetrameric enzymes as judged by a comparison of their subunit and native M_r values (Section 4.1.1). The quaternary structures of other bacterial aromatic alcohol and aldehyde dehydrogenases have not been investigated although their native M_r values have been reported to range from 27 000 to 200 000 and 160 000 to 200 000 respectively (Stachow et al., 1967; Jaeger et al., 1981; Kiyohara et al., 1981; Yamanaka & Mioshima, 1984). The fermentative alcohol dehydrogenase (Brändén et al., 1975) and the K^+-activated aldehyde dehydrogenase (Bostian & Betts, 1978a) from bakers' yeast are tetramers with native M_r values of 140 000 - 150 000 and 240 000 respectively. Horse liver alcohol dehydrogenase is a dimer of native M_r approximately 80 000 (Green and McKay, 1969). There are two isozymes of aldehyde dehydrogenase in horse liver, one cytosolic and the other mitochondrial, and both are tetrameric, with native M_r values of 230 000 for the cytosolic enzyme and 240 000 for the mitochondrial enzyme (Eckfeldt & Yonetani, 1982). The subunit M_r values of BADH and BZDH II are therefore comparable with those of the alcohol and aldehyde dehydrogenases from both horse liver and, especially, yeast. BADH falls into the class of "long" subunit alcohol dehydrogenases as designated by Jornvall et al. (1981).

4.5.2. Amino acid sequence analysis

Determination of the N-terminal amino acid sequences allows analysis at four levels: (a) comparison of the two enzymes, (b) comparison with other known and archived sequences, (c) possible identification of a specific sequence of amino acids which could result in production of a corresponding oligonucleotide sequence to probe DNA "libraries" of A. calcoaceticus and so aid possible future studies of the genes for BADH and BZDH II, and (d) comparison with amino acid sequences derived from DNA sequences which would help identify the start of the protein coding region of the genes for the enzymes.

Single N-terminal sequences were obtained from the analysis of the enzymes (Table 4.1) indicating the presence of only one subunit type in each case. At first inspection, the two sequences are quite different, with no obvious homologous regions over what corresponds to approximately 10% of each total sequence The sequences were subjected to further analysis using a computer program to compare them with an archived data base of amino acid sequences (NBRF data bank). However, no good homologies with other proteins could be identified. This data bank contains over 3000 amino acid sequences but few bacterial alcohol or aldehyde dehydrogenases sequences are listed.

The sequences were also analysed in an attempt to identify a run of amino acids whose corresponding DNA coding region would show minimal degeneracy, thus being suitable for the construction of a specific oligonucleotide "probe". Using the known codon utilization of E. coli (Lewin, 1974a) as a guide for that in A. calcoaceticus no suitable probe sequence could be

identified for BADH; however, a reasonably useful sequence was found for BZDH II:

	13	14	15	16	17	18	19
Amino acids	-Phe	Asn	Gly	Ser	Trp	Gln	Asp-
Degeneracy	2	2	4	6	1	2	2

This sequence should at least be useful in helping to narrow down the population of DNA fragments which might contain the BZDH II gene.

A preliminary comparison of the amino acid compositions of BADH and BZDH II was also made (data not shown). A procedure has been developed to determine similarity between proteins using composition data (Cornish-Bowden, 1983) but it is not recommended for comparison of two proteins such as BADH and BZDH II which differ considerably in subunit size and so it was not pursued. Once the genes for BADH and BZDH II have been cloned and sequenced, knowledge of the amino acid composition of the proteins will be useful for checking the accuracy of complete amino acid sequences deduced from DNA sequences.

4.5.3 Possible cofactors

In addition to simple $NAD(P)^+$-linked soluble dehydrogenases, several bacterial alcohol and aldehyde dehydrogenases have been found to contain bound flavin, haem, or PQQ and many of them are membrane-associated with electron transfer via the prosthetic group direct to the cytochrome system (e.g. O'Keefe & Anthony, 1980; MacKintosh & Fewson, 1987).

No indication of any bound prosthetic group of cofactor was obtained from the absorption spectra of the purified BADH and BZDH II (Figure 4.4). NAD^+ appeared to be the only electron acceptor used by these enzymes (Section 4.2.8), which both behaved as entirely soluble enzymes (Section 3.1.3). BADH and BZDH II can therefore be considered as cytoplasmic, NAD-linked dehydrogenases; electron transfer occurring from alcohol or aldehyde to the non-covalently bound cofactor in the same manner as that postulated for the horse liver and yeast alcohol and aldehyde dehydrogenases (Jakoby, 1963; Feldman & Weiner, 1972b; Branden et al., 1975; Bostian & Betts, 1978b).

4.5.4 Sulphydryl groups

The inhibition of BADH and BZDH II by sulphydryl reagents (Figure 4.12 and 4.13) is a common feature shared with all bacterial aromatic alcohol and aldehyde dehydrogenases and also with the mammalian and yeast alcohol and aldehyde dehydrogenases. The requirement for DTT in buffers to maintain activities (Section 3.1.5) is consistent with the enzymes' sensitivity to sulphydryl reagents. Protection of BADH and BZDH II against inhibition by these reagents (Figure 4.14) suggests (but does not prove) that the inhibition is active-site directed.

The reaction mechanism proposed for horse liver and yeast aldehyde dehydrogenases involves a nucleophilic attack on the aldehyde by a reactive sulphydryl group (Jakoby, 1963; Feldman & Weiner, 1972b) which is sensitive to modification by sulphydryl reagents. The sensitivity of BZDH II to sulphydryl reagents may therefore be indicative of it having a similar

reaction mechanism. This idea is strengthened by the observation that BZDH II exhibits esterase activity (Section 4.3.6) with 4-nitrophenyl acetate as substrate, as do both horse liver and yeast aldehyde dehydrogenases (e.g. Feldman & Weiner, 1972b). There has been much debate as to whether the dehydrogenase and esterase activities are at one or two active sites, and whether a common reactive thiol group is involved in their reaction mechanisms (Duncan, 1985). Recently, however, the argument has come down on the side of the single active site model with the reactions having a common thio-ester acyl intermediate step (Duncan, 1985; Loomes & Kitson, 1986).

It was initially proposed that sulphydryl inhibition of horse liver and yeast alcohol dehydrogenases was due to chemical modification of a reactive sulphydryl group at the cofactor binding site (Sund & Theorell, 1963); more recently, it has been suggested that the inhibition is caused by modification of a cysteine residue which is a ligand to the metal atom at the active site (Branden et al., 1975). BADH is particularly sensitive to inhibition by iodoacetate (Table 4.8). Horse liver alcohol dehydrogenase is also inhibited by iodoacetate (Sund & Theorell, 1963). The model proposed for the mammalian enzyme is that the iodoacetate is site-directed by the positive charge of an arginine residue at the active site (Arg-47), so that an ion-pair is set up with the negative charge of the acid. The iodo-group of the reagent is then strategically positioned to specifically interact with one of the zinc ligating cysteine residues (Cys-46) at the active site, inhibiting the enzyme (Branden et al., 1975). Several bacterial aromatic alcohol

dehydrogenases are also sensitive to inhibition by iodoacetate but not to the same extent as BADH. Thus benzyl alcohol dehydrogenase purified from Pseudomonas putida T-2 is much more sensitive to 4-chloromercuribenzoate than it is to iodoacetate (Suhara et al., 1969). BZDH II (Table 4.8) and other bacterial aromatic aldehyde dehydrogenases (Ballal et al., 1967; Kiyoharo et al., 1981) are also much less sensitive to inhibition by iodoacetate.

Both BADH and BZDH II can be inhibited by low concentrations of 4-chloromercuribenzoate (Table 4.8). Several bacterial aromatic alcohol and aldehyde dehydrogenases are especially sensitive to this sulphydryl reagent (Ballal et al., 1967, 1968; Suhara et al., 1969; Kiyohara et al., 1981). As the reagent contains a benzene ring structure its potency could perhaps also be an active-site directed phenomenon.

4.5.5 Active site metals

The activities of both BADH and BZDH II are unaffected when assayed in the presence of a range of metal chelators (Section 4.4.3). The insensitivity of BADH to inhibition by metal chelators is another property it shares with other bacterial aromatic alcohol dehydrogenases (Suhara et al., 1969; Jaeger et al., 1981). In contrast, horse liver and yeast alcohol dehydrogenases are both sensitive to chelating agents and this is known to be a result of the presence of zinc atoms at the active sites (Sund & Theorell, 1963).

The lack of inhibition of BADH could result from the absence of a metal atom at its active site; if so, this would mean that the enzyme has a different mechanism from the metal-containing

alcohol dehydrogenases. This situation would be precedented by the Class I and II aldolases. The Class I aldolases do not contain metal atoms and have a different reaction mechanism from the Class II aldolases which have an active site zinc atom (Horecker et al., 1972). Alternatively, any active site metal might be protected from chelation; the micro-environment developed for the binding of hydrophobic aromatic substrates perhaps being unsuited for the electrostatic binding of the chelators. If the inhibition of BADH by sulphydryl reagents (Figure 4.12) is a result of modification of a cysteine residue involved in ligating a metal atom, then the lack of inhibition by metal chelators is surprising.

There has been no suggestion that metal atoms are involved in the reaction mechanism of horse liver or yeast aldehyde dehydrogenases (Jakoby, 1963; Feldman & Wiener, 1972b; Bostian & Betts, 1978b) and similarly BZDH II was unaffected by chelating agents.

4.5.6 Substrate specificity

The specificities of BADH and BZDH II were estimated by determining the so-called "specificity constants" (k_{cat}/K'_m) for various substrates. It is usually considered that the higher the k_{cat}/K'_m ratio the closer the structure of the substrate transition-state intermediate is to the enzyme's active site (Fersht, 1984).

(a) BADH

BADH is in general specific for aromatic alcohols although perillyl alcohol (XIV), a cyclohex-1-ene compound, is also a

good substrate (Tables 4.5 and 4.6). The benzene ring can be replaced by a thiophene or furan ring, but not apparently by a pyridine ring (Table 4.4).

There appears to be a preference for aromatic alcohols with small substituent groups, preferably away from the reactive carbinol group i.e. at the para - rather than the ortho-position; 2-bromobenzyl alcohol was the only substituted benzyl alcohol tested which was not oxidised (Table 4.4). This suggests that the active site of the enzyme may be a cleft structure, substituents in the para- position on the aromatic ring being least sterically and electrostatically hindered for binding. The electron-withdrawing properties of the substituent groups on the aromatic ring may also be involved in dictating the acceptability of a particular substrate (Klinman, 1972).

Cinnamyl alcohol (VI) and coniferyl alcohol (VIII), which have an alkenyl group between the reactive carbinol and the aromatic ring, were oxidised by BADH (Tables 4.5 and 4.6). In the trans- configuration the carbinol group may be correctly positioned for reactivity within the active site. The alkenyl structure along with part of the benzene ring possibly mimics half an aromatic ring structure, thus allowing substrate binding. 2-Phenylethanol, which was not a substrate for BADH (Table 4.4), does not have the alkenyl group near the carbinol. The lack of a double bond along with the length difference of the $-CH_2CH_2OH$ group compared to the $-CH_2OH$ and $-CH{:}CH.CH_2OH$ groups might indicate a wrong alignment of the carbinol.

The specificity of the reductase activity of BADH was not investigated. This activity of BADH is likely to have limited physiological significance (see p. 135) although an investigation of the substrate specificity might give information regarding enzyme mechanism.

The alcohol specificity of A. calcoaceticus BADH resembles that of other aromatic alcohol dehydrogenases such as the benzyl alcohol dehydrogenase (Suhara et al., 1969) and perillyl alcohol dehydrogenases (Ballal et al., 1966) from Pseudomonas spp. and the coniferyl alcohol dehydrogenase from Rhodococcus erythropolis (Jaeger et al., 1981). Benzyl alcohol and perillyl alcohol give the highest k_{cat}/K'_m values with BADH from A. calcoaceticus (Table 4.6) and this along with other shared properties (see p.128) suggests a similarity between BADH and the benzyl alcohol dehydrogenase purified from the toluene-grown Pseudomonas putida T-2 (Suhara et al., 1969), which oxidises cyclohex-1-ene ring compounds (e.g. perillyl alcohol) as well as aromatic alcohols.

Horse liver alcohol dehydrogenase has an exceptionally broad substrate specificity and will oxidise both aliphatic and aromatic alcohols; benzyl alcohol is oxidised at approximately the same rate as ethanol (Sund & Theorell, 1963). The enzyme's multi-purpose oxidoreductase properties are probably an advantage in the liver of a large mammal. Horse liver alcohol dehydrogenase, although probably one of the most studied of all enzymes, is now considered rather unusual in its broad specificity and may not be a good model for comparison with other dehydrogenases (Jeffery, 1980).

The classical fermentative yeast alcohol dehydrogenase oxidises a much smaller number of substrates; chiefly the straight-chain alcohols. Benzyl alcohol is a very poor substrate, although cinnamyl alcohol is oxidised at almost 40% of the rate for ethanol (Sund & Theorell, 1963). It has been shown that the active site of yeast alcohol dehydrogenase contains bulkier residues than does the active site of the horse liver enzyme, and this may explain the more restricted substrate specificity of the yeast enzyme (Jornvall et al., 1978).

(b) BZDH II

BZDH II appears to have a much more restrictive active site than does BADH. Aromatic (benzene, furan, pyridine, or thiophene rings) aldehydes are preferred substrates, although hexan-1-al and octan-1-al are oxidised, albeit poorly (Tables 4.5 and 4.6). Cinnamaldehyde (IV) with its propen-1-al group and perillaldehyde (XII), which is non-planar and has a bulky isopropenyl group, were also relatively poor substrates although the former had the higher k_{cat}/K'_m ratio (Table 4.6). Coniferaldehyde was not tested as it is not commercially available. Aromatic aldehydes with more than one substituent group were not oxidised (Table 4.4). However, aromatic aldehydes with only one substituent group were substrates, and those with the highest k_{cat}/K'_m ratios were the ones with the smaller substituent groups (Table 4.6). For example, the specificity constant of benzaldehyde was by far the highest, and then mono-fluorobenzaldehydes were better substrates than mono-hydroxybenzaldehydes, which were in turn better than mono-methoxybenzaldehydes.

BZDH II is particularly sensitive to inhibition by several of the non-substrate aldehydes, especially by ortho-halogenated benzaldehydes (Table 4.9). The substitution at the ortho-position of the ring may cause the aldehyde to bind incorrectly for oxidation but with an increased affinity thus competing out the active substrates. Alternatively, the inhibitor may have a high affinity for a possible second binding site that could be involved in the observed substrate inhibition by benzaldehyde (see p. 138), resulting in inhibition of BZDH II at very low concentrations of the non-substrate aldehydes. The potency of the substituted aldehydes to inhibit BZDH II appears to be lowered as the size and number of the substituent groups on the ring increases.

The ability of BZDH II to oxidise certain aldehydes probably relates to their hydration properties. Aromatic aldehydes are essentially unhydrated in solution (e.g. Klinman, 1972), whereas aliphatic aldehydes exist to varying degrees in solution as the hydrated gem-diol form (Bodley & Blair, 1971); pyridinecarboxaldehydes (Gregory et al., 1972), thiophenecarboxaldehydes, and furancarboxaldehydes may also be hydrated to some extent in solution.

The specificity of BZDH II suggests that the active site may be a tight pocket structure rather than the cleft postulated for BADH. However, an interpretation of the ability of an enzyme to bind (and oxidise) a substrate in relation to that substrate's structure is a complex function of not only steric effects, but also hydrogen bonding, electronic, and hydrophobic effects (Klinman, 1972). For example, the aromatic ring of

the substrates of BADH and BZDH II appears to confer specificity. Binding of the ring may contribute greatly to the binding energy and therefore the energy for catalysis. The reactive carbinol or carbinyl group may not participate in substrate-enzyme recognition. Furthermore, the hydrophobicity of the aromatic ring would be very acceptable to a hydrophobic active site. The lack of oxidation of aliphatic alcohols and most aliphatic aldehydes may result from little or no energy for catalysis being obtained from any binding without a ring structure. Hexan-1-al and octan-1-al might be oxidised by BZDH II because of their hydrophobicity and appropriate chain length. With horse liver alcohol dehydrogenase, which appears to have minimal steric hinderance compared to the yeast alcohol dehydrogenase, it may be that the presence of the carbinol group is more important than other structures in substrate-enzyme interactions. This may be the cause of the low activity of the horse liver enzyme compared to the aromatic alcohol dehydrogenases; low binding energy resulting in little energy available for catalysis.

The specificity of a dehydrogenase may well influence the survival fitness of an organism, e.g. the ability of a soil microorganism to degrade aromatic alcohols and aldehydes is to its advantage and so is the ability of a mammalian enzyme to oxidise a wide variety of ingested and synthesised alcohols. Natural selection may well operate on that specificity; minor changes in an enzyme's structure could greatly alter its specificity.

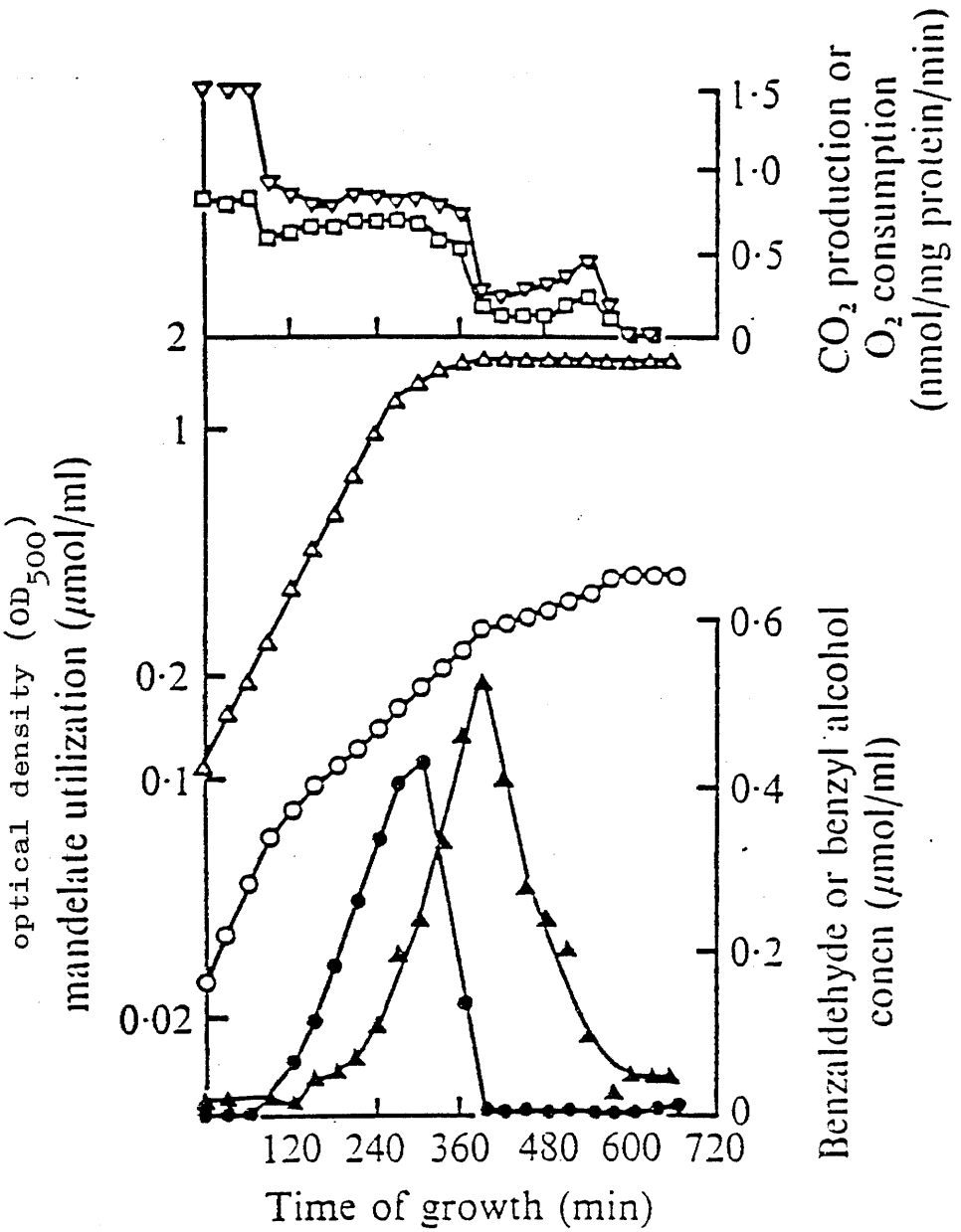
Those alcohol dehydrogenases from mammals, yeasts, and bacteria that have been studied in enough detail appear to have evolved from a single ancient origin, diverging greatly, and resemble each other more in their tertiary structure rather than in their primary structure (Jornvall, 1977). The tertiary structure may principally confer the dehydrogenase properties of catalysis and the primary structure the specificity determining which alcohols can bind and be oxidised.

4.5.7 The kinetic properties of BADH and BZDH II in relation to the metabolism of benzyl alcohol in A.calcoaceticus

When A.calcoaceticus NCIB 8250 is grown on mandelate there is a transient accumulation of benzaldehyde and then of benzyl alcohol (Figure 4.15 Cook et al., 1975). The low activity of benzaldehyde dehydrogenase I (BZDH I) induced by mandelate seems to be responsible for the accumulation of its substrate (Cook et al., 1975). As mandelate and phenylglyoxylate concentrations in the medium fall, the benzaldehyde induces BADH and BZDH II, and it is the reductase activity of BADH (Section 4.3.1), which is 4 to 5 times greater than the dehydrogenase activity of BZDH II (Table 4.3 and Section 4.3.2), that results in the production of benzyl alcohol. The BZDH II activity, along with the remaining BZDH I activity, then clear the accumulated benzaldehyde. The conversion of benzaldehyde to benzyl alcohol may be a specific mechanism preventing toxification or may simply be an accidental consequence of the equilibrium constant (K_{eq}) of the process. The reduction of aldehyde to alcohol is thermodynamically favoured even at high

Figure 4.15. Growth of A.calcoaceticus in mandelate-salts medium (from Cook et al., 1975).

The culture (21) used for inoculation was grown from a nutrient broth inoculum (9 ml) in 10 mM mandelate-salts medium for 9h at 30°C. The bacteria were harvested, resuspended in ice-cold basal medium and added to 3 mM (carboxy-^{14}C) mandelate-salts medium (800 ml, 40 μCi). The culture was grown at 30°C and rates of oxygen consumption (▽) and carbon dioxide production (□) were measured. At intervals samples were assayed for optical density OD_{500} (○) utilisation of (carboxy-^{14}C)-mandelate (△), and accumulation of benzaldehyde (●) and benzyl alcohol (▲).


optical density (OD_{500})
mandelate utilization (μmol/ml)
CO_2 production or
O_2 consumption
(nmol/mg protein/min)
Benzaldehyde or benzyl alcohol
concn (μmol/ml)
Time of growth (min)
2
1
0·2
0·1
0·02
1·5
1·0
0·5
0
0·6
0·4
0·2
0
120 240 360 480 600 720

pH, as can be seen from the K'_{eq} and $\Delta G'_{o}$ values obtained at pH 9.2 (Section 4.2.6); at intracellular pH (presumably approximately 7.0) the equilibrium of the reaction will even more favour benzyl alcohol accumulation.

Other aromatic alcohol dehydrogenases exhibit reductase activity, for example, 3-aminobenzyl alcohol dehydrogenase from Mycobacterium tuberculosis has a reductase activity which is 3 times that of the dehydrogenase activity (Sloane, 1973) and 3-hydroxybenzyl alcohol dehydrogenase from Penicillin urticae reduces aldehyde 6 times faster than it oxidises alcohol (Gaucher & Forrester, 1972). Aldehyde dehydrogenases do not exhibit reductase activity at spectrophotometrically detectable levels (Section 4.2.6) as the reduction of acid to aldehyde is thermodynamically highly unfavourable and indeed reduction of aromatic carboxylic acids to their aldehydes (e.g. in lignin biosynthesis) occurs via - CoA derivatives (Higuchi, 1980).

The V_{max} values of BADH and BZDH II and other bacterial aromatic alcohol and aldehyde dehydrogenases are approximately two orders of magnitude higher than the values for the corresponding mammalian enzymes (Feldman & Weiner, 1972a; Weiner et al., 1974). The alcohol and aldehyde dehydrogenases from yeast are also much more active than the mammalian enzymes (Wills, 1976; Bostian & Betts, 1978a). It is likely that the high activity of the bacterial enzymes allows the organisms to have high growth rates when using aromatic alcohols or aldehydes as sole carbon sources. The greater efficiency of the enzymes no doubt results in lower energy requirements for biosynthesis of the enzymes. The same arguement would apply to the yeast

enzyme, where alcohol is produced in stoicheometric amounts; in mammals, however, the reactions are likely to be quantitatively relatively much less important and so there would be minimal selection pressure on evolving maximum turnover numbers.

The K'_m of BZDH II for benzaldehyde is very low (0.68 μM) and this, along with the substrate inhibition of the enzyme by benzaldehyde above 10 μM (Figure 4.11), suggests that the enzyme has evolved to utilise low benzaldehyde concentration; the enzyme being saturated at concentrations not toxic to the enzyme or the cell. Several other prokaryotic and eukaryotic aldehyde dehydrogenases have K'_m values for their substrates of less than 1 μM and also are substrate inhibited. These include the Pseudomonas putida benzaldehyde dehydrogenase which has a K'_m for benzaldehyde of 0.2 μM and is inhibited by benzaldehyde at concentrations above 0.1 mM (Stachow et al., 1967) One possible mechanism for inhibition by substrate for yeast aldehyde dehydrogenase is thought to involve the binding of a second substrate molecule to the enzyme after a molecule has already bound, forming an abortive complex (Jakoby, 1963). An alternative mechanism was suggested by Gregory et al. (1972) from studying the aldehyde inhibition of xanthine oxidase; inhibition could result from the increasing effect of the hydrated form of the aldehyde at higher substrate concentrations, although this would be unlikely to be the mechanism with the unhydrated aromatic aldehydes.

Neither BADH nor BZDH II was affected by the possible metabolic regulatory compounds tested (Section 4.4.5). No signs of feed-back inhibition of the enzymes activities had

previously been observed during investigations of the regulation of benzyl alcohol metabolism in A.calcoaceticus (Beggs et al., 1976). This pathway is clearly not subject to feed-back inhibition.

4.5.8 Induction and substrate specificities

Benzyl alcohol and benzaldehyde both induce BADH and BZDH II (Livingstone et al., 1972) but in general the substrate specificities of both BADH and BZDH II appear to be different from their induction specificities. For example, 2-thiophenemethanol (XXIII), 3-pyridinemethanol (XVII), and 4-pyridinemethanol (XX) will induce the enzymes (Livingstone et al., 1972) but only 2-thiophenemethanol (XXIII) is a substrate for BADH (Table 4.5). Cinnamyl alcohol (VI) on the other hand is a substrate for BADH (Table 4.5) but is not an inducer (Livingstone et al., 1972), and 3-pyridinecarboxaldehyde (XV) is both an inducer (Livingstone et al., 1972) and a substrate of BZDH II (Table 4.5). In other bacterial systems (e.g. the lac system of E.coli (Lewin, 1974b) or the ami system of P. aeruginosa (Clarke, 1984)) inducer and substrate specificities are often very different suggesting that in general the relevant enzymes and repressors had different evolutionary origins (Baumberg, 1981).

BZDH II was inhibited by a range of aromatic aldehydes (Table 4.9). Those substrate analogues which are not oxidised by the enzymes could perhaps be anti-inducers of BADH and BZDH II expression. Anti-inducers can be used to isolate constitutive

strains and enzyme inhibitors can be used to isolate over-expressing strains, as has previously been achieved with the enzymes specific for mandelate metabolism in P.putida and A.calcoaceticus (Hegeman & Root, 1976; Fewson et al., 1978). Unfortunately, in preliminary experiments I found the substituted benzaldehydes to be very toxic to growth and so this is not an area which can be readily pursued.

4.5.9 Substrate specificity and growth

The combined substrate and inducer specificities of both BADH and BZDH II will determine which substrates can be utilised as carbon sources for A.calcoaceticus, provided that there is the ability to take up the compound and that the enzymes of the ortho ring-cleavage pathway will accept their products. Fewson (1967a) found that A.calcoaceticus NCIB 8250 would grow on benzyl alcohol, benzaldehyde, 2-hydroxybenzyl alcohol (salicyl alcohol), 2-hydroxybenzaldehyde (salicyl aldehyde), 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol), 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 3,4-dihydroxybenzaldehyde. No other substituted benzyl alcohols or benzaldehydes would serve as growth substrates. As 4-hydroxy-3-methoxybenzaldehyde and 3,4-dihydroxybenzaldehyde are not substrates for BZDH II (Table 4.4), growth must occur as a result of the oxidation of these compounds by another aldehyde dehydrogenase, probably benzaldehyde dehydrogenase I. Other aromatic alcohols, such as 3-hydroxy- or 3-methoxybenzyl alcohols, although oxidised via BADH and BZDH II (Tables 4.5 and 4.6) are not utilised

by A. calcoaceticus as growth substrates (Fewson, 1967a). Oxygen utilisation patterns indicate that these compounds are not metabolised past the corresponding acid (Kennedy & Fewson, 1968b) the specificity of the ring-cleavage enzymes dictating which acids will be further metabolised. The specificity of the ring-cleavage enzymes also appear to be responsible for the inability of A. calcoaceticus to grow on cinnamyl alcohol (VI) (Fewson, 1967a), which is oxidised only to cinnamic acid (V) (Kennedy & Fewson, 1968b). Although cinnamyl alcohol (VI) or presumably coniferyl alcohol (VIII) cannot serve as sole carbon sources, their oxidation via BADH and BZDH II could give some energy to the organism and the acids produced would then be available for metabolism by other organisms. As cinnamyl alcohol (VI) and coniferyl alcohol (VIII) are both known intermediates of lignin biosynthesis and degradation (Crawford, 1981) it might be expected that a soil microorganism, such as A. calcoaceticus, would metabolise them to some extent. It may well be that in the natural environment the mixed population of microorganisms contains individual species which can metabolise compounds only partially, excreting or releasing those compounds which are then available to be utilised by other organisms. It is presumably by this route that A. calcoaceticus and other soil bacteria encounter aromatic alcohols and aldehydes, as a result of fungal degradation of lignins (Cain, 1980).

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Introduction

One might expect two dehydrogenases that catalyse sequential steps in a pathway and use NAD^+ to oxidise substrates of a similar structure to be related and to have evolved from a common ancestor. One precedent for such a hypothesis, would be Ornston's work on the evolution of the enzymes of the ortho ring-cleavage pathway (Yeh & Ornston, 1980; Ornston & Yeh, 1982). However, without complete primary and tertiary structures the deduction of such an evolutionary relationship between BADH and BZDH II would be premature.

BADH and BZDH II differ in several respects: N-terminal amino acid sequence (Section 4.1.3), amino acid composition (Section 4.5), pI values (Section 4.1.2), subunit M_r values (Section 4.1.1). The enzymes also have certain features in common: NAD^+ dependency (Section 4.2.8), some aspects of substrate specificity (Section 4.3.4), sulphydryl reagents sensitivity (Section 4.4.1), and metal chelator insensitivity (Section 4.4.3). However, comparisons of such physical and kinetic properties as BADH and BZDH II do or do not share are not really a sufficient basis for extensive arguments about their origin and subsequent evolution. They do, however, allow comparison with other extensively characterised alcohol and aldehyde dehydrogenases and thus give a tentative insight into the possible structures, mechanisms and functions of the enzymes. Further, some of the results obtained in the course of this work will be useful in future cloning and sequencing of the genes for BADH and BZDH II and the results of that work will be crucial to evolutionary speculations.

From these physical and chemical comparisons of BADH and BZDH II (discussed in Sections 3.4 and 4.5) certain areas of future investigation are highlighted in this chapter. If pursued, some of these studies should give a much more detailed picture of the evolution of the pathways for the catabolism of aromatic compounds in A. calcoaceticus and other soil micro-organisms and the molecular enzymology of the processes involved.

5.2 Does BADH contain a metal atom at the active site?

BADH is unaffected by metal chelators (Section 4.4.3). To determine if it does contain a metal atom then at least three avenues of attack are open. Firstly, a metal analysis could be done on the purified enzyme e.g. by subjecting the protein to atomic absorption spectroscopy (Vallee & Galdes, 1984). The results from these sorts of analyses sometimes, however, result in a doubtful conclusion. The presence or absence of a metal atom can always be attributed to the acquisition or loss of the metal during preparation of the sample. A more conclusive method is to grow the organism in the presence of radioactive metals and purify the enzyme. If the purified enzyme contains stoichiometric amounts of a specific metal then this is fairly good proof that it is an intrinsic component. Another line of attack is to use exhaustive dialysis against a range of chelating agents followed by specific reactivation.

If BADH does not contain a metal atom then it may resemble the alcohol dehydrogenase from Drosophila melegaster which is metal-free (Winberg et al., 1986) and appears to be only distantly related to the liver and yeast alcohol dehydrogenase (Jornvall, 1977). However, if BADH does contain a metal atom

then it would be important to know how it is protected from the metal chelators.

5.3 Can sulphydryl reagents be used to probe further the active sites of BADH and BZDH II?

It would appear at least possible that the inhibition by sulphydryl reagents is caused by reaction at the active sites of BADH and BZDH II for the enzymes can be protected from inhibition by their substrates (Section 4.4.2). If so, this would open the way to selective labelling of the active sites and the isolation of active site peptides. This would be particularly useful because comparison of active site sequences tends to give a clearer indication of relationships between proteins than the N-terminal sequences.

As Yeh & Ornston (1980) have observed with the enzymes of the ortho ring-cleavage pathway, N-terminal sequences can be very variable due, in part, to the possibility of shuffling of sequences between enzymes, and the lack of non-catalytic function of the terminal regions. Yeh & Ornston (1984) have done preliminary experiments aimed at opening up the possibility of using 3-chloromercuribenzoate specifically to modify thiol groups associated with the active sites of β-ketoadipate enol-lactose hydrolase and succinyl-CoA β-ketoadipate-CoA transferase.

5.4 How do the structures of the active sites of BADH and BZDH II dictate their substrate specificity?

BADH and BZDH II appear to have evolved to oxidise aromatic alcohols or aldehydes efficiently (Section 4.3.4). To elucidate

the structure of the active sites of the enzymes a full scale determination of the primary and tertiary structures would have to be undertaken. Only when a 3-dimensional picture is available can a full interpretation of the substrate profiles of the enzymes be made. It would be interesting to know if the aromatic ring specificity results from particular residues being positioned in the substrate binding sites. Bulky residues may have been excluded and replaced with residues which can accommodate and interact with the aromatic ring of the substrates, but at the same time retain the hydrophobicity of the active site.

5.5 How are the aromatic alcohol and aldehyde dehydrogenases from A. calcoaceticus related to their counterparts in Pseudomonas putida?

Now that BADH and BZDH II have been characterised, purification and examination of the mandelate-induced BZDH I from A. calcoaceticus is important. In particular, the primary sequences of BZDH I and BZDH II should be examined for clues as to how these isofunctional enzymes co-evolved. It would also be most fascinating to compare BADH and BZDH II with the benzyl alcohol and benzaldehyde dehydrogenases which are both chromosomally- and plasmid-encoded in Pseudomonas putida (Collins & Hegeman, 1984; Williams & Murray, 1974). The benzyl alcohol dehydrogenase purified from P. putida T-2 grown on toluene (Suhara et al., 1969) may perhaps be the enzyme encoded on the TOL plasmid, and its similarities to BADH have already been discussed (see Section 4.5). Comparison of these enzymes might

give some indication of how the pathways have been acquired by the organisms i.e. could they have been transferred from one chromosomal genome via plasmids to different organisms?

5.6 Could antibodies be used to study relationships between the aromatic alcohol and aldehyde dehydrogenases?

The immunological studies of Patel & Ornston (1976) were a useful complement to later amino acid sequence information in the quest to trace evolutionary relationships among the enzymes of the ortho ring-cleavage pathway (Ornston & Yeh, 1982; also see Section 1.4 of this thesis). However, immunological studies with other proteins, especially the very elegant work of Cohen and colleagues (Zakin et al., 1978; Ferrara et al., 1984) on aspartate kinases/homoserine dehydrogenases, suggest that antibodies raised against denatured antigens may give more valuable results than antibodies raised against native antigens.

After the purification of BADH and BZDH II I raised antibodies in rabbits against each native enzyme. Preliminary studies showed that antibodies raised against BADH cross-reacted with BZDH II. However, antibodies raised against BZDH II did not cross-react with BADH. Since other enzymes of the convergent benzyl alcohol and mandelate pathways have been, or soon will be, purified then these immunological studies could be further extended to investigate possible evolutionary relationships, particularly between the two benzaldehyde dehydrogenases (BZDH I and BZDH II) and between them and BADH.

The antibodies may also be useful in screening bacterial colonies containing cloned BADH and BXDH II genes, since antibodies to BADH and BZDH II do not appear to interact with other proteins from crude extracts.

5.7 Can medically and industrially useful aromatic compounds be produced from these catabolic pathways?

Another area of research which is of growing importance is the use of microbial pathways of aromatic metabolism for applied industrial processes (Franklin *et al.*, 1981). Wild-type strains can be used or strains can be genetically engineered to combine the genes for the pathway of interest plus any additionally required enzymic steps. The desirability of using microbial transformations to obtain useful products is very dependent on economic circumstances. However, these natural pathways have the potential to allow the production of a range of valuable compounds.

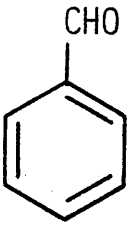
Aromatic alcohol and aldehyde dehydrogenases could be used in several procedures. Toxic aromatic compounds, especially benzaldehydes, can be catabolised to less toxic alcohols and acids (Wisniewski *et al.*, 1983). Aromatic compounds, for example from the biodegradation of lignin or from industrial wastes, could be used to support growth of microorganisms, which in turn could be used as food sources in agriculture.

5.8 Future goals

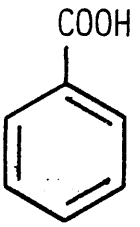
Probably the most important goal, on which all other future work hinges, is the cloning and, overexpression of the genes for BADH and BZDH II. This will allow sequencing of the genes and would greatly enhance any evolutionary speculations about the enzymes. Overexpression of the enzymes will afford sufficient protein to attempt X-ray crystallography studies, which should solve the questions raised in this thesis concerning the nature of the active sites of BADH and BZDH II.

APPENDIX

benzaldehyde	I
benzoic acid	II
benzyl alcohol	III
cinnamaldehyde	IV
cinnamic acid	V
cinnamyl alcohol	VI
coniferaldehyde	VII
coniferyl alcohol	VIII
2-furancarboxaldehyde	IX
2-furancarboxylic acid	X
2-furanmethanol	XI
perillaldehyde	XII
perillic acid	XIII
perillyl alcohol	XIV
3-pyridinecarboxyaldehyde	XV
3-pyridinecarboxyaldehyde	XVI
3-pyridinemethanol	XVII
4-pyridinecarboxaldehyde	XVIII
4-pyridine carboxylic acid	XIX
4-pyridinemethanol	XX
2-thiophenecarboxaldehyde	XXI
2-thiophene carboxylic acid	XXII
2-thiophenemethanol	XXIII



I



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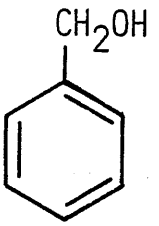

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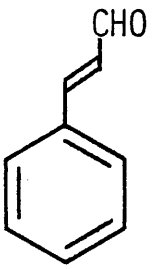

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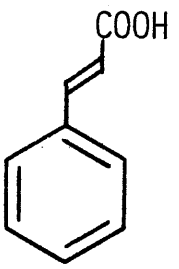
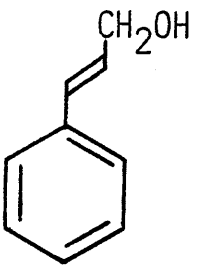


V



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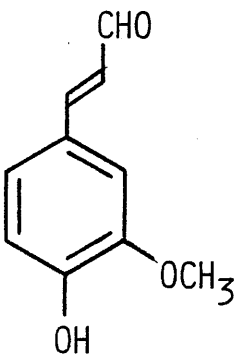

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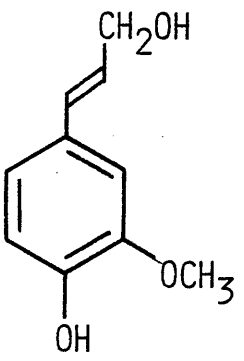

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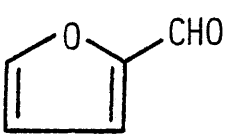

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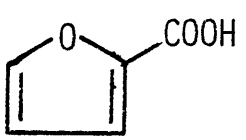

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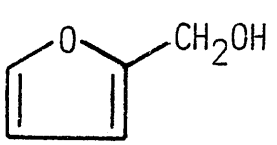

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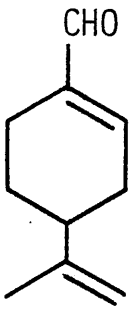

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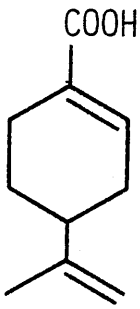

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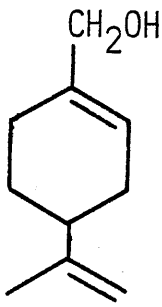

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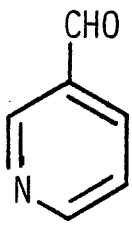

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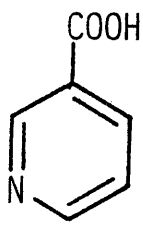

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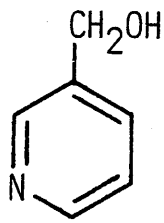

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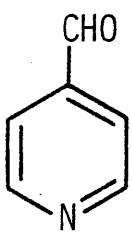

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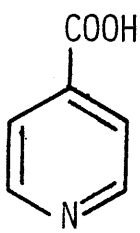

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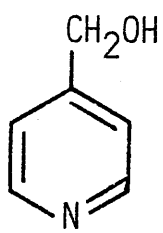

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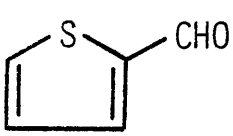

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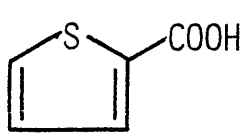

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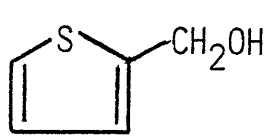

XXIII

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MICROBIAL AROMATIC ALCOHOL AND ALDEHYDE DEHYDROGENASES

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INTRODUCTION: THE VARIETY OF MICROBIAL ENZYMES

The metabolism of alcohols and aldehydes by eukaryotic and prokaryotic microorganisms is achieved by the expression of an extensive array of constitutive and inducible dehydrogenases and oxidases. Most of these enzymes have relatively narrow inducer and substrate specificities and their variety, as well as their astonishingly diverse characteristics, reflect the range of alcohols and aldehydes that may be encountered in the environment.

Catabolism and Biosynthesis

Alcohol and aldehyde dehydrogenases enhance the flexibility of microbial degradative, fermentative and biosynthetic pathways:
(a) Degradative enzymes allow growth substrates (e.g. alcohols derived from alkanes, benzyl alcohol, vanillin, p-hydroxybenzaldehyde) to be converted into amphibolic intermediates and to provide energy. The catabolic role of alcohol and aldehyde dehydrogenases may be particularly important in the carbon cycle that operates in Nature. Biodegradation of lignin, for example, provides many aromatic alcohols and aldehydes that may serve as growth substrates for soil and aquatic microorganisms.
(b) Anaerobic fermentation in yeast principally gives rise to ethanol but in bacteria a great range of end-products, including a variety of alcohols (e.g. butanol, isopropanol, 2,3-butanediol), can be produced depending upon the species,

the environmental conditions and the enzyme complement (Doelle, 1975). The production of some of these compounds is of industrial importance.
(c) The biosynthetic role can be illustrated by the bifunctional NAD-dependent dehydrogenase of Salmonella typhimurium which oxidises histidinol, via histidinal, to histidine (Loper and Adams, 1965). Alcohol and aldehyde dehydrogenases sometimes provide key links between primary and secondary metabolism; thus, synthesis of the antibiotic patulin by Penicillium urticae requires an NADP-dependent m-hydroxybenzyl alcohol dehydrogenase (Forrester and Gaucher, 1972). Biotechnological alterations of some of these enzymes involving DNA modifications and gene transfer will no doubt play a significant part in extending the biosynthetic capacities of microorganisms so that new and improved products can be made.

Intracellular Location

Most bacterial alcohol and aldehyde dehydrogenases are so-called 'soluble' enzymes and are located in the cytoplasm, but some have been shown to be membrane-bound. For example, an NADP-dependent aldehyde dehydrogenase of Acinetobacter calcoaceticus (Aurich et al., 1985), a 2,7,9-tricarboxy-1H-pyrrolo[2,3f]quinoline-4,5-dione (pyrroloquinoline quinone: PQQ)-dependent polyvinyl alcohol dehydrogenase of a Pseudomonas sp. (Shimao et al., 1986), and the alcohol and aldehyde dehydrogenases of the acetic acid producing bacteria Acetobacter aceti and Gluconobacter suboxydans (Ameyama and Adachi, 1982a,b) are all associated with membranes. In addition, the methanol dehydrogenases (often referred to as 'dye-linked dehydrogenases') of certain methylotrophic bacteria, which are assayed in vitro using N-methylphenazonium methosulphate (phenazine methosulphate: PMS) as electron acceptor, are thought to be associated with cytochrome c in vivo (O'Keeffe and Anthony, 1980).

The intracellular compartmentation of eukaryotes gives rise to greater possibilities of variation in location. Thus, Saccharomyces cerevisiae contains three alcohol dehydrogenases of which two are cytoplasmic and the third is mitochondrial (Heick et al., 1969). In addition, alcohol oxidase, which is involved in methanol oxidation by certain yeasts, has been located in the microbodies of Kloeckera sp. (Fukui et al., 1975).

Electron Acceptors

The most commonly encountered alcohol and aldehyde dehydrogenases are those that are dependent on NAD or NADP. Recently, however, several PQQ-dependent alcohol dehydrogenases have been isolated from methylotrophic (Duine and Frank, 1980) and non-methylotrophic (Groen et al., 1984) bacteria and it has been suggested that some PMS-dependent alcohol dehydrogenases may utilize PQQ as electron acceptor *in vivo* (Duine and Frank, 1981).

FAD or FMN-linked dehydrogenases or oxidases have been isolated from a few microorganisms. For example, alcohol oxidases of the yeasts *Candida boidinii* and *Kloeckera* sp. are flavoproteins and transfer electrons directly to molecular oxygen to form hydrogen peroxide (Sahm and Wagner, 1973; Kato et al., 1976).

Several dehydrogenases contain haem centres which are involved in electron transfer, for example the PMS-dependent aldehyde dehydrogenase from *Methylomonas methylovora* (Patel et al., 1980) and the PQQ-dependent alcohol dehydrogenase from *Pseudomonas testosteroni* (Groen et al., 1986) are both haemoproteins. A flavocytochrome oxidoreductase which can oxidise *p*-hydroxybenzyl alcohol has been purified from *Pseudomonas putida* (Hopper and Taylor, 1977).

Substrate Specificity

It is possible to classify microbial alcohol and aldehyde dehydrogenases according to their substrate specificities. This serves to illustrate the enormous diversity of substrates that can be utilised but yields a rather imprecise and overlapping set of categories which may not represent true homologies or genuine evolutionary relationships. This difficulty is highlighted by the classification recommended by I.U.B.-I.U.P.A.C. (1979) which involves broad groupings, stemming in part from the lack of enzyme specificity.

The major types of substrate specificity seem to be:
(a) Preference for aliphatic primary alcohols. Example: methanol dehydrogenases from methylotrophic bacteria. These enzymes rapidly oxidise primary alcohols and have a high affinity for aliphatic substrates. Formaldehyde and other

hydrated aliphatic aldehydes can also be oxidised (Sperl et al., 1974).

(b) Oxidation of secondary aliphatic alcohols only. Examples: secondary alcohol dehydrogenases of C_1-utilizing bacteria (Hou et al., 1979) and secondary alcohol dehydrogenase from Pseudomonas sp. (Niehaus et al., 1978).

(c) Preference for secondary aliphatic alcohols. Examples: an alcohol dehydrogenase from Comamonas terrigena which has a preference for L-stereoisomers of secondary alcohols (Barrett et al., 1981), and the thermostable secondary alcohol dehydrogenase from Pseudomonas fluorescens (Hou et al., 1983).

(d) Oxidation of primary and secondary aliphatic alcohols. Example: alcohol dehydrogenase from the white-rot fungus Sporotrichum pulverulentum (Rudge and Bickerstaff, 1986). Alcohol dehydrogenase from bakers' yeast Sacchoromyces cerevisiae can also be included in this class although it has a preference for primary alcohols (Sund and Theorell, 1963).

(e) Oxidation of aliphatic aldehydes: (i) by enzymes with a relatively narrow specificity, such as NAD(P)-dependent formaldehyde dehydrogenase from Methylococcus capsulatus (Bath) (Stirling and Dalton, 1978), and the magnesium-activated NADP-dependent bakers' yeast aldehyde dehydrogenase (Jakoby, 1963), and (ii) by enzymes with a broad specificity, for example, PMS-dependent formaldehyde dehydrogenase from Hyphomicrobium X (Marison and Attwood, 1980) and potassium-activated NAD(P)-dependent bakers' yeast aldehyde dehydrogenase (Jakoby, 1963).

(f) Preference for aromatic alcohols, although some of these enzymes also oxidise aliphatic alcohols. Examples: benzyl alcohol dehydrogenase from Pseudomonas putida (Suhara et al., 1969), coniferyl alcohol dehydrogenase from Rhodococcus erythropolis (Jaeger et al., 1981), perillyl alcohol dehydrogenase from Pseudomonas sp. (Ballal et al., 1966), and p-hydroxybenzyl alcohol dehydrogenase from Rhodopseudomonas acidophila (Yamanka and Minoshima, 1984).

(g) Preference for aromatic aldehydes; more specific than aromatic alcohol dehydrogenases, in that fewer will oxidise aliphatic aldehydes. Examples: benzaldehyde dehydrogenase from Pseudomonas fluorescens (Stachow et al., 1967), perillaldehyde dehydrogenase from Pseudomonas sp. (Ballal et al., 1967), and o-carboxybenzaldehyde dehydrogenase from Alcaligenes faecalis (Kiyohara et al., 1981).

It is not unusual to find that more than one type of alcohol or aldehyde dehydrogenase can be expressed in a single organism depending on the growth substrate. For example, Acinetobacter calcoaceticus contains aromatic, primary aliphatic, or diol-specific alcohol dehydrogenases when grown on benzyl alcohol, ethanol or 2,3-butanediol respectively (Fewson, 1966). The same species also possesses two aliphatic and two aromatic aldehyde dehydrogenases (Livingstone et al., 1972; Fixter and Nagi, 1984; Aurich et al., 1985). The ability to express several types of enzyme obviously increases the potential of microorganisms to grow and survive under different environmental conditions.

Isoenzymes

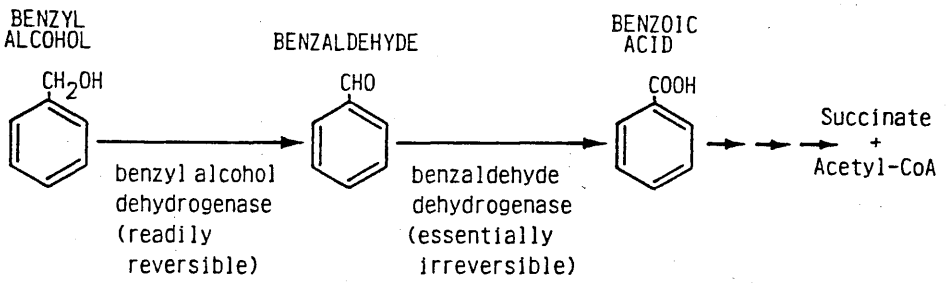
Several microbial isofunctional alcohol and aldehyde dehydrogenases have been characterized. The regulatory and kinetic properties of some of these isoenzymes suggest that one form may be involved in catabolism, the other in anabolism. For example, in bakers' yeast the two cytoplasmic alcohol dehydrogenases (known as ADH-I and ADH-II) have different physiological roles; the constitutive ADH-I (the classical yeast alcohol dehydrogenase; Sund and Theorell, 1963; Branden et al., 1975) has a K_m value for ethanol of 24mM, and is involved in alcohol formation, whereas the inducible ADH-II oxidises ethanol to acetaldehyde under aerobic conditions and has a K_m value for ethanol of 2.7mM (Wills, 1976). A similar situation exists in the ethanol-producing bacterium Zymononas mobilis in which one alcohol dehydrogenase requires zinc ions and the other is activated by ferrous ions. It is the former enzyme which is thought to be responsible for aldehyde reduction in vivo (Neale et al., 1986). By contrast, Acinetobacter calcoaceticus has two NAD-dependent benzaldehyde dehydrogenases which are both involved in convergent catabolic pathways. One enzyme is heat-stable, potassium-activated and is induced by phenylglyoxylate. The other is heat-labile and is induced by benzyl alcohol (Livingstone et al., 1972).Pseudomonas putida also has two NAD-dependent benzaldehyde dehydrogenases but it also has an NADP-dependent enzyme which is induced during growth on mandelate (Stachow et al., 1967).

Genetic Location and Regulation

The majority of microbial alcohol and aldehyde dehydrogenases are inducible. However, only a rather limited number of studies have been carried out to determine the location and regulation of the relevant genes. It has been demonstrated that in Pseudomonas putida there are both chromosomally and plasmid-encoded benzyl alcohol and benzaldehyde dehydrogenases. The plasmid, designated TOL, encodes the enzymes which enable P. putida to grow on toluene and m- and p-xylenes (Williams, 1981).

BENZYL ALCOHOL METABOLISM IN ACINETOBACTER CALCOACETICUS

Acinetobacter calcoaceticus is a ubiquitous soil microorganism of clinical and industrial importance. It can grow on a large number of aromatic compounds, including benzyl alcohol (Fewson, 1967). The pathway of benzyl alcohol catabolism is:



This catabolic pathway may have evolved to allow A. calcoaceticus to utilize as carbon sources some of the aromatic alcohols and aldehydes which are derived from the biodegradation of lignin.

Both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are 'soluble' NAD-dependent enzymes. They appear to be chromosomally encoded and are induced by either benzaldehyde or benzyl alcohol or by various ring-substituted analogues of these compounds.

PURIFICATION AND CHARACTERIZATION OF BENZYL ALCOHOL DEHYDROGENASE AND BENZALDEHYDE DEHYDROGENASE

Purification of both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase has now been achieved using a common purification procedure involving ion-exchange and triazine dye affinity chromatography and gel filtration using the Pharmacia fast protein liquid chromatography system. Sodium dodecyl sulphate polyacrylamide gel electrophoresis indicates that both enzymes are homogeneous. The purification procedure results in good yields of both enzymes and they are stable for several months at -20°C

Determination of physical and kinetic properties of the enzymes (Table 1) allows comparison with respect to (a) each other, (b) other bacterial aromatic alcohol and aldehyde dehydrogenases, and (c) alcohol and aldehyde dehydrogenases from eukaryotes.

Quaternary Structure

Both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are tetrameric enzymes as judged by a comparison of their native and subunit M_r values. Little information is available as to the quaternary structure of other bacterial aromatic alcohol and aldehyde dehydrogenases although their native M_r values range from 27 000 to 200 000 and from 160 000 to 200 000 respectively (Stachow et al., 1967; Jaeger et al., 1981; Kiyohara et al., 1981; Yamanaka and Mioshima, 1984). Horse liver alcohol dehydrogenase is a dimer of native M_r about 80 000 (Green and McKay, 1969), while the classical fermentative yeast alcohol dehydrogenase is a tetramer of native M_r 140 000-150 000 (Bränden et al., 1975). Potassium-activated aldehyde dehydrogenase from yeast is also tetrameric and has a native M_r of 240 000 (Bostian and Betts, 1978).

Substrate Specificity

Acinetobacter calcoaceticus benzyl alcohol dehydrogenase does not oxidise aliphatic alcohols and in this respects resembles other aromatic alcohol

TABLE 1. Physical and Kinetic Properties of Benzyl Alcohol Dehydrogenase and Benzaldehyde Dehydrogenase from *Acinetobacter calcoaceticus*

	Benzyl alcohol dehydrogenase	Benzaldehyde dehydrogenase
Native M_r	155 000	223 000
Subunit M_r	39 700	55 000
Quaternary structure	Tetrameric	Tetrameric
pH optimum	Oxidation:pH9.2 Reduction:pH8.9	Oxidation:pH9.5
pI value	5.0	4.6
Cofactor requirement	NAD	NAD
Substrates	Aromatic alcohols and aldehydes	Aromatic aldehydes and some aliphatic aldehydes
Sulphydryl reagents(e.g. chloromercuribenzoate, *N*-ethylmaleimide)	Sensitive	Sensitive
Metal chelators (e.g. pyrazole, EDTA)	Insensitive	Insensitive
Esterase activity	None	Present

dehydrogenases, e.g. the benzyl alcohol dehydrogenase (Suhara et al., 1969) and perillyl alcohol dehydrogenase (Ballal et al., 1966) from *Pseudomonas* spp. and the coniferyl alcohol dehydrogenase from *Rhodococcus erythropolis* (Jaeger et al., 1981). Benzyl alcohol and perillyl alcohol give the highest k_{cat}/K_m' ratios for the *A. calcoaceticus* enzyme, suggesting a close similarity

between it and the benzyl alcohol dehydrogenase from toluene-grown P. putida (Suhara et al., 1969) which oxidises cyclohex-1-ene ring compounds, such as perillyl alcohol, as well as aromatic alcohols. The A. calcoaceticus dehydrogenase also oxidises cinnamyl alcohol and coniferyl alcohol, known intermediates of lignin biodegradation. NAD-dependent horse liver alcohol dehydrogenase is well known for having a very broad substrate specificity, oxidising a variety of aliphatic or aromatic primary and secondary alcohols; it also oxidises some aliphatic aldehydes to the corresponding acids. Benzyl alcohol is oxidised at approximately the same rate as ethanol (Sund and Theorell, 1963). The classical fermentative NAD-dependent yeast alcohol dehydrogenase is much more specific than the mammalian liver enzyme, chiefly oxidising primary straight chain alcohols. Benzyl alcohol is a very poor substrate, although cinnamyl alcohol is oxidised at about 40% of the rate of ethanol (Sund and Theorell, 1963).

Acinetobacter calcoaceticus benzaldehyde dehydrogenase oxidises some aliphatic aldehydes, such as hexanal and octanal, but much less effectively than aromatic aldehydes. Benzaldehyde gives the highest k_{cat}/K_m' value, with a K_m' of approximately 1μM. In general the activity appears to depend on the size of the substituents on the aromatic ring, e.g. methoxy- and hydroxy-benzaldehydes are poor substrates compared with fluoro-benzaldehydes. Cinnamaldehyde and perillaldehyde are both oxidised slowly. Similary, other bacterial aromatic aldehyde dehydrogenases all preferentially oxidise only a small group of substrates. The aromatic aldehyde dehydrogenase from Alcaligenes faecalis is specific for o-carboxybenzaldehyde (Kiyohara et al., 1981), and the NADP-dependent benzaldehyde dehydrogenase from Pseudomonas putida oxidises only aromatic aldehydes, e.g. benzaldehyde has a K_m' value of 0.2μM (Stachow et al., 1967). Horse liver contains two aldehyde dehydrogenases. Both are NAD-dependent and dehydrogenate a range of substrates, e.g. they oxidise benzaldehyde with K_m' values of less than 0.1μM (Eckfeldt and Yonetani, 1982). Of the two aldehyde dehydrogenases in bakers' yeast, the potassium-activated enzyme is the least specific and it oxidises short chain aliphatic aldehydes as well as aromatic aldehydes such as benzaldehyde. The magnesium-activated enzyme oxidises only short chain aliphatic aldehydes (Jakoby, 1963). The

specificity of the enzymes no doubt relates to the hydrophobic, steric and hydration characteristics of the aldehydes. In no case is the detailed interaction of substrate and enzyme clear at the molecular level. In some cases substrate ambiguity would seem to have a selective advantage in allowing a single enzyme to convert several substrates, but in other cases it is probably merely a fortuitous consequence of the properties of the active site.

Inhibitors

Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are both sensitive to sulphydryl reagents. This is a common property of all the bacterial aromatic alcohol and aldehyde dehydrogenase examined to date as well as of the mammalian and yeast alcohol and aldehyde dehydrogenases. It was originally thought that sulphydryl inhibition of the horse liver and yeast alcohol dehydrogenases was due to modification of a reactive thiol group involved in co-enzyme binding (Sund and Theorell, 1963); however, it is now believed that the inhibition is caused by modification of the cysteine residues which are ligands to the zinc atom at the active site (Bränden et al., 1975). It has been suggested on the basis of kinetic studies that a free thiol is involved in the catalytic mechanism of the aldehyde dehydrogenase from yeast (Jakoby, 1963) and horse liver (Feldman and Weiner, 1972). Modification of active site thiols may therefore be involved in the inhibition of bacterial aromatic alcohol and aldehyde dehydrogenases but much more detailed work is necessary before this can be established.

The insensitivity of benzyl alcohol dehydrogenase to inhibition by metal chelators is another property shared with most other bacterial aromatic alcohol and aldehyde dehydrogenases. By contrast, horse liver and yeast alcohol dehydrogenases are both sensitive to chelating agents and this is known to be due to the presence of zinc atoms at the active sites (Sund and Theorell, 1963). The lack of inhibition observed with the bacterial aromatic alcohol dehydrogenase could be due to the absence, or protection, of a metal atom at the active site. Protection could result from the micro-environment developed for the binding of hydrophobic aromatic compounds not favouring the binding of chelating agents. Unfortunately, no metal analysis has

yet been undertaken for any of the bacterial aromatic alcohol dehydrogenases. There is no suggestion that metals are involved in mammalian or yeast aldehyde dehydrogenases and the benzaldehyde dehydrogenase from A. calcoaceticus was unaffected by any of the chelating agents tested.

Esterase Activity

Acinetobacter calcoaceticus benzaldehyde dehydrogenase exhibits esterase activity with p-nitrophenylacetate as substrate. Other bacterial aromatic aldehyde dehydrogenases do not appear to have been tested for esterase activity. However, both horse liver and yeast aldehyde dehydrogenase also exhibit esterase activity (e.g. Feldman and Weiner, 1972). It has been proposed that a single active site involving a thio-ester acyl intermediate is involved in both the dehydrogenase and the esterase activities (Duncan, 1985) so it may be that the A. calcoaceticus benzaldehyde dehydrogenase possesses a similar reaction mechanism and this would be consistent with the sulphydryl inhibition studies.

OUTLOOK FOR FUTURE RESEARCH

Microbial alcohol and aldehyde dehydrogenases are a particularly fertile field for studying comparative molecular biological aspects of enzymology. Comparison of benzyl alcohol and benzaldehyde dehydrogenases with other alcohol and aldehyde dehydrogenases will allow further insights into the evolutionary, functional, and mechanistic aspects of these enzymes. Why, for example, can potassium (but not magnesium)-activated yeast aldehyde dehydrogenase oxidise benzaldehyde, whereas benzyl alcohol is a poor substrate for yeast alcohol dehydrogenase but a good substrate for mammalian alcohol dehydrogenase? Do the aromatic alcohol dehydrogenases lack a metal atom at their active sites; if so, how does their reaction mechanism differ from that of enzymes which do possess a metal? Is there any structural homology between chromosomally-encoded and plasmid-encoded benzyl alcohol and benzaldehyde dehydrogenases? Finally, will sequencing studies reveal whether similar or isofunctional alcohol and aldehyde dehydrogenases arose by gene duplication?

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