REACTIVITY AND FUNCTION OF THIOL GROUPS
IN LIVER ALCOHOL DEHYDROGENASE

by

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Ph.D.

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1970
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Enzyme activity of liver alcohol dehydrogenase is inhibited by a number of compounds which can react with thiol groups. This study includes work with parachloromercurobenzoate, 5,5'-dithiobis-(2-nitrobenzoic acid), iodoacetate, iodoacetamide, 3-iodopropionate, 3-(2-bromoacetyl)-pyridine, 2-chloroethanol and 2-iodoethanol. Iodoacetate and 3-(2-bromoacetyl)-pyridine were found to alkylate one thiol group per subunit of inactivated enzyme.

Inactivation of the enzyme with iodoacetate was found to follow Michaelis-Menten kinetics. This was used to study the binding of other ligands, and to examine their mutual interactions. Fatty-acids, adenine nucleotides, chloride and 4-biphenyl-carboxylic acid protected the enzyme competitively, while bromide and iodide showed mixed kinetics. Orthophenanthroline protected non-competitively. Imidazole stimulated inactivation, and weakened the binding of iodoacetate. Imidazole is thought either to bind at more than one site, or to mediate a conformation-change in the enzyme, perhaps by altering the co-ordination geometry of zinc. Decanoate and ADP-ribose were found to be mutually competitive, as was imidazole with decanoate and orthophenanthroline. Imidazole strengthened the binding of AMP and ADP-ribose at high pH, but binding was still weaker than at pH 7.4; it is suggested that the phosphate of AMP may be bound to an imidazole-insensitive, pH-sensitive "positive centre" on the enzyme - possibly a lysine residue. Binding of chloride was found to be complex, and several different complexes can probably be formed.

It is pointed out that the Michaelis-Menten kinetics of
inactivation with iodoacetate could be produced by reversibly-bound iodoacetate protecting the enzyme from alkylation by iodoacetate molecules free in solution: it is suggested that this may be more likely than the more obvious interpretation whereby reversibly-bound iodoacetate alkylates a thiol group adjacent to the site of reversible binding.

Carboxymethyl enzyme was found to have 2 - 2.5% residual activity, which was not due to a minor isoenzyme component. It formed some disulphide bonds on standing, and in this and other respects had some similarities with low-zinc alcohol dehydrogenase. Carboxymethyl enzyme formed binary complexes with NADH, AMP, imidazole, decanoate and 4-biphenyl-carboxylic acid; ternary complexes were formed with NAD\(^+\) and pyrazole, NAD\(^+\) and decanoate, and NADH and imidazole. However, stable, highly-fluorescent complexes with NADH and amides (which are a characteristic of the native enzyme) were not formed. It is suggested that the formation of these highly-fluorescent complexes may take place by a mechanism also necessary for catalysis, which carboxymethylation disrupts. The introduction of an anion into the active centre by carboxymethylation would be expected to considerably reduce the binding of other anions. It is suggested that either the carboxymethylation causes no net change of charge, or that the thiol group is not in the active centre.

Bromoacetyl-pyridine has been shown to be a strong, specific alkylating agent for the enzyme, but no evidence was found for non-covalent interaction with the active centre. Halogenated ethanol derivatives, although powerful reversible inhibitors, were very weak alkylating agents.
Some comparisons with yeast alcohol dehydrogenase have been made. Its reactive thiol group is more reactive than that of the liver enzyme, but reaction with 5,5'-dithiobis-(2-nitrobenzoic acid), protection from iodoacetate by AMP and labilisation by imidazole were qualitatively similar.
The experiments described in this thesis were carried out in the Department of Biochemistry, University of Edinburgh, between December 1966 and June 1970. I am grateful to Professor R.B. Fisher, C.B.E., for providing laboratory facilities. The work was carried out with the aid of a Research Grant (No. C 966/188/B) from the Medical Research Council to Dr. J.S. McKinley-McKee, and was concluded while the author held a University Demonstratorship. I wish to thank Dr. J.S. McKinley-McKee not only for supervision and direction but also for generous provision of chemicals, equipment and literature, and for always being willing to discuss things. I am also grateful to many other members of this Department for their geniality and helpfulness, especially Mr. (now Dr.) Li-Yao Cheng and Mr. David L. Morris.

Dr. D.K. Apps (of this Department) and Professor F. Hütte (München) kindly provided gifts of chemicals. Dr. J.W. Minnis performed the elemental analyses. I also thank Mr. John Kay (and Dr. A.P. Ryle, Mr. Jack McGowan and Mrs. Frances Falla) for assistance with the amino-acid analysis, and Mr. I.A. Nimmo for help with use of the Olivetti Programma P.101 desktop computer. Some of the Figures are reproduced from the European Journal of Biochemistry, with the Editor's permission.

All the experiments described in this thesis were the work of the author alone, except in occasional instances, of which due acknowledgement is made in the text. The whole of the thesis, and also of the papers in the Appendix, were composed by the author alone.
ABBREVIATIONS AND SPECIAL EXPRESSIONS

Non-standard abbreviations have been kept to a minimum. A number are used in equations, tables and figures, but definitions are given. The following are used more generally:

- **ADH** Alcohol Dehydrogenase (tables and figures only)
- **DTNB** 5,5'-Dithiobis-(2-nitrobenzoic acid)
- **PCMB** Parachloromercurobenzoic Acid

The expression "carboxymethylation", unless otherwise stated, refers to specific carboxymethylation of one thiol per subunit (cysteine 46) in liver alcohol dehydrogenase. It does not refer to exhaustive carboxymethylation of all potentially-reactive groups made available by denaturation.
"All things were made through Him, and without Him was not anything made that was made."

John 1. v. 3. (R.S.V.).
CHAPTER 1

Introduction

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1.1. Preface.

The purpose of this chapter is to give a concise account of the present state of knowledge of alcohol dehydrogenase (E.C. 1.1.1.1.) from horse liver. The enzyme has been studied extensively. Reviews by Theorell (1958), Sund and Theorell (1963), McKinley-McKee (1964) and Theorell (1964, 1967, 1970) cover most of the various aspects of the past work. More detailed discussion of previous work will be deferred to the experimental chapters (4-8), or to the General Discussion (Chapter 9).

1.2. Historical.

Alcohol-oxidase activity was observed in 1910 by Batelli and Stern (1910), in various mammalian tissues. Horse liver had the highest activity, and the bulk of the work since then has been performed on enzyme from this source. Quibell (1938) showed that NAD was a cofactor for it, and so did Lutwak-Mann (1938), who also partially purified it by precipitation with acetone or ammonium sulphate. The enzyme was crystallised by Bonnichsen and Wassen (1948). This marked the beginning of over two decades of intensive study on the purified enzyme, first in Theorell's laboratory in Stockholm, and later elsewhere as well.

1.3. Purification, and Isoenzymes.

Bonnichsen and Wassen (1948) used several precipitation
reactions (after initial heat-denaturation) and a final crystallisation from ammonium sulphate (50-55% saturation). Bomrichsen and Brink (1955) modified this, using chloroform-ethanol to remove haemoglobin, followed by precipitation by 30% ethanol, and crystallisation (in more dilute ethanol). Dalziel (1958) reversed the order of the first three steps, and introduced a chromatography step using carboxymethyl-cellulose. This removed an acidic, enzymically-active subfraction, but even after several recrystallisations it did not yield an electrophoretically homogeneous protein. Taniguchi, Theorell and Åkeson (1957) purified it to homogeneity by further chromatography on carboxymethyl-cellulose.

Electrophoresis of liver alcohol dehydrogenase preparations (McKinley-McLee and Moss, 1965) also showed that it contained several ethanol-active components. Pietruszko, Clark, Graves and Ringold (1966) found that steroid 3β-ol dehydrogenase activity of liver alcohol dehydrogenase (Ungar, 1960) was associated with a minor, basic component of the enzyme. The main steroid-active component was purified on carboxymethyl-cellulose, and crystallised from 25% ethanol (Theorell, Taniguchi, Åkeson and Skursky, 1966). As well as steroid dehydrogenase activity, it had about half the normal ethanol activity, and the alcohol and steroid binding-sites appeared to be different and independent. Further work (Pietruszko, Ringold, Li, Vallee, Åkeson and Theorell, 1969; Pietruszko and Theorell, 1969) has shown that steroid activity is due to a different subunit type, the S subunit. Ethanol-active subunits have been named E subunits. Normal ethanol-active enzyme is type EE; the steroid-active isoenzyme described above is type
ES. Another more basic form, SS, has been purified to homogeneity (Theorell, 1970), and has been crystallised (quoted by Jornvall, 1970a). The type SS has a little activity towards ethanol, as well as substantial steroid activity. The S subunit has been found to have a very similar amino-acid sequence to the E subunit (Jornvall, 1969, 1970a).

In addition to these complexes, one or more minor, more acidic components migrate near EE and ES on electrophoresis. These have been named EE', EE", ES', ES", etc. (Pietruszko and Theorell, 1969). Theorell (1970) reported that EE could be converted to EE' and EE", but that EE' could not be converted to EE; he suggested that loss of amide nitrogen was responsible. However, Lutstorf and von Wartburg (1969) showed that EE' and EE" can be converted to EE, and suggested that the different EE types are "conformers" - i.e. have the same primary structure but different conformations of the polypeptide chain.

The experiments described in this thesis were performed on enzyme consisting very largely of the form EE.

1.4. Physical and Chemical Properties.

Horse-liver alcohol dehydrogenase is a globular protein: Theorell and Bonnichsen (1951) found its molecular weight to be 73,000. Ehrenberg and Dalziel (1957, 1958), using enzyme with a higher specific activity, redetermined this: its sedimentation coefficient was 5.11 S (at infinite dilution), its diffusion coefficient was 5.96 F, its partial specific volume was 0.750, and
its molecular weight was 83,300. Later results have shown that the molecular weight is about 80,000 from physical measurements (Green and McKay, 1969) and also from chemical constituents (Jornvall and Harris, 1970).

The ethanol-active enzyme (EE) contains two subunits which are very probably identical. Theorell and Bonnichsen (1951) showed that approximately two molecules of NADH were bound per 73,000 molecular weight units. Two molecules of NADH were bound very tightly in the presence of amides (Winer and Theorell, 1960; Theorell and McKinley-McKee 1961b), and two molecules of NAD$^+$ in the presence of pyrazole (Theorell and Yonetani, 1963). The molecule can be split into separate subunits (with molecular weight of approximately 40,000) by sodium dodecyl sulphate (Blomquist, Smith and Martinez, 1967) and guanidine hydrochloride (Green and McKay, 1969; Butler, Jornvall and Harris, 1969). Harris (1964) showed that two cysteine residues per molecule gave only one labelled peptide with radioactive iodoacetate. Extensive sequence work (Jornvall and Harris, 1970; Jornvall, 1970b) has shown that only one type of polypeptide chain is present, with 374 amino-acids and a molecular weight of approximately 40,000. This shows that the subunits are identical, with one polypeptide chain in each. X-ray crystallography, too, indicates two symmetrically related halves per molecule, after allowing for the solvent content of the crystal (Branden, 1965). There have been reports of liver alcohol dehydrogenase containing more than two subunits (Drum, Harrison and Li, 1967a; Drum, Harrison, Li, Bethune and Vallee, 1967b; Cheng, McKinley-McKee, Greenwood and Hourston, 1968). These results may be due to particularly labile peptide bonds, as found in aldolase (Sine and Hass, 1969); or to contamination by proteolytic enzymes, as found
with yeast hexokinase (Pringle, 1970) and yeast alcohol dehydrogenase (Buhner and Sund, 1969).

The amino-acid composition of the enzyme has been reported by Theorell et al. (1938), and by Cannon and McBay (1969). Each subunit contains 14 cysteine residues, no cystine, 12 arginines, 7 or 8 histidines, 2 tryptophans and 4 tyrosines. The enzyme is quite rich in acidic residues and amides, lysine, proline, phenylalanine, aliphatic amino-acids and hydroxy-amino-acids. The results of sequence studies have been published, with at least half the amino-acids located in cysteine- and tryptophan-containing peptides (Jornvall and Harris, 1970; Jornvall, 1970b). Recently the complete sequence has been announced (Jornvall, 1970c). The differences between the E and the S polypeptide chains have been mentioned above (Section 1.3).

The thiol groups have been studied for reactivity and effect on catalytic activity and coenzyme binding. PCMB reacts with 28 thiol groups per molecule (Witter, 1960), abolishing activity and coenzyme-binding but reacting nonspecifically with essentially all of the thiol groups. A derivative of N-ethylmaleimide (Witter, 1960), iodine (Li and Vallee, 1965) and silver ions (Bonnichsen, 1953; Wallenfels, Sund, Zarnitz, Malhotra and Fischer, 1959) react with 25 to 30% of the enzyme's thiol groups. Iodoacetate (Li and Vallee, 1963, 1964a, 1965; Harris, 1964; Evans and Rabin, 1968) does react specifically with one thiol group per subunit; it was protected by coenzymes and other ligands, and was considered to be in the active centre. This reaction was studied in more detail, and much of this thesis describes the results. Jornvall (1970b) has located the thiol as residue 46 in the sequence. Witter (1960) suggested that thiol groups were probably mainly involved in
maintaining the tertiary structure of the protein. It has not proved possible to reversibly block all the thiol groups, and then release them and regain activity, although this has been done successfully on lactate dehydrogenase (Pfleiderer and Jeckel, 1967). This may be because the alcohol dehydrogenase subunit has 14 thiol groups, while pig heart lactate dehydrogenase has only four. Therefore even if the thiol groups themselves do not play a central role in maintaining the protein's conformation, modifying them will cause a bigger disruption by the groups introduced. More specific roles of thiol groups in the catalytic mechanism of the enzyme have been suggested: see, for example, Evans and Rabin (1968). However, evidence for such mechanisms is slender.

It has been suggested that histidine may be important in dehydrogenase mechanisms (Rabin and Whitehead, 1962; Ringold, 1966; Evans and Rabin, 1968). Photo-oxidation (with Methylene Blue or Rose Bengal) causes changes in activity of liver alcohol dehydrogenase, and also of lactate dehydrogenase (Robinson, Stollar, White and Kaplan, 1963), α-glycerophosphate dehydrogenase (Apitz-Castro and Suarez, 1970) and glyceraldehyde phosphate dehydrogenase (Bond, Francis and Park, 1970). The changes were correlated with a loss of histidine. Two histidine residues were lost in alcohol dehydrogenase without loss of catalytic activity with NAD⁺ or any observed change in secondary or tertiary structure. However, with acetylpyridine adenine dinucleotide, reaction with the first two histidines did curtail catalytic activity. When more than two histidines were modified, the activity was affected — but the tertiary structure was too. Therefore one cannot draw firm conclusions at present about the presence or role of histidine in the active site.
Lysine groups have been maleylated in denatured protein, to stabilise and solubilise separate polypeptide chains (Butler et al., 1969). Pyridoxal phosphate inhibits the enzyme (Cheng, 1970; Morris, 1970). However, Flapp (1970) has found that modification of about three lysine groups per subunit is associated with an increase in catalytic activity; the reaction of these lysine groups, and the effect on activity, is largely abolished in ternary complexes (NAD$^+$ and pyrazole, or NADH and isobutyramide) while most of the rest of the enzyme's lysine groups are still free to react. The enhancement of activity is attributed to faster dissociation of the binary enzyme-coenzyme complexes; at least one lysine group is considered to be near the active centre, although probably not playing a direct rôle in binding or catalysis. Glyceraldehyde phosphate dehydrogenase (Polgar and Harris, 1965), glutamate dehydrogenase (Holbrook and Jeckel, 1969; Freedman and Radda, 1969) and lactate dehydrogenase (Schwert, 1970) have lysine groups which affect activity; but in these cases specialist functions (such as acylation or binding of anionic substrates) are their suggested rôles.

Schellenberg and co-workers have found that a tryptophan can be tritiated when labelled substrates (tritiated at the hydrogen which is transferred) are used, for several dehydrogenases (Schellenberg, 1966, 1967; Chan and Schellenberg, 1967); however, the exact significance of this is not clear, at present.

Apart from amino-acids in the peptide chain (and the N-terminal acetyl groups: Jornvall, 1967), the only other component of liver alcohol dehydrogenase is zinc. Yeast alcohol dehydrogenase contains four atoms of zinc i.e. one per subunit (Vallee and Hoch, 1955). Theorell, Nygaard and Bonnichsen (1955) and Vallee and
Hoch (1957) reported that the liver enzyme contained two atoms of zinc (also one per subunit). However, Åkesson (1964) found that many enzyme samples and several methods of analysing for zinc consistently gave four atoms of zinc per molecule, or two per subunit. This has since been confirmed (Oppenheimer, Green and McKay, 1967; Drum et al., 1967b; Drum, Li and Vallee, 1969a,b). As well as the main component EE, other, minor, components contain four zinc atoms, e.g. ES (Theorell et al., 1966) and what is probably EE' (Sandler and McKay, 1969).

The chemistry and function of zinc in the enzyme has been studied by three main methods:

(a) The effect on the enzyme of known zinc-complexing agents. These are discussed briefly in Section 1.7, and more fully in Chapter 6.

(b) Measuring the rate of zinc-exchange. The zinc in the enzyme can be exchanged with zinc ions in solution, and the rate of this can be measured using $^{65}$Zn. Substrates, inhibitors and coenzymes decrease the exchange rate (Druyan and Vallee, 1964). Most of these experiments were performed before it was realised that each subunit contains two different zinc atoms, presumably in different environments. However, it has been shown that acetate buffer prevents half of the zinc atoms from being exchanged, and the inactivating the enzyme other half can be removed by diethylidithiocarbamate; the zinc atoms stabilised by acetate are labilised by carboxymethylation of the cysteine residue which is reactive towards iodoacetate (Drum et al., 1967b).

(c) Removal of the zinc, and studying the apo-enzyme. The apo-enzyme is inactive but still binds coenzyme (Hoagstrom, Iweibo and Weiner, 1969) and has been crystallised (Branden, Zeppezauer,
Boiwe, Söderlund, Söderberg, and Nordström, 1970); but other experiments with substrates and inhibitors have not yet been reported.

A fourth method has been useful for other zinc metalloenzymes. In carboxypeptidase A (Vallee and Riordan, 1969), carbonic anhydrase (Edsall, 1968; Lindskog, 1966), and alkaline phosphatase (Simpson and Vallee, 1968) the zinc can be replaced by other metals, e.g. cobalt. The resulting enzymes are active but with modified catalytic properties. Furthermore, the spectral properties of cobalt can be used to deduce features of the metal ligands in the enzyme (Dobray-Duclaux and May, 1968). Recently, Drum (1970) has reported that all the zinc in liver alcohol dehydrogenase can be replaced by cobalt or cadmium, giving enzyme with 70% and 28% activity, respectively. Earlier, cadmium derivatives that were inactive were described (Witter, 1960; Druyan and Vallee, 1962). Cobalt can replace part of the zinc of yeast alcohol dehydrogenase when incorporated during biosynthesis (Curdel and Iwatsubo, 1968). Many workers have suggested that a zinc atom is in the active centre of liver alcohol dehydrogenase, and that it binds the substrate, and possibly the nicotinamide of the coenzyme as well (Theorell et al., 1955; Theorell and McKinley-McKee, 1961b,c; Plane and Theorell, 1961; Dalziel, 1963a; Yonetani, 1963a,b; Theorell and Yonetani, 1963; Taniguchi et al., 1967; Sigman, 1967; Evans and Rabin, 1968). However, preliminary X-ray data (Branden et al., 1970) suggest that at least one zinc atom may not be at the active centre.
Alcohol dehydrogenase catalyses the following overall reaction:

\[ R_1-\text{CHOH}-R_2 + \text{NAD}^+ \rightarrow R_1-\text{C(\text{OH})}-R_2 + \text{NADH} + \text{H}^+ \]

The equilibrium constant, \( K \), is given by:

\[ K = \frac{[\text{Aldehyde}] \cdot [\text{NAD}^+] \cdot [\text{H}^+]}{[\text{Alcohol}] \cdot [\text{NADH}]} \]

\( K \) has been measured for ethanol and acetaldehyde (Backlin, 1958). At 20\(^\circ\) and ionic strength 0.1, it is 0.601 \((\pm 0.014)\) \(10^{-11}\). It increases with increasing ionic strength. Therefore at pH values below 11, the equilibrium lies in favour of ethanol.

The enzyme is rather non-specific for the alcohol substrate: a wide variety of primary and secondary alcohols (Winer, 1958), and also cyclic alcohols (Merritt and Tomkins, 1959) serve as substrates. Primary alcohols are in general better substrates the longer the aliphatic chain. The activity of the enzyme with methanol is a disputed question (Winer, 1958; Kini and Cooper, 1961), although formaldehyde can be reduced. Farnesol (Waller, 1965) and Vitamin A (Bliss, 1951) are substrates.

Attempts have been made to use the wide substrate-specificity of liver alcohol dehydrogenase to define the geometry of the substrate-binding site. Dickinson and Dalziel (1957a,b) showed that the (+) isomers of butan-2-ol and octan-2-ol were the preferred substrates, although specificity was not absolute. Prelog (1964) put forward a "lattice theory" suggesting that carbon atoms in substrates could occupy positions in a diamond-like lattice of defined shape and size. Elliott, Jacob and Tao (1969) discussed possible ways in which substrates could be positioned relative to the nicotinamide of the coenzyme, and correlated this with observed reactivities of methyl-cyclohexanols and -cyclohexanones. Graves,
Clark and Ringold (1965) carried out a similar study using substituted cyclohexanol and decalones. Ringold, Bellas and Clark (1967) showed, however, that substrate geometry was not the only important factor: adamantone, which from its geometry should be a good substrate, was in fact a very poor one. They suggested that this was because the enzyme undergoes a conformation-change when the substrate binds.

Coenzyme specificity is also comparatively low. Liver alcohol dehydrogenase is active with a large number of analogues of NAD (see review by Sund and Theorell, 1963). The α-isomer of NAD is inactive. Hypoxanthine can replace adenine (Fawcett and Kaplan, 1962; Shore, 1969). Inserting an extra phosphate moiety between the two ribose residues gives a coenzyme with some activity (Fawcett and Kaplan, 1962). Almost all modifications of the amide of the nicotinamide which preserve a carbon (with π-orbitals) attached to the ring at the 3-position are active: exceptions are β-acrylamido-pyridine and nicotinic acid (Colowick, Van Eys and Park, 1966; Biellmann and Jung, 1970). NADP can also act as a very weak substrate (Dalziel and Dickinson, 1965a).

Liver alcohol dehydrogenase transfers hydrogen stereospecifically to the A-face of the dihydropyridine ring of NADH (Levy and Vennesland, 1957). These experiments also show that the hydrogen atom is itself transferred between coenzyme and substrate, without equilibration with solvent protons.

Crystalline preparations of liver alcohol dehydrogenase usually contain some triose phosphate isomerase activity; however, this can be separated by chromatography with crude carboxymethyl-cellulose (Snyder and Lee, 1966). The separated triose phosphate isomerase has properties very similar to the enzyme from muscle.
It appears therefore that it is not an activity of the alcohol dehydrogenase protein.

Steroid 3β-ol dehydrogenase activity has been shown to be due to certain isoenzymes of alcohol dehydrogenase (see Section 1.3, above).

Liver alcohol dehydrogenase also shows aldehyde mutase activity: two molecules of aldehyde are converted to one each of alcohol and acid. Acetaldehyde or formaldehyde are substrates. This does seem to be a genuine activity of the alcohol dehydrogenase enzyme (Abeles and Lee, 1960). These authors, and also Dalziel and Dickinson (1965b), have suggested that the aldehyde mutase reaction involves hydrated aldehyde which is oxidised similarly to a normal alcohol, while aldehyde-reduction would be of the non-hydrated aldehyde. The apparent $K_m$ for aldehyde in the mutase reaction is higher than for aldehyde reduction. The overall reaction would be:

1. E-NAD$^+$ + hydrated aldehyde = E-NADH + acid
2. E-NADH + non-hydrated aldehyde = E-NAD$^+$ + alcohol

1.6. Kinetics.

In some senses it is creating artificial divisions to include a separate section on kinetics. However, kinetic studies have played an important part in work on liver alcohol dehydrogenase, and contributed to general concepts of enzymology.

Theorell and Bonnichsen (1951) showed that alcohol dehydrogenase can bind NADH, in the absence of aldehyde. Theorell and Chance (1951) suggested the following kinetic mechanism:
\[
E + \text{NAD}^+ \xrightarrow{k_+}{k_-} E\text{-NAD}^+
\]
\[
E\text{-NAD}^+ + \text{alcohol} \xrightarrow{k_2}{k_-} E\text{-NADH} + \text{aldehyde}
\]
\[
E\text{-NADH} \xrightarrow{k_{-1}'}{k_{+1}'} E + \text{NADH}
\]

This mechanism assumes not only that there is a compulsory order of addition of substrates (coenzyme being added first and lost last), but that the concentration of ternary complexes (between enzyme, coenzyme and substrate) is very low. At high concentrations of coenzyme and substrate, the rate-limiting step will be the release of coenzyme-product.

Initial-rate measurements at various concentrations of substrates and products, using both aldehyde reduction and ethanol-oxidation, allow all six rate-constants to be measured, and also checked for constancy and hence for adherence to the Theorell-Chance mechanism (Dalziel, 1957a). Furthermore, direct determination of \( \frac{k_{-1}}{k_{+1}} \) and \( \frac{k_{-1}'}{k_{+1}'} \) (the dissociation-constants of NAD\(^+\) and NADH) under equilibrium conditions (see 1.7) can be made and compared with the kinetically-determined values. Theorell and Chance (1951) found only moderate agreement between their theory and their results. Theorell et al., (1955), in another kinetic study, found quite close agreement with the Theorell-Chance mechanism at high pH, but some discrepancy at lower pH. Theorell and McKinley-McKee (1961a,b,c) found that Theorell-Chance kinetics were approximately followed, but that enzyme-alcohol and enzyme-aldehyde complexes were also formed.
Dalziel (1963b) showed that with ethanol/acetaldehyde and purified coenzymes, the observed initial-rate kinetics were very close to the Theorell-Chance mechanism. Impurities in NAD$^+$ and NADH (Dalziel, 1963c, 1961a, 1962a-c) caused deviations, especially at lower pH values.

Ethanol causes high-substrate inhibition (Theorell et al., 1955; Theorell and McKinley-McKee, 1961c; Dalziel and Shore and Theorell, 1966b). It forms an "abortive" ternary complex with enzyme and NADH, which breaks down more slowly than the binary complex of enzyme and NADH. However, with cyclic secondary alcohols, high-substrate activation is observed at high coenzyme concentrations (Dalziel and Dickinson, 1966a,b), and inhibition at low coenzyme concentrations. They suggested that activation was because NADH dissociated more rapidly from the "abortive" ternary complex than from the enzyme-NADH binary complex; and they attributed inhibition at low coenzymes levels to the existence of an enzyme-alcohol binary complex, which combined more slowly with NAD$^+$ than did free enzyme.

The overall picture, therefore, is one approximating to the Theorell-Chance mechanism, but with a preferred-order rather than compulsory-order of addition of substrates; ternary complexes interconvert quickly, but enzyme-alcohol complexes can exist, and "abortive" complexes can form as well.

As well as steady-state kinetics, pre-steady-state or transient kinetics of liver alcohol dehydrogenase have been studied. Theorell and Chance (1951) measured the rate of combination of NADH with the enzyme using dual-wavelength spectrophotometry. Theorell, Ehrenberg and de Zalenski (1967), using stopped-flow fluorimetric measurements, reported that this reaction took place in two stages; but Geraci and Gibson (1967) found no such effect. Czerlinski
(1962), using temperature-jump experiments on liver alcohol dehydrogenase with NADH and imidazole, obtained evidence for a rearrangement of the enzyme-NADH-imidazole ternary complex.

Shore (1960) measured the rate of formation and breakdown of the enzyme-NADH complex, and also those of two coenzyme analogues. Nicotinamide-modified coenzymes can have higher maximum rates of reduction than the native coenzyme. The Theorell-Chance mechanism predicts that this will be because the breakdown of the enzyme-NADH complex is faster: Shore confirmed this, for acetyl-pyridine adenine dinucleotide. The activation of the enzyme produced by modification of lysine residues (Flapp, 1970; see Section 1.4) was also attributed to an increased rate of breakdown of enzyme-coenzyme complexes. Theorell, Åkeson, Liszka-Kobec and de Zalenski (1970) found good agreement between the measured rate-constants for formation and breakdown of the enzyme-NADH complex, and the observed dissociation-constant of this complex.

Stopped-flow experiments have also shown that the two catalytic sites are kinetically not independent and identical (Bernhard, Dunn, Luisi and Schack, 1970): when substrates are in excess, there is a rapid "burst" of product formation, equivalent to half the number of active sites. When enzyme is in excess over one of the substrates (NADH or aldehyde), the burst is equivalent to half the limiting substrate. Therefore the rate of formation of ternary enzyme-coenzyme-substrate complexes is the same for each site, but the rate of catalysis is not. The second (slower) reaction corresponds to the steady-state rate, and so may control it. Dutler (1970) showed that this "burst", however, depends on which substrates, and what buffer solutions are used.

As well as initial-rate steady-state kinetics, and kinetics of
complex formation and breakdown, some useful work has been done on the kinetics at equilibrium. This method was developed by Silverstein and Boyer (1964). The interconversion of NAD$^+$ and NADH, or alcohol and acetaldehyde, were measured using radioactive tracers under equilibrium conditions at a range of coenzyme and substrate concentrations. Substrate interconversion was always greater than coenzyme interconversion; and, whereas substrate interconversion rose to a maximum, coenzyme interconversion reached a peak and dropped off again as the substrate concentration was raised. The results suggested that coenzyme was preferentially bound before substrate, but were not compatible with a strict compulsory-order mechanism. Recently this has been extended to mixtures including imidazole (Silverstein, 1970), which increases coenzyme turnover and decreases substrate turnover. However, the substrate concentration was very high - sufficient to give much high-substrate inhibition; and imidazole chloride or nitrate was used, and both these ions affect the enzyme (Theorell et al., 1955).

1.7. Ligand Binding.

A large number of compounds - substrates or modifiers - have been shown to bind to liver alcohol dehydrogenase. The effect of most modifiers was observed, in the first instance, on the rate of the enzyme-catalysed reaction. Binding can sometimes be measured directly by using equilibrium dialysis, ultracentrifugation, ultrafiltration or gel-filtration; or indirectly by, for example, protection from denaturation or irreversible inhibitors; or (with
a metallo-enzyme such as liver alcohol dehydrogenase) retardation of exchange of metal-ion (Druyan and Vallee, 1964). However, specific methods are available to study the binding of particular ligands, and are given below: the effect of other ligands on the binding of the specific ligand concerned can also be studied.

(1) NADH. When NADH binds to liver alcohol dehydrogenase, the absorption-spectrum undergoes a blue-shift and a slight decrease in intensity (Theorell and Bonnichsen, 1951); the fluorescence emission is intensified and shifted to shorter wavelengths (Boyer and Theorell, 1956); the polarisation of fluorescence is increased (Weiner, 1968); and an extrinsic Cotton-effect is produced (Ulmer, Li and Vallee, 1961). Of these four methods, the fluorescence methods are the most sensitive, and therefore the most useful, because NADH is bound tightly. This method can also be used for substances which form ternary complexes with enzyme and NADH: the ternary complex may have altered optical properties, compared with the enzyme-NADH binary complex.

(2) Adenine Nucleotides. The absorption-spectrum of the adenine is modified when bound to the enzyme (Theorell and Yonetani, 1964). This has been used to measure the binding of ADP-ribose (Theorell and Yonetani, 1964) and of NAD+ (Taniguchi et al., 1967) to the enzyme. This method is not very sensitive, and so cannot be used to measure very small dissociation-constants. It also requires rather a lot of enzyme, and of care.

(3) NAD+ plus Pyrazole. NAD+ and pyrazole react together on the enzyme surface, perhaps giving an addition-complex, with an optical absorption at 290 nm (Theorell and Yonetani, 1963). Both NAD+ and pyrazole are firmly bound with dissociation-constants of about 0.1 µM. This is useful for measuring the binding-site
concentration, but is of limited use in studying the effect of other ligands on the enzyme. Ternary complexes of enzyme, $\text{NH}_2^+$ and amides have been reported (Sigman and Winer, 1970), which may be similar to pyrazole complexes.

(4) **Spin-Labelled Compounds.** Weiner (1969) studied the effect of a nitroxide-labelled coenzyme analogue on liver alcohol dehydrogenase. He could measure the binding of this to the enzyme in two ways: by the decrease in ESR signal when the analogue bound to the enzyme, and by the effect of free and bound radical on the proton relaxation rate of the water (Mildvan and Weiner, 1969a). The second method could also be used to deduce properties of the water around the bound radical. Perturbation of protons on other ligands bound to the enzyme was also observed, and their distances from the unpaired electron estimated (Mildvan and Weiner, 1969b). Although these studies gave a wealth of information, some of it conflicted with earlier, hitherto accepted data. An alternative but similar approach has been described: a nitroxide radical can be covalently attached to the protein (Griffith and McConnell, 1966), and the resonance properties of this are modified by ligand-binding.

(5) **Halide-Binding NMR Measurements.** The NMR spectra of $^{35}\text{Cl}^-$, $^{79}\text{Br}^-$ and $^{81}\text{Br}^-$, are broadened considerably when bound to proteins (Stengle and Baldeschweiler, 1966). Line broadening is also shown by liver alcohol dehydrogenase (Zeppezauer, Lindman, Forsen and Lindqvist, 1969).

(6) **Metal-Chelating Agents.** Orthophenanthroline binds well to liver alcohol dehydrogenase (Vallee, Coombs and Williams, 1958). Sigman (1967) has used spectrophotometric observations of the binding of $2,2'$-bipyridyl to the enzyme to study the binding of many ligands.

(7) **Fluorescent Dyes.** Anilino-naphthalene sulphonate, Rose
Bengal and Auramine O bind to the enzyme (Brand, Gohlke and Rao, 1967; Turner and Brand, 1968; Conrad, Heitz and Brand, 1970). The fluorescence emission of these dyes is enhanced and undergoes a blue-shift, enabling their binding to be measured.

(8) Iodoacetate. This thesis describes the demonstration and use of reversible binding of iodoacetate. In some ways this is an extension of the work of Evans and Rabin (1968), who used protection or labilisation to study the effects of ligands on the enzyme; however, they did not observe reversible binding of iodoacetate.

Compounds which have been shown (directly or indirectly) to form complexes with liver alcohol dehydrogenase can be classified as follows:

1. **Coenzymes, coenzyme analogues and fragments** (e.g. adenine nucleotides; Theorell and Yonetani, 1964).

2. **Aliphatic fatty-acids** (Winer and Theorell, 1960). These act as "alcohol analogues". They form binary complexes with the enzyme, and also strong ternary complexes with enzyme and NAD\(^+\), but not NADH.

3. **Aromatic fatty-acids**. These include 4-biphenyl-carboxylic acid and phenanthroic acid (Sigman, 1967); salicylic acid (Dawkins, Gould, Sturman and Smith, 1967); and thyroxine and derivatives (McCarthy, Lovenberg and Sjoerdsma, 1968; McCarthy and Lovenberg, 1969; Gilleland and Shore, 1969). These are competitive with coenzyme, but unlike aliphatic acids they do not form ternary complexes with NAD\(^+\). Fluorescent dyes (e.g. anilinonaphthalene sulphonate, see above) should probably be included here as well. Auramine O may be an exception, being cationic.
"Aldehyde analogues." Under this heading it is useful to include all compounds which form complexes of the type enzyme-NADH-I. Amides are perhaps the prime example (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961b,c; Woronick, 1961, 1963a,b). Dimethyl sulphoxide (Perlman and Wolff, 1968) and alcohols (see Section 1.6) also form such complexes. Compounds of this type can be studied using the optical properties of NADH. Woronick (1963a,b) used fluorescence to study a wide variety of amides, which affected the fluorescence of enzyme-bound NADH.

Metal-complexing agents. A number of reagents which could complex with zinc bind to the enzyme. For bidentate ligands, e.g. orthophenanthroline (Vallee and Coombs, 1959) and 2,2'-bipyridyl (Sigman, 1967), spectral changes provide evidence that a zinc atom is chelated. For monodentate bases (Theorell, Yonetani and Sjoberg, 1969), the evidence is less strong. Imidazole and derivatives can stimulate breakdown of the enzyme-coenzyme complex under some conditions; pyrazole and derivatives, and also amides, appear to form adducts with NAD+, as mentioned above. Thiol compounds can also inhibit powerfully (Van Eys, Stolzenbach, Sherwood and Kaplan, 1958; Lambe and Williams, 1965; Cheng, 1970; Morris, 1970), and hydroxylamine (Kaplan and Ciotti, 1954; Kaplan Ciotti and Stolzenbach, 1954) forms adducts with NAD+. All these may well form ligands with a zinc-atom in the enzyme: but so may alcohols, aldehydes, amides, fatty-acids and other anions, so this category is not to be considered exclusive. Further consideration is given to this in Chapter 6.

Miscellaneous. Other compounds known to bind to alcohol dehydrogenase include a variety of anions (Theorell et al., 1955; Plane and Theorell, 1961), folate derivatives (Snyder, Vogel and
The binding properties of each subunit of the dimeric enzyme have usually been found to be independent. Even the ES isoenzyme behaves just like a mixture of EE and SS (Theorell, et al., 1970). However, only one NADH molecule per dimeric enzyme molecule reduced the line-broadening of $^{35}$Cl$^-$ in NMR experiments (Lindman, Zeppezauer and Aheson, 1970); in the presence of isobutyramide, this is increased to two. Högström et al., (1963) reported that the zinc-free (apo) enzyme contained dissimilar binding sites for NADH.

These results, and also the kinetic results discussed above (Bernhard et al., 1970) suggest that subunit interactions may occur.

1.8. Liver Alcohol Dehydrogenase from Other Species

Most of the work on liver alcohol dehydrogenase has used the enzyme from horse, which appears to be the richest source (Batelli and Stern, 1910; Krebs and Perkins, 1970). It has also been purified and crystallised from human liver (von Wartburg, Bethune and Vallee, 1964; Mourad and Woronick, 1967), and highly purified from rhesus monkey (Papenberg, von Wartburg and Aebi, 1965). Partial purifications have been reported from rat (Merritt and Tomkins, 1959; Lambe and Williams, 1965), fish (Boeri, Bonnichsen and Tosi, 1955), rabbit and lamb (Bliss, 1951). The horse enzyme appears to be more stable than the enzyme from other sources.

The human enzyme is similar to the horse one in specific activity (Mourad and Woronick, 1967); in being heterogeneous (Blair and Vallee, 1966); in molecular weight ( provisionally 87,000) and in containing zinc (von Wartburg et al., 1964). However, some humans
can contain an atypical alcohol dehydrogenase, with a higher activity and a lower pH-optimum (von Wartburg, Pupenberg and Aebi, 1965).

Apart from liver, alcohol dehydrogenase is found in several other tissues, but the only one with appreciable activity is kidney; each tissue seems to have a different pattern of isoenzymes (von Wartburg and Pupenberg, 1966). The enzyme found in gastric mucosa may be of bacterial origin (Krebs and Perkins, 1970).

1.9. Physiological Significance.

Although liver alcohol dehydrogenase has been studied so extensively, and exists in high concentration in liver cytoplasm (Nyberg, Schuberth and Angard, 1953), no obvious physiological rôle for it was apparent. Ethanol is not usually a significant content of the diet of mammals (except in the recent history of "civilised" man). It can occur in fermented fruit in tropical countries; very small quantities are produced in peripheral tissues (McManus, Contag and Olson, 1966) and it can also be produced by bacterial fermentation in the intestines (Krebs and Perkins, 1970). Farnesol oxidation (Waller, 1965) may be catalysed by alcohol dehydrogenase, but this would require perhaps 6% of the alcohol dehydrogenase activity actually found. Vitamin A can be oxidised by the enzyme, and a similar enzyme is involved in retinol oxidation in the eye (Koen and Shaw, 1966). Alcohol dehydrogenase has also been implicated in steroid demethylation (Okuda and Takigawa, 1968). Indeed the broad substrate specificity for large primary and secondary alcohols (Winer, 1958) suggests that the enzyme might be important in many
detoxification reactions. However, it is not obvious why these processes should require the high concentrations of the enzyme found in liver tissue (in horse, 20 μM; Waller, Theorell and Sjovall, 1953). Krebs and Perkins (1970) consider that ethanol oxidation is the (or the major) physiological function of liver alcohol dehydrogenase.

A different suggestion for the high content of alcohol dehydrogenase in liver has been put forward by Reynolds (1970). Most of the intracellular NADH is enzyme-bound (Bucher and Klingenberg, 1958; Bucher, 1970). Although alcohol dehydrogenase is not the only major dehydrogenase present, it could contribute significantly to the binding of NADH; this would help to buffer the concentration of cytoplasmic NADH in liver. Because of the strong complexes formed with fatty-acids, and with AMP, the buffering range could alter in the same way that the NADH concentration alters, under different physiological conditions. Unfortunately, it is beyond the scope of this thesis to discuss such theories in more detail.

1.10. Non-Mammalian Alcohol Dehydrogenases.

Alcohol dehydrogenase from brewer's yeast was the first nicotinamide-nucleotide-linked dehydrogenase to be crystallised (Negelein and Wulff, 1937). The enzyme from baker's yeast (Racker, 1950, 1955) has however attracted much more investigation: see the review by Sund and Theorell (1963). It is simple to prepare, and like the brewer's yeast enzyme is much more active than the liver enzyme. It has a molecular weight of 140-150,000 (Hayes and Velick, 1954; Buhner and Sund, 1969) and probably contains four subunits, each of molecular weight 36,000 (Ohta and Ogura, 1965;
Each subunit contains one zinc atom (Vallee and Hoch, 1955) and one coenzyme-binding site (Hayes and Velick, 1954). The substrate specificity is rather low, but in general it is more specific for smaller alcohols (except methanol) than is the liver enzyme (Dickinson and Dalziel, 1967a,b).

It might appear that the yeast enzyme is similar to a dimer of liver enzyme molecules. However, there are many significant differences. The yeast enzyme is inhibited by orthophenanthroline at much higher concentrations than is the liver one, and appears not to chelate the zinc atom (Anderson, Reynolds and Anderson, 1966).

Thiols (e.g. mercaptoethanol) are powerful inhibitors of the liver enzyme but not of the yeast enzyme (Cheng and McKinley-McKee, 1968; Cheng, 1970). The kinetic mechanism of the yeast enzyme is in dispute, but it is unlikely to be Theorell-Chance. Hayes and Velick (1954) and Nygaard and Theorell (1955a,b) suggested that it was a random-order mechanism, with the rate-limiting step being interconversion of the ternary complexes. Dalziel (1957a), however, pointed out that Nygaard and Theorell's data were equally consistent with a compulsory order mechanism, with the interconversion of ternary complexes being rate-limiting. Wratten and Cleland (1963), using product inhibition, claimed that the mechanism was clearly compulsory-order. Silverstein and Boyer (1964), however, using their equilibrium-rate determination method, claimed to have excluded interconversion of ternary complexes as the rate-determining step.

Another point of difference with the liver enzyme concerns the thiol groups. The yeast enzyme contains eight or nine per polypeptide chain (Wallenfels and Sund, 1957a). One is very reactive towards iodoacetate and iodoacetamide (Rabin and Whitehead, 1962; Hashed and Rabin, 1968) - much more reactive than in the liver.
enzyme. Crystalline preparations of the liver enzyme usually have constant, maximal specific activity, and a constant number of thiol groups (28). However, the yeast enzyme varies from preparation to preparation in both activity and thiol content, although the two are related (Wallenfels and Sund, 1957a). The yeast enzyme is much more unstable, although it is not clear how much this is due to proteases (Buhner and Sund, 1969). It is considerably protected by thiol-containing compounds (Atkinson, Eckermann and Lilley, 1967). These problems help to explain why the liver enzyme is better understood than the yeast one.

Recently, multiple forms of yeast alcohol dehydrogenase have been reported (Schimpfessel, 1968; Lutstorf and Megnet, 1968). Probably, different enzymes are biosynthesised for alcohol-production in fermentation, and alcohol-utilisation as carbon-source.

Alcohol dehydrogenase also occurs in plants, particularly embryos as they germinate, which may be anaerobic, such as barley (Duffus, 1968), wheat (Stafford and Vennesland, 1953), rice (App and Meiss, 1958), pea (Cossins, Kopala, Blawacky and Spronk, 1968) and corn (Hageman and Flesher, 1960). The enzyme is also found in latex (d'Auzac and Jacob, 1968).

Drosophila contains alcohol dehydrogenase, which has been purified (Sofer and Ursprung, 1968).

Many micro-organisms contain alcohol dehydrogenase, including *E. coli* (Still, 1940), *Leuconostoc* (DeMoss, 1955) and Neurospora (Nason, Kaplan and Colowick, 1951). *Pseudomonas* contains an NAD-linked, unstable benzyl-alcohol dehydrogenase when grown on toluene as carbon-source (Suhara, Takemori and Katagiri, 1969): *Pseudomonas* can also contain a non-NAD-linked alcohol dehydrogenase,
with no metal component, and a prosthetic group which is probably a pteridine derivative (Anthony and Zatman, 1967a,b).

The above only represent a sample of sources of alcohol dehydrogenase. Others are given in Sund and Theorell (1963).
### Materials and Methods

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2.1. Materials.

Enzymes. Most of the liver alcohol dehydrogenase used was purchased from C.F. Boehringer und Soehne (Mannheim), who had prepared it by the method of Dalziel (1958). However, some of the earlier experiments, described in Chapter 4, used enzyme prepared in Stockholm by Dr. McKinley-McKee, by the method of Bonnichsen and Brink (1955). In either case, enzyme was stored as a crystalline suspension (8 mg/ml) in 30% ethanol at -14°C. Before use, crystals from approximately 1.5 ml of suspension were collected by centrifuging, dissolved in 1 ml glycine buffer (pH 10) and dialysed for 3 days at 0 - 4°C in Visking dialysis tubing (which had been soaked in distilled water), against 3 changes (1.7 litres each) of 40 mM phosphate buffer (ionic strength 0.1) (pH 7.4). The enzyme was centrifuged to remove a small amount of denatured, precipitated protein.

Yeast alcohol dehydrogenase was obtained from C.F. Boehringer und Soehne (Mannheim), as a crystalline suspension (30 mg/ml) in ammonium sulphate, and stored frozen at approximately -20°C. Freshly purchased enzyme was used except where otherwise stated. The crystals were collected by centrifuging, dissolved (in 40 mM phosphate, pH 7.4) and dialysed against two changes of 10 mM phosphate (pH 7.4) at 0 - 4°C for two days, and centrifuged before use. Sometimes, bacteria grew rapidly in the dialysis buffer as judged from the odour produced. This may be due to proteolysis (Buhner and Sund, 1969) providing peptides and amino-acids for bacteria to utilise.

The activity and purity of the enzyme preparations are discussed in Section 2.4.
Coenzymes, Nucleotides and Nucleosides. NAD\(^+\) (used mainly in the enzyme assays) was obtained from Sigma Chemical Co. (St. Louis), C.F. Boehringer und Soehne (Mannheim), and P.-L. Biochemicals (Milwaukee). No differences in activity were observed. Morris (1970) found that most of the samples from P.-L. Biochemicals contained alcohol. This was serious when doing pyrazole titrations, coenzyme from this source was not used for them; but it was quite satisfactory for enzyme assays in an excess of ethanol.

NADH, 3',5'-cyclic AMP, ADP, ATP and adenosine were obtained from Sigma Chemical Co. (St. Louis). AMP and ADP-ribose were obtained from Sigma Chemical Co. (St. Louis) and from P.-L. Biochemicals (Milwaukee). The concentration of NADH was determined from absorbance at 340 nm (millimolar extinction coefficient = 6.22; Horecker and Kornberg, 1948); and of the adenine nucleotides and adenosine, and GMP, from the absorbance at 260 nm. The millimolar extinction coefficient for adenine was taken to be 15.4 (Morell and Bock, 1954), and for guanine, 11.8 (Volkin and Cohn, 1954).

Sephadex. Sephadex G-25 (Medium) was obtained from Pharmacia (Uppsala).

Other Chemicals. Other chemicals were the purest commercially available, and were used without further purification. Exceptions were as follows:

Iodoacetate (for the majority of the experiments) was recrystallised (by Dr. J.S. McKinley-McKee or by the author) from 60°–80° petroleum ether. It was white, with melting-point 81.1–81.7°, and iodine content 68.26%. In some experiments it was used as obtained from Sigma Chemical Co. It was also white, with iodine
content 68.25%. (Elemental analysis of iodoacetate and iodoacetamide was kindly performed by Dr. J.W. Minnis.)

Iodoacetamide was recrystallised (by Dr. J.S. McKinley-McKee) from ethanol-water and water. It was white, with melting-point 92.2-92.4°, and iodine content 68.30%.

3-Iodopropionate (Eastman) was recrystallised from water.

PCMB was dissolved at neutral pH and precipitated twice with acid (by Dr. J.S. McKinley-McKee), as described by Boyer (1954).

Imidazole and isobutyramide were recrystallised by Dr. J.S. McKinley-McKee (Theorell and McKinley-McKee, 1961b).

4-Biphenyl-carboxylic acid (Fluka AG, Buchs SG) was recrystallised from methanol and methanol-water (by Dr. J.S. McKinley-McKee).

Bromoacetil-pyridine hydrobromide was a generous gift from Dr. D.K. Apps. It was prepared by brominating 3-acetylpyridine, and recrystallised from pyridine.

4-Bromo- and 4-iodo-pyrazole were generous gifts from Prof. R. Huttel (Munchen).

**Distilled Water.** Tap-water was distilled in a Manesty still, and then redistilled in an all-glass apparatus. Its pH was normally between 5.5 and 6.

**Glassware.** Glassware was usually cleaned with "chromic acid" (chromic oxide in concentrated sulphuric acid), soaked in water, and rinsed several times in distilled water before being oven-dried at 70° (for non-volumetric glassware). Carlsberg micropipettes were used (1-300 µl), and cleaned by filling with chromic acid, and then soaked in water. Then distilled water was sucked through followed...
by air to dry.

2.2. Solutions.

Buffer Solutions. These were made up as follows:

Phosphate, pH 7.37. This was made up with 10 mM NaH₂PO₄ and 30 mM Na₂HPO₄, giving an ionic strength of 0.100. Other ionic strengths at this pH were made up *pro rata*.

Phosphate, pH 6.0. This was made up with 70 mM NaH₂PO₄ and 10 mM Na₂HPO₄, giving an ionic strength of 0.1.

Phosphate, pH 8.0. This was made up with 2 mM NaH₂PO₄ and 31.7 mM Na₂HPO₄, giving an ionic strength of 0.1.

Tris-phosphate, pH 7.0. A solution of NaH₂PO₄ (to give 34.5 mM finally) was brought to pH by adding tris (free base), giving an ionic strength of 0.1. Other ionic strengths were made up *pro rata*.

Phosphate, pH 7.9. This was made up with 2.4 mM NaH₂PO₄ and 31 mM Na₂HPO₄, giving an ionic strength of 0.1. Other ionic strengths were made up *pro rata*.

Tris-phosphate, pH 8.6. This was made up as for pH 7.9, but the concentration of phosphate was 33 mM.

Glycine-NaOH. If a given glycine concentration was required (e.g. for enzyme assay solutions), glycine was weighed out, dissolved in water, and brought to pH by adding a solution of 2M NaOH. If a given ionic strength was required (taken to be equal to the concentration of NaOH added, i.e. ignoring the zwitterion), then 2M NaOH was pipetted out, diluted, and brought to pH by dissolving solid
glycine. Unless otherwise stated, the buffer at pH 9.35 (ionic strength 0.1) contained 36 mM glycinate anion, with phosphate and other indicated anions making up the ionic strength. At pH 10.0 (ionic strength 0.1), glycinate anion was 15–20 mM, with phosphate and other indicated anions making up the ionic strength.

PCMB. This was weighed out, dissolved in water with two drops of 2M NaOH, neutralised with NaH₂PO₄, and any faint precipitate redissolved by adding a trace more NaOH. The concentration was determined by absorbance at 232 nm (millimolar extinction coefficient = 16.9; Boyer, 1954).

Decanoate and 4-Biphenyl-Carboxylic Acid. Weighed samples were dissolved in water with two drops of 2M NaOH, and brought to the required volume with either water or phosphate buffer.

NAD⁺ and NADH. NAD⁺ was dissolved in water, giving an acidic solution (pH 3.4), in which it is stable for several days. NADH was dissolved in glycine-NaOH buffer, pH 10, diluted with water, and used within 2–3 days.

Iodoacetate and 3-Iodopropionate. It was necessary to neutralise these reagents so that they would not significantly affect the pH of solutions, and give known ionic strength contributions. Neutralised, 0.20M solutions were used, usually 5 ml being made up. Weighed samples were dissolved in water, and carefully neutralised with 2M NaOH to pH 4.7–5.3 for iodoacetate; since the pKₐ for iodoacetic acid is 3.12, this represents 95–99% neutralisation.
3-Iodopropionate would not dissolve completely until it had been largely neutralised: approximately pH 6 was used. Any samples that were over-neutralised were discarded, since iodoacetate hydrolysates at higher pH values.

2.3. Instruments.

pH. Measurements of pH were made on a Cambridge bench-type pH-meter, with a glass electrode and a reference calomel electrode.

Spectrophotometry. Spectrophotometric measurements were all made using at least 2.5 ml of solution in 1 cm cells. Only quartz cells were used below 340 nm. A Hilger Uvispek spectrophotometer or a Gilford Model 2000 recording spectrophotometer coupled to a Unicam SP 500 monochromator were used. Both had thermostatted sample-chambers. The temperature was 23.5°.

Fluorimetry. A recording spectrophotofluorimeter (Farrand Optical Co. Inc., New York) with grating monochromators for both excitation and emission was used. The light source was a 150-watt Xenon-arc lamp. Silica cells (1 cm-square), with approximately 3 ml of solution were used. The sample-chamber was thermostatted at 23.5° in all experiments.

Amino-Acid Analysis. A Locarte automatic amino-acid analyser was used.
Assay of Liver Alcohol Dehydrogenase. The protein concentration was
determined from its absorbance at 280 nm. A solution of 1 mg/ml has
an absorbance of 0.42 (Dalziel, 1958) or 0.455 (Bonichsen, 1950;
Taniguchi et al., 1967). In the present work, a value of 0.45 was
assumed, and a molecular weight of 84,000 (Section 1.4).

The catalytic activity of the enzyme was assayed as described
by Dalziel (1957b). The absorbance at 340 nm of NADH produced in a
1 cm cell was observed. The cuvette contained 1.0 ml NAD+
0.47 m/l in assay cuvette
(1 mg/ml), 150 μl ethanol (95% ethanol diluted 100-fold), and 1.85 ml
glycine-NaOH buffer (glycine 0.1M) pH 10.0, at 23.5°C. The assay was
started by stirring in a small volume of enzyme on a glass rod, and
the time for an increase in absorbance of 0.2 units (t0.2) was
determined: this is proportional to enzyme activity (Dalziel, 1957b).

The enzyme concentration in the added solution was calculated as
follows:

\[
\text{Enzyme (μM)} = \frac{1.13}{t_{0.2}} \cdot \frac{1}{84,000} \cdot \frac{3.00 + v}{3.00} \cdot 0.83
\]

where \( v \) = volume of enzyme added (in ml). The first term gives the
concentration in mg/ml (Dalziel, 1958). The second term converts
to molarity, by dividing by the molecular weight. The third term
allows for dilution of the NADH produced, by the volume of enzyme
added. The fourth term (0.83) was introduced to bring the values
into line with the concentration of NADH-binding-sites (Theorell and
McKinley-McKee, 1961a).

The concentration of coenzyme binding-sites was determined, in
some samples, by fluorescence titration with NADH in the presence of
excess isobutyramide (Theorell and McKinley-McKee, 1961b); or by
titration with NAD$^+$ in the presence of pyrazole (Theorell and Yonetani, 1963).

Compared with the concentration determined from absorbance at 280 nm, activity gave an apparent purity of 83-94%, but most samples were 88-93%. Binding-sites concentration (assuming 2.0 per molecule) gave an apparent purity of 90-100%.

**Assay of Yeast Alcohol Dehydrogenase.** The absorbance at 280 nm was used to determine protein concentration, a solution of 1 mg/ml having an absorbance of 1.26 (Hayes and Velick, 1954). A molecular weight of 150,000 was assumed (Hayes and Velick, 1954).

The catalytic activity was determined from the initial rate of production of NADH (measured by increase in absorbance at 340 nm). The cuvette contained, in 3 ml, 1 mg NAD$^+$, 0.2M ethanol and 57 mM glycine buffer, pH 8.8. The highest activity found was 230 international units per mg.

**Determination of Thiol Content in Proteins.**

**PCMB.** Thiol groups in liver and yeast alcohol dehydrogenases react rapidly with PCMB (Wallenfels and Sund, 1957a; Witter, 1960; Dalziel, 1961b). Therefore, they could be titrated, and the formation of the mercury-mercaptide bond monitored at 255 nm (Boyer, 1954). Aliquots of PCMB were added both to the solution containing enzyme, and to the spectrophotometer blank. This method is accurate and widely used, but is rather tedious. It was used in this study mainly for yeast alcohol dehydrogenase.

**DTNB.** The method used was that of Ellman (1959). Thiol groups give disulphide exchange with DTNB, and the liberated thiophenylate ion has a millimolar extinction coefficient of 13.6
cm$^{-1}$ at 412 nm; pH 8.0 or 7.4 was used, the reaction proceeding somewhat faster at pH 8.

Since DTNB reacted rather slowly with the alcohol dehydrogenases (see Chapter 4), it was necessary to use a denaturing agent. In 5M guanidine hydrochloride, pH 8, liver alcohol dehydrogenase appeared to react in about five seconds, and yeast alcohol dehydrogenase in about ten seconds. However, the yellow colour produced was seen to fade rapidly (half-time 14 min.), presumably due to aerobic oxidation of the thiophenylate ion, catalysed by impurities (perhaps metal ions) in the guanidine hydrochloride. For this reason, other denaturing agents were sought. The fading reaction occurred slowly under most conditions, and necessitated fairly rapid denaturation of the enzymes to give a reliable estimate of thiol content. Urea (8M) was ideal for liver alcohol dehydrogenase, the reaction with DTNB reaching completion after 4 min. However, it was too slow a denaturant for the yeast enzyme. Sodium dodecyl sulphate was too slow with each enzyme. Therefore, PCMB was used for thiol determinations on the yeast enzyme, and DTNB in 8M urea for the liver enzyme.

Another problem was that iodoacetamide (but not iodoacetate) appeared to react with the liberated thiophenylate ion, producing fairly rapid fading. Instead of 8M urea as denaturant, a mixture of 2.5M guanidine hydrochloride and 4M urea was used, with liver enzyme. This gave a maximal absorbance at 412 nm after 30 sec.; although subject to a certain systematic error, it still proved a useful method of monitoring enzyme thiol content.

Inactivation Experiments. These were carried out in 1 cm quartz or glass cuvettes, total volume 3 ml. Buffer and ligands being
studied were added first, and the cuvette kept at 23.5°. Then enzyme was added and stirred with a glass rod, and finally the inactivating agent added and stirred, and the stop-clock started. Aliquots were withdrawn for activity assay as described above. Usually, the range 100-40% activity (or lower) was followed, with usually six (or more) assays. DTNB itself absorbs strongly at 340 nm; iodoacetamide inhibits the assay (Woronick, 1961). Both these effects will be constant for a particular inactivation.

The results were plotted on semi-log graph-paper (see Fig. 5.1) which gave a linear graph except where otherwise stated. The half-time, and hence the rate-constant of inactivation (= 0.693/half-time) were determined from this.

When DTNB was the inactivating agent, direct measurement of absorbance at 412 nm gave the concentration of reacted thiol groups. With PCNB, this was taken to be equal to the concentration of PCNB added. With iodoacetate and iodoacetamide, aliquots were removed and added to DTNB + 8M urea, or to 4M urea–2.5M guanidine-HCl, as described above, to give the unreacted thiol content. (The concentration of iodide ion, and therefore of reacted alkylating agent, can be followed using the iodide electrode: Rabin, Ruiz Cruz, Watts and Whitehead, 1964; Evans and Rabin, 1968.)

Preparation of Carboxymethylated Liver Alcohol Dehydrogenase.
Dialysed samples of native enzyme (1 ml, 80-110μM) were alkylated with iodoacetate (4–6 mM) at room-temperature (approx. 20°). Advantage was taken of the enhanced sensitivity of the enzyme to iodoacetate conferred by imidazole (Evans and Rabin, 1968), and 1-4 mM imidazole was added to the enzyme. Since it is probable that only the "essential" thiol is rendered more sensitive to alkylation,
side-reactions with other residues in the enzyme should be minimised. After 1-2 hours, when the remaining activity was 2-2.5% (see Chapter 7), imidazole, iodoacetate and iodide were removed by dialysis, or by passage through a column (1.4 x 12 cm) of Sephadex G-25.

**Fluorescence Titrations.** As far as possible, conditions were used which exactly duplicated those for inactivations. Buffer, ligands and enzyme, total volume 3 ml, were placed in the Farrand spectrophotofluorimeter sample-chamber, thermostatted at 23.5°. Excitation was at 325-335 nm (isobestic point = 328 nm: Theorell and Bonnichsen, 1951), and emission was usually measured at 410 nm. The spectrophotofluorimeter had four slits - before and after each monochromator. For scanning spectra, these were of 10, 5, 10, and 5 nm (in order in the light-path). For dissociation-constant determinations, they were 20, 20, 10 and 10 nm.

Aliquots of NADH solution (usually giving concentration increases of 10 x 0.06 μM, 7 x 0.19 μM, and 5 x 1.2 μM) were added on a glass rod, and the fluorescence monitored continuously on the recorder. The fluorescence of a perspex standard was measured immediately before and after each titration, to check that the fluorescence remained constant. The standard was never left in the light-path, as the fluorescence tended to decrease with time. Sudden decreases of light intensity were noticed, on occasions; when this happened during a titration, the results were discarded.

The method of calculation is described and discussed in Chapter 3, Section 3.4.
Amino-Acid Analyses. Protein samples were hydrolysed for 24 hours in approximately 6N HCl at 105°, after freezing and thawing, and sealing under vacuum, to remove oxygen. Automatic amino-acid analysis was carried out by the accelerated method of Spackman, Moore and Stein (1958) on 1–2 mg protein, in the Locarte amino-acid analyser, with the assistance of Mr. John Kay and Mr. Jack McGowan.
Chapter 3

Theoretical

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In Chapter 5, it is shown that the inactivation of liver alcohol dehydrogenase by iodoacetate has kinetics of the Michaelis-Menten type (Michaelis and Menten, 1913). By assuming a general model for this, involving reversible binding of iodoacetate, an equation will be derived, which is used in Chapter 5.

The model.

Let $E$ represent enzyme (alcohol dehydrogenase).

"I" inhibitor (iodoacetate).

"EI" the reversible complex of $E$ and I.

"K" the dissociation-constant of EI.

"E'-I and E"-I represent irreversibly-inactivated enzyme.

"k'" represent the first-order rate-constant for the reaction between $E$ and bound I.

Let $k''$ and $k_z$ represent the second-order rate-constants for the reactions of $E$ and EI respectively with molecules of I in free solution.

Let brackets indicate concentrations.

"$E'_t$" represent free (uncomplexed) enzyme, and $E_t$ total enzyme (but excluding irreversibly-inactivated enzyme).

As shown in the scheme above, a reversible complex between
E and I is postulated; but no particular assumptions are made regarding the rates of inactivation, except that they obey the Law of Mass Action.

Now, \( [I] \cdot k_s \ll k' \) and \( k_s \ll k'' \); otherwise, Michaelis-Menten kinetics would not be obeyed. In addition, it will be assumed that \( k' \) and \( k'' \) are too small to upset the equilibrium between \( E \) and \( EI \).

The rate of formation of \( E'-I \), therefore, will be:

\[
k'' \cdot \left[ \frac{[E_t] \cdot [I]}{K + [I]} \right] + k' \cdot [I]
\]

Now, \( \left[ \frac{[E_t] \cdot [I]}{K + [I]} \right] \)

and \( \frac{[E_t] \cdot K}{K + [I]} \)

\[
\therefore \text{Inactivation rate} = \left[ \frac{[E_t] \cdot [I]}{K + [I]} \right] \cdot (k'' \cdot X + k')
\]

-(1.1)

Since \( [E_t] \) will be continuously decreasing as inactivation proceeds, one may divide both sides by \( [E_t] \) to give the rate-constant of inactivation, \( v \):

\[
v = \frac{[I]}{K + [I]} \cdot (k'' \cdot X + k')
\]

-(1.2)

This equation is used and discussed in Chapter 5. Its derivation has been summarised previously (Reynolds and McKinley-McKee, 1959), and is similar to one given by Baker (1967).
3.2. Binding of an Effector (which Is Not a Complete Inhibitor) to an Enzyme.

In Chapter 6, it is shown that imidazole increases the rate of reaction of liver alcohol dehydrogenase with iodoacetate. The kinetics seem to be analogous to enzyme kinetics in the presence of a modifier which alters both $K_M$ and $V_{max}$.

Consider an enzyme, with one substrate, where both substrate and modifier can bind reversibly to the enzyme:

\[
\begin{align*}
E & \xleftrightarrow{K_{E,S}} ES \\
K_{E,M} & \xleftrightarrow{K_{ES,M}} E + Products \\
EM & \xleftrightarrow{K_{EM,S}} EMS \\
K_{EM,S} & \xleftrightarrow{K_{EM,S}} E + M + Products
\end{align*}
\]

The equilibria between enzyme complexes will be assumed to be maintained.

Let $E$ represent enzyme (one active centre).
" $S$ " substrate.
" $M$ " modifier.
" $V_1$ " maximum velocity in the absence of modifier.
" $V_2$ " maximum velocity with an infinite concentration of modifier.

Let brackets indicate concentrations.
" $ES$, $EM$ and $EMS$ represent reversible complexes.
" $E_1$ represent free (uncomplexed) enzyme, and let $E_t$ represent total enzyme.

Let us define the following dissociation-constants:

\[
K_{E,S} = \frac{[E_1] \cdot [S]}{[ES]}
\]
\[
K_{E, M} = \frac{E_r \cdot [I]}{[E_t]}
\]

\[
K_{EM, S} = \frac{[E_r] \cdot [S]}{[E_t] [S]} = \frac{[M] \cdot [S] \cdot [E_r]}{K_{E, M} \cdot K_{EM, S}}
\]

Since enzyme concentration is conserved,

\[
[E_t] = [E_r] + [ES] + [EM] + [EM, S]
\]

Substitution of concentrations of enzyme complexes by use of functions of the dissociation-constants defined above, gives:

\[
\frac{[ES]}{[E_t]} = \frac{1}{1 + \frac{[S]}{K_{E, S}} + \frac{[M]}{K_{E, M}} + \frac{[M]}{K_{EM, M}} \cdot \frac{[S]}{K_{EM, S}}}
\]

The observed rate of the enzyme-catalysed reaction, \( v \), will be:

\[
v = \frac{[ES]}{[E_t]} \cdot v_1 + \frac{[EM]}{[E_t]} \cdot v_2
\]

Now,

\[
\frac{[ES]}{[E_t]} = \frac{[S]}{K_{E, S}} \cdot \frac{[E_r]}{[E_t]}
\]

and

\[
\frac{[EM]}{[E_t]} = \frac{[M]}{K_{E, M}} \cdot \frac{[S]}{K_{EM, S}} \cdot \frac{[E_r]}{[E_t]}
\]

\[
\therefore \quad v = \left\{ \frac{v_1 \cdot [S]}{K_{E, S}} + \frac{v_2 \cdot [M]}{K_{E, M}} \cdot \frac{[S]}{K_{EM, S}} \right\}
\]

\[
\therefore \quad v = \frac{v_1 \cdot [S] + v_2 \cdot [M] \cdot [S]}{1 + \frac{[S]}{K_{E, S}} + \frac{[M]}{K_{E, M}} + \frac{[M]}{K_{EM, M}} \cdot \frac{[S]}{K_{EM, S}}}
\]

However, a convenient way of estimating \( K_{E, M} \) is to determine \( v \) at a series of values of \([M]\). To be useful for this, Equation (2.2) requires rearranging. Cross-multiplication, and
collection of the terms containing $\frac{[M]}{K_{E,M}}$, gives:

$$\frac{[M]}{K_{E,M}} \cdot \left\{ v(1 + \frac{[S]}{K_{E,M,S}}) - v_{2} \cdot \frac{[S]}{K_{E,M,S}} \right\} = v_{1} \cdot \frac{[S]}{K_{E,S}} - v(1 + \frac{[S]}{K_{E,S}})$$

$$\therefore K_{E,M} = \frac{V_{1} \cdot \frac{[S]}{K_{E,S}} - v(1 + \frac{[S]}{K_{E,S}})}{v(1 + \frac{[S]}{K_{E,M,S}}) - v_{2} \cdot \frac{[S]}{K_{E,M,S}}}$$

- (2.3)

The above derivation has been published (Reynolds, Morris and McKinley-McKee, 1970). A more general derivation, under steady-state conditions (rather than equilibrium conditions assumed above), has been given by Reiner (1969).

3.3. **Mutual Interaction on an Enzyme of Two Inhibitors, One Being Competitive and One Non-competitive with Substrate**

An analysis has been given (Yonetani and Theorell, 1964) describing the mutual interaction of two inhibitors, each of which is competitive with substrate. In Chapter 6, the interaction of AMP and orthophenanthroline is examined. Although AMP is competitive with iodoacetate (the "pseudo-substrate"), orthophenanthroline is approximately non-competitive. Therefore, an analysis similar to that of Yonetani and Theorell (1964) is given here, only for one substrate-competitive and one substrate-noncompetitive inhibitor.

Equilibrium conditions will be assumed, in the binding of ligands (including substrate) to the enzyme.
Let $I_c$ represent a substrate-competitive inhibitor.

" $I_{nc}$ " " " -noncompetitive inhibitor.

" $E$ " enzyme (one active centre).

" $S$ " substrate.

" $V_{max}$ " maximum velocity (infinite substrate, no inhibitors).

Let brackets indicate concentrations.

" $ES$, $EI_c$, $EI_{nc}$, $EI_cI_{nc}$ and $ESI_{nc}$ represent reversible complexes.

" $E_f$ and $E_t$ represent free (uncomplexed) enzyme and total enzyme, respectively.

Let us define the following dissociation-constants:

$$K_s = \frac{[E_f] \cdot [S]}{[ES]}$$

$$K_c = \frac{[E_f] \cdot [I_c]}{[EI_c]}$$

$$K_{nc} = \frac{[E_f] \cdot [I_{nc}]}{[EI_{nc}]} = 1. \frac{[EI_c] \cdot [I_{nc}]}{[EI_{c nc}]} = \frac{1}{\beta}. \frac{[ES] \cdot [I_{nc}]}{[ESI_{nc}]}$$

In these equations, $\alpha$ represents the interaction constant between the two inhibitors; when one is bound to the enzyme, the other is bound with a dissociation-constant $\alpha$ times that with free enzyme. $\beta$ represents, analogously, the interaction constant between $I_{nc}$ and substrate. If $I_{nc}$ is a perfectly non-competitive inhibitor, then $\beta = 1$. If it is partially uncompetitive, $\beta < 1$, and if partially competitive, $\beta > 1$.

Conservation of total enzyme concentration requires:

$$[E_t] = [E_f] + [ES] + [ESI_{nc}] + [EI_c] + [EI_cI_{nc}] + [EI_{nc}]$$

Using the dissociation-constants defined above and substituting for the enzyme-complex concentrations gives:
\[
[B_t] = [E^*_r] \left\{ 1 + \frac{[S]}{K_s} + \frac{[S]}{K_s} \cdot \frac{[I_{nc}]}{K_{nc}} + \frac{[I_{nc}]}{K_{nc}} + \frac{[I_c]}{K_c} + \frac{1}{\alpha} \cdot \frac{[I_{nc}]}{K_{nc}} \cdot \frac{[I_c]}{K_c} \right\}
\]

\[
= [E^*_r] \times R \quad \text{(for simplicity of handling)}.
\]

Since only ES can react to form product, the reaction velocity, \( v \), will be:

\[
v = V_{\text{max}} \cdot \frac{[ES]}{[E^*_r]} = V_{\text{max}} \cdot \frac{[E^*_r]}{[B_t]} \cdot \frac{[S]}{K_s}
\]

However, \([E^*_r]/[B_t]\) is known (in terms of ligand concentrations and dissociation-constants) from above. When this is substituted, one obtains:

\[
v = V_{\text{max}} \cdot \frac{[S]}{K_s} \cdot \frac{1}{R}
\]

\[
\therefore \quad \frac{1}{v} = \frac{V_{\text{max}}}{[S]} \cdot \frac{K_s}{R}
\]

When \( R \) is expressed in full, but with the terms regrouped and factorised, one obtains:

\[
\frac{1}{v} = \frac{K_s}{[S]} \cdot \frac{1}{V_{\text{max}}} \left\{ (1 + \frac{[S]}{K_s}) \cdot (1 + \frac{[I_{nc}]}{K_{nc}}) + \frac{[I_c]}{K_c} \cdot (1 + \frac{[I_{nc}]}{\alpha K_{nc}}) \right\} - (3.1)
\]

If \( [I_c] \) is varied at different fixed values of \( [I_{nc}] \) and \([S]\), straight lines will be produced when \( \frac{1}{v} \) is plotted against \( [I_c] \).

Let us define the intercept on the abscissa as \( [I_c]' \), and on the ordinate as \( \frac{1}{v} \), and calculate them:

a) \( [I_c]' \) (i.e. when \( \frac{1}{v} = 0 \))

\[
(1 + \frac{[S]}{K_s}) \cdot (1 + \frac{[I_{nc}]}{K_{nc}}) = - \frac{[I_c]}{K_c} \cdot (1 + \frac{[I_{nc}]}{\alpha K_{nc}})
\]
\[
\text{Gradient} = \frac{K_s}{[S]} \cdot \frac{1}{V_{\text{max}}} \cdot \frac{1}{V_c} \cdot (1 + \frac{[I_{nc}]}{K_{nc}}) 
\]

\( - \left[ \frac{1}{V} \right] = \frac{K_c \cdot (1 + \frac{[S]}{K_s}) \cdot (1 + \frac{[I_{nc}]}{K_{nc}})}{(1 + \frac{[I_{nc}]}{\alpha K_{nc}})} \quad -(3.2) \)

b) \( \frac{1}{V} \), (i.e. when \( [I_c] = 0 \))

\[
\frac{1}{V} = \frac{K_s}{[S]} \cdot \frac{1}{V_{\text{max}}} \cdot (1 + \frac{[S]}{K_s}) \cdot (1 + \frac{[I_{nc}]}{\alpha K_{nc}}) 
\]

The gradient of the line can be found from the negative of the ratio of these two:

\[
\text{Gradient} = \frac{K_s}{[S]} \cdot \frac{1}{V_{\text{max}}} \cdot \frac{1}{V_c} \cdot (1 + \frac{[I_{nc}]}{\alpha K_{nc}}) 
\]

\( - \left[ \frac{1}{V} \right] = \frac{K_c}{[S]} \cdot \frac{1}{V_{\text{max}}} \cdot \frac{1}{V_c} \cdot (1 + \frac{[I_{nc}]}{\alpha K_{nc}}) \quad -(3.4) \)

The point of intersection of these lines (with different fixed values of \( [I_{nc}] \) but constant \([S] \)) can be found by using the fact that at this point \( \frac{1}{V} \) is independent of \( [I_{nc}] \). This will be at a particular value of \( [I_c] \), which we can call \( [I_c]^\prime \).

In Equation (3.1), putting \( [I_c] = [I_c]^\prime \), and putting \( \frac{1}{V} \) equal for two values of \( [I_{nc}] \)(which we can call \( [I_{nc}]_1 \) and \( [I_{nc}]_2 \)), one obtains:

\[
(1 + \frac{[S]}{K_s}) \cdot (1 + \frac{[I_{nc}]_1}{K_{nc}}) + \frac{[I_c]^\prime}{K_c} \cdot (1 + \frac{[I_{nc}]_1}{\alpha K_{nc}}) - (1 + \frac{[S]}{K_s}) \cdot (1 + \frac{[I_{nc}]_2}{K_{nc}}) - \frac{[I_c]^\prime}{K_c} \cdot (1 + \frac{[I_{nc}]_2}{\alpha K_{nc}}) = 0
\]

\[
\left[ [I_{nc}]_1 - [I_{nc}]_2 \right] \cdot \left( \frac{1}{K_{nc}} \cdot (1 + \frac{[S]}{K_s}) + \frac{[I_c]^\prime}{K_c} \cdot \frac{1}{\alpha K_{nc}} \right) = 0
\]

∴ \( [I_c]^\prime = -(1 + \frac{[S]}{K_s}) \cdot K_c \cdot \alpha \quad -(3.5) \)

The corresponding expression for interaction of two
substrate-competitive inhibitors is very similar; it contains no
\( 1 + \frac{[S]}{K_s} \) term (Yonetani and Theorell, 1964).

Therefore, knowing \([S]\), \(K_s\) and \(K_c\), one can calculate \(\alpha\) from the observed value of \([I_c]^n\).

It is worthwhile examining the two special cases of \(\alpha = \infty\)
(complete interaction, i.e. \(I_c\) and \(I_{nc}\) are mutually competitive);
and \(\alpha = 1\) (complete independence, i.e. \(I_c\) and \(I_{nc}\) are mutually non-
competitive).

a) If \(\alpha = \infty\) (mutually competitive). In this case,
\(I_c^n = -\infty\), and the family of lines with different values of \([I_{nc}]\)
are parallel. (This is also found with a mutually competitive
pair of substrate-competitive inhibitors (Yonetani and Theorell,
1964).

b) If \(\alpha = 1\) (mutually non-competitive). In this case,
\( [I_c]^n = -(1 + \frac{[S]}{K_s})K_c \)
Comparison with Equation (3.2) shows that \([I_c] = [I_c]^n\) - i.e. the
point of intersection of the family of lines at different values of
\([I_{nc}]\) is identical with the intercept of any line on the abscissa.
This is different from the case of two substrate-competitive
inhibitors (unless \([S] \ll K_s\)), when the point of intersection will
be in the second quadrant.

The above derivations have been published (Reynolds et al.,
1970).
3.4. Titrations of Enzyme with NADH, Measured by Fluorescence Enhancement.

Boyer and Theorell (1956) found that when NADH was bound to liver alcohol dehydrogenase, the fluorescence intensity of the coenzyme was increased, and the emission maximum, normally 462 nm (Duysens and Amesz, 1957), was shifted towards shorter wavelengths by about 20 nm. At a wavelength of about 410 nm, enzyme-bound NADH fluoresces quite strongly, while free NADH fluoresces comparatively weakly: enhancement of fluorescence, on binding to enzyme, is about 10 to 14-fold (Theorell and McKinley-McKee, 1961b). At shorter wavelengths, although the enhancement factor would be increased, the intensity of emitted light decreases markedly. Emission at 410 - 430 nm has been the basis of several studies of coenzyme-binding (Theorell, 1958; Theorell and Winer, 1959; Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961b; Woronick, 1963a,b; Theorell et al., 1970).

The methods used for calculation of the dissociation-constants for NADH have differed. It is usually assumed that each subunit behaves independently of the other - both regarding dissociation-constant, and fluorescence-enhancement; also, that free NADH (and other substances in solution) do not quench the fluorescence of bound or free coenzyme. Sometimes the data required that these assumptions be revised; and it therefore seems desirable that the method of calculation should be able to reveal inconsistencies.

Theorell and Winer (1959), and Theorell and McKinley-McKee (1961b) used numerical methods, adding six or eight equal aliquots
of NADH, and determining $K_{E,R}$ (the dissociation-constant of the enzyme-NADH complex). Knowing the enhancement-factor when NADH binds to the enzyme, and the concentration of binding-sites, $K_{E,R}$ could be calculated for each addition of NADH. However, if only one was known, the other could be calculated together with $K_{E,R}$ for any pair of values of added NADH. These methods gave constant values of $K_{E,R}$ at all concentrations of NADH used: therefore it does seem that the assumptions above are validated.

In the present work (Chapter 7), carboxymethylated alcohol dehydrogenase was examined. Weiner and co-workers (Hoagstrom et al., 1969) produced evidence that zinc-free alcohol dehydrogenase had two different binding-sites, with different dissociation-constants for NADH; it was therefore conceivable that carboxymethylated enzyme would too. A graphical method was considered preferable. The calculation was in two stages: firstly, calculation from the fluorescence values observed, and from the quantities of NADH added, of the concentrations of enzyme complex, and of free NADH: and secondly, graphical representation of this to estimate the dissociation-constant. The second stage is exactly analogous to enzyme kinetics. It was decided to use plots of the concentration of the enzyme-NADH complex versus enzyme-NADH/free NADH, analogous to $v$ vs $v/s$ plots (Woolf-Hofstee plots: Hofstee, 1959). Such plots are the same as Scatchard plots (Scatchard, 1949), used by Hoagstrom et al., (1969), except that the axes are reversed. Other graphical methods for calculation of fluorescence-titrations have been used by Anderson and Weber (1965), Cassman and England (1966) and Winer, Schwert and Millar (1959). Illingworth and Tipton (1970) used a simple (but comparatively imprecise) graphical
method given by Dixon (1965).

Fluorescence of solutions with and without enzyme were measured as aliquots of NADH were added (described in Chapter 2, Section 2.4). These were then divided by the value of the perspex fluorescent standard, and the values of fluorescence before adding any NADH were subtracted. The standardised fluorescence values thus produced for each NADH concentration were subtracted from each other, to give the increase in fluorescence due to binding of some of the NADH to some of the enzyme.

Before proceeding, it was necessary to know (a) the enzyme concentration, and (b) the fluorescence enhancement that would have been produced when the enzyme was saturated with NADH. In practice, (a) was known (pyrazole titrations), and (b) was estimated from the enhancement values obtained. If the estimate was inaccurate, this was shown up by the graph not extrapolating to the known enzyme concentration, at an infinite concentration of NADH. It was a simple matter to recalculate it with a revised estimate of the saturated fluorescence enhancement.

This enabled the concentration of the enzyme-NADH complex to be calculated. Subtraction gave the concentration of free NADH.

Calculation was greatly simplified by the use of an Olivetti Programma P101 desktop computer. The author is indebted to Mr. I.A. Nimmo for his help in the use of this, and for writing the program used. The logical steps outlined above were used: in the first stage, the fluorescence standards and initial fluorescence values were typed in, followed by pairs of values (with and without enzyme), and the fluorescence enhancement was printed out. The estimated enhancement at saturation was typed
in together with the enzyme concentration; then, values of NADH added and fluorescence enhancement were typed in; values of the concentration of the enzyme-NADH complex, and of the ratio enzyme-NADH/free NADH, were printed out directly. These were then plotted graphically as described above, and the apparent dissociation-constant was determined from the negative of the gradient.

It should be noted that this calculation requires (a) that the enzyme concentration be known, with fair accuracy; and (b) the assumption that fluorescence enhancement is the same at each coenzyme-binding site. If the second assumption is invalid, then the method is invalid unless modified. The linear graphs usually obtained (see Chapter 7) indicate that, if it is not valid, the dissociation-constants for the two sites must be sufficiently different to cancel the expected non-linearity; this must be considered unlikely. Hoagstrom et al. (1969), who used fluorescence polarization, were less liable to this kind of criticism.
CHAPTER 4

PCMB and DTNB

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Fig. 4.1. Inactivations of liver ADH (A) and yeast ADH (B) with PCMB. In phosphate buffer, ionic strength 0.1 (pH 7.4) at 23.5°. Liver enzyme, 2.9 μM; yeast enzyme, 0.96 μM aged enzyme (o), and 1.5 μM fresh enzyme (●). Arrows indicate points where all the thiol groups had been titrated with PCMB.
4.1. Introduction.

Both liver and yeast alcohol dehydrogenase are inhibited by compounds which react with thiol groups (Sund and Theorell, 1963). Both PCMB and DTNB are specific, widely-used reagents for thiol groups. PCMB had been used previously in several studies on the yeast enzyme (Wallenfels and Sund, 1957a; Wallenfels and Muller-Hill, 1964). PCMB (Witter, 1960; Dalziel, 1961b) and the equivalent sulphonic acid compound (Yonetani and Theorell, 1962) have been used on the liver enzyme. PCMB was re-examined, and DTNB was examined, to see if selective reaction with some of the thiol groups could be achieved, and thus to try to learn something about their function in the enzyme.

4.2. Results.

PCMB. The effect of PCMB on the activity of liver and yeast alcohol dehydrogenases is shown in Figs. 4.1a and 4.1b. These are similar to earlier data of Witter (1960) and Wallenfels and Muller-Hill (1964) respectively. Liver alcohol dehydrogenase shows initially a certain amount of selectivity – i.e. the proportional loss of activity is more than the proportional loss of thiol groups. However, this may be because reaction of any of several thiol groups will reduce activity, rather than because some are more reactive than others. Activity is not completely lost until all thiols have reacted. There was no indication of any anomalous effects when small amounts of PCMB were added, indicating that no one thiol group (essential or non-essential) reacted preferentially with the reagent.
Fig. 4.2. Inactivations of liver ADH (A) and yeast ADH (B) with DTNB. In phosphate buffer, ionic strength 0.1 (pH 8.0) at 23.5°. Liver enzyme, 2.9 μM; yeast enzyme, 1.0 μM aged enzyme (o), and 1.5 μM fresh enzyme (•).
The linear loss of activity shown by yeast alcohol dehydrogenase extrapolates to zero activity when about four thiol groups per molecule of enzyme (one per subunit, on average) remain, in fresh enzyme. In aged enzyme, the value was nearer 2.5; as will be seen below, approximately 40% of the thiol groups in this preparation were in inactive enzyme. The fresh sample of enzyme had a turnover number of 34,500 min.\(^{-1}\), and the aged sample, 13,600 min.\(^{-1}\).

DTNB. Liver and yeast alcohol dehydrogenases were reacted with an excess of DTNB. The loss of activity followed pseudo-first-order kinetics, and (with one or two provisos—with below) so did the loss of thiol groups. At times during the reaction, activity and thiols reacted were measured, and the results are shown in Figs. 4.2a and 4.2b. The liver enzyme shows no selectivity at all, the proportion of thiol groups reacted being the same as the proportion of activity lost. The reaction was slow, with a half-time of 5 hours: simple thiols e.g. cysteine, or enzyme in 5M guanidine hydrochloride, reacted in a few seconds.

Fresh yeast alcohol dehydrogenase (Fig. 4.2b) produced a similar, near-linear relationship. However, the aged preparation (stored frozen for several months) produced a rapid burst of reaction with DTNB, which did not affect activity. The burst was complete in ten minutes, while the half-time for activity loss was one hour. The burst reaction may be with dissociated enzyme (Buhner and Sund, 1969).

Dithiothreitol was able to partially reactivate liver alcohol dehydrogenase. Enzyme (3 pM) was inactivated to 32% of its initial activity with DTNB (300 pM). Dithiothreitol (1.3mM) was added, which produced a slow reactivation: after 35 min., activity was 44%,
**Fig. 4.3.** Rates of inactivation of liver ADH with 0.67 mM DTNB.

In phosphate buffer, ionic strength 0.1 (pH 7.4) at 23.5°.

Enzyme, 0.6 μM. Without additions, ●. With imidazole (base form, 26 mM), △. With orthophenanthroline (27 μM), ○. With AMP (132 μM), ▼.
Fig. 4.4. Reaction of liver ADH and carboxymethylated liver ADH with DTNB (0.33 mM). In phosphate buffer, ionic strength 0.1 (pH 7.4) at 23.5°. Circles represent native liver ADH (0.75 μM), and triangles represent carboxymethylated ADH (0.75 μM). Open symbols, with imidazole (base form, 26 mM); filled symbols, without imidazole.
and after 2½ hours, 52%. It was noticed that dithiothreitol, like mercaptoethanol, was a powerful inhibitor of liver alcohol dehydrogenase (Lambe and Williams, 1965; Pietruszko and Theorell, 1969; Cheng and McKlinley-McKee, 1968; Cheng, 1970).

No "fading" of yellow colour was observed when native enzymes were being reacted with DTNB, until almost all the thiol groups had reacted. (See Chapter 2, Section 2.4).

Imidazole partially protects liver alcohol dehydrogenase from PCMB (Witter, 1960). It was found that imidazole, orthophenanthroline and AMP protect the enzyme from DTNB (Fig. 4.3). Chloride and formate also protect, but are less effective. Guanidine hydrochloride (1M) was found (under conditions analogous to Fig. 4.2a) to increase the rate of reaction ten-fold, but the proportionality shown in Fig. 4.2a was maintained. At higher pH (9.6), the reaction rate was increased by a factor of 3, compared with pH 7.4 (both with 0.67 mM DTNB).

At higher concentrations of DTNB, a saturation-effect was observed with liver alcohol dehydrogenase: concentrations of DTNB of 0.67, 1.33 and 3.33 mM gave half-times of 76, 71 and 71 minutes, for activity loss.

The reaction of carboxymethylated liver alcohol dehydrogenase with DTNB is shown in Fig. 4.4. The rate of reaction is somewhat higher than with native enzyme, and imidazole protects it as well. This was an aged preparation of carboxymethylated enzyme (see Chapter 7), containing approximately 16 free thiol groups per molecule.
4.3. Discussion.

The experiments with PCMB produced virtually no new information about the enzymes. The curious observation that yeast alcohol dehydrogenase loses all activity when it still has four thiol groups left has been made previously (Wallenfels and Sund, 1957a; Wallenfels and Muller-Hill, 1964). Coenzyme-binding, on the other hand, is directly proportional to the thiol groups remaining (Wallenfels and Muller-Hill, 1964). For the liver enzyme, too, coenzyme-binding seems to be nearly proportional to thiol-content, while activity is proportionally lower (Witter, 1960; see also Yonetani and Theorell, 1962).

The lack of selectivity of DTNB was disappointing. The burst reaction with an inactive component in aged yeast enzyme is interesting, but not particularly useful in aiding understanding of thiol function in the native enzyme. The proportionality between activity lost and thiols reacted may indicate that the first thiol to react on a particular enzyme molecule may cause some denaturation, enabling the remaining thiol groups of that molecule to react more rapidly. This has also been suggested by Yonetani and Theorell (1962). The labilising effect of 1M guanidine hydrochloride may be because it weakens the overall tertiary structure of the enzyme, while ligands such as imidazole, orthophenanthroline and AMP may contribute to strengthening it. It is unlikely that direct, specific shielding of thiol groups takes place. The similarity of carboxymethylated liver alcohol dehydrogenase with native enzyme, in reactivity with DTNB and protection by imidazole, indicated that the thiol which is carboxymethylated does not play a very significant role in the
reaction of the enzyme with DTNB.

The slow, partial reactivation of the liver enzyme with dithiothreitol may indicate that renaturation and disulphide exchange, and not just simple thiol regeneration, is occurring.

The saturation-effect found for rate of reaction of DTNB with the liver enzyme, as the DTNB concentration was raised, suggests that a reversible enzyme-DTNB complex may form. This is perhaps not surprising, since many aromatic compounds can bind to the enzyme (see Chapter 1, Section 1.7). The enzyme is protected comparatively strongly by orthophenanthroline, and weakly by AMP, formate and chloride (compared to their dissociation-constants with the enzyme—see Chapter 6). Therefore, DTNB may bind non-competitively with orthophenanthroline, but perhaps competitively with AMP (and possibly also formate and chloride). This is exactly the pattern found with some other aromatic anions (see Chapter 6). Thiol groups in the reversible enzyme-DTNB complex (if it exists) could either react ("internally") with the enzyme-bound DTNB, or be protected from ("external") attack by other molecules of DTNB free in solution (this is considered in following chapters in more detail, for iodoacetate).

The rate of reaction of liver enzyme with DTNB is faster at high pH, but the reaction with iodoacetate is slower (see Chapter 5). Also, imidazole effectively protects the enzyme (native or carboxymethylated) from reaction with DTNB: but iodoacetate reacts faster with native enzyme in the presence of imidazole. This suggests that, even if reversible binding of iodoacetate and DTNB should be at the same site on the enzyme, the reaction with thiol groups is very different. If the rate-limiting step for reaction with DTNB
is the reaction with the first thiol, then this must proceed by a
different mechanism from the reaction with iodoacetate, and
probably involves a different thiol group.
CHAPTER 5

Iodoacetate, Iodoacetamide and 3-Iodopropionate

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5.3. Discussion .............................................. 66
5.1. Introduction.

Liver alcohol dehydrogenase has two thiol groups per molecule which are reactive towards iodoacetate (Li and Vallee, 1963, 1965; Harris, 1964), and yeast alcohol dehydrogenase has four which are reactive towards iodoacetamide (Rabin et al., 1964) and iodoacetate (Harris, 1964). Initially, it was intended to study the effect of pH on alkylation of the liver enzyme (as had been done on the yeast enzyme using iodoacetamide; Rabin and Whitehead, 1962). After a series of such observations had been made, results of a similar investigation on liver alcohol dehydrogenase using both iodoacetate and iodoacetamide were published (Evans and Rabin, 1968) and also on the yeast enzyme (Rashed and Rabin, 1968).

However, during the present experiments it was noticed that, at higher concentrations of iodoacetate, a saturation effect was occurring; the rate-constant of inactivation showed Michaelis-Menten-type kinetics at various concentrations of iodoacetate. This seemed important, and the primary purpose of this chapter is to demonstrate that iodoacetate forms a reversible complex with active liver alcohol dehydrogenase.

Some of the results described in this Chapter have been published (Reynolds and McKinley-McKee, 1969; see Appendix).

5.2. Results.

Iodoacetate. Liver alcohol dehydrogenase is inactivated by iodoacetate; the reaction is first-order with respect to enzyme
Fig. 5.1. Inactivations of liver ADH with iodoacetate (semi-expressed as % of activity in the absence of iodoacetate log graph). In 25 - 40 mM phosphate buffer (pH 7.4), ionic strength 0.1, at 23.5°C. Enzyme, 0.6 - 1.6 μM. Iodoacetate concentrations: o, 1.33 mM; Δ, 2.0 mM; ●, 4.0 mM; ▲, 13.3 mM. (From Reynolds and McKinley-McKee, 1969, with publishers' permission.)
Fig. 5.2. Double-reciprocal plots of inactivation rate-constants of liver ADH as a function of iodoacetate concentration. Conditions as in Fig. 5.1, but with added anions (as sodium salts): o, none (calculated from Fig. 5.1); △, chloride (26.7 mM); •, formate (26.7 mM); ▼, acetate (26.7 mM); and ▲, decanoate (60 μM). (From Reynolds and McKinley-McKee, 1969, with publishers' permission).
Table 5.1. Dissociation-constants at pH 7.4, ionic strength 0.1, determined from Fig. 5.2 (by ratio of slopes, for protecting ions).

<table>
<thead>
<tr>
<th>Ion</th>
<th>Dissociation-Constant</th>
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<tr>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>4.5 mM</td>
</tr>
<tr>
<td>Chloride</td>
<td>23 mM</td>
</tr>
<tr>
<td>Formate</td>
<td>24 mM</td>
</tr>
<tr>
<td>Acetate</td>
<td>51 mM</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Decanolate</td>
<td>39 μM</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

References:  
A = Winer and Theorell (1960).  
B = Sigman (1967).  
C = Theorell and McKinley-McKee (1961b).
Fig. 5.3. Inactivation kinetics of liver ADH at pH 7.9.
Ionic strength 0.104, at 23.5°C. Enzyme, 0.6 - 1.6 μM.
o, in tris-phosphate buffer; V, in phosphate buffer; V, in tris-chloride buffer; Δ, in tris-phosphate buffer + sodium chloride (26.7 mM); A, in tris-phosphate buffer + sodium formate (26.7 mM).
Fig. 5.4. Inactivation kinetics of liver ADH at pH 8.6 (trisphosphate buffer). Ionic strength 0.104, at 23.5°. Enzyme, 0.6 - 1.6 μM. o, no additions; Δ, + sodium formate (40 mM); V, + sodium chloride (40 mM).
Fig. 5.5. Inactivation kinetics of liver ADH at pH 9.35
(glycine-NaOH buffer + phosphate). Ionic strength 0.104,
at 23.5°. Enzyme, 0.6 - 1.6 μM. ○, no additions; △, + sodium
formate (40 mM); ▲, + sodium chloride (40 mM).
Fig. 5.6. Dissociation-constants of iodoacetate, chloride and formate, as functions of pH, relative to their value at pH 7.4. The line represents theoretical curve with pK = 9.0 (acid asymptote, 1.0 and alkaline asymptote infinity, i.e. no binding). Points calculated from Figs. 5.2 - 5.5: o, iodoacetate; △, chloride; •, formate. (From Reynolds and McKinley-McKee, 1969, with publishers' permission).
activity (Fig. 5.1). Using 1.9 mM iodoacetate, the stoichiometry was 1 – 1.2 thiol groups lost for each subunit of inactivated enzyme, between pH 6 and 9, in accordance with Li and Vallee (1963, 1965). However, at pH 10 a much greater loss of thiol groups was observed, the proportion of which was nearly equal to the proportion of activity lost. Also, the log (activity) vs. time plot levelled off. It is suggested that hydrolysis of iodoacetate, or reaction with the glycine buffer, may be responsible. At all these pH's, the enzyme was stable in the absence of iodoacetate (losing less than 5% activity in several hours).

The rate-constants of inactivation follow Michaelis-Menten-type kinetics, as shown by the Lineweaver and Burk (1934) plot in Fig. 5.2. This is in contrast to the linear dependence found by Evans and Rabin (1968). The most likely explanation of these kinetics is that a reversible enzyme-iodoacetate complex can form.

Fatty-acids, and chloride, protect the enzyme from alkylation, and show excellent competitive kinetics (Fig. 5.2); their dissociation-constants (calculated from ratios of slopes) agree quite well with literature values, as is seen in Table 5.1. The reversible binding of fatty-acids, therefore, seems to be competitive with the reversible binding of iodoacetate. However, at high concentrations of decanoate, non-linear Dixon plots (Dixon, 1953) were obtained, indicating that protection was not quite complete: however, it was about 98%, and so should not significantly affect results at low concentrations of decanoate. The enzyme was stable in the presence of decanoate and absence of iodoacetate.

The kinetics of inactivation were also followed at three other pH values (Figs. 5.3 – 5.5). Dissociation-constants are summarised in Fig. 5.6, and rate-constants (at infinite iodoacetate concentration)
Table 5.2. The effect of pH on the extrapolated maximum rate of inactivation of liver alcohol dehydrogenase, determined from Figs. 5.2 - 5.5.

<table>
<thead>
<tr>
<th>pH</th>
<th>Max. Inactivation Rate-Constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>$7.8 \times 10^{-2}$ min$^{-1}$</td>
</tr>
<tr>
<td>7.9</td>
<td>$7.45 \times 10^{-2}$ min$^{-1}$</td>
</tr>
<tr>
<td>8.6</td>
<td>$7.5 \times 10^{-2}$ min$^{-1}$</td>
</tr>
<tr>
<td>9.35</td>
<td>$6.1 \times 10^{-2}$ min$^{-1}$</td>
</tr>
</tbody>
</table>
Fig. 5.7. Reaction of liver ADH with iodoacetamide: semi-log graph of loss of activity, and of thiol groups. Ionic strength 0.1, at 23.5°. Enzyme, 4 μM; iodoacetamide, 13.3 mM. Circles, pH 7.4 (phosphate buffer); triangles, pH 6.2 (phosphate buffer). Open symbols represent activity, and filled symbols represent thiol content.
The dissociation-constants of iodoacetate and protecting fatty-acids vary similarly, and the maximum velocity of inactivation varies only slightly (and probably not significantly). The pH-variation may indicate a pH on the enzyme of around 9. However, this is estimated very largely from the values at pH 9.35. These experiments were the most difficult to perform, due to the non-linearity of the semi-log graphs (see above). These inactivations were all performed in duplicate, with up to ten enzyme assays being used to follow carefully the first part of the inactivation. The glycinate anion binds to the enzyme (Theorell and McKinley-McKee, 1961b) but should have very little effect at the low concentrations used here.

**Iodoacetamide.** Iodoacetamide inactivates liver alcohol dehydrogenase much more slowly than does iodoacetate (Li and Vallee, 1965). A preliminary report has stated (McKinley-McKee, 1963a) that iodoacetamide reacts with most of the thiol groups of the enzyme when it inactivates, and not just one per subunit like iodoacetate.

The loss of activity and thiol groups on reaction of the enzyme with iodoacetamide at pH 7.4 and pH 6.2 is shown in Fig. 5.7. Between pH 7 and 9, the loss of activity is pseudo-first-order. However, the loss of thiol groups was always found to follow the pattern shown — a relative increase in rate with time. This may be because the remaining thiols on the inactivated enzyme can react faster than on native enzyme. At pH 6, the same phenomenon (a downward-curving semi-log graph) for loss of activity was observed. It may be that there is progressive reaction of non-essential groups, which however after reaction cause the essential groups to react faster. Even initially, there is more than one thiol group lost per
**Fig. 5.8.** Inactivation kinetics of liver ADH with iodoacetamide. At 23.5\(^\circ\), pH 8.8 (glycine-NaOH buffer, 0.1 M in glycine).

Enzyme, 1.5 - 2 \(\mu\)M. Circles and triangles represent different experiments.
Fig. 5.9. Inactivation kinetics of liver ADH with 3-iodopropionate. Ionic strength 0.104, pH 7.4 (phosphate buffer), at 23.5°. Enzyme, 1 μM.
subunit of inactivated enzyme; and as the inactivation proceeds it becomes less and less selective.

Attempts were made to look for a saturation-effect with iodoacetamide, shown in Fig. 5.8. The effect is possibly non-existent, and at best very weak, with an apparent dissociation-constant greater than about 50 mM.

3-Iodopropionate. 3-Iodopropionate also shows a saturation-effect with liver alcohol dehydrogenase (Fig. 5.9), with a dissociation-constant of 3 mM. It is a much less powerful alkylating agent than iodoacetate. 3-Iodopropionate protected the enzyme from iodoacetate, but the calculated dissociation-constant (1 mM) did not agree well with the value (above) found directly. In Fig. 5.9, it appears that the rate of inactivation deviates from strict Michaelis-Menten kinetics at high concentrations of 3-iodopropionate.

5.3. Discussion.

The above results show that iodoacetate follows Michaelis-Menten-type kinetics when it inactivates liver alcohol dehydrogenase. This suggests that a reversible complex between iodoacetate and the enzyme can form, and the rate-constant of inactivation is proportional to the fraction of enzyme present as this reversible complex.

In Chapter 3, Section 3.1, a model is described in which free enzyme can be alkylated by free iodoacetate (with second-order rate-constant \( k'' \)); and the iodoacetate which is reversibly bound to the enzyme can itself alkylate it (with a first-order rate-constant \( k' \)). The observed rate-constant of alkylation, \( v \), was shown to be (equation 1.2):

\[ v = (equation 1.2) \]
\[ v = \frac{[1]}{K + [1]} \cdot (k''k + k') \]

Two comments can be made concerning this: firstly, the observed dissociation-constant, \( K \), will be identical to the real dissociation-constant of the reversible enzyme-iodoacetate complex, provided that \( k' \) and \( k'' \) do not disturb the reversible binding condition equilibrium. This is probably valid because (a) the rate of inactivation is comparatively low; (b) semi-log plots are linear from the outset; and (c) in the enzyme assays linear initial progress curves are found, indicating rapid dissociation of the enzyme-iodoacetate binary complex. Secondly, Michaelis-Menten kinetics will be observed, whatever the relative values of \( k' \) and \( k'' \). If \( k'' \) is zero, then the reversible enzyme-iodoacetate complex will be a necessary intermediate in the alkylation process, and the reactive thiol group is presumably located near the reversible binding-site of iodoacetate. It is convenient to describe this process as internal alkylation. However, if \( k' \) is zero, then the formation of the reversible enzyme-iodoacetate complex protects the enzyme completely from alkylation by iodoacetate. It is convenient to describe this as external alkylation. In this case, presumably the reactive thiol group would be further from the reversible binding-site, or perhaps sterically positioned so that reaction was impossible.

Therefore, kinetic measurements are completely unable to distinguish between internal and external alkylation. In the general case, the overall reaction will be a combination of the two. However, the reversible complex must be unable to react with another molecule of free iodoacetate (i.e. \( k_z \) must be zero), otherwise Michaelis-Menten kinetics will not be obeyed. 3-Iodopropionate, which does appear to deviate at high concentrations, may have a non-zero value.
for $k_z$.

The competitive protection by chloride, and by other carboxylic acids, and also their similar pH-dependence, is circumstantial evidence which helps to confirm the observation of formation of reversible complexes. Attempts to measure the formation of the reversible complex by competition with NADH (observed fluorimetrically) failed, because a rapid, time-dependent loss of NADH-fluorescence occurred in the presence of iodoacetate: it was quite rapid in the absence of enzyme, but much more rapid with enzyme present. The reason for this is unknown. Some iodine-containing compounds catalyse the hydration (Schreier and Cilento, 1969) and autoxidation (da Silva Araujo and Cilento, 1969) of NADH. However, iodoacetate itself is unlikely to cause such a rapid effect (Cilento, 1970). Iodoacetate inhibits the aldehyde mutase reaction (Abeles and Lee, 1960), and is competitive with formaldehyde; its apparent dissociation-constant is approximately 5 mM. This may be coincidental, since a high concentration of NAD was present. Iodoacetate may perhaps react with NADH or NAD$^+$, or may be able also to form a complex of enzyme, NAD$^+$ and iodoacetate.

Michaelis-Menten kinetics would also be given if the iodoacetate solution contained an impurity which bound reversibly to the enzyme and protected it from attack by iodoacetate. However, this is unlikely because two different samples of iodoacetic acid, and also sodium iodoacetate, gave indistinguishable inactivation-kinetics. Provided that the iodoacetate concentration was not high enough to significantly affect the pH, neutralised and un-neutralised samples gave the same results, too. Ethylene diamine tetra-acetate (1 mM) also produced no change in the inactivation-kinetics. Enzyme obtained from Seravac (as used by Evans and Rabin, 1968) also gave
the same results. The reasons for the discrepancies between their results and the present ones are not known; however at some pH values they used buffers containing chloride, without realising that it protected the enzyme.

The binding of fatty-acids agrees quite well with literature values, although it may be significantly stronger. Quantitative figures for chloride-binding are not available, but from kinetics (Theorell et al., 1955), effect on NADH-binding (Theorell, 1958; Theorell and Winer, 1959; Li, Ulmer and Vallee, 1963) and NMR (Zeppezauer et al., 1969), chloride is known to bind to liver alcohol dehydrogenase.

The much weaker binding by iodoacetamide is in keeping with the generally weaker binding of amides, compared with acids, to the enzyme (Winer and Theorell, 1960; Woronick, 1961, 1963a,b). Some preliminary experiments with isobutyramide (see Chapter 6) indicated that at high iodoacetate concentrations it stimulated alkylation by iodoacetate. Iodoacetamide may be binding reversibly to the enzyme, but the binding may be having only a small effect on reactivity of the thiol group (or groups). Chloride did give a certain amount of protection against iodoacetamide. Iodoacetamide probably does react with the same thiol groups that iodoacetate alkylates, but it also reacts with others. The specificity decreases as the reaction progresses. Iodoacetamide forms reversible complexes with the enzyme and NADH (Woronick, 1961, 1963a,b), from which iodoacetamide has a dissociation-constant of approximately 2 mM. It was also estimated that the dissociation-constant of the enzyme-iodoacetamide binary complex was 24 mM (Woronick, 1963b).

The ionisation (with a pK of around 9) implicated in the binding of anions is not far from the value of 8.75 which affects NAD+.
binding (Taniguchi et al., 1967), and may be the same one. It was suggested that this was ionisation of a water molecule bound to zinc (Theorell and McKinley-McKee, 1961b,c; Taniguchi et al., 1967).
The relative constancy of the rate-constants at infinite iodoacetate concentration (Table 5.2) requires different explanations depending on whether alkylation is internal or external. If internal \( (k'' = 0) \), then \( k' \) must be essentially independent of pH. If external \( (k' = 0) \), then \( k'' \) must decrease with the same pH-dependence as does the affinity of the enzyme for anions. Each of these explanations is feasible. However, it is perhaps rather unlikely that alkylation would be a combination of internal and external mechanisms. Further discussion of the relative probability of internal and external alkylation is deferred until Chapter 9, when other evidence is considered as well.

The Michaelis-Menten kinetics of inactivation found when iodoacetate inactivates liver alcohol dehydrogenase are very similar to results found for another zinc-enzyme, carbonic anhydrase. Bromoacetate has a dissociation-constant with human carbonic anhydrase B of 3.8 mM, and iodoacetamide, 20 mM; acetate and chloride, which protect competitively with bromoacetate, have dissociation-constants of 22 mM and 15 mM respectively, at ionic strength 0.075 (Whitney, Nyman and Malmstrom, 1967). Iodoacetate also shows Michaelis-Menten kinetics, with a dissociation-constant of 1.6 mM (Bradbury, 1969a). The maximum rate of inactivation is 2 to 3 times slower than for alcohol dehydrogenase, and the residue alkylated is a histidine, not a thiol; most of the carboxymethyl-histidine was found in one peptide (Bradbury, 1969b) but some was present in a different sequence. Carbonic anhydrase has an
ionisation with a pK of 6.4 - 8.2 (depending upon which isoenzyme, and what ionic conditions, are used: Ward, 1970). Only the acid form of the enzyme binds anions well; replacing the zinc by cobalt alters the dissociation-constants for anions; and the binding of anions (but not iodoacetamide) is nearly abolished by removal of zinc (Whitney et al., 1967). It seems that the metal is intimately involved in the binding of anions, and anions may bind to the metal. The ionisation is probably due to zinc-bound water molecule, possibly in conjunction with a histidine residue (Whitney et al., 1967; Coleman, 1967). Carboxymethylated carbonic anhydrase has some residual activity (Bradbury, 1969a), like alcohol dehydrogenase (Chapter 7); but since the reactions catalysed are so different, it would be as well not to put too much weight on this observation. Therefore, although the two enzymes are very different, they show some remarkable parallels; this is probably attributable to their zinc.
### CHAPTER 6

**Ligand-Binding and Interactions**

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Fig. 6.1. Effect of bromide and iodide on inactivation of liver ADH by iodoacetate. In sodium phosphate buffer (pH 7.4), ionic strength 0.104, at 23.5°. Enzyme, 0.4 - 0.6 µM.

○, + sodium bromide (20 mM); Δ, + potassium iodide (20 mM);
line without points, no additions (see Fig. 6.8).
6.1. **Introduction.**

In Chapter 1, Section 1.7, different methods of measuring ligand-binding to liver alcohol dehydrogenase have been described. Since the enzyme can bind a wide variety of substances, specific measuring of one can be used to examine interactions between ligands. In the previous chapter, it was shown that reversible binding of iodoacetate can be measured by determining the rate-constant of inactivation at different concentrations of iodoacetate. Compared with other methods, it is very simple to perform and to obtain highly self-consistent and repeatable results. It requires reasonably small quantities of enzyme. But it is somewhat tedious.

Using the large collection of previous knowledge about ligand-binding to the enzyme, many interactions have been investigated. This is a logical continuation of the work described in the previous chapter, concerning effect of pH and protecting ligands. Adenine nucleotides, orthophenanthroline and imidazole were used by Evans and Rabin (1968). Most of the results described in this chapter have been published (Reynolds *et al.*, 1970).

6.2. **Results.**

**Halide Ions.** In the previous chapter, chloride was shown to protect liver alcohol dehydrogenase from inactivation by iodoacetate, competitively with iodoacetate: the estimated dissociation-constant of the enzyme-chloride binary complex was 23 mM. Fig. 6.1 shows the effect of bromide and iodide. Neither is perfectly
Fig. 6.2. Effect of AMP, ADP, and ATP on inactivation of liver ADH by iodoacetate. Conditions as for Fig. 6.1. Enzyme, 0.5 μM, o, + AMP (91.2 μM); Δ, + ADP (425 μM); ∇, + ATP (565 μM); line without points, no additions (see Fig. 6.8). (From Reynolds et al., 1970, with publishers' permission.)
Fig. 6.3. Effect of adenosine, ADP-ribose and GMP on inactivation of liver ADH by iodoacetate. Conditions as for Fig. 6.1. Enzyme, 0.5 µM. o, + adenosine (27.4 mM); Δ, + ADP-ribose (38.5 µM); v, + GMP (313 µM); line without points, no additions (see Fig. 6.8). (From Reynolds et al., 1970, with publishers' permission).
Table 6.1. Dissociation-constants of complexes of liver ADH with adenosine and nucleotides. The values were calculated from Figs. 6.2 and 6.3 by ratio of slopes.

<table>
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<tr>
<th>Protecting Ligand</th>
<th>Dissociation-Constant, ( K )</th>
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<td>This York (pH 7.4)</td>
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<tr>
<td>Adenosine</td>
<td>17,200 ( \mu \text{M} )</td>
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<tr>
<td>AMP</td>
<td>32 ( \mu \text{M} )</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ADP</td>
<td>180 ( \mu \text{M} )</td>
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<td>ATP</td>
<td>708 ( \mu \text{M} )</td>
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<tr>
<td>ADP-ribose</td>
<td>10.5 ( \mu \text{M} )</td>
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</tr>
<tr>
<td>GMP</td>
<td>110 ( \mu \text{M} )</td>
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</table>

References:  
A = Reynolds et al. (1970).  
B = Yonetani and Theorell (1964).  
C = Li and Vallee (1964b).  
D = Theorell and Yonetani (1964).  
E,F = Yonetani (1963a,b).
Fig. 6.4. Effect of cyclic 3',5' AMP on inactivation of liver ADH by iodoacetate. Conditions as in Fig. 6.1. Enzyme, 0.5 μM; iodoacetate, 3.33 mM.
Fig. 6.5. Effect of orthophenanthroline on inactivation of liver ADH by iodoacetate. Conditions as in Fig. 6.1. Enzyme, 0.5 μM. ○, + orthophenanthroline (8.67 μM); Δ, + ortho-phenanthroline (4.33 μM); line without points, without orthophenanthroline (see Fig. 6.8). (From Reynolds et al., 1970, with publishers' permission.)
Fig. 6.6. Effect of 2,2' bipyridyl (Δ) and pyridine (○) on inactivation of liver ADH by iodoacetate. Conditions as in Fig. 6.1. Enzyme, 1.1 μM; iodoacetate, 3.33 mM. (From Reynolds et al., 1970, with publishers' permission).
competitive with iodoacetate, indicating that ternary enzyme-iodoacetate-halide complexes can form. The calculated dissociation-constants for the enzyme-halide complexes are 16 mM for bromide and approximately 9 mM for iodide.

Adenine Nucleotides. The effect of AMP, ADP and ATP is shown in Fig. 6.2, and of ADP-ribose, GMP and adenosine in Fig. 6.3. The nucleotides are competitive with iodoacetate, while adenosine shows mixed kinetics. The experiments with adenosine were made difficult by the low solubility of adenosine, and its weak protection. The calculated dissociation-constants are shown in Table 6.1. As with fatty-acids, (Chapter 5, Section 5.2), the apparent binding is somewhat tighter than determined by other methods. However, the high value obtained for adenosine, 17 mM compared with 6 mM by substrate kinetics (Reynolds et al., 1970; see Appendix) suggests that protection may not be complete. Also, a weak enzyme-adenosine-iodoacetate ternary complex appears to be formed.

3',5'-Cyclic AMP was only a weak protector of liver alcohol dehydrogenase (Fig. 6.4). If it is assumed to be competitive with iodoacetate, its apparent dissociation-constant was 2 mM.

Bidentate Chelating Agents. Unlike adenine nucleotides, the protection of the enzyme by orthophenanthroline (Evans and Rabin, 1968) is approximately non-competitive with iodoacetate (Fig. 6.5). Dissociation-constants for the two concentrations were 5.8 and 6.3 pM, in reasonable agreement with Yonetani (1963b) who obtained 8 pM at pH 7. 2,2'-Bipyridyl also protects (Fig. 6.6) with a dissociation-constant estimated to be 500 pM (assumed non-competitive): Sigman (1967) obtained 400 pM, from direct
Fig. 6.7. Effect of imidazole on inactivation of liver ADH by iodoacetate. At 23.5°, ionic strength 0.10. Enzyme, 0.6 μM. ○, in imidazole-phosphate buffer, pH 7.4 (base form of imidazole, 29 mM); △, imidazole (63 mM) in glycine-NaOH buffer + phosphate, pH 10.0 (glycinate anion, 16 mM); △, imidazole (0.46 M) in glycine-NaOH + phosphate buffer, pH 10.0 (glycinate anion, 16 mM); line without points, in phosphate buffer (pH 7.4) without imidazole (for points, see Fig. 6.8). (From Reynolds et al., 1970, with publishers' permission.)
Table 6.2. Dissociation-constants of the liver ADH-imidazole complex. Ionic strength 0.1, 23.5°. Enzyme, 0.4 μM, and iodoacetate, 3.33 mM. The method of calculation is given in Chapter 3, Section 3.2; constants used are given in the text.

<table>
<thead>
<tr>
<th>pH</th>
<th>Imidazole (base form)</th>
<th>Half-Time</th>
<th>Dissociation Constant (calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>0</td>
<td>21.42 min</td>
<td>-</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.36 mM</td>
<td>15.0</td>
<td>1.06 mM</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.72 &quot;</td>
<td>11.08 &quot;</td>
<td>0.72 &quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.20 &quot;</td>
<td>9.67 &quot;</td>
<td>0.74 &quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>2.40 &quot;</td>
<td>8.62 &quot;</td>
<td>0.93 &quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>28.6 &quot;</td>
<td>6.35 &quot;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td></td>
<td>0.86 mM</td>
</tr>
</tbody>
</table>

| 10.0| 0                     | 80.5 min | -                                 |
| "  | 1.60 mM               | 26.67 "  | 5.7 mM                            |
| "  | 2.667 "               | 22.33 "  | 6.2 "                             |
| "  | 5.33 "                | 14.25 "  | 5.9 "                             |
| "  | 10.67 "               | 11.86 "  | 5.6 "                             |
| "  | 63.5 "                | 8.55 "   | -                                 |
|    | Average               |           | 5.9 mM                            |
spectrophotometric observation. Neither 2,2'-bipyridyl nor ortho-
phenanthroline appears to protect the enzyme completely. This was
not due to slow inactivation by the chelating agent itself, because
when the iodoacetate was omitted, the enzyme was found to be stable.

Monodentate Neutral Ligands. Evans and Rabin (1968) found that
imidazole stimulated inactivation of liver alcohol dehydrogenase by
iodoacetate. As shown in Fig. 6.7, the half-time at infinite iodo-
acetate concentration decreased approximately ten-fold, and the
binding of iodoacetate became approximately five-fold weaker, at
pH 7.4. Results at pH 10 were very similar, but alkylation was
around 30% slower. This was not due to incomplete saturation of the
enzyme with imidazole at pH 10; increasing the concentration of
imidazole from 60 mM to 460 mM caused a further slight fall in
reactivity. This may indicate that imidazole is binding, more
weakly, at extra sites.

Using Equation 2.3 (Chapter 3, Section 3.2), the dissociation-
constant of imidazole from the enzyme-imidazole binary complex was
determined at pH 7.4 and pH 10: results are shown in Table 6.2.
The following values were used in the calculation (M, or modifier,
being imidazole, and S, the "substrate", being iodoacetate):

\[
\begin{align*}
\text{pH 7.4: } & \quad K_{E,S} = 4.4 \text{ mM} \\
& \quad V_1 = 0.0767 \text{ min}^{-1} \quad \text{From Fig. 6.8} \\
& \quad K_{E,M,S} = 24.2 \text{ mM} \\
& \quad V_2 = 0.925 \text{ min}^{-1} \quad \text{From Fig. 6.7} \\
\text{pH 10: } & \quad K_{E,S} = 22.7 \text{ mM (From } V_1 \text{ and value without imidazole,} \\
& \quad \text{see Table 6.2).} \\
& \quad V_1 = 0.06 \text{ min}^{-1} \text{ (Assumed, see Table 5.2).}
\end{align*}
\]
Fig. 6.8. Effect of ethanol and 4-biphenyl-carboxylic acid on inactivation of liver ADH by iodoacetate. Conditions as in Fig. 6.1. Enzyme, 0.6 μM. o, without additions (iodoacetate, enzyme and phosphate buffer only); Δ, + ethanol (0.84 M); □, + 4-biphenyl-carboxylic acid (58.5 μM). (From Reynolds et al., 1970, with publishers' permission).
\[
\begin{align*}
K_{2M,S} &= 30.0 \text{ mM} \\
V_2 &= 0.770 \text{ min}^{-1}
\end{align*}
\]

From Fig. 6.7

The average values for the dissociation-constant of the enzyme-imidazole binary complex (0.86 mM at pH 7.4, and 5.9 at pH 10, for the base form of imidazole) are only approximate, because the values of the above constants critically determine the values obtained, and several are only known approximately themselves. Theorell and McKinley-McKee (1961b) obtained 0.55 mM at pH 7, 0.68 mM at pH 8 and 0.67 mM at pH 9. Although this agrees reasonably with the value found here at pH 7.4, their results gave no indication of a significant weakening at high pH. Evans and Rabin (1968) obtained 0.7 mM at pH 7.2 and 5.4 at pH 9.0. However, they were unaware of the reversible binding of iodoacetate to the enzyme. If the results in Table 6.2 are recalculated by their method, dissociation-constants of 1.14 mM (pH 7.4) and 6.03 mM (pH 10) are obtained.

Pyridine can also stimulate alkylation of the enzyme by iodoacetate (Fig. 6.6), and so can ethanol (Fig. 6.8) but only at high concentrations of iodoacetate; at lower concentrations of iodoacetate it protected. Preliminary experiments suggested that isobutyramide had an effect similar to ethanol in this respect. Isobutyramide inhibited the enzyme assay and gave non-linear progress curves; imidazole was included in the assay cuvettes, to partially overcome the inhibition by isobutyramide. Ethanol (0.17 M) also protected the enzyme slightly (30%) against iodoacetamide.

Aromatic acids. A number of aromatic acids can bind to alcohol dehydrogenase (Chapter 1, Section 1.7). Only one was used in the present study, 4-biphenyl-carboxylic acid. It protected,
Fig. 6.9. Effect of AMP on inactivation of liver ADH by iodoacetate, in excess imidazole. At 23.5°, ionic strength 0.1. 0, at pH 7.4, in imidazole-phosphate buffer, (base form of imidazole, 29 mM), enzyme, 0.5 μM, iodoacetate, 1.33 mM; Δ, at pH 10.0, in glycine-NaOH buffer + phosphate (19 mM in glycinate anion) containing imidazole (98 mM), enzyme 1.0 μM, iodoacetate 1.33 mM. (From Reynolds et al., 1970, with publishers' permission).
Fig. 6.10. Effect of ADP-ribose on inactivation of liver ADH by iodoacetate, in excess imidazole. Conditions as in Fig. 6.9. Enzyme, 0.5 μM. O, at pH 7.4, in imidazole-phosphate buffer (base form of imidazole, 29 mM), iodoacetate 1.33 mM; △, at pH 10.0, in glycine-NaOH buffer containing phosphate and imidazole (63 mM), and iodoacetate (2.0 mM). (From Reynolds et al., 1970, with publishers' permission.)
Table 6.3. Dissociation-constants of AMP and ADP-ribose from complexes with liver ADH in the presence of excess imidazole. From Figs. 6.9 and 6.10.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>pH</th>
<th>Dissociation-Constant</th>
<th>Excess Imidazole</th>
<th>No Imidazole</th>
<th>(Ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>7.4</td>
<td>54.2 µM</td>
<td>32 - 140 µM</td>
<td>(A)</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>10.0</td>
<td>169 &quot;</td>
<td>460 &quot;</td>
<td>(B)</td>
<td></td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>7.4</td>
<td>24.6 &quot;</td>
<td>10.5 - 36 &quot;</td>
<td>(A)</td>
<td></td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>10.0</td>
<td>84 &quot;</td>
<td>100 - 145 &quot;</td>
<td>(C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400 &quot;</td>
<td>(D)</td>
<td></td>
</tr>
</tbody>
</table>

References:  
A = see Table 6.1.  
B = Reynolds et al. (1970).  
C = Yonetani (1963a,b).  
D = Theorell and Yonetani (1964).
competitively with iodoacetate (Fig. 6.8), giving a dissociation-constant of 45 μM; Sigman (1967) found 60 μM. The situation may be rather complicated, however, since Dixon (1953) plots showed a biphasic pattern, with weaker binding at higher concentrations of biphenyl-carboxylic acid. It may be that it can bind in more than one place per subunit: or perhaps there are inter-subunit interactions in the dimeric enzyme molecule. The conditions in Fig. 6.8 represent the lower concentration range.

Interactions Between Imidazole and Other Ligands. Since imidazole activates the alkylation reaction with iodoacetate, one can compare the effect of protecting ligands in the presence of a near-saturating concentration of imidazole with the protection without imidazole. If the factor by which binding of the protecting ligand is weakened is the same as the ratio of imidazole-concentration to that of its dissociation-constant from the enzyme, then imidazole and the protecting ligand are very likely to be mutually competitive.

Adenine nucleotides (AMP, Fig. 6.9, and ADP-ribose, Fig. 6.10) still protect the enzyme considerably. Dissociation-constants (assuming the nucleotides are still competitive with iodoacetate, and allowing for the small amount of enzyme-imidazole-iodoacetate ternary complex formed) are shown in Table 6.3. When compared with Table 6.1, it is seen that imidazole only weakens the binding of these nucleotides by a factor of 1.7 – 2.5, at pH 7.4. Direct comparison at pH 10 could not be carried out since inactivation without imidazole was not feasible at pH 10. However, substrate kinetics gave an inhibitor-constant for AMP at pH 10 of 460 μM (Reynolds et al., 1970; see Appendix), which was 6.7 times higher.
Table 6.4. Effect of excess imidazole on the protection of liver ADH against iodoacetate by orthophenanthroline, decanoate and 4-biphenyl-carboxylic acid. Ionic strength 0.1, 23.5°.

Enzyme, 0.5 - 1.0 µM; iodoacetate, 1.33 mM at pH 7.4 and 2.0 mM at pH 10.0.

<table>
<thead>
<tr>
<th>Added Protector</th>
<th>pH</th>
<th>Half-Time (min.)</th>
<th>$K_{EIm,L}$ (µM)</th>
<th>$K_{E,L}$ (µM)</th>
<th>Ratio</th>
<th>Imidazole (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.4</td>
<td>15.17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>27</td>
</tr>
<tr>
<td>OP (52 µM)</td>
<td>&quot;</td>
<td>21.08</td>
<td>133</td>
<td>6.1</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Dec (64.5 µM)</td>
<td>&quot;</td>
<td>24.08</td>
<td>1040</td>
<td>39</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>BPCA (240 µM)</td>
<td>&quot;</td>
<td>26.17</td>
<td>340</td>
<td>44.6</td>
<td>7.6</td>
<td>27</td>
</tr>
<tr>
<td>&quot; (720 µM)</td>
<td>&quot;</td>
<td>44.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>10.0</td>
<td>13.58</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>63</td>
</tr>
<tr>
<td>BPCA (360 µM)</td>
<td>&quot;</td>
<td>25.17</td>
<td>420</td>
<td>?</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>&quot; (720 µM)</td>
<td>&quot;</td>
<td>34.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$K_{EIm,L}$ = estimated dissociation-constant of the ligand concerned, from its ternary complex with enzyme and imidazole.

$K_{E,L}$ = dissociation-constant of the ligand concerned, from its binary complex with the enzyme (from Chapters 5 and 6).

Ratio = $K_{EIm,L}/K_{E,L}$, i.e. the factor by which imidazole weakens the binding of the ligand.

OP, Dec and BPCA represent orthophenanthroline, decanoate and 4-biphenyl-carboxylic acid respectively.
than at pH 7.2. Theorell and Yonemoto (1964) determined the inhibitor-constant of ADP-ribose at pH 10 to be 400 pM. The presence of imidazole does appear to strengthen the binding of these nucleotides at pH 10 (and weaken it at pH 7.4), and therefore reduce their pH-dependence; however, there is still a difference of at least three-fold between their binding at pH 7.4 and pH 10. Theorell and Yonemoto (1964) found that the binding of ADP-ribose to the enzyme was weaker when investigated using difference spectrophotometry than found in earlier investigations using kinetics, and fluorescence of NADH. They suggested that small amounts of strongly-binding impurities in the ADP-ribose could account for these low values in the earlier investigations (when a large excess of ADP-ribose over enzyme was used), which would be less evident in their spectrophotometric experiments, where a much higher ratio of enzyme to ADP-ribose was used. In the present inactivation experiments, a large excess of ADP-ribose over enzyme was used, and a low value for the dissociation-constant with ADP-ribose was found (Table 6.1). The considerable increase in the presence of imidazole would be consistent with the binding of the postulated impurities being weakened by imidazole; imidazole weakens the binding of NADH (at pH 7 - 8) by nearly ten-fold (Theorell and McKinley-McKee, 1961b).

Imidazole weakens the binding of orthophenanthroline, decanoate and 4-biphenyl-carboxylic acid to liver alcohol dehydrogenase (Table 6.4). Orthophenanthroline and decanoate give excellent agreement with what is predicted if they are competitive with imidazole (see above). On the other hand, biphenyl-carboxylic acid is a better protector than expected if competitive: an
Table 6.5. Effect of excess imidazole on protection of liver ADH from iodoacetate by chloride and formate. Ionic strength 0.1, at 23.5°. Enzyme, 0.6 μM; imidazole (base form) 29 mM, at pH 7.4 and 63 mM at pH 10.0; iodoacetate, 1.33 mM at pH 7.4 and 2.0 mM at pH 10.0.

<table>
<thead>
<tr>
<th>Added Protector</th>
<th>pH</th>
<th>Half-Time (min.)</th>
<th>$K_{EIm,L}$ (mM)</th>
<th>$K_{E,L}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.4</td>
<td>14.75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloride (38.7 mM)</td>
<td>&quot;</td>
<td>27.17</td>
<td>44</td>
<td>23</td>
</tr>
<tr>
<td>Formate (&quot; )</td>
<td>&quot;</td>
<td>17.0</td>
<td>(250)</td>
<td>24</td>
</tr>
<tr>
<td>None</td>
<td>10.0</td>
<td>13.33</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloride (38.0 mM)</td>
<td>&quot;</td>
<td>23.67</td>
<td>46</td>
<td>(?)</td>
</tr>
<tr>
<td>Formate (&quot; )</td>
<td>&quot;</td>
<td>16.7</td>
<td>(152)</td>
<td>(?)</td>
</tr>
</tbody>
</table>

$K_{EIm,L}$ = estimated dissociation-constant of the ligand concerned from its ternary complex with enzyme and imidazole.

(Values in brackets are unreliable.)

$K_{E,L}$ = dissociation-constant of the ligand concerned from its binary complex with the enzyme (see Chapter 5).
Fig. 6.11. Effect of both ADP-ribose and decanoate on inactivation of liver ADH by iodoacetate. Conditions as in Fig. 6.1. Enzyme, 0.6 μM; iodoacetate, 3.33 mM. O, without decanoate; V, + decanoate (80.7 μM); Δ, + decanoate (161.5 μM).

(From Reynolds et al., 1970, with publishers' permission).
Fig. 6.12. Effect of increased ionic strength on inactivation of liver ADH by iodoacetate, and on protection by AMP and chloride. At 23.5° and ionic strength 0.60, in sodium-potassium phosphate buffer, pH 7.4. Enzyme, 0.5 μM. O, without protecting compound; Δ, + chloride (140 mM); V, + AMP (161 μM). (From Reynolds et al., 1970, with publishers' permission).
Fig. 6.13. Effect of AMP together with decanoate or chloride on inactivation of liver ADH by iodoacetate. Conditions as in Fig. 6.12. Enzyme, 0.5 μM; iodoacetate, 6.67 mM. o, without decanoate or chloride; Δ, + sodium chloride (140 mM); V, + decanoate (198 μM). (From Reynolds et al., 1970, with publishers' permission).
enzyme-biphenylcarboxylate-imidazole ternary complex may therefore be formed. The pH-dependence in the presence of excess imidazole is seen to be small, since protection is almost as effective at pH 10.

The protecting effect of chloride is not abolished by imidazole (Table 6.5), although its binding is weakened by a factor of two. This binding now appears to be pH-independent. Formate, however (which binds very similarly to chloride, in the absence of imidazole: see Chapter 5), becomes a much weaker protector in excess imidazole.

**ADP-Ribose and Decanoate.** Since both these are protectors, competitive with iodoacetate, the method of Yonetani and Theorell (1964) was used to examine their interactions. Results are shown in Fig. 6.11. Parallel lines are obtained, indicating that decanoate and ADP-ribose are competitive with each other. The deviation at high concentrations of ADP-ribose and decanoate is probably due to slight instability of the enzyme: a small effect would make a noticeable difference, since the rate of inactivation was slow anyway, on that part of the graph.

**Increased Ionic Strength.** Interactions which are due to electrostatic attraction or repulsion should become weaker in more polar surroundings. Some experiments were done to investigate this. At an ionic strength of 0.6, AMP and iodoacetate are still competitive, with each of their dissociation-constants increased by a factor of 2.7 (Fig. 6.12). Protection by chloride, however, became mixed: the dissociation-constant of the enzyme-chloride binary complex was increased by a factor of 3.3.

AMP is nearly competitive with decanoate and chloride (Fig. 6.13), but more so with chloride than decanoate.
Fig. 6.14. Effect of both AMP and orthophenanthroline on the inactivation of liver ADH by iodoacetate. Conditions as in Fig. 6.1. Enzyme, 0.5 μM; iodoacetate, 3.33 mM. ○, without orthophenanthroline; △ and ▽, + orthophenanthroline (4.33 μM and 8.67 μM respectively). (From Reynolds et al., 1970, with publishers' permission.)
Fig. 6.15. Effect of both chloride and orthophenanthroline on the inactivation of liver ADH by iodoacetate. Conditions as in Fig. 6.12. Enzyme, 0.5 μM; iodoacetate, 6.67 mM. o, without orthophenanthroline; Δ, + orthophenanthroline (7.49 μM).

(From Reynolds et al., 1970, with publishers' permission.)
Table 6.6. Some inactivation experiments on yeast ADH with iodoacetate. Ionic strength 0.1, pH 7.4, at 23.5°. Enzyme, 0.1 µM.

<table>
<thead>
<tr>
<th>Added Ligand</th>
<th>Iodoacetate (mM)</th>
<th>Half-Time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>(approx. 90)</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.67</td>
<td>6.63</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.67</td>
<td>0.84</td>
</tr>
<tr>
<td>Imidazole (base, 29 mM)</td>
<td>0.67</td>
<td>4.22</td>
</tr>
<tr>
<td>AMP (1.58 mM)</td>
<td>0.67</td>
<td>9.07</td>
</tr>
<tr>
<td>Na Chloride (40 mM)</td>
<td>0.67</td>
<td>6.77</td>
</tr>
<tr>
<td>Na Formate (40 mM)</td>
<td>0.67</td>
<td>6.15</td>
</tr>
</tbody>
</table>
Orthophenanthroline and AMP. AMP is competitive with iodoacetate, but orthophenanthroline is approximately non-competitive. To study their interaction, a method of analysis was devised, and is given in Chapter 3, Section 3.3. It is very similar to that of Yonetani and Theorell (1964), but is for one substrate-competitive inhibitor (varied) and one substrate-noncompetitive inhibitor (different fixed concentrations used). The lines meet just below the abscissa, indicating that the binding of AMP and orthophenanthroline are almost independent of one another, but with perhaps a slight weakening effect, (Fig. 5.14).

Orthophenanthroline and Chloride. In order to be able to have a sufficiently high concentration of chloride, an ionic strength of 0.6 was used. The results are shown in Fig. 6.15. At low concentrations of chloride, it seems to be competitive with orthophenanthroline; but at higher concentrations of chloride, complexes involving enzyme, orthophenanthroline and one or more chloride ions seem to be formed. The evidence of one curve, containing few experimental points, is not very convincing: but Morris (1970) has obtained exactly similar curves from substrate kinetics (varying chloride, at several fixed concentrations of orthophenanthroline, and maintaining the ionic strength with phosphate). It should be borne in mind that the varied phosphate concentration may itself be having an effect.

Comparison with Yeast Alcohol Dehydrogenase. Table 6.6 shows the results of some preliminary experiments on yeast alcohol dehydrogenase. The thiol group is much more reactive, and scarcely any saturation-effect was discernible at the highest concentrations it
was possible to measure. Imidazole stimulated inactivation, and AMP protected it. Chloride and formate had no effect at the concentrations used (40 mM). In the absence of iodoacetate, the enzyme was rather unstable at low concentrations. However, more concentrated solutions appeared to be much more stable.

6.3. Discussion.

When interpreting these experiments, it is necessary to bear two points in mind: firstly, that the complexes postulated are only ones that do exert an effect measurable by inactivation with iodoacetate. Other complexes may well form which have no effect on this. Secondly, what appears to be one simple reversible complex may in fact be several different complexes between the enzyme and a ligand combining in several different ways. If only one molecule of ligand is bound at once (i.e. all the complexes are mutually competitive) then the overall result will be one "hybrid" dissociation-constant. However, the different complexes may have different interactions with other types of ligand. It is distinctly probable that this kind of behaviour is occurring with some of the ligands used in the experiments described above.

Adenine nucleotides are perhaps comparatively simple. Under all conditions they seem to be competitive with iodoacetate. Therefore, it seems reasonable to postulate that adenylate compounds and iodo-acids (and also aromatic acids – see below) bind, if not at the same site, at least at overlapping sites.

Adenosine is more complex. Its ability to act as a metal-
chelating agent may enable it to bind non-competitively with iodoacetate (like orthophenanthroline), as well as competitively (or partly competitively) at the adenylate site. However, its much (3-fold) weaker apparent dissociation-constant for protection compared with substrate-kinetics (Reynolds et al., 1970: see Appendix) suggests that protection may be only partial.

A number of previous studies have used bivalent chelating-agents (orthophenanthroline or bipyridyl) to investigate liver alcohol dehydrogenase, following the discovery (Theorell et al., 1955) that, like the yeast enzyme, it contains zinc. Orthophenanthroline inhibits the enzyme reversibly (Vallee and Hoch, 1957) and difference spectra (Vallee et al., 1958; Vallee and Coombs, 1959) indicate that it binds to zinc. The dissociation-constant for orthophenanthroline from the enzyme (33 μM, Vallee and Coombs, 1959; 8 μM, Yonetani, 1963b; 9 μM, Yonetani and Theorell, 1964; 6 μM, this work, above) agrees quite well with the third molecule forming Zn(o-phenanthroline)₃ of orthophenanthroline chelating inorganic zinc ions (Kolthoff, Leussing and Lee, 1951; Vallee and Coombs, 1959; Plane and Long, 1963). Dalziel (1963a) has pointed out that published inhibition kinetics (Vallee, Williams and Hoch, 1959; Plane and Theorell, 1961) are quite compatible with a compulsory-order mechanism (coenzyme binding before substrate) with orthophenanthroline competing with coenzyme.

2,2'-Bipyridyl has been shown by Sigman (1967) to bind to alcohol dehydrogenase; the inspiration for a number of the present experiments with iodoacetate came from his work. As with orthophenanthroline, two molecules are bound per molecule of enzyme, and both spectra and magnitude of the dissociation-constant suggest
that it binds to zinc. However, Sigman (1967) was able to show that bipyridyl could, under certain conditions (high substrate concentrations) activate the enzyme-catalysed reaction. Therefore it seems that bipyridyl, unlike orthophenanthroline (but like imidazole) can form ternary complexes with enzyme and coenzyme.

The present experiments have shown that imidazole and decanoate are competitive with orthophenanthroline, while AMP is approximately non-competitive: these results are in accordance with the effect of imidazole, decanoate and adenine nucleotides (Sigman, 1967) on bipyridyl-binding; however, acetate did not compete effectively with bipyridyl, indicating that (in the absence of NAD$^+$) it may bind away from the zinc, possibly like aromatic acids. The incomplete protection given by decanoate (Chapter 5) also would indicate that its binding may be somewhat different from that of iodoacetate. Yonetani and Theorell (1964) found that orthophenanthroline tightened the binding of AMP and ADP (but bound independently of ADP-ribose). With AMP in particular, they found it to be a weaker inhibitor than did other workers (Li and Vallee, 1964b; Reynolds et al., 1970); but its binding became three-fold stronger in the presence of orthophenanthroline, and was then similar to the value found here. The binary complexes of enzyme with ADP-ribose, and with orthophenanthroline, and also the ternary complex with both ligands bound simultaneously, have been crystallised (Yonetani, 1963a). The effect of pH, and of imidazole, is rather complex. Theorell and McKinley-McKee (1961b,c) suggested that imidazole was binding to zinc in the enzyme, displacing a water molecule. They observed that it also abolished or reduced some of the effects
normally observed on raising the pH: therefore it was suggested that the ionisation at high pH was that of the zinc-bound water molecule. When ionised, the hydroxide ion was considered to neutralise the one positive charge on the zinc (the other was presumed to be neutralised by an anionic ligand from the protein). This explained well the greater affinity of the enzyme for NAD$^+$ at higher pH; the positive zinc would repel the nicotinamide’s positive charge in the presence of a neutral ligand (imidazole or water), but not if it carried one negative charge (hydroxide, or carboxylate).

This provided a nice explanation for the mutual stabilisation of NAD$^+$ and fatty-acids in ternary complexes with the enzyme – the extra positive charge of the nicotinamide assisting binding of the anionic carboxylate group, as well as vice versa (see above). However, NADH and fatty-acids were observed to be competitive (Winer and Theorell, 1960). This could scarcely be due to steric interaction, because of the ready formation of complexes with NAD$^+$ and fatty-acids. The competition observed between decanoate and ADP-ribose (Fig. 6.11) is perhaps due to electrostatic repulsion between the bound carboxylate group and the phosphates of the nucleotide. The slightly weaker interaction at higher ionic strength (Fig. 6.13) between AMP and decanoate may support this concept, although using AMP instead of ADP-ribose may have been responsible.

Both imidazole and orthophenanthroline (Yonetani, 1963b) are bound much more weakly at high pH. If they displace a zinc-bound water-molecule at neutral pH, but a zinc-bound hydroxide ion at high pH, the latter could well be bound much more tightly.

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The stimulation of inactivation by pyridine, by ethanol (at high iodoacetate concentrations) and possibly also by isobutyramide suggests that it may be a general phenomenon of neutral monodentate ligands; it is therefore unlikely that imidazole stimulates inactivation by a specific mechanism such as that suggested by Rabin, Evans and Hashed (1970). Binary enzyme-ethanol complexes have been reported previously (Sigman, 1967), but may have no kinetic significance (Theorell and McKinley-McKee, 1961a,c; Shore and Theorell, 1966a,b; Dalziel and Dickinson, 1966a,b). McKinley-McKee (1963b) has stated that ethanol does not affect inactivation by iodoacetamide.

The binding of NADH (Theorell and Winer, 1959; Theorell and McKinley-McKee, 1961b; Theorell et al., 1970), and of ADP-ribose (Yonetani, 1963a,b; Theorell and Yonetani, 1964), and probably of AMP (Reynolds et al., 1970) show similar pH-dependence, being bound much more weakly above pH 9. It would be of interest to know whether this was due to the same ionisation as described above or not. If the same, the pH-dependence should be suppressed by imidazole. The results in Figs. 6.9 and 6.10 and in Table 6.3 indicate that the pH-dependence is reduced quite appreciably by imidazole - but a three-fold difference in binding still exists between pH 7.4 and pH 10 for AMP and ADP-ribose. This is in contrast to adenosine, which (even without imidazole) appears to bind equally well at the two pH values (Reynolds et al., 1970). Therefore, although the phosphates of ADP-ribose and AMP probably are attracted to a certain extent by the (postulated) zinc's positive charge, one may tentatively postulate another, imidazole-insensitive positive charge (perhaps a lysine) with a pH probably
Table 6.7. Summary of dissociation-constants of coenzymes and coenzyme fragments from liver ADH, at pH 7 - 7.5.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Dissociation-Constant (µM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>6,200</td>
<td>Reynolds et al. (1970).</td>
</tr>
<tr>
<td>AMP</td>
<td>32 - 140</td>
<td>Table 6.1.</td>
</tr>
<tr>
<td>ADP</td>
<td>180 - 390</td>
<td>Table 6.1.</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>10.5 - 36</td>
<td>Table 6.1.</td>
</tr>
<tr>
<td>NADH</td>
<td>0.3</td>
<td>Theorell and McKinley-McKee, (1961b); Anderson and Weber, (1965).</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>130</td>
<td>Taniguchi et al. (1967).</td>
</tr>
<tr>
<td>NMN</td>
<td>10,000</td>
<td>Sigman (1967).</td>
</tr>
<tr>
<td>NRPPR</td>
<td>(approx. 3,000)</td>
<td>Fawcett and Kaplan (1962).</td>
</tr>
</tbody>
</table>

NMN = Nicotinamide mononucleotide.
NRPPR = Nicotinamide-β-riboosyl-pyrophosphoryl-ribose (i.e. NAD⁺ with the adenine removed).
below 10, which is involved in binding the phosphate groups – mainly the phosphate nearer adenine. It is not clear whether this could be one of the lysine groups responsible for activating the enzyme when modified (Plapp, 1970).

Table 6.7 contains a summary of dissociation-constants of coenzymes and coenzyme fragments with the enzyme. They serve to emphasize the importance of the phosphate of AMP in binding (as discussed by Li and Vallee, 1964b), and suggest that the phosphate nearer nicotinamide may play only a minor role in coenzyme binding. The oxidised form of the coenzyme analogue acetyl-pyridine adenine dinucleotide binds as strongly as NAD$^+$ (Shore and Theorell, 1967; Shore and Gilleland, 1970), but the reduced form was bound more weakly than NADH. The nicotinamide of NAD$^+$ seems to be a hindrance to binding, at neutral pH. As discussed above, this may be due to repulsion by a positive charge (probably zinc) in the enzyme, which is neutralised at high pH. Nicotinamide mononucleotide does not displace bipyridyl from the enzyme (Sigman, 1967), and the observed inhibition by it might be due to adenine-containing impurities, or perhaps due to nicotinamide binding at the adenine site on the enzyme. In this respect, yeast alcohol dehydrogenase is different: quaternary ammonium and pyridinium compounds can bind (Fonda and Anderson, 1967), and binding is strengthened by adenine nucleotides. Dogfish lactate dehydrogenase only binds nicotinamide mononucleotide in the presence of AMP (McPherson, 1970): the phosphate of AMP might trigger off a conformation change, forming the nicotinamide binding-site (Adams et al., 1970a,b; McPherson, 1970).

Dalziel (1963d) has suggested that the large negative entropy
of activation when NAD$^+$ or NADH dissociate from the enzyme may be due to separation of electrostatic charges in the transition-complex.

The pH-dependence of NADH-binding to the steroid isoenzyme appears to be less than for the ethanol-active form (Theorell et al., 1970). Since none of the altered amino-acids (Jornvall, 1970a) has a pK in the pH 9 – 10 region, the effect may be an indirect one.

The binding of iodoacetate itself, and of 4-biphenyl-carboxylic acid, may be similar to each other. This is shown by pH-dependence with and without imidazole, competition with adenine nucleotides and non-competition with orthophenanthroline and bipyridyld (Sigman, 1967). The pH-dependence, with and without imidazole, suggests that their binding is largely dependent on the zinc (i.e. imidazole-sensitive positive charge). The weakening effect of imidazole on their binding, although not competitive, is understandable. However, if this is the case, it is hard to see why orthophenanthroline and bipyridyl, which are larger than imidazole, have virtually no effect on their binding. One can postulate that the carboxylate anion of these acids is held near whose positive charge zinc, provides electrostatic binding (rather than the with the carboxylate group lysine postulated above), but far enough away (or positioned at such an angle) that orthophenanthroline does not affect its binding.

The effect of imidazole, in exerting an effect which bidentate ligands do not, is also difficult to explain satisfactorily. One possibility is that it causes a conformation-change in the enzyme (perhaps mediated by a change in co-ordination geometry of zinc). This would be supported by the protection which imidazole gives to
the enzyme from PCMB (Witter, 1960); it is considerably more effective than the very strong enzyme-NADH-isobutyramide ternary complex. However, both imidazole and orthophenanthroline provide good protection against PCMB (Chapter 4). Another possibility is that, unlike orthophenanthroline, imidazole is able to bind at more than one site. Rather high concentrations of imidazole were used, and binding to secondary sites, perhaps other than zinc, is a possibility; indeed, it was suggested above (by the data in Fig. 6.7) that this was probably occurring. Temperature-jump studies (Czerlinski, 1962) showed two different enzyme-NADH-imidazole ternary complexes, but this could be interpreted in terms of either of the above mechanisms.

The binding of halide ions is complex. The results defy rational explanation unless one postulates that they bind in a number of different ways. Bromide and iodide are the only anionic compounds found not to be competitive with iodoacetate at ionic strength 0.1. Chloride and bromide can form ternary complexes with enzyme and NADH (Theorell et al., 1955; Plane and Theorell, 1961), stimulating the dissociation of NADH from the enzyme. In this sense, they are acting like imidazole. However, they are unlikely to bind simply and solely like imidazole, because fatty acids (which are competitive with imidazole, while chloride is not) are competitive with NADH. The near-competition with AMP (Fig. 6.13) and largely non-competition with imidazole (Table 6.5) indicate that chloride-binding has much in common with that of the phosphate of AMP. However, imidazole renders chloride-binding independent of pH, but not nucleotide-binding. With orthophenan-throline (Fig. 6.15) it appears that there is competition at low
concentrations but not at high concentrations of chloride, suggesting some comparatively strong binding to zinc (or near it), and weaker binding elsewhere as well, probably at more than one site. Carbonic anhydrase B can bind up to about six chloride ions simultaneously (Verpoorte, Mehta and Edsall, 1967).

Further evidence for the complex binding of chloride, and of its interactions with other ligands, is obtained from the recent NMR investigations of Lindman et al., (1970). NADH (with or without isobutyramide) partially reduces the line-broadening of $^{35}$Cl$^{-}$ when it interacts with liver alcohol dehydrogenase. In the absence of isobutyramide, the effect is complete when one equivalent of NADH has bound per dimeric enzyme molecule; but in the presence of isobutyramide, two molecules of NADH are required, i.e. one per active site.

In all these experiments no effect of inorganic phosphate on alkylation (except by altering the ionic strength) has been noticed. However, it does bind to the enzyme competitively with NADP (Dalziel and Dickinson, 1965a), and it is very difficult to dialyse the enzyme free of phosphate (Reynolds, 1967). Therefore phosphate very probably is bound to the enzyme, although its effect on alkylation is unknown. Several phosphate ions can bind to glyceraldehyde phosphate dehydrogenase (Velick and Furfine, 1963); anions protect this enzyme (Boross, Cseke and Vas, 1969), and also creatine kinase (Milner-White and Watts, 1970) from alkylation.

The brief experiments with yeast alcohol dehydrogenase suggest that in some senses it may be similar to the liver enzyme. AMP provides protection, and imidazole appears to stimulate the reaction
slightly; further studies would be required, to reach any definite conclusions. The enzyme only has one zinc atom per subunit (Vallee and Hoch, 1955). Binding by phenanthroline compounds (Anderson et al., 1960) is much weaker than to the liver enzyme; it is equally good for derivatives that cannot act as chelating agents; those obtained on and difference spectra are similar to transferring the compounds into less polar environments (as well as to the formation of chelates). Therefore, it seems likely that the yeast enzyme does not readily form zinc chelates. Other, more comprehensive studies using inactivation by alkylation have been made by Rabin and Whitehead (1962); Whitehead and Rabin (1964); Rabin et al., (1964); Rashed and Rabin (1968); Eisele and Wallenfels (1968); Plapp, Woenckhaus and Pfleiderer (1968), and Tipton (1968). The last two references provide some positive evidence that a thiol group near the active centre is being alkylated.

Eisele and Wallenfels (1968) showed that the different optical isomers of 2-iodopropionate react at different rates with yeast alcohol dehydrogenase. This is perhaps not surprising: proteins themselves are optically active, being made up of the L isomer of optically-active amino-acids. The reaction of the liver enzyme with too slow 2-iodopropionate was to be useful (Eisele, 1969); it also showed a saturation-effect.
Chapter 7

Carboxymethyl Alcohol Dehydrogenase

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7.3. Discussion ........................................... 99
7.1. Introduction

In the preceding chapters, doubts were raised as to whether the thiol group which is reactive towards iodoacetate (cysteine 16; Jornvall, 1970b) is at the active site of the enzyme. As mentioned in Chapter 1, Section 1.4, Li and Vallee (1965) showed by optical rotatory dispersion that the position, sign and shape of the extrinsic Cotton effect of enzyme-bound NADH is the same in carboxymethylated as in native enzyme; the reduced amplitude and breadth was attributed to increased flexibility of the bound dihydropyridine group, but the environment was considered to be unaltered. They also showed, by spectrophotometric titration, that NADH was bound by the carboxymethylated enzyme, but much more weakly than by native enzyme.

The discussion of Chapter 6 indicated what kind of effects might be expected if an extra anionic group was introduced into the active centre. In this Chapter, experiments are described using carboxymethyl alcohol dehydrogenase, to examine the effect of carboxymethylation on ligand binding.

Part of this work has been published (Reynolds and McKinley-McKee, 1970).

7.2. Results.

Residual Activity. Samples of liver alcohol dehydrogenase were carboxymethylated with iodoacetate, as described in Chapter 2, Section 2.4. In all preparations, it was found that activity was
Fig. 7.1. Extensive inactivation of liver ADH by iodoacetate. Ionic strength 0.1, in sodium phosphate buffer, containing imidazole (3.6 mM), at 23.5°. Enzyme, 13 μM; iodoacetate, 6 mM. o, without AMP; △, + AMP (170 μM); ▽, + AMP (340 μM).
Fig. 7.2. Starch-gel electrophoresis of native and carboxymethylated liver ADH, in 10 mM sodium phosphate buffer, pH 7.4. The electric field was 13 volts/cm. 1.5 x actual size.
lost rapidly until about 2 - 2.5% of the initial activity remained; but the last trace of activity was much more resistant to inactivation. This was true whether imidazole was present or not. The kinetics of this are shown in Fig. 7.1.

It was thought that a likely explanation of this residual activity would be that a minor isoenzyme component was resistant to iodoacetate. Starch-gel electrophoresis, performed exactly as by McKinley-McKee and Moss (1965), and using the same apparatus, gave the results shown in Fig. 7.2. The native enzyme showed one main protein band, EE, and a very faint minor protein band, ES. Activity staining showed an intense band, EE, and fainter bands ES, EE' and EE". The carboxymethylated enzyme showed one band of activity and of protein, both having approximately the same mobility as EE in the native enzyme. This shows that the residual activity is a property of protein with the same mobility as the bulk of the protein present, and hence is unlikely to be due to a minor isoenzyme in the native protein.

Residual activity might not be a genuine catalytic activity of the carboxymethylated enzyme, but might instead represent a small fraction of the protein being alkylated at some other residue; this might be more resistant, now, to alkylation at cysteine 46. If this postulated "other" residue is not protected effectively by AMP, then inactivations in the presence of AMP should enable more of this "variant" modified enzyme to accumulate, and give an increased residual activity. This clearly does not occur (Fig. 7.1). Therefore, if this "other" residue does exist, it must also be protected by AMP: however, this does lessen the likelihood of this explanation being correct; it appears that residual activity probably is a genuine catalytic activity of liver alcohol dehydrogenase (EE) carboxymethylated at residue 46 (Jornvall), 1970b).
Fig. 7.3. Titrations of native and (24 hours old) carboxymethyl liver ADH with 4-iodopyrazole. Ionic strength 0.08, in sodium phosphate buffer (pH 7.4), at 23.5°. NAD⁺, 136 μM. o, native enzyme (2.45 μM); Δ, carboxymethyl enzyme (2.6 μM).
When ethanol (8 mM) was added to carboxymethylated enzyme (0.7 μM) and NAD⁺ (460 μM) at pH 10, no "burst" of increased fluorescence was observed, indicating no sudden production of (enzyme-bound) NADH. Therefore, if the bulk of the protein does have residual activity, and the affinity for NAD⁺ and ethanol is not reduced too drastically, it does not obey the Theorell-Chance mechanism (Theorell and Chance, 1951).

Amino-Acid Analysis. When the thiol content was measured immediately after carboxymethylation, a loss of 1 - 1.2 thiol groups per subunit was found (see Chapter 5). However, after carboxymethylated enzyme had been kept for at least 24 hours, or if the reaction with iodoacetate was terminated by dialysis, up to 30% or more of the thiol groups were lost, i.e. about 5 out of 14 per subunit. Amino-acid analysis showed that one S-carboxymethylcysteine was present per subunit, but also some cystine (approximately 2.5 residues per subunit). The column of the amino-acid analyser had been recently repacked, and was giving excellent separations: otherwise, the cystine peak would not have been resolved. It is possible (but unlikely) that the cystine was an artifact due to oxidation during hydrolysis. Native liver alcohol dehydrogenase (unlike the yeast enzyme) is comparatively stable, and preparations normally have (and retain) full activity and a full complement of thiol groups when stored for a few days at 0 - 4°C.

Complexes with NAD⁺ and Pyrazole. As shown in Fig. 7.3, titrations of native and carboxymethyl enzyme with 4-iodopyrazole in excess NAD⁺ were very similar. The same gradient at low pyrazole concentrations indicates that the complex formed has the same extinction
Fig. 7.4. Titrations of (aged) carboxymethyl liver ADH with pyrazole: effect of decanoate. Conditions were as in Fig. 7.3. Carboxymethyl ADH, 2 μM; NAD⁺, 136 μM. ○, without decanoate, except as indicated (●); Δ, + decanoate (33 μM).
coefficient at 290 nm. The curvature as the end-point of the
titration is reached is similar, and estimated dissociation-
constants of 4-iodo-pyrazole from the (assumed) ternary complex of
enzyme, NAD$^+$ and pyrazole derivative were also similar (approxi-
mately 0.1 μM), which is rather higher than obtained from kinetic
estimates by Theorell et al., (1969). However, the NAD$^+$ concen-
tration (136 μM) will not be sufficient to saturate the enzyme, in the
absence of pyrazole; the dissociation-constant of the native
enzyme-NAD$^+$ binary complex is approximately 130 μM at pH 7 and
30 μM at pH 8 (Taniguchi et al., 1967).

Titrations with 4-iodo-pyrazole did enable an estimate of the
coenzyme-binding-site concentration to be made. It was usually
about 10% less than on a sample of native enzyme with the same
absorbance at 280 nm, suggesting a small loss of binding-sites
either by denaturation or by chemical reaction of other groups in
the enzyme. When the alkylation of native enzyme was terminated
by dialysis (instead of by passage through Sephadex G-25), or on
keeping, the loss was 20 - 30%.

Fatty-acids form strong ternary complexes with enzyme and
NAD$^+$ (Winer and Theorell, 1960). Fig. 7.4 shows that decanoate
does appear to displace pyrazole from the carboxymethyl-enzyme-
NAD$^+$-pyrazole ternary complex. The titration curve in the presence
of a constant concentration of decanoate may be misleading because
pyrazole could only displace decanoate to form its own ternary
complex rather slowly (see Theorell et al., 1969). Native enzyme
showed similar effects when decanoate displaced pyrazole.
Estimates of the dissociation-constant for decanoate from the
ternary complex of carboxymethyl-enzyme, NAD$^+$ and decanoate were
Fig. 7.5. Uncorrected fluorescence-emission spectrum of NADH in the presence of carboxymethyl liver ADH. Ionic strength 0.1, in sodium phosphate buffer (pH 7.4), at 23.5°. Excitation at 330 nm. NADH, 0.8 μM; carboxymethyl enzyme concentrations as follows: a, zero; b, 0.7 μM; c, 2.1 μM.
Fig. 7.6. Anomalous fluorescence intensity of NADH at low concentrations. Ionic strength 0.1, in sodium phosphate buffer (pH 7.4), at 23.5°. Excitation at 330 nm, emission at 410 nm. The dashed line represents an extrapolation of the experimental line between 1 and 7 μM (which was linear).
rather erratic, and varied between 1 and 9 pM. The value for native enzyme was 2 pM (Theorell and McKinley-McKee, 1961b).

**Fluorescence Experiments with NADH.** The fluorescence emission of NADH is enhanced considerably, and the peak undergoes a blue shift, when bound to native liver alcohol dehydrogenase (Boyer and Theorell, 1956; Theorell and McKinley-McKee, 1961b). However, as shown in Fig. 7.5, carboxymethylated liver alcohol dehydrogenase only slightly enhances the peak height of the emission spectrum of NADH. The considerable broadening of the shorter-wavelength part of the spectrum does give an enhancement of 7 to 8-fold at 410 nm, and this may be used to estimate the dissociation-constant of NADH from its complex with the carboxymethylated enzyme.

Fluorescence titrations were found to be comparatively difficult to do accurately, and many refinements could with advantage have been adopted to the method which was used. The main problem was that at very low concentrations of NADH (without any enzyme), the fluorescence observed was greater, proportionately, than at higher concentrations. This effect is shown in Fig. 7.6. The effect was only observed when the NADH concentration was below about 1 pM; from 1 to over 6 pM, fluorescence increased linearly with concentration of NADH. The effect was always observed, but its magnitude was not precisely reproducible. It occurred in the presence of protein too; if sufficient allowance was not made for it, anomalous (stronger) binding of NADH at low concentrations appeared to take place. The reason for this is unknown; one suggestion (made by Dr. G.K. Radda) was that it was due to adsorption of NADH to the walls of the cuvette; the fluorescence measured should, however, be from the centre of the cuvette.
Fig. 7.7. Fluorescence titrations of native liver ADH with NADH. Ionic strength 0.1, in sodium phosphate buffer (pH 7.4), at 23.5°. o, without AMP; ▲, + AMP (61 μM); ▼, + AMP (122 μM). Ethanol (8 mM) was present.
Contaminating metal ions (or other impurity) may also be a possibility. Mercuric ions bind to NADH and abolish its absorption at 340 nm (Doukin and King, 1968); it is probable that other metals can bind too, but unlikely that they would increase fluorescence.

Other sources of error might be scattered light within the emission monochromator; this could be cut out by using optical filters. Scattered light from the cuvette could be caused by particles present in the solution in it, and might be removed by centrifugation or filtration, or using optical filters as above.

The method of calculation is described and discussed in Chapter 3, Section 3.4.

The technique was tested using native enzyme (Fig. 7.7). A considerable amount of NADH-oxidising material (presumably aldehyde) was found to completely prevent useful titrations being performed. A low concentration of ethanol (8 mM) was used to suppress this. In the absence of AMP, a good titration-curve was given, with dissociation-constant 0.296 μM. This is in excellent agreement with literature values (0.29 μM: Anderson and Weber, 1965: 0.31 μM, Theorell and McKinley-McKee, 1961b). With AMP present (61 or 122 μM), dissociation-constants for the enzyme-AMP binary complex were calculated to be 53.2 and 69.7 μM: the lack of agreement between these figures was rather disappointing.

A small but noticeable amount of NADH-oxidation was observed during fluorescence titrations of carboxymethyl enzyme. This could either be corrected for by the method of Theorell and Winer (1959), or abolished by adding ethanol (2 - 8 mM). The latter method was adopted, as it was considered very unlikely that this
Fig. 7.7. Fluorescence titrations of freshly-prepared carboxymethyl liver ADH with NADH; effect of AMP. Conditions as in Fig. 7.7. o, no other ligand; △, + AMP (122 μM).
Fig. 7.9. Fluorescence titrations of carboxymethyl liver ADH (stored 24 hours) with NADH: effect of decanoate. Conditions as in Fig. 7.7. o, no other ligand; △, + decanoate (50 μM); ▽, + decanoate (100 μM).
Fig. 7.10. Fluorescence titrations of carboxymethyl liver ADH (stored 24 hours) with NADH: effect of 4-biphenyl-carboxylic acid and of lower pH. Conditions as in Fig. 7.7. Dashed line without points, no added ligand at pH 7.4 (see Fig. 7.9); o and Δ, + 4-biphenyl-carboxylic acid (50 μM and 100 μM respectively) at pH 7.4; v, no added ligand (pH 6.1, sodium phosphate buffer).
Fig. 7.11. Fluorescence titrations of carboxymethyl liver ADH (stored 24 hours) with NADH: effect of acetamide, and of imidazole. Conditions as in Fig. 7.7. Dashed line without points, no added ligand (pH 7.4); ○, + acetamide (0.37 M) at pH 7.4; ●, + imidazole (base form, 29 mM) at pH 7.4; △, + imidazole (97 mM) at pH 9.8 (glycine-NaOH buffer + sodium phosphate, 19 mM in glycinate anion).
Table 7.1. Apparent dissociation-constants of NADH from carboxymethyl liver ADH in the presence of other ligands, determined by fluorescence enhancement. Evaluated from Figs. 7.7 - 7.11.

<table>
<thead>
<tr>
<th>Added Ligand</th>
<th>pH</th>
<th>Q</th>
<th>D (pM)</th>
<th>K_{E,L} (pM)</th>
<th>Native ADH (pM)</th>
<th>(Ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.4</td>
<td>7.2-7.6</td>
<td>1.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP (122 pM)</td>
<td>“</td>
<td>8.2</td>
<td>3.50</td>
<td>115</td>
<td>32 - 140</td>
<td>(A)</td>
</tr>
<tr>
<td>Dec (50 pM)</td>
<td>“</td>
<td>7.7</td>
<td>3.16</td>
<td>58.5</td>
<td>39 - 230</td>
<td>(B)</td>
</tr>
<tr>
<td>Dec (100 pM)</td>
<td>“</td>
<td>8.0</td>
<td>4.59</td>
<td>57.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPCA (100 pM)</td>
<td>“</td>
<td>3.1</td>
<td>(8.49)</td>
<td>(25)</td>
<td>45 - 60</td>
<td>(A,C)</td>
</tr>
<tr>
<td>Acetamide (0.4 M)</td>
<td>“</td>
<td>8.8</td>
<td>2.3</td>
<td></td>
<td></td>
<td>(see D)</td>
</tr>
<tr>
<td>Imidazole (base, 29 mM)</td>
<td>“</td>
<td>5.5</td>
<td>2.9</td>
<td></td>
<td></td>
<td>(see E)</td>
</tr>
<tr>
<td>None</td>
<td>6.1</td>
<td>6.8</td>
<td>1.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>9.8</td>
<td></td>
<td>(no binding of NADH detected)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imidazole (97 mM)</td>
<td>9.8</td>
<td>3.5</td>
<td>4.42</td>
<td></td>
<td></td>
<td>(see E)</td>
</tr>
</tbody>
</table>

Q = factor by which NADH fluorescence is enhanced upon binding to carboxymethyl enzyme.

D = apparent dissociation-constant of NADH from carboxymethyl-ADH.

K_{E,L} = calculated dissociation-constant of the binary enzyme-ligand complex, assuming ligand is competitive with NADH.

BPCA = 4-biphenyl-carboxylic acid.

Dec = decanoate.

CM- = carboxymethyl-.

Brackets around values (D and K_{E,L}) indicate large experimental error.

References:
- A, see Table 6.1.
- B, see Table 5.1.
- C, Sigman (1967)
- D, Winer and Theorell (1960)
- E, Theorell and McKinley-C, McKee (1961b)
would make a significant difference to binding of NADH to the carboxymethyl enzyme, when it made none to the native enzyme. Results are shown in Figs. 7.8 - 7.11. The effects of AMP, decanoate and 4-biphenyl-carboxylic acid respectively are shown in Figs. 7.8, 7.9 and 7.10. Fig. 7.11 shows the effect of imidazole at pH 7.4 and at pH 9.8, and of acetamide. At pH 9.8, but without imidazole, no binding could be detected. The experiment with 4-biphenyl-carboxylic acid was particularly erratic. Anomalous results at low saturation of the enzyme are probably due to the non-enzymic enhancement described above. At pH 6, binding of NADH is slightly stronger than at pH 7.4 (Fig. 7.10).

The results of these fluorescence titrations with carboxymethyl enzyme are summarised in Table 7.1. It is seen that AMP and decanoate still bind well to the carboxymethylated enzyme. The close agreement of the two figures for decanoate must be considered coincidental, as is seen from inspection of Fig. 7.9. Imidazole seems to quench the fluorescence of bound NADH, indicating a ternary complex of carboxymethylated enzyme, NADH and imidazole; NADH, however, is bound quite firmly to this complex; in fact, the strength of binding of NADH is very similar to the native enzyme (Theorell and McKinley-McKee, 1961b), even though NADH is bound six times more weakly to the binary complex. Acetamide appears (like imidazole) to make NADH bind a little more weakly, but without changing the fluorescence of bound NADH. Isobutyramide was also found to do this. This is considerably different from the native enzyme, where imidazole and many amides cause enhancement of fluorescence of bound NADH; imidazole weakens the binding of NADH (Theorell and McKinley-McKee, 1961b) while amides strengthen
NADH-binding (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961b; Woronick, 1961, 1963a,b).

Ethanol in high concentrations (Shore and Theorell, 1966a,b) can form a ternary (abortive) complex with native enzyme, to which NADH is bound more strongly than to the enzyme-NADH binary complex, and in which the fluorescence enhancement is reduced. Dimethyl sulphoxide (Perlman and Wolff, 1968) forms a tight, highly fluorescent complex with enzyme and NADH, just as some amides do. Both ethanol (3%) and dimethyl sulphoxide (0.3%) had only a small effect on carboxymethyl enzyme.

The results with 4-biphenyl-carboxylic acid, although erratic, show that it does bind quite tightly to the enzyme. The reduced value of Q (enhancement factor of bound NADH) may be an experimental error. If not, it could indicate that ternary complexes of carboxymethyl enzyme, NADH and biphenyl-carboxylic acid can form. Normally, aromatic acids are competitive with coenzyme (Sigman, 1967); therefore the molecule is binding at some other, secondary site; such a possibility was raised in Chapter 6, because of biphasic Dixon plots. The quenching of the fluorescence of the binary native enzyme-NADH complex by NAD⁺ (Theorell and McKinley-McKee, 1961b) could indicate secondary sites for NAD⁺. Secondary binding-sites have been found for the spin-labelled coenzyme analogue used by Weiner (1969).

7.3. Discussion.

The residual activity found appears to be a genuine catalytic activity of liver alcohol dehydrogenase carboxymethylated at
cysteine 46 in the amino-acid sequence (Jornvall, 1970b). It is apparently not due to a minor isoenzyme component which is resistant to iodoacetate, and probably not due to a small amount of "variant" modified enzyme, modified at a different residue and now resistant to alkylation at residue 46. The apparently unaltered electrophoretic mobility suggests that carboxymethylation may not alter the charge on the enzyme. Carboxymethylation does not detectably alter the enzyme conformation (Li and Vallee, 1965). The finding that residual activity (if due to the bulk of the protein) may not obey the Theorell-Chance mechanism is not surprising. A reduction of 40-fold or more in the dissociation velocity constant of the enzyme-NADH complex would probably result in tighter binding of NADH, yet in practice it was observed to be weaker.

Amino-acid analysis confirmed the specificity of alkylation by iodoacetate, since only one mole of S-carboxymethyl-cysteine per subunit was found. However, the unexpected provisional finding of cystine indicates that four or more cysteines become able to form disulphide bonds in the carboxymethylated enzyme. This may be due to a general loosening of structure. The disulphide bonds could be within subunits, or between subunits in the dimeric enzyme molecule, or between enzyme molecules leading to aggregation. No attempt was made to determine which of these mechanisms was operating. It is interesting to note that Hoagstrom et al., (1969) found that zinc-free liver alcohol dehydrogenase (prepared by dialysis) only contained 8 out of an initial 26 thiol groups per molecule. Also, Oppenheimer et al., (1967) found that, over a period of a few days, 5 disulphide bonds per molecule (i.e. 2.5 per subunit) formed. Green and McKay (1969) found that low-zinc enzyme
tended to aggregate, even in the presence of reducing agent. It may be, therefore, that zinc-removal and carboxymethylation have similar effects on the general structure of the enzyme molecule.

It is possible that some of the properties of carboxymethylated alcohol dehydrogenase could be due to secondary loss of zinc, rather than to the carboxymethylation itself. During the course of a preparative inactivation, it was found that after 15 min., both activity and formation of the highly-fluorescent enzyme-NADH-isobutyramide complex had decreased to 20% of their initial values. Secondary loss of zinc is unlikely to occur so rapidly; but it may occur more slowly, associated with the formation of disulphide bonds. Drum et al., (1967b) found that two of the four zinc atoms in the enzyme could be removed much more easily after carboxymethylation. No difference in binding properties was noticed when fresh or aged samples of carboxymethylated enzyme were used, apart from the decrease in binding-sites.

The disulphide bonds formed may not be equally distributed among the modified enzyme molecules. Some enzyme molecules may be denatured, be unable to bind NADH, and contain more than five disulphide bonds per molecule.

The carboxymethylated enzyme is seen to be qualitatively and quantitatively similar to the native enzyme in the following properties:

(i) Formation of ternary complexes, absorbing at 290 nm, with NAD$^+$ and pyrazole.

(ii) Formation of ternary complexes with NAD$^+$ and decanoate.

(iii) Formation of binary complexes with NADH, AMP, decanoate, imidazole (see also Chapter 4), orthophenanthroline (Drum et al.,

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1967b) and (probably) 4-biphenyl-carboxylic acid. In the presence of imidazole, the pH-dependence of binding of NADH appears to be similar to that of AMP and ADP-ribose in the native enzyme.

The main differences, however, are:

(i) Near-abolition of catalytic activity (as described above).
(ii) Inability to enhance fluorescence of bound NADH, except at the short-wavelength part of its emission spectrum. Compounds which produce highly fluorescent ternary complexes with native enzyme (imidazole, amides and dimethyl sulphoxide) have little effect on the binding of NADH.

In the previous chapter, it was shown that introduction of a negative charge (as a reversibly-bound anion) at the postulated zinc site has the following effects: abolition (or considerable weakening) of binding of NADH, adenine nucleotides and iodoacetate; strengthening of binding of NAD⁺; and competition with imidazole, orthophenanthroline and decanoate. A reversibly-bound anion at the postulated nucleotide-phosphate site might have the following effects: prevention of binding of NADH, adenine nucleotides, aromatic acids and (by charge-repulsion) decanoate; on the other hand, it should have little effect on imidazole-binding, or on orthophenanthroline-binding.

It is seen, however, that covalent introduction of a carboxy-methyl anion has only a small effect on the binding of any of these compounds, although the largest effect is on NADH itself. Therefore, either carboxymethylation does not take place at the active centre; or, if it does, no net change of charge is brought about. This could occur if the thiol was present as the anion: this is unlikely because of its low reactivity with iodoacetate and

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(more especially) iodoacetamide: see Chapter 9, Section 9.1. It could also be produced by protonation of a neighbouring group taking place concomitantly with carboxymethylation of the thiol, for example:

It is not clear why the present experiments should show NADH-binding to be quite strong (dissociation-constant $1.7 \mu M$) while Li and Vallee (1965) found it to be $40 \mu M$, by spectrophotometry.

Yeast alcohol dehydrogenase reacts with iodoacetamide; its reactive thiols are sufficiently sensitive for specific alkylation with iodoacetamide to be possible. The resulting carboxamidomethylated enzyme is still able to bind NADH, as shown by gel-filtration in the presence of NADH (Auricchio and Bruni, 1969). Inactivation with 2-bromo-2-phenyl-acetaldehyde (Tipton, 1968) is very probably due to reaction with a thiol at the active site of the enzyme; yet the inhibited enzyme was still able to bind NADH with unaltered fluorescence enhancement. The phenyl-acetaldehyde group introduced in this reaction is however uncharged, which may explain the lack of effect on NADH-binding and fluorescence enhancement. Unfortunately, iodoacetamide did not react specifically enough with liver alcohol dehydrogenase for specific carboxamidomethyl enzyme to be attainable. It might perhaps be possible in the presence of imidazole. Lactate dehydrogenase (from pig heart, $H_4$ enzyme) can bind NADH, and NAD$^+$ and sulphite, after inhibition by maleiminide, but about ten times more weakly

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(Holbrook, 1966); however a larger maleimide derivative, N(N-acetyl-
sulphamoyl-phenyl) maleimide, inactivates lactate dehydrogenase and
reduces the stoichiometry of binding of NADH without altering its
dissociation-constant. Coenzyme also protects lactate dehydro-
genase. Thus lactate dehydrogenase and the two alcohol
dehydrogenases do show some similarities.
Chapter 8

Bromoacetyl-pyridine, Iodoethanol and Chloroethanol

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   Bromoacetyl-Pyridine: Incorporation into Protein .... 107
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**Fig. 8.1.** Inactivations of liver ADH by bromoacetyl pyridine. At 23.5°, ionic strength 0.1, in sodium phosphate buffer. Enzyme, 0.5 µM; bromoacetyl pyridine, 0.67 mM. Filled symbols, no further additions; ○, + imidazole (base form, 29 mM); ▽, + orthophenanthroline (15 µM); △, + AMP (136 µM).
Earlier chapters of this thesis, and references cited, have shown that nitrogen-base compounds (e.g. imidazole and pyridine) and also ethanol can form binary complexes with liver alcohol dehydrogenase. It was hoped that by using halogenated compounds based on these, it might be possible to specifically alkylate any reactive group that was close to the reversible binding-site of the parent compounds.

Bromoacetyl-pyridine (which will be used instead of its full name, 3(2-bromo-acetyl)-pyridine) was chosen for several reasons: being a nitrogen base, it should be able to bind reversibly to the enzyme, as described above; it has already been used on another dehydrogenase, lactate dehydrogenase from pig heart, in which it reacted with a histidine as well as with a cysteine residue (Woecnckhaus, Berghauser and Pfleiderer, 1969); and also, due to the kindness of Dr. D.K. Apps, it was available pure in this laboratory. 2-Iodoethanol and 2-chloroethanol were used because of their ready commercial availability.

8.2. Results.

Bromoacetyl-Pyridine: Kinetics of Inactivation. Bromoacetyl-pyridine hydrobromide was dissolved in phosphate buffer, pH 7.4, and used in the inactivation experiments shown in Fig. 8.1. It proved quite a powerful alkylating agent, as found by Apps (1969). The stock solution probably also hydrolysed slightly, as shown by the
Table 8.1. Half-times of inactivation of liver ADH with bromoacetylpyridine. Values are calculated from Fig. 8.1, and are given in the order in which the inactivations were performed.

<table>
<thead>
<tr>
<th>Added Ligand</th>
<th>Half-Time (min.)</th>
<th>Factor of Increase</th>
<th>Factor Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.65</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Imidazole (base, 27 mM)</td>
<td>4.8</td>
<td>1.2</td>
<td>30</td>
</tr>
<tr>
<td>Decanoate (119 μM)</td>
<td>4.9</td>
<td>1.23</td>
<td>3.0 *</td>
</tr>
<tr>
<td>AMP (136 μM)</td>
<td>12.7</td>
<td>3.2</td>
<td>4.1</td>
</tr>
<tr>
<td>OP (15 μM)</td>
<td>6.3</td>
<td>1.58</td>
<td>2.7</td>
</tr>
<tr>
<td>Chloride (40 mM)</td>
<td>5.05</td>
<td>1.26</td>
<td>1.7 *</td>
</tr>
<tr>
<td>None</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* not shown in Fig. 8.1.

OP represents orthophenanthroline.

"Factor of Increase" represents the ratio: \( \frac{\text{Half-time + added ligand}}{\text{Half-time - added ligand}} \)

"Factor Expected" is calculated assuming that the binary enzyme-ligand complex is totally resistant to alkylation: the concentration of the enzyme-ligand complex is calculated from its dissociation-constant for protection against iodoacetate (Chapters 5 and 6).
decrease in rate of inactivation in the two cases without other ligands (Fig. 8.1): these were performed first and last. Table 8.1 shows the half-times, and fractional increase in half-times, caused by the protecting ligand. The main disappointment was that imidazole protected only very slightly. If irreversible alkylation was preceded by reversible binding of the pyridine moiety, then imidazole should provide considerable protection. On the other hand, if alkylates by a mechanism similar to that of iodoacetate or iodoacetamide (Evans and Rabin, 1969), then imidazole should stimulate inactivation. It may be that the two effects are tending to cancel each other out. None of the ligands protects as much as would be expected on the basis of their known dissociation-constants (Chapter 6), but AMP is the most effective, followed by orthophenanthroline.

Bromoacetyl-Pyridine: Incorporation into Protein. Woenckhaus et al. (1969) found that bromoacetyl-pyridine reacted with a specific histidine group as well as a cysteine in pig heart lactate dehydrogenase. Therefore, attempts were made to determine which groups in liver alcohol dehydrogenase were alkylated, by means of hydrolysis and amino-acid analysis.

N-acetyl-cysteine reacted rapidly with bromoacetyl-pyridine. Using the procedure of Woenckhaus et al. (1969), N-acetyl,S-(2-hydroxy-2-(pyridyl-(3))-ethyl)-cysteine was prepared, by borohydride reduction after alkylation. After hydrolysis in 6N HCl in vacuo for 24 hours, its elution profile was examined in the Locarte amino-acid analyser, and an estimate of its colour constant made (7.8). Attempts to make the histidine derivative, N<sup>Im</sup>-(2-hydroxy-2(pyridyl-(3))-ethyl)-histidine were not successful due to the very low yield. However, a very small peak, considerably more basic than
ammonia and histidine, was observed after hydrolysis in the amino-acid analyser.

Liver alcohol dehydrogenase (0.5 ml containing approximately 4 mg) was reacted with 60 µl of 3mM bromoacetyl-pyridine solution at room-temperature (pH 7.4) for about 1 hour; this reduced the activity to 40% of the initial value. The enzyme was cooled to 0°, and reduced with 1 mg of sodium borohydride. After 1 hour, it was dialysed against 2x2 litres of phosphate buffer (pH 7.4, 20 mM) for 2 hours and 14 hours respectively. The specific activity of the enzyme was now 43%, and so borohydride treatment had had almost no effect on activity. A greater degree of inactivation was not used because it was feared that this would lessen the specificity of reaction, as was found with iodoacetamide (Chapter 5).

Hydrolysis and amino-acid analysis gave one small extra peak compared with native enzyme, which eluted in exactly the same position as the S-cysteine derivative. Approximately 1.0 - 1.3 residues per inhibited subunit were estimated, correlating very well therefore with the loss of activity. No extra basic peak (attributable to modified histidine) was observed. However, had there been one, it may have eluted with the arginine peak.

**Iodoethanol and Chloroethanol.** In the presence of 13 mM iodoethanol, no significant inactivation occurred in one hour; however, the enzyme assay was inhibited 40%. The concentration of iodoethanol in the assay cuvette would be 213 µM. The estimated apparent inhibitor constant was 250 - 300µM; for 2-chloroethanol, which also inhibited the assay system, it was estimated to be 450 - 500µM.

Imidazole (base form approximately 30 mM) did not labilise the enzyme to chloroethanol (50 mM). In fact it seemed to protect it:
after 90 min., activity was 97% as compared with 73% without it (40 mM phosphate buffer, pH 7.4, at 23.5⁰).

The presence of NAD⁺ made almost no difference to the inactivation. Addition of one aliquot of NAD⁺ (0.9 or 9 μM) to enzyme (0.7 μM) plus iodoethanol (13 mM) caused a rapid drop of 13 - 16% in enzyme activity, but after that the enzyme was just as resistant to inactivation as before. Addition of ethanol (5 mM) to generate NADH caused no change in enzyme activity.

8.3. Discussion.

The protection effects of several ligands against bromoacetyl-pyridine gave no clear indication that it inactivated the enzyme after first forming a reversible complex. Imidazole only protected very slightly, indicating no competition for binding by the two compounds. However, as mentioned above, it may be that the stimulation-effect shown by imidazole (Evans and Rabin, 1968) on inactivation by iodoacetate and iodoacetamide was tending to annul the protecting effect that could also be occurring.

It should be noted that bromoacetyl-pyridine is unlikely to bind analogously to the nicotinamide portion of NAD. The nicotinamide moiety appears to be unable to bind properly to liver alcohol dehydrogenase in the absence of the adenylate moiety, as discussed earlier, in Chapter 6, Section 6.3 (see also McPherson, 1970).

The stoichiometry of acetyl-pyridine incorporation into the enzyme is interesting. The specificity is considerably greater than that of iodoacetamide (Chapter 5); this may be due to its
greater reactivity, or to a specific property of the protein. Although one may perhaps presume that the same thiol group is modified by iodoacetate, by bromoacetyl-pyridine and (among a few others) by iodoacetamide, there is no positive evidence to support this.

The failure to find any modified histidine in the bromoacetyl-pyridine-inactivated enzyme was disappointing, and may be due to inadequate techniques. Woenckhaus et al. (1969) used radioactive bromoacetyl-pyridine, which is a considerably more satisfactory method. They found that the thiol was much more reactive than the histidine, and the reactive thiol in alcohol dehydrogenase is considerably more reactive than the essential thiol of lactate dehydrogenase (Fondy et al., 1965). Also Woenckhaus et al. (1969) were able to reversibly block all the thiol groups of lactate dehydrogenase with mercuric ions. Reversible blocking has not yet proved possible for liver alcohol dehydrogenase.

The most interesting feature of chloroethanol and iodoethanol is their powerful inhibition of the enzyme assay. The concentrations giving 50% inhibition were about 250 - 300 pM for iodoethanol, and 450 - 500 pM for chloroethanol. If the inhibition is by formation of ternary complexes of enzyme, NADH and ethanol-derivative, then the dissociation constant of ethanol from this complex will be approximately equal to the above figures: if NADH can dissociate from this complex at a significant rate, then the dissociation-constants for ethanol derivatives from it will be lower. If inhibition is by forming complexes of enzyme, NAD^+ and ethanol-derivative (presumably competitive with ethanol), then the dissociation-constants of ethanol derivatives from these complexes would be much lower. Sigman (1967) has shown that trifluoroethanol

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is competitive with ethanol, with an inhibitor constant of 7.2 \mu M, so the second possibility, above, is quite feasible.

The sudden small drop in enzyme activity on adding NAD$^+$ was repeatable, and only observed for the first aliquot of NAD$^+$ added to enzyme plus iodoethanol. The reason for this is unknown. If iodoethanol or chloroethanol were substrates, then the generated halogenated aldehyde should be a much more powerful alkylating agent than the parent compound, due to the electron-withdrawing properties of the carbonyl group. However, fluorethanol is not a substrate for horse-liver alcohol dehydrogenase (Treble, 1962): it was an inhibitor, competitive with ethanol (inhibitor constant 2 mM).

Van Eys et al. (1962) used a thiazole-analogue of NAD to covalently modify a thiol group of liver alcohol dehydrogenase. Other attempts to use affinity-labelling to demonstrate reactive groups at the active site of this enzyme have been unsuccessful (Tipton, 1968). It is not clear whether the thiol residue modified by Van Eys et al. (1962) is the same one alkylated by iodoacetate (cysteine 46).
Chapter 9

General Discussion

9.1. Internal versus External Alkylation .................. 113

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9.3. Function of the Reactive Thiol Group ................. 120
9.1. Internal versus External Alkylation

In Chapter 5, it was shown that alkylation of liver alcohol dehydrogenase by iodoacetate could occur in two possible ways, or a combination of them: **internal alkylation**, in which the reversibly-bound iodoacetate then alkylates the thiol group; and **external alkylation**, in which the reversibly-bound iodoacetate protects the enzyme from alkylation by iodoacetate in the surrounding solution. Inactivation-kinetics alone are unable to distinguish between the two mechanisms. However, using the other experimental evidence presented in this thesis, and drawing analogies from the literature, one can examine the relative likelihood of each mechanism. In the case of internal alkylation, the reactive thiol must be close to the reversibly-bound iodoacetate: this could also be the case with external alkylation, but need not be.

The chemical reactivity of the thiol group of liver alcohol dehydrogenase with iodoacetate is between that of a simple thiol (SH) and a simple mercaptide ion (S⁻) (Evans and Rabin, 1968); its reactivity with iodoacetate and iodoacetamide as a function of pH is anomalous, and does not appear to represent a normal thiol ionisation (Evans and Rabin, 1968). Enhanced reactivity upon binding is one criterion for internal alkylation (Baker, 1967). However, the uncertainty of the ionisation state of the thiol in the enzyme prevents this being good evidence in favour of internal alkylation. In Chapter 5, Section 5.3, it was suggested that the comparative constancy of the rate-constants of inactivation at different pH values indicated it to be unlikely that a combination of internal and external alkylation was taking place.

The specificity of iodoacetate's reaction with one group on
liver alcohol dehydrogenase, compared with less or much less specificity shown by all the other thiol reagents used (except bromoacetyl-pyridine), but including PCMB and DTNB (Chapter 4), iodoacetamide (Chapter 5), iodine (Li and Vallee, 1965), a derivative of N-ethyl-maleimide (Witter, 1960) and silver ions (Bonnichsen, 1953; Wallenfels et al., 1959), may be considered to favour internal alkylation: none of these other reagents (whether anions, cations or neutral molecules) react specifically with just the one thiol per subunit. Therefore, the specificity may be due to an orientation effect (due to binding), rather than to a greater chemical reactivity of the thiol group itself.

Protection of reactive groups by substrates, inhibitors or coenzymes is often taken as evidence that they are near the active site: however, this is not always the case, as has been found for lactate dehydrogenase (see below). Also, it could be invoked for external alkylation just as readily as for internal alkylation. Therefore, in this case, protection by substrates, inhibitors and coenzymes is of no help in distinguishing between internal and external alkylation.

The experiments with carboxymethyl enzyme (Chapter 7) suggested that the carboxymethyl group is probably not at the binding-site of aliphatic acids (possibly zinc), or of the phosphate of AMP, or of the carboxyl group of 4-biphenyl-carboxylic acid. Although the thiol group could perhaps be near these sites in the native enzyme and swing away after carboxymethylation, there is no evidence for a conformation-change (Li and Vallee, 1965). The finding of some residual activity also implies that the thiol group may not be actually in the active centre.
Imidazole both weakens the binding of iodoacetate (5-fold) and stimulates inactivation (10-fold, at infinite iodoacetate concentration). Part of the stimulation may be due to iodoacetate now being less able to protect the enzyme by reversible binding, which would point towards external alkylation. Similarly, ethanol's stimulation at high iodoacetate concentrations may be for this reason.

The non-competitive protection shown by orthophenanthroline shows that iodoacetate can be bound without alkylation taking place: but protection by orthophenanthroline from bound or unbound iodoacetate would be equally possible. In general, for all these experiments on inactivation kinetics, a feasible explanation of all phenomena on the basis of either internal or external alkylation can be produced.

A number of other systems show Michaelis-Menten kinetics of inactivation. Baker, Lee and Tong (1962) found that 4-(iodoacetamido)-salicylic acid and 4-(iodoacetamido)-benzoic acid gave saturation-effects when inactivating glutamate and lactate dehydrogenases: enzyme inactivation paralleled binding ability and not chemical reactivity alone, indicating internal rather than external alkylation. Michaelis-Menten kinetics were also found for acetylcholinesterase and organophosphorus compounds (Main, 1964; Aldridge and Reiner, 1969); α-chymotrypsin and phenylmethane sulphonyl fluoride (Gold and Fahnrey, 1964); triose phosphate isomerase and 1-hydroxy-3-iodo-2-propanone phosphate (Hartman, 1968); carbonic anhydrase and iodoacetate (Bradbury, 1969a), bromoacetate and iodoacetamide (Whitney et al., 1967), and bromoacetazolamide (Kandel, Wong, Kandel and Gornall, 1968); ribonuclease and a uridine analogue (Pincus and Carty, 1970); hexokinase and iodoacetate under
some conditions (Jones, 1970); and D-amino-acid oxidase and N-alkyl maleimides (Fonda and Anderson, 1969). None of these authors appear to have considered the possibility of external alkylation: all (except Jones, 1970) considered the reactions to be internal. Fonda and Anderson (1969), however, found the same first-order rate-constant for internal alkylation for a series of N-alkyl maleimides which had similar chemical activities for alkylation but showed large differences in binding: this is good evidence for internal alkylation.

For yeast alcohol dehydrogenase, N-(2-bromoacetamidoethyl) nicotinamide gave Michaelis-Menten kinetics of inactivation, and ADP enhanced its reactivity (Plapp et al., 1968). These authors considered that alkylation was probably internal. Tipton (1968) found that 2-bromo-2-phenyl acetaldehyde is a potent inhibitor of the yeast enzyme; it alkylates a thiol group which is very probably at the enzyme's active site. The reagent was unreactive against the liver enzyme. Iodoacetate reacts with one thiol group per subunit of liver and yeast alcohol dehydrogenase, and these are contained in similar sequences (Harris, 1964). It is not known whether iodoacetate and 2-bromo-2-phenyl acetaldehyde react with the same thiol group.

Lactate dehydrogenase has one essential thiol group per subunit (Fondy et al., 1965; Holbrook and Jeckel, 1967) which is protected by coenzyme; yet X-ray crystallography has shown (Adams et al., 1970a,b) that the essential thiol group in dogfish \( \text{Mg} \) lactate dehydrogenase is 13.5 A\(^0\) from the nearest part of the coenzyme molecule, the nicotinamide. It appears to be on a polypeptide backbone which is near to the coenzyme, but the cysteine is pointing
Fig. 9.1. Schematic representation of possible topographical features of liver ADH. For discussion, see text.
away from it (Adams et al., 1970c).

On balance, it seems that the thiol group is probably more likely not to be actually at the active centre, or at the reversible binding-site of iodoacetate. This study appears to be the first in which the favoured explanation for Michaelis-Menten kinetics of inactivation is external rather than internal alkylation.

9.2. Ligand-Binding.

In Chapter 6, the binding of different ligands and the nature of their possible binding-sites was investigated, and discussed extensively. This section will only summarise the main findings, and discuss some of them in relation to work in other chapters.

Some of the results are summarised in Fig. 9.1. Halide ion sites are difficult to allocate: they appear to bind in many different ways, as discussed in Chapter 6. Some evidence that would favour the binding of AMP by a lysine is that AMP protects one lysine per subunit from pyridoxal phosphate (Morris, 1970). The hydrophobic (adenine) site would also bind aromatic anions and dyes, and the iodine of iodoacetate. Another, "thinner" hydrophobic site (not shown) would bind substrates and substrate analogues (e.g. decanoate). The zinc would bind metal-complexing agents, alcohol and decanoate, and presumably aldehyde and alkyl amides too. The ionisations of the two postulated positive charges would be precise required to interact in a rather complex manner; the pH data in earlier chapters is quite insufficient to elucidate this in detail.

The principle of only one of these "positive centres" being able to bind anions at a time (see Chapter 6) would also apply to the pK's
of these groups: if either was in the base form, the pK of the other would be required to be raised considerably; this could explain why the binding of NAD\(^+\) as a function of pH follows a monovalent dissociation-curve (Taniguchi et al., 1967); the phosphate-site's pK would otherwise lessen the binding of NAD\(^+\) at pH 10. This interaction of positive charges is not what would be expected, for two adjacent positive groups; in fact, they would be expected to show a strong preference not to be both charged at the same time. Therefore, their interaction is presumably mediated by the protein (with an anionic group possibly involved).

Fig. 9.1 certainly represents an oversimplification, because it takes no account of the effect shown by imidazole (but not ortho-phenanthroline) on the binding of iodoacetate and 4-biphenyl-carboxylic acid. Either imidazole can bind in another place as well; or the binding of each of the two complexing agents produces a different conformation of the enzyme. The latter effect could be mediated by altering the co-ordination geometry of the zinc. Also, the concept of two anion-binding sites (one imidazole-dependent, and one independent) fails to account for the pH-insensitive binding of NAD\(^+\) (or chloride) in the presence of imidazole (Theorell and McKinley-McKee, 1961b). Perhaps the only way to rationalise these observations is by postulating that imidazole does cause a conformation-change (as discussed above and in Chapter 6). More work is clearly needed on this.

The dissociation-constants determined in Chapters 5 and 6 for anions (fatty-acids and AMP, in particular) were usually somewhat smaller than those determined by other methods. In Chapter 7 the dissociation-constant for AMP, determined by fluorescence titrations
with NADH, agreed with kinetic determinations (Reynolds et al., 1970) but was approximately twice as large as determined from competition with iodoacetate: yet the same conditions, and the same sample of AMP, were used. Presumably, this could be due to the presence of ethanol (8 mM) during the kinetics and fluorescence titrations: or to the presence of iodoacetate during the inactivations. Binding of some of these compounds to sites other than their normal ones (Weiner, 1969) might account for this. Anderson and Weber (1965) suggested that unequal binding of NADH to M-type subunits of lactate dehydrogenase was probably due to relaxation effects: that is, an enzyme subunit that has just parted with a molecule of NADH may be in a different conformation (with different binding properties) than enzyme which has been uncomplexed for a "long" time. They found no such effect with M₄ lactate dehydrogenase, or with liver alcohol dehydrogenase. Adams et al. (1970a,b) found that the dogfish M₄ lactate dehydrogenase subunits became non-equivalent in the presence of coenzyme, separating into two crystallographically-different pairs. Relaxation effects may perhaps occur after AMP-binding (and fatty-acid binding) or after iodoacetate-binding, to liver alcohol dehydrogenase. This may account for the interesting positive interaction between AMP (or ADP) and orthophenanthroline (Yonetani and Theorell, 1964) which was mentioned in Chapter 6: it was only shown in substrate-kinetics experiments. Relaxation effects also occur in the binding of carbon monoxide to haemoglobin (Gibson, 1959); in this case, they probably involve association-dissociation reactions of the protein subunits (Gibson and Antonini, 1967).
9.3. **Function of the Reactive Thiol Group.**

This study has served, like many predecessors, to underline one of the truisms of enzyme investigations: the disparity between the complexity and intricacy of enzyme structure and mechanism on the one hand, and the difficulty of using experimental methodology to answer important questions about the enzyme, on the other. This work has provided some small clues towards understanding the function of the reactive thiol group, but much more information will be required before a reasonably satisfactory (let alone complete) description can be given.

When chemical modification of an amino-acid residue in an enzyme abolishes activity, it is normally considered "essential". Another definition of an "essential" residue is one which is retained in the same protein from different species, as shown by sequence work. In neither case does it necessarily follow that this residue plays a positive rôle in catalysis, or substrate-binding, or even maintaining the conformation of the protein. What does follow is that the modification (chemical, or (presumed) mutational) has altered something, rendering the protein no longer active. However, even some knowledge of the properties of a chemically-modified protein may be useful in indicating what sort of disruption has been brought about; this may help one to understand more about the way the enzyme functions. Such caution of interpretation has been urged many times (for example: Cecil, 1963; Boyer and Schulz, 1959; Boyer, 1959).

The binding studies on carboxymethyl-enzyme (Chapter 7) have shown that carboxymethylation probably produces no net change of charge at the active centre: this is considered to favour the
possibility that the introduced carboxymethyl group (and therefore probably the thiol group in the intact enzyme) is not at the active site. However, the similarity of optical rotatory dispersion in native and carboxymethyl enzymes (Li and Vallee, 1965) indicates that no great conformational differences are produced by carboxymethylation. It may be that catalytic activity, and fluorescence properties, are affected by very subtle changes of enzyme structure and interactions. The finding of residual activity does indicate that, as in aldolase (Szajáni, Friedrich and Szabolcsi, 1969; Szajáni, Sajgo, Biszku, Friedrich and Szabolcsi, 1970) and possibly creatine kinase (Noda, Nihei and Moore, 1961; Kimudavalli, Moreland and Watts, 1970), the free thiol is not absolutely required for catalysis.

The effect of carboxymethylation is in some ways similar to that of removing zinc from liver alcohol dehydrogenase. An average of approximately 5 disulphide bonds can form per molecule of enzyme in each case (discussed in Chapter 7). Fluorescence enhancement of bound NADH is considerably reduced (by carboxymethylation; Chapter 7) or abolished (by zinc removal; Hoagstrom et al., 1969). Carboxymethylation labilises the zinc atom to which orthophenanthroline does not bind (Drum et al., 1967b) ("structural" zinc), to removal or to exchange with radioactive zinc. In fact, it is possible that the thiol group is a ligand for this zinc atom. The thiol group shows abnormal pH-dependence in its reactivity (Evans and Rabin, 1968) which was interpreted as possibly due to hydrogen-bonding to an imidazole side-chain; complexing to zinc would also explain it. Drum (1970) has commented that the spectrum of the cadmium derivative of liver alcohol dehydrogenase might indicate cysteine to be a metal ligand. The zinc to which orthophenanthroline
binds appears not to have a sulphur ligand (Plane and Long, 1963), suggesting that the "structural" zinc is responsible.

The greater reactivity of the thiol group with iodoacetate as compared with iodoacetamide suggests that iodoacetate may be drawn in by a positive charge. If alkylation is internal, then it is probably by the active-centre zinc atom (Chapter 6); but if external, it is probably another positive charge on the enzyme. This could be a guanidine or amino group (or possibly imidazole), or perhaps the other ("structural") zinc atom. At higher ionic strength, the maximum rate of inactivation (at infinite iodoacetate concentration) is somewhat decreased, which might indeed indicate external alkylation facilitated by an ionic interaction.

The protection of this "structural" zinc from exchange with $^{65}$Zn by acetate is probably not due to the complex described in Chapter 5 and by Winer and Theorell (1960), because its effect would be too weak. Instead, acetate probably binds tightly elsewhere on the enzyme, and may cause a local conformation-change (Drum et al., 1967, 1968a,b). Jornvall (1967) found that it was very difficult to remove acetate completely from the enzyme.

The near-loss of catalytic activity caused by carboxymethylation may have a close connection with the reduction of ability to produce coenzyme fluorescence-enhancement, and in particular the loss of ability to form stable complexes with NADH and amide. These complexes have been considered to be analogous to complexes of enzyme, NADH and aldehyde. The enzyme-NADH complex probably has a somewhat different conformation from free enzyme: although its optical rotatory dispersion is very similar (Rosenberg, Theorell and Yonetani, 1964, 1965), its crystal structure is
Table 9.1. Comparisons of parts of the sequences of alcohol (ADH), lactate (LDH) and glyceraldehyde phosphate (GPDH) dehydrogenases.


1 represents yeast ADH around reactive cysteine (Harris, 1964).
2 " liver ADH around cysteine 46 (Harris, 1964; Jörnvall, 1970b).
3 " pig GPDH around tyrosine 46 (Harris and Perham, 1968; see also Harris et al., 1970).
4 " lobster GPDH around tyrosine 45 (Davidson et al., 1967).
5 " LDH (several species) around cysteine 149 (Holbrook et al., 1967; Pfleiderer and Mella, 1970; see Adams et al., 1970c).
6 " pig GPDH around cysteine 149 (Harris and Perham, 1968).
7 " lobster GPDH around cysteine 148 (Davidson et al., 1967).
different (Brandén, 1965; Brandén et al., 1965, 1970). Ternary complexes of enzyme with NADH and isobutyramide, and NAD$^+$ and pyrazole are distinctly different from free enzyme by both these criteria. On the other hand, complexes of enzyme with ADP-ribose or orthophenanthroline, or both, are similar to free enzyme rather than to these ternary complexes. Carboxymethyl enzyme apparently cannot form ternary complexes with amides, but can with pyrazole. This conformation-change may also be necessary for catalysis; carboxymethylation may disrupt both complex-formation (with NADH and amides) and catalysis by interfering with the conformation-change.

Another possibility is that the small change in enzyme structure introduced by carboxymethylation prevents transfer of vibrational energy by the enzyme molecule (Phillipson, 1968). However, if this were the only effect, carboxymethylation would not be expected to abolish the formation of stable ternary enzyme-NADH-amide complexes and yet not enzyme-NAD$^+$-pyrazole complexes.

If the thiol group plays an important rôle in alcohol dehydrogenase, then one might expect other nicotinamide-nucleotide-linked dehydrogenases to show similar properties. Most of these enzymes can be inhibited by reagents which react with thiol groups: the main exception appears to be cytoplasmic malate dehydrogenase (Siegel and England, 1962; Guha, England and Listowsky, 1968).

Glyceraldehyde phosphate dehydrogenase has one very reactive thiol group per subunit: it is at position 149 in the pig enzyme, and 148 in the lobster enzyme (see Table 9.1). The surrounding sequences are somewhat related to those around the reactive thiols in yeast and liver alcohol dehydrogenases, and also around the
essential thiol in lactate dehydrogenase. Although the alcohol and lactate dehydrogenase sequences around the thiol group are similar, the thiols are not at equivalent positions in the amino-acid sequence of the polypeptide chains; in dogfish \( \text{M}_4 \) lactate dehydrogenase, high-resolution X-ray studies (Adams et al., 1970c) have shown that the thiol is at position 149 (as in glyceraldehyde phosphate dehydrogenase), while chemical studies of liver alcohol dehydrogenase show that the thiol is at position 46 (Jornvall, 1970b). In alcohol dehydrogenase, residue 149 is a tyrosine (Jornvall, 1970c). The chemical reactivity of the alcohol dehydrogenase thiol (position 46) is intermediate between that of glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase (both being at positions 148 - 149); however the thiol is much more reactive in heart than in muscle lactate dehydrogenase (Pfleiderer, 1970). Table 9.1 compares the sequences around cysteine 149 in lactate and 46 in alcohol dehydrogenases, and also shows both these parts of the glyceraldehyde phosphate dehydrogenase sequences. Residue 46 in pig glyceraldehyde phosphate dehydrogenase (45 in lobster enzyme) is tyrosine, not cysteine, showing that cysteine is not "essential" here, either, for dehydrogenases in general. In dogfish lactate dehydrogenase, residue 46 (identity unknown) is part of a parallel pleated sheet structure, not particularly close to the coenzyme-binding site (Adams et al., 1970c). Therefore by analogy (and the two enzymes do have similar topology: see Sund, 1970) it would appear that cysteine 46 in liver alcohol dehydrogenase may not be at the active centre; chemical modification of it may affect activity mainly by disrupting some aspect of the protein's conformation.
The reason for the different chemical reactivities of the thiol groups in these different enzymes is unknown. In glyceraldehyde phosphate dehydrogenase, the reactive cysteine can be acylated by substrates (see Velick and Furfine, 1963), and therefore the exceptional chemical reactivity may well be a necessary part of the catalytic mechanism. Earlier work (Li and Vallee, 1963, 1964a, 1965; Harris, 1964) established that the thiol group was the only major site of reactivity of iodoacetate with liver alcohol dehydrogenase. However, in other proteins iodoacetate (and iodoacetamide) can react with other residues; for example, in pancreatic ribonuclease it reacts with histidine, methionine and lysine residues (Gundlach, Stein and Moore, 1959); in ribonuclease T1 it reacts with a glutamate residue (Takahashi, Stein and Moore, 1967). Iodoacetamide inactivates mitochondrial malate dehydrogenase, reacting specifically with one histidine per subunit (Anderton, 1970; Anderton and Rabin, 1970), but iodoacetate had no effect. Cytoplasmic malate dehydrogenase, however, is inactivated slowly by iodoacetate; methionine (two per subunit) is carboxymethylated (Leskovac and Pfleiderer, 1969). Methionine is specifically carboxymethylated in NADP-linked isocitrate dehydrogenase, producing partial inactivation (Coleman, 1968). The reactivity of these three different types of amino-acid in different dehydrogenases is perhaps best considered as due to unique properties of the environment of the group concerned (in the case of liver alcohol dehydrogenase, a positive charge may be near the thiol). These properties may well have no direct connection with the functioning of the enzyme concerned: however, as in the studies described in this thesis, the reactivity can sometimes be
exploited to learn more about the enzyme.
References

Abbreviations used are based on the system used by the Journal of Biological Chemistry (in accordance with the U.S.A. Standard for Periodical Title Abbreviations). The following journal titles, however, have been further abbreviated:

ABB, Arch. Biochem. Biophys.
BBA, Biochem. Biophys. Acta
JACS, J. Amer. Chem. Soc.
JBC, J. Biol. Chem.

References are listed alphabetically by first author.


Åkeson, Å. (1964). BBRC 17 211.


Biochemistry 9 185.
Middlebrook, W.R., Szent-Györgyi, A.G. and Schwarz, D.R.), 
Brandén, C.-I., Larsson, L.-M., Lindqvist, I., Theorell, H. and 
Brandén, C.-I., Zeppezauer, E., Boiwe, T., Söderlund, G., 
Nucleotide Dependent Dehydrogenases" (ed. Sund, H.), 
Springer-Verlag, Heidelberg, p.129.
Bücher, Th. (1970). In "Pyridine Nucleotide Dependent 
Dehydrogenases" (ed. Sund, H.), Springer-Verlag, Heidelberg, 
p.439.
Letters 2 239.


Dalziel, K. (1957a,b). ACS 11 1706, 397.


Dalziel, K. (1962b,c). Biochem. J. 84 240, 244.


Dalziel, K. (1963d). ACS 17 Suppl. 1 8.27.


Dalziel, K. and Dickinson, P.M. (1966a,b). Biochem. J. 100 34, 491.


Fondy, T.P., Everse, J., Driscoll, G.A., Castillo, F.,


Li, T.-K. and Vallee, B.L. (1964b). JBC 239 792.


Hoppe-Seyler's Z. Physiol. Chem. 350 473.


Woronick, C.L. (1963a,b). ACS 17 1789, 1791.


BBRC 37 137.
ADDENDA

After the bulk of this thesis had been typed, the following important papers appeared:

(1) Jörnvall, H. (1970). EJB 16 25. The complete amino-acid sequence has been determined. Except for the N-terminal portion (one-sixth of the polypeptide chain), there is no apparent homology with glyceraldehyde phosphate dehydrogenase. Jörnvall also describes (ibid., p. 41) the experimental details of his characterisation of the differences between the E and S-type subunits.

(2) Whitney, P.L. (1970). EJB 16 126. Experiments on carboxymethylated carbonic anhydrase B, described here, underline the similarity of this enzyme with liver alcohol dehydrogenase. Carboxymethylated carbonic anhydrase, as well as having residual activity, still forms reversible complexes with ligands (including bromoacetate itself) which are very similar to those formed by the native enzyme. These results show that the carboxymethyl group is in different positions in the reversible complex and in the covalently-modified enzyme. However, the author concluded that the alkylation mechanism is internal (i.e. alkylation proceeds via the reversible complex). This is the opposite of the tentative conclusion advanced in this thesis, even though the experimental results in each case show very considerable similarities.

The question of activity of alcohol dehydrogenase towards methanol is largely resolved by this work. The enzymes from yeast, horse liver and human liver all have a similar (low) catalytic activity for methanol oxidation, the human liver enzyme having the greatest affinity for the substrate.

(4) Holbrook, J.J. and Stinson, R.A. (1970). Biochem. J. 120 289. The binding properties of pig heart H₄ lactate dehydrogenase, inactivated by thiol modification, have been studied in detail. The results may be compared with those described in Chapter 7, for liver alcohol dehydrogenase, although the modified thiol is probably residue 149 in lactate dehydrogenase, and 46 in alcohol dehydrogenase. The two modified dehydrogenases form similar binary complexes with NADH; but lactate dehydrogenase can form no ternary complexes (except with NAD⁺ and sulphite, $10^3 - 10^4$ fold weaker than with native enzyme), while alcohol dehydrogenase does form some ternary complexes but not others.
APPENDIX

The following papers have been published, describing some of the work in Chapters 5, 6 and 7. The substrate-kinetics experiments described in the second paper were kindly performed by Mr. David L. Morris.
Anion-Binding to Liver Alcohol Dehydrogenase,
Studied by Rate of Alkylation

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Department of Biochemistry, University of Edinburgh

(Received May 3/July 11, 1969)
Iodoacetate alkylates and irreversibly inhibits horse-liver alcohol dehydrogenase. A reversible enzyme-iodoacetate complex also forms, as deduced from the following evidence:

1. Rate of inactivation is not proportional to iodoacetate concentration, but, rather, follows Michaelis-Menten-type kinetics.
2. The enzyme is protected competitively by other fatty-acids, and also by chloride.
3. The dissociation-constants of iodoacetate, and formate, and chloride all vary similarly with pH (increasing at high pH, and suggesting a $pK = 8.9-9.1$).

These results are discussed in terms of a model, and it is suggested that the reversible enzyme—iodoacetate complex may be stable, rather than a necessary intermediate for alkylation of the enzyme.

The great similarity of the results to those of another zinc-enzyme, carbonic anhydrase, is discussed; and it is suggested that anions bind to a zinc atom in the enzyme.

Alcohol dehydrogenase from horse-liver has two cysteine groups per molecule which are reactive towards iodoacetate [1, 2]. Evans and Rabin [2] used this to study the binding of several ligands to the enzyme. They reported that the rate of loss of enzyme activity was directly proportional to the concentration of iodoacetate; however, the experiments reported below show a saturation effect, and Michaelis-Menten-type kinetics apply. Moreover, the enzyme is competitively protected by fatty acids, and by chloride. This enables the dissociation constants of complexes between enzyme and these anions to be calculated. Compounds which form binary complexes also tend to form ternary complexes with the enzyme and one or both forms of the coenzyme [3, 4]. Therefore a simple independent method for measuring the formation of binary complexes may prove very useful.

**MATERIALS AND METHODS**

Horse-liver alcohol dehydrogenase was purchased as a crystalline suspension from C. F. Boehringer & Soehne (Mannheim); before use, samples were centrifuged, the enzyme was dissolved, and dialysed against three or four changes of phosphate buffer (ionic strength 0.1, pH 7.4) for 3 days at 4°C. The enzyme was centrifuged, and assayed as described previously [5, 6]. Assuming an extinction coefficient ($E_{1mg/ml}$) at 260 nm of 0.45, the apparent purity was 87-93%/o.

Iodoacetate acid was purchased from Sigma Chemical Co. (St. Louis, Mo.) and recrystallised twice from petroleum ether. It was white, with melting-point 81.1-81.7°C; total iodine content was 68.26%/o. Before use it was neutralised to pH 4.7-5.3 with sodium hydroxide. Iodoacetamide was purchased from Sigma Chemical Co. and recrystallised from ethanol—water and then water before use. It was white with melting-point 92.2-92.4°C and total-iodine content 68.30%/o. NAD+ was purchased from P-L Biochemicals (Milwaukee, Wis.) and used without further purification. Other chemicals were of reagent grade. Distilled water was redistilled in quartz apparatus.

Enzyme inactivations were carried out in stoppered 1 cm cuvettes with enzyme concentration 0.6 to 1.6 μM: all experiments (inactivations and enzyme assays) were carried out at 23.5°C: since the rate of inactivation was found to be strongly dependent on ionic strength, this was kept constant by adding phosphate. When glycine buffer was used, the zwiterion was considered not to contribute to the ionic strength. Enzyme activity was measured on a Gilford Model 2000 spectrophotometer, by withdrawing 50 μl samples from the inactivation mixture, adding these to assay cuvettes on a glass rod, and measuring the initial rate of production of NADH ($E_{1mg/ml}$ = 6.22 at 340 nm). The assay cuvettes contained 1 mg NAD+; 8 mM ethanol, and 62 mM glycine/NaOH.
buffer, pH 10.0, in 3.0 ml. Normally, six enzyme assays per inactivation were carried out, covering the range 100—40% activity. At pH 9.35, iodoacetate hydrolyses appreciably; inactivations were done in duplicate, and up to ten assays used to follow carefully the first part of the inactivation.

RESULTS

The loss of enzyme activity was first-order with respect to enzyme, as shown by a linear relationship of log (activity) versus time (Fig.1). Semi-log graphs such as this were used to determine the half-time,

![Fig.1. Semi-log graph of inactivation with time at different iodoacetate concentrations. Inactivations were carried out in stoppered glass or quartz cuvettes, at 23.5°, total volume 3.0 ml. Buffer was 0.025 M phosphate, pH 7.4, enzyme concentration 0.6—1.6 μM. Ionic strength 0.104, maintained by adding phosphate where necessary. Iodoacetate concentrations: ○, 1.3 mM; △, 2.0 mM; ●, 4.0 mM; ▲, 13.3 mM](image1.png)

and hence the rate constant of the reaction, using the relationship: rate constant = (ln 2)/half-time.

The variation in rate of inactivation with iodoacetate concentration is shown in Fig.2. This is a double-reciprocal plot; it shows a linear relationship, and the lines do not pass through the origin. In the absence of iodoacetate, the enzyme is stable under these conditions. Moreover, other fatty acids (formate, acetate and decanoate) and also chloride protect the enzyme competitively. With chloride, the protection was not quite perfectly competitive; the intercept on the ordinate was very slightly above those for the fatty acids alone. This may not be significant: if it is, it suggests that chloride-binding may be slightly different from fatty acid binding, with a very weak enzyme—chloride—fatty acid complex being able to form.

These results appear to constitute strong evidence that iodoacetate, as well as the protecting ions, form reversible binary complexes with the enzyme. The dissociation constants for these complexes were calculated from the slopes of the graph (Fig.2) and are shown in Table 1. The table also shows values obtained from previous work (by competition with NADH, measured by fluorescence enhancement [3, 7]; or by competition with 2,2'-bipyridyl, measured spectrophotometrically [8]). No values are available for chloride: but Theorell and Winer [9] found a decreasing affinity of the enzyme for NADH which (allowing for the difference in ionic strength) would indicate a figure similar to the one obtained here. Li, Ulmer and Vallee [10] showed that chloride, formate and other ions and bases (including Tris) reduce the extrinsic Cotton effect of NADH: but they did not evaluate enzyme-ligand dissociation constants, and interpretation is difficult because of apparently non-

![Fig.2. Double-reciprocal plot of inactivation-rate versus iodoacetate concentration. Conditions as in Fig.1 but with added ions (as sodium salts) as follows: ○, none (calculated from Fig.1); △, chloride (26.7 mM); ●, formate (26.7 mM); ▲, acetate (26.7 mM); and ▲, decanoate (60 μM)](image2.png)

Table 1. Dissociation constants at pH 7.4

<table>
<thead>
<tr>
<th>Ion</th>
<th>Dissociation constant</th>
<th>This work*</th>
<th>Previous work (other methods)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>μM</td>
<td>mM</td>
<td>μM</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>4.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Chloride</td>
<td>23</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Formate</td>
<td>24</td>
<td>47</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Acetate</td>
<td>51</td>
<td>100</td>
<td>900</td>
<td>[3]</td>
</tr>
</tbody>
</table>

* Evaluated from Fig.2 (from ratio of slopes, for protecting ions).
constant ionic strength, and also the possibility of ternary complexes being formed.

Iodoacetamide, on the other hand, showed no such saturation effect (at pH 8.9). If there was one, the dissociation constant would be greater than 50 mM.

Although these results suggest that the enzyme—fatty acid binary complex cannot react with iodoacetate, they do not rule out a small residual reactivity. This was investigated in the following experiment. A constant and rather high iodoacetate concentration was used, with various decanoate concentrations, from zero to very high. (Decanoate was chosen because of its high affinity for the enzyme.)

The reciprocal of the reaction velocity was plotted against decanoate concentration, after the method of Dixon [11]. If protection by decanoate was complete, a straight line would be obtained even at very high decanoate concentrations. But at high decanoate concentrations, inactivation was faster than expected on this basis, indicating some residual sensitivity of the enzyme—decanoate complex to (external) iodoacetate. Under these conditions, but without iodoacetate, the enzyme was found to be stable. This residual activity has a second order rate constant (estimated from the above experiment) of approximately 0.35 M\(^{-1}\) \times \text{min}^{-1}, compared with 20 M\(^{-1}\) \times \text{min}^{-1} under these conditions for free enzyme, assuming external alkylation (see Discussion). So, assuming that the protecting effect is similar for the other enzyme—fatty acid complexes, it is small enough not to affect the results of the experiments. Also, if the reversible enzyme—iodoacetate complex could react with another iodoacetate molecule, the line in Fig. 2 would deviate towards higher velocities at high iodoacetate concentrations. Therefore \(k_2\) (see Discussion) appears to be negligible.

The effect of pH on the reaction of the enzyme with iodoacetate, and the dissociation constants of chloride and formate were determined. The results are shown in Fig. 3: the ratios of the dissociation constants at pH 7.4 (Table 1) are plotted versus pH, so that a direct comparison of the way that they vary with pH can be made. The line is a theoretical curve calculated assuming an ionisation on the enzyme with a \(pK\) of 9.0, with no binding to the alkaline form. It is evident that the binding of all three ions varies similarly with pH, and a \(pK\) of approximately 8.9 to 9.1 would appear to be involved. However, this value may be slightly inaccurate: glycine has been shown to complex with the enzyme, with a dissociation constant of 230 mM at pH 9.0 [7]; this would correspond to 106 mM for the anionic form, which was thought to be the species which binds. In the experiments reported here, anionic glycine would be 36 mM. Previous work has shown that binding of \(\text{NAD}^+\) to the enzyme is affected by an ionisation with a \(pK\) on the free enzyme of 8.6 [7] and more recently 8.75 [12]. It may be the same ionisation which affects anion-binding here.

Tris has been shown [10] to have an effect on liver alcohol dehydrogenase. However, at pH 7.9, very similar results were obtained when phosphate buffer was used instead of Tris-phosphate. Therefore, it seems that specific buffer effects at pH 7.9 and 8.6 due to Tris will not be very significant.

In contrast to the variation of dissociation constants with pH, the velocity extrapolated to infinite iodoacetate concentration varied only slightly, as shown in Table 2.

![Image](https://example.com/image.jpg)

**Fig. 3.** The dissociation constants of iodoacetate, chloride and formate at different pH values, shown as ratios of their values at pH 7.4 (see Table 1). Enzyme, 0.6—1.6 \(\mu\)M. Buffers used: pH 7.4, phosphate; pH 7.9 and 8.6, Tris-NaH_2PO_4; pH 9.35, glycine-NaOH. Ionic strength 0.104, at 23.5°C throughout. Concentrations at pH 7.9 and 8.6 were the same as at pH 7.4 (see legend to Fig. 2). At pH 9.35, formate and chloride were 40 mM, and glycine-NaOH buffer was 0.036 M in NaOH. Continuous line: theoretical titration curve for \(pK\) = 9.0, with ratio of dissociation constants 1.0 at acid pH and infinity at alkaline pH. Points are experimental: O, iodoacetate; A, chloride; and +, formate.

<table>
<thead>
<tr>
<th>pH</th>
<th>Maximum Inactivation Rate (\text{min}^{-1})</th>
</tr>
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<tbody>
<tr>
<td>7.4</td>
<td>(7.8 \times 10^{-2})</td>
</tr>
<tr>
<td>7.9</td>
<td>(7.45 \times 10^{-2})</td>
</tr>
<tr>
<td>8.6</td>
<td>(7.5 \times 10^{-2})</td>
</tr>
<tr>
<td>9.35</td>
<td>(6.1 \times 10^{-2})</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Theorell and co-workers have shown that complexes form between liver alcohol dehydrogenase and fatty acids [3, 7] and therefore it is not surprising
that the experiments described here should show them too; the values obtained (Table 1) are in general lower than those previously obtained with other methods, although the re-determined value for decanoate [7] does agree well. However, the occurrence of a reversible enzyme—iodoacetate complex does not prove that it is a necessary intermediate in the formation of carboxymethyl-enzyme. The following scheme shows, in a general way, the various possibilities:

\[ E + I \rightleftharpoons K \rightleftharpoons EI \]

\[ E' - I \rightleftharpoons K' \rightleftharpoons EI' \]

I represents the inhibitor (i.e., iodoacetate); EI the reversible complex; \( E' - I \) and \( EI' \) irreversibly-inhibited enzyme; \( k' \) and \( k'' \) the first- and second-order rate constants respectively and \( K \) the dissociation constant of EI. If one assumes: (a) that \( k_2 \) is negligible (as suggested above in Results) and (b) that \( k' \) and \( k'' \) are too small to upset the equilibrium between \( E \) and EI [this is reasonable since the inactivation rate is low, and inactivations (Fig. 1) are linear from the outset], then the rate of formation of \( E' - I \) can be shown to be:

\[ \text{rate} = \frac{(k'' \cdot K + k') \cdot [E] \cdot [I]}{K + [I]} \] (1)

Where \( [E] \) represents the total concentration of active enzyme remaining (i.e., \( E + EI \)), and therefore is what the enzyme assay will measure. This scheme represents essentially a summary of that of Baker [13].

From Eqn. (1) the following statements concerning the model may be made: (a) It will obey Michaelis-Menten-type kinetics, being of exactly the same form as the Michaelis equation, (b) The \( K \) which will be obtained experimentally is the dissociation constant for EI, regardless of the relative values of \( k' \) and \( k'' \). Either \( k'' \) or \( k' \) could be zero—in the latter case the inhibitor is a protecting enzyme from itself, by forming a reversible complex. It will be convenient to describe alkylation via \( k'' \) (or \( k_2 \)) as external alkylation, and via \( k' \) as internal alkylation.

The data presented here do not permit a definite decision to be made as to which process is predominating. The following would point to external alkylation:

a) Iodoacetamide reacts very similarly to iodoacetate, including enhancement of reactivity in the presence of imidazole [2]. No saturation effect was observed with iodoacetamide.

b) Yeast alcohol dehydrogenase has thiol groups which are considerably more reactive than the liver enzyme, and which are contained in similar sequences [14]; yet it shows no saturation effect with 2-iodoacetate [15]. Liver alcohol dehydrogenase does show reversible binding with 3-iodoacetate [15a].

However, internal alkylation may be suggested by the selectivity which iodoacetate (and, to a lesser extent, iodoacetamide) shows towards these two sulphydryl groups (one per subunit), out of the total of 28 per molecule [16]. Parachloromercuribenzoate [16] shows little selectivity, and parachloromercuri-sulphonate [17] and 5,5'-dithiobis (2-nitrobenzoate) show none [15a]. Iodine [1], a derivative of N-ethylmaleimide [16], and silver ions [18] react with 25 to 30% of the sulphydryl groups.

The \( pK \) implicated in the binding of anions (8.9 to 9.1) is higher than the value of 7.9 suggested by Evans and Rabin [2] but nearer the values of 8.6 [7] and more recently 8.75 [12] found by Theorell and co-workers which affected NAD+-binding. However, Evans and Rabin used Tris-chloride buffer between pH 7.9 and 8.6, which may account for this discrepancy, because of the binding and protecting effect of chloride. Both groups of workers suggested that the ionisation is of a zinc-bound water molecule. This might be expected to have a \( pK \) of approximately 8.75 [19].

Therefore the results reported here might be considered to strengthen, rather than weaken, the conclusions of Evans and Rabin [2]. These workers used enzyme obtained from Seravac, whereas in this study C. F. Boehringer & Soehne (Mannheim) enzyme was used. However, two samples of Seravac enzyme (specific activity 32% and 50% compared with Boehringer's 90%) were found to give inactivation kinetics which were indistinguishable from those reported above. Therefore it appears that these differences in \( pK \), as well as the apparent lack of a saturation effect, are unlikely to be due to the enzyme being from a different source.

The relative constancy at different pH values of the inactivation-rate at infinite iodoacetate concentrations requires different explanations depending on whether alkylation is internal or external. If it is internal then if \( k'' = 0 \), \( k' \) must be independent of pH. If it is external then if \( k' = 0 \), \( k'' \) must decrease with increasing pH, probably being affected by the same ionisation which increases \( K \).

The results reported here bear a remarkable similarity to experiments on human carbonic anhydrase B [20]. Inactivation by bromoacetate (and iodoacetamide) follows Michaelis-Menten type kinetics, in this case a histidine residue being alkylated: dissociation constants were 3.8 mM for bromoacetate and 20 mM for iodoacetamide. Acetate and chloride bind reversibly and protect the enzyme (\( K = 22 \) mM and 15 mM respectively, at ionic strength 0.075). Carbonic anhydrase can be prepared as the apo-enzyme (i.e., without the zinc); this still binds iodo-
acetamide but not the anions: and also the zinc can be replaced with cobalt, when the ions are bound tighter. As with liver alcohol dehydrogenase, only the acid form of the enzyme (pK = 7.4) binds anions appreciably [21]. Thus the metal is involved intimately in the binding of anions.

One molecule of liver alcohol dehydrogenase contains four zinc atoms and binds two molecules of coenzyme [22] and so it seems likely that in this case too, zinc binds these anions. Orthophenanthroline inhibits the enzyme [4, 23], and spectroscopic evidence suggests that it binds to the zinc [24]. It has been suggested that substrates and substrate analogues (amides and fatty acids) bind to zinc in the enzyme [7, 8]. The observations reported in this paper would appear, for anions at least, to add strength to this hypothesis.

The authors thank the Medical Research Council for grants aiding this work, and for personnel remuneration for C. H. R.

REFERENCES

1. Li, T-K., and Vallee, B. L., Biochemistry, 4 (1965) 1195.

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Complexes of Liver Alcohol Dehydrogenase
Further Studies on the Rate of Inactivation

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(Received November 24, 1969/February 3, 1970)
The Michaelis-Menten-type kinetics of inactivation of horse-liver alcohol dehydrogenase with iodoacetate have been used to study the binding of a number of known ligands. Adenine nucleotides protect competitively with iodoacetate as do halides and aromatic acids. Uncharged chelating-agents (orthophenanthroline, and 2,2'-bipyridyl) protect non-competitively. Uncharged monodentate ligands (e.g. imidazole) stimulate inactivation.

The effect of imidazole on the binding of various ligands at pH 7.4 and at pH 10 was studied. Multiple inhibition analysis was used to examine the mutual interaction of pairs of substances which protected the enzyme from iodoacetate. ADP-ribose and decanoate were mutually competitive, while AMP and orthophenanthroline were non-competitive. Increasing the ionic strength was found to affect differently the mutual interactions of pairs of anions, as well as to weaken their binding to the enzyme.

In some cases, kinetic studies of reduction of NAD+ were used to complement the inactivation studies.

It is concluded that, in the coenzyme, the phosphate group nearer to adenosine is important for binding. Most, but not all, of the ligand-ligand interactions can be explained by steric or electrostatic effects; it is tentatively suggested that conformational changes may also be involved. Precise interpretation, however, is complicated: there are two different types of zinc in the enzyme, and anions might bind to either, or neither.

It has already been shown [1] that iodoacetate binds reversibly to horse-liver alcohol dehydrogenase, as well as inactivating it by alkylation two essential sulphydryl groups, i.e. one per subunit [2-4]: at ionic strength 0.1, various anions were found to protect the enzyme competitively with iodoacetate. In this study, the work is extended to include nucleotides and zinc-binding agents known to bind to alcohol dehydrogenase. Multiple inhibition kinetics are used to examine the mutual effect of different pairs of ligands. Since these inactivation studies cannot be carried out satisfactorily at pH 10 (in the absence of imidazole), substrate-kinetics studies are also used in some cases, to complement the inactivation studies.

MATERIALS AND METHODS

Horse-liver alcohol dehydrogenase was purchased as a crystalline suspension from Boehringer Mannheim GmbH (Mannheim). It consisted largely of the isomer EE [5]. The apparent purity [1] was 87 - 93%.

Iodoacetate acid was obtained from Sigma Chemical Co. (St. Louis, Mo.): melting point 80 - 82°.

Enzyme. Alcohol dehydrogenase, or alcohol: NAD+ oxidoreductase (EC 1.1.1.1).

iodine content 68.25 °/0 (theoretical = 68.26 °/0). Before use it was neutralised to pH 4.7 - 5.3 with sodium hydroxide.

Nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo.) or P-L Biochemicals (Milwaukee, Wis.). Imidazole was recrystallised as described previously [6]. 4-Biphenyl-carboxylic acid was purchased from Fluka AG (Buchs, Switzerland) and recrystallised from methanol and then methanol-water: melting point 223 - 225°. Other chemicals were of reagent grade.

Inactivations were always carried out at 23.5°, at constant ionic strength, maintained by adding phosphate where necessary. At pH 10, glycine buffer was used, concentration approximately 20 mM in glycinate anion. With imidazole present, hydrolysis of iodoacetate (found previously, at high pH [1]) was negligible, as inactivation experiments were usually completed comparatively quickly. For experiments at pH 7.4 with excess imidazole, sodium dihydrogen phosphate and imidazole (free base) were used, to give the same pH and the same phosphate concentration as in experiments without imidazole [6]. Enzyme assays (usually six) normally spanned the range 100 - 40 °/0 activity: a Gilford Model 2000 spectrophotometer recorded the initial increase in absorbance at 340 nm, for details see [1].
Kinetics of NAD\(^+\) reduction by ethanol were observed using a sensitive filter fluorimeter to measure the fluorescence of NADH, after the instrument had been calibrated with known concentrations of NADH. Under the experimental conditions, response was linear.

**RESULTS**

**Adenine Nucleotides**

Evans and Rabin [2] showed that AMP and ADP protected liver alcohol dehydrogenase from iodoacetate. As shown in Figs. 1 and 2, protection by AMP, ADP, ATP, GMP and ADP-ribose is competitive with iodoacetate. Adenosine protection, however, shows mixed kinetics. The dissociation-constants of these compounds, calculated from the ratio of slopes (see Eq. 1 for points): 

\[
\text{Protecting ligand} = \frac{1}{[\text{iodoacetate}]} \times \text{Dissociation constant}
\]

are listed in Table 1.

Reduction of NAD\(^+\) by ethanol was used to determine inhibition constants for AMP and adenosine, at pH 7.14 and 10.0 as shown in Fig. 3 and Table 2. For AMP, as with other nucleotides [7], it is usually found that substrate-kinetics studies give larger values for the dissociation constants than inactivation studies. Adenosine is bound very much more weakly than AMP, and is pH-independent, unlike AMP. This indicates that the phosphate group has a strong influence on binding, and that its binding is pH-dependent. It could be that a positive charge on the enzyme is involved, which is deprotonated at high pH. ADP is bound more weakly than AMP, and ATP more weakly still. Inactivation experiments

![Graph](image1.png)

**Fig. 1. Double-reciprocal plot showing protection of liver alcohol dehydrogenase from iodoacetate by adenine nucleotides.** Enzyme, 0.5 μM, 23.5°C. Phosphate buffer approximately 40 mM, pH 7.4. Ionic strength 0.10 (maintained by varying phosphate concentration). Line without points, no protecting compound (see Fig. 7 for points); O, AMP (91.2 μM); Δ, ADP (326 μM); v, ATP (665 μM).

![Graph](image2.png)

**Fig. 2. Double reciprocal plots showing protection from iodoacetate.** Conditions as for Fig. 1. O, Adenosine (27.4 mM); Δ, ADP-ribose (38.5 μM); v, GMP (313 μM).

<table>
<thead>
<tr>
<th>Protecting ligand</th>
<th>Dissociation constant (K) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>17200</td>
</tr>
<tr>
<td>AMP</td>
<td>32</td>
</tr>
<tr>
<td>ADP</td>
<td>180</td>
</tr>
<tr>
<td>ATP</td>
<td>708</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>10.5</td>
</tr>
<tr>
<td>GMP</td>
<td>110</td>
</tr>
</tbody>
</table>

![Table](image3.png)

**Table 1. Dissociation-constants of complexes of liver alcohol dehydrogenase with adenosine and nucleotides, determined by protection from iodoacetate.** The results were obtained from Fig. 1 and 2 by ratio of slopes.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>pH 7.14</th>
<th>pH 10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>69 μM</td>
<td>140 μM</td>
</tr>
<tr>
<td>ADP</td>
<td>80 μM</td>
<td>5800 μM</td>
</tr>
</tbody>
</table>

![Table](image4.png)

**Table 2. Kinetic determination of inhibition constants, \(K\), for AMP and adenosine at pH 7.14 and pH 10.0.** At pH 10, evaluated from Fig. 3 by ratio of slopes. At pH 7.14, an analogous set of experiments was performed, using phosphate buffer, ionic strength 0.1, at 23.5°C; enzyme 1.4 to 2.8 nM.
Complexes of Alcohol Dehydrogenase


1/NAD⁺(mM) - Effect of AMP and adenosine on NAD⁺-reduction by ethanol and liver alcohol dehydrogenase at pH 10.0. Enzyme, 90 µM; ethanol, 8.25 mM; buffer, 10 mM glycine-NaOH plus phosphate (pH 10.0), ionic strength 0.1, at 23.5°C; O, without inhibitor; △, + AMP, (660 µM); v, + adenosine (14.6 mM)

Fig. 3. Effect of AMP and adenosine on NAD⁺-reduction by ethanol and liver alcohol dehydrogenase at pH 10.0. Enzyme, 90 µM; ethanol, 8.25 mM; buffer, 10 mM glycine-NaOH plus phosphate (pH 10.0), ionic strength 0.1, at 23.5°C; O, without inhibitor; △, + AMP, (660 µM); v, + adenosine (14.6 mM)

Fig. 4. Double-reciprocal plot showing effect of orthophenanthroline on rate of alkylation of alcohol dehydrogenase by iodoacetate. Enzyme, 0.5 µM at 23.5°C. Phosphate buffer (pH 7.4) approx. 40 mM (ionic strength 0.1, maintained by varying phosphate concentration). Line without points, without orthophenanthroline (for points see Fig. 7); △, orthophenanthroline (4.33 mM); O, orthophenanthroline, (8.67 µM)

Orthophenanthroline inhibits liver alcohol dehydrogenase [8, 9], and spectroscopic evidence suggests that it binds to zinc [10]. Sigman [11] has shown that 2,2'-bipyridyl is bound to the enzyme, with a stoichiometry of two molecules of bipyridyl per molecule of enzyme, although one molecule of enzyme (with two coenzyme-binding sites) contains four zinc atoms [12].

Evans and Rabin [2] showed that orthophenanthroline protects the enzyme from inactivation by iodoacetate, and so the kinetics of this were investigated: Fig. 4 shows that orthophenanthroline is approximately non-competitive with iodoacetate: it does not prevent iodoacetate binding to the enzyme, but does protect the sulphydryl group from being alkylated. 2,2' Bipyridyl (Fig. 5) also protected the enzyme, but not quite totally. A control experiment omitting iodoacetate showed that this was not due to inactivation by bipyridyl alone, but to incomplete protection of the reactive sulphydryl groups. Orthophenanthroline also did not protect totally.

The two concentrations of orthophenanthroline (Fig. 4) gave dissociation-constants of 5.8 and 6.3 µM (average 6.1 µM). 2,2' Bipyridyl was found to have a dissociation-constant of 550 µM. Sigman [11] found 400 µM, spectroscopically. This could explain why his values for dissociation-constants of several ligands which competed with bipyridyl (e.g. decanoate), were rather higher than usual.
As well as displacing bipyridyl [11], aliphatic fatty acids form strong ternary complexes with liver alcohol dehydrogenase and NAD$^+$. [13]. However, an aromatic acid, 4-biphenyl-carboxylic acid, acted as a coenzyme-competitive inhibitor but it did not displace bipyridyl from the enzyme, and did not form a ternary complex with enzyme and NAD$^+$. Iodoacetate, perhaps because of its bulky iodine atom, would appear to be binding in a manner similar to aromatic acids rather than aliphatic ones.

![Graph](image)

**Fig. 6. Double-reciprocal plot showing the effect of imidazole on inactivation of liver alcohol dehydrogenase by iodoacetate.**

Enzyme 0.6 µM, at 23.5°C and ionic strength, 0.1. Line without points, without imidazole (in phosphate buffer, pH 7.4). For experimental points, see Fig. 7. O, imidazole (base form 29 mM)-phosphate buffer (pH 7.4); ×, imidazole (63 mM) in glycine/NaOH/Na$_2$HPO$_4$ buffer (NaOH, 16 mM), pH 10.0; Δ, imidazole (0.46 M) in glycine/NaOH/Na$_2$HPO$_4$ buffer (NaOH, 16 mM), (pH 10.0)

**Monodentate Neutral Ligands**

Evans and Rabin [2] found that imidazole increased the rate of reaction of iodoacetate with liver alcohol dehydrogenase, and rendered it almost pH-independent. The kinetics of inactivation with iodoacetate in the presence of excess imidazole are shown in Fig. 6. At pH 7.4, imidazole weakens iodoacetate-binding by about 5-fold, and increases the maximum rate of inactivation (extrapolated to infinite iodoacetate concentration) about 10-fold. These figures are approximate only, since considerable extrapolation was required. At pH 10, results were similar, except that alkylation was a little slower, or binding a little weaker. When the imidazole concentration was raised even further, the rate of inactivation decreased; this shows that the difference between rates at pH 7.4 and pH 10 was not due to incomplete saturation of the enzyme with imidazole at pH 10: and it indicates that imidazole can bind weakly at a second site (perhaps competitively with iodoacetate), this time protecting the sulphhydryl group.

Using the above data, and also a series of inactivations with constant iodoacetate (3.33 mM) and varying imidazole concentrations, an attempt was made to calculate the dissociation-constant of the imidazole-enzyme complex at pH 7.4 and pH 10. The method of calculation was as follows.

Let [E] represent total (unmodified) enzyme concentration; let E represent free enzyme (one subunit, since the two subunits are independent): E-1m and E-IAc, the binary reversible complexes of enzyme with imidazole and iodoacetate respectively; E-Im-IAc the reversible ternary complex of enzyme, imidazole and iodoacetate; and let [IAc] and [Im] represent the iodoacetate and imidazole concentrations (always in large excess over [E], when present). Let us define the following dissociation constants:

$$K_1 = \frac{[E][IAc]}{[E-IAc]} \Rightarrow [E-IAc] = \frac{[E][IAc]}{K_1}$$

$$K_2 = \frac{[E-Im][IAc]}{[E-Im-IAc]} \Rightarrow [E-Im-IAc] = \frac{[E-Im][IAc]}{K_2}$$

$$K_3 = \frac{[E][Im]}{[E-Im]} \Rightarrow [E-Im] = \frac{[E][Im]}{K_3}.$$  

Also:

$$[E] = [E] + [E-IAc] + [E-Im] + [E-Im-IAc].$$

Substitution, followed by rearrangement of the equation, gives:

$$
\frac{[E]}{[E]} = 1 \div \left\{ 1 + \frac{[IAc]}{K_1} + \frac{[Im]}{K_2} + \frac{[Im]}{K_2} \times \frac{[IAc]}{K_3} \right\}.
$$

Since

$$\frac{[E-IAc]}{[E]} = \frac{[IAc]}{K_1} \times \frac{[E]}{[E]}$$

and

$$\frac{[E-Im-IAc]}{[E]} = \frac{[Im]}{K_2} \times \frac{[IAc]}{K_3} \times \frac{[E]}{[E]}$$

the proportion of E-IAc and E-Im-IAc present can be calculated. Let $V_1$ and $V_2$ represent the rate constants of alkylation (assumed internal [11]) of E-IAc and E-Im-IAc respectively, in the presence of infinite iodoacetate. Then, assuming Michaelis-Menten kinetics [1], the experimental rate-constant of inactivation, $v$, will be:

$$v = \frac{[E-IAc]}{[E]} \times V_1 + \frac{[E-Im-IAc]}{[E]} \times V_2.$$
Substituting the values derived above for the relative concentration of these enzyme complexes,

\[
v = \frac{V_1 \frac{[\text{Acar}]}{K_3} + V_2 \frac{[\text{Im}]}{K_9} \frac{[\text{Iac}]}{K_8}}{1 + \frac{[\text{Acar}]}{K_1} + \frac{[\text{Im}]}{K_5} + \frac{[\text{Im}]}{K_9} \frac{[\text{Iac}]}{K_8}}.
\]

When rearranged, this gives:

\[
K_3 = \frac{[\text{Im}]}{\frac{1}{V_1} - v (1 + \frac{[\text{Acar}]}{K_3} - \frac{[\text{Iac}]}{K_8} \times V_2)}.
\]

\[
K_5 = \frac{[\text{Im}]}{\frac{1}{V_1} - v (1 + \frac{[\text{Iac}]}{K_5})}\]

\[
K_9 = \frac{[\text{Iac}]}{\frac{1}{V_1} - v (1 + \frac{[\text{Acar}]}{K_9})}.
\]

\(v\) was determined at various concentrations of imidazole (\(v = 0.693/\text{half-time of enzyme activity}\)); the results are given in Table 3. At pH 7.4, \(K_1 = 4.4\) mM; \(V_2 = 0.0767\) min⁻¹; \(K_2 = 24.2\) mM and \(V_3 = 0.925\) min⁻¹. At pH 10.0, \(V_1 = 0.06\) min⁻¹ (assumed, see [1]); \(K_3 = 22.7\) mM (from \(V_3\) and the value without imidazole present, see Table 3); \(K_5 = 30.0\) mM; \(V_4 = 0.770\) min⁻¹. It must be emphasised that these figures for \(K_3\), given in Table 3, are only approximate: the values of the constants, given above, critically determine the values obtained, and several are not known accurately.

The mean values obtained for \(K_3\), the dissociation-constant of the enzyme-imidazole binary complex, were 0.96 mM at pH 7.4 and 5.9 mM at pH 10: these refer to the concentration of the base form of imidazole, determined as described above (Materials and Methods). Theorell and McKinley-McKee [6], using fluorescence enhancement of NADH, found this dissociation-constant to be 0.55 mM at pH 7, 0.68 mM at pH 8 and 0.67 mM at pH 9, thus agreeing reasonably with the above value at pH 7.4. However, they gave no indication of an increase at higher pH-values. Evans and Rabin [2] found 0.7 mM at pH 7.2 and 5.4 mM at pH 9.0; however, in their calculation, they made no allowance for reversible binding of iodoacetate to the enzyme. When the results in Table 3 are calculated by their method, values of 1.14 mM (at pH 7.4) and 6.06 mM (pH 10) were obtained.

Imidazole is not the only compound which stimulates alkylation. Pyridine does (Fig. 5) at quite low concentrations. High concentrations of ethanol gave the results shown in Fig. 7. At low concentrations of iodoacetate, ethanol protected the enzyme, while at high concentrations of iodoacetate it stimulated alkylation. Preliminary experiments indicated that isobutyramide had a similar effect. (Experiments with isobutyramide were difficult because low concentrations inhibited the enzyme assay, and also gave non-linear progress curves in the assay. This was partially alleviated by including imidazole in the assay mixtures.) Therefore it seems that, in general, neutral molecules which bind to the enzyme (and which are not polyvalent metal-chelating agents) can stimulate alkylation by iodoacetate.

**Imidazole and Adenine Nucleosides**

The effect of imidazole on the binding of AMP and ADP-ribose was studied. Using a constant (excess) concentration of imidazole, and constant iodoacetate, the rates of inactivation at various concentra-
iodoacetate, inactivation studies (Table 1) gave dissociation-constants smaller than those in the presence of imidazole, at pH 7.4, but similar to the kinetically-determined values (Table 2). At pH 10, in the absence of imidazole, only kinetic data are available for comparison: inactivation studies with excess imidazole (Table 4) gave smaller dissociation-constants. Therefore, the variation with pH of binding of these adenine nucleotides is less in the presence of imidazole than in its absence, but is still quite appreciable (more than three-fold between pH 7.4 and pH 10).

**Imidazole and Orthophenanthroline**

In the presence of excess imidazole, orthophenanthroline becomes a much weaker protector of liver alcohol dehydrogenase from iodoacetate (Table 5). The factor by which it became weaker agrees quantitatively with what would be expected if orthophenanthroline was competitive with imidazole [assuming that the dissociation-constant of the enzyme-imidazole complex was approximately 0.9 mM (see above)]. Imidazole was also found to displace 2,2'-bipyridyl from the enzyme [11].

**Imidazole and Decanoate**

Decanoate, like orthophenanthroline, becomes a weaker protector in the presence of imidazole: and, here again, the results indicate that imidazole and decanoate are competitive (Table 5).
Table 5. Effect of excess imidazole on the protection of liver alcohol dehydrogenase from iodoacetate by orthophenanthroline, decanoate and 4-biphenylcarboxylic acid

<table>
<thead>
<tr>
<th>Additions</th>
<th>pH</th>
<th>Half-time</th>
<th>$K_{inact}$ (apparent)</th>
<th>$K_{E,L}$</th>
<th>Ratio</th>
<th>Imidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without protector</td>
<td>7.4</td>
<td>15.17</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>27</td>
</tr>
<tr>
<td>+ Orthophenanthroline (52 μM)</td>
<td>7.4</td>
<td>21.08</td>
<td>133</td>
<td>6.1</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>+ Decanoate (645 μM)</td>
<td>7.4</td>
<td>24.08</td>
<td>1040</td>
<td>39</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>+ Biphenylcarboxylic acid (240 μM)</td>
<td>7.4</td>
<td>20.17</td>
<td>340</td>
<td>44.6</td>
<td>7.6</td>
<td>27</td>
</tr>
<tr>
<td>+ Biphenylcarboxylic acid (720 μM)</td>
<td>7.4</td>
<td>44.08</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Without protector</td>
<td>10.0</td>
<td>13.58</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>63</td>
</tr>
<tr>
<td>+ Biphenylcarboxylic acid (360 μM)</td>
<td>10.0</td>
<td>25.17</td>
<td>420</td>
<td>?</td>
<td>—</td>
<td>50</td>
</tr>
<tr>
<td>+ Biphenylcarboxylic acid (720 μM)</td>
<td>10.0</td>
<td>34.50</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Estimated dissociation constant of the ligand concerned, from its ternary complex with enzyme and imidazole.

** Dissociation constant of the ligand concerned, from its binary complex with the enzyme. (From this work, and [1]).

Table 6. Protection of liver alcohol dehydrogenase from iodoacetate by chloride and formate in the presence of excess imidazole

<table>
<thead>
<tr>
<th>Additions</th>
<th>pH</th>
<th>Half-time</th>
<th>$K_{inact}$ (apparent)</th>
<th>$K_{E,L}$</th>
<th>Ratio</th>
<th>Imidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>No protector</td>
<td>7.4</td>
<td>14.75</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>27</td>
</tr>
<tr>
<td>+ Chloride (38.7 mM)</td>
<td>7.4</td>
<td>27.17</td>
<td>44</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Formate (38.7 mM)</td>
<td>7.4</td>
<td>17.0</td>
<td>(250)</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No protector</td>
<td>10.0</td>
<td>12.33</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>63</td>
</tr>
<tr>
<td>+ Chloride (38.0 mM)</td>
<td>10.0</td>
<td>23.67</td>
<td>46</td>
<td>(?)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Formate (38.0 mM)</td>
<td>10.0</td>
<td>16.7</td>
<td>(152)</td>
<td>(?)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Estimated dissociation constant of the ligand concerned from its ternary complex with enzyme and imidazole.

** Dissociation constant of the ligand concerned from its binary complex with the enzyme [1].

† Iodoacetate 1.33 mM and imidazole, base form 20 mM.

‡ Iodoacetate 2.0 mM and imidazole 63 mM.

Imidazole and 4-Biphenylcarboxylic Acid

Results with 4-biphenylcarboxylic acid were complicated by the fact that it gave biphasic Dixon plots [14], indicating apparently a higher dissociation-constant at higher biphenylcarboxylic acid concentrations. Fig. 7 shows that (in the lower concentration range) it is competitive with iodoacetate. However, Table 5 shows that protection from iodoacetate, although weaker than in the absence of imidazole, is nevertheless greater than predicted by competition with imidazole: raising the pH to 10 weakens the protection further, but only slightly. These results are both very similar to the effect of imidazole on iodoacetate-binding. This strengthens the above suggestion that iodoacetate binds in a manner similar to 4-biphenyl-carboxylic acid, and different from decanoate.

Imidazole and Chloride

Excess imidazole weakens the protecting effect of chloride (Table 6), but only by a factor of two (at pH 7.4). At pH 10, the effect is almost identical to that at pH 7.4—the pH dependence [1] of chloride-binding has been abolished by imidazole.

ADP-Ribose and Decanoate

Since both these ligands protect liver alcohol dehydrogenase (competitively) from iodoacetate, their mutual interactions cannot be studied by saturating the enzyme with one ligand (as was done with imidazole, above). Instead, the method of Yonetani and Theorell [7] was used: results are shown in Fig. 10. The parallel lines obtained indicate that decanoate and ADP-ribose are competitive: the deviation at
high concentrations of decanoate and ADP-ribose (where inactivation was slow) may be due to instability of the enzyme. This difficulty was not experienced in other experiments. Since strong enzyme-
NAD\textsuperscript{+}-decanoate ternary complexes can form, it seems likely that this competition is due to electrostatic repulsion of the negative charges on the phosphate groups and the carboxylic acid group.

**Effects of Increased Ionic Strength**

If the binding of anions, as well as their mutual competition, is due to electrostatic effects, then raising the ionic strength should weaken them. Fig. 11 shows that AMP is still competitive with iodoacetate could be due to using AMP instead of ADP-ribose; but it seems likely that the higher ionic strength has weakened the interaction of adenine nucleotides with decanoate. Chloride, however, appears to interact more with AMP than does decanoate.

**Orthophenanthroline and Chloride**

At ionic strength 0.1, it was not possible to use a sufficiently high concentration of chloride to examine its interaction with orthophenanthroline. In Fig. 13, the results are shown of such an experiment, at ionic strength 0.6. They appear to indicate complex interactions, with orthophenanthroline being competitive with chloride at low concentration and

![Graph showing effects of increased ionic strength on inactivation of liver alcohol dehydrogenase with iodoacetate, and protection by AMP and chloride.](image)

**Orthophenanthroline and AMP**

Yonetani and Theorell [7] analysed the interaction of AMP, ADP and ADP-ribose with ortho-

at ionic strength 0.6: chloride, on the other hand, now shows mixed competitive-non-competitive protection, compared with almost perfectly competitive protection when the ionic strength was 0.1 [1]. Dissociation-constants were 2.7-fold weaker for AMP, and 3.3-fold weaker for chloride, compared with ionic strength 0.1, indicating the importance of electrostatic interactions in their binding; however, if phosphate (buffer) was to bind to the enzyme and was competitive with AMP or Cl\textsuperscript{-}, it could contribute to weakening the binding. AMP was examined for competition with decanoate and chloride (Fig. 12). Both are nearly competitive with AMP, but chloride more so than decanoate. This difference with Fig. 11 becoming non-competitive at higher ones. This suggests that chloride binds strongly, competitively with orthophenanthroline, and weakly non-competitively. However, it should also be pointed out that at higher chloride concentrations, the phosphate concentration was lowered: if phosphate had a specific effect, then it could alter the results considerably. However, the linear protection in the absence of orthophenanthroline suggests that this is probably not significant.
phenanthroline: all these inhibitors are competitive with coenzyme, and their calculations were based on this assumption. However, since orthophenanthroline is approximately non-competitive with iodoacetate, a modification to their method of calculation is required.

Let I represent a substrate-competitive inhibitor, \( I_{\text{nc}} \) a substrate-non-competitive inhibitor, \( S \) the substrate and \( E \) the enzyme (one active centre, as above). Assume that the enzyme obeys Michaelis-Menten kinetics, with the dissociation-constant of the ES complex equal to the Michaelis constant. Define dissociation-constants as follows:

\[
K_{\text{nc}} = \frac{[E][I_{\text{nc}}]}{[E][I_{\text{nc}}]} = \frac{1}{\beta} \frac{[E][S][I_{\text{nc}}]}{[E][S][I_{\text{nc}}]}. 
\]

(If inhibition is perfectly non-competitive, which is the case we shall consider, \( \beta = 1 \); if partially competitive, \( \beta > 1 \), and if partially uncompetitive, \( \beta < 1 \).)

\[
K_e = \frac{[E][I]}{[E][I]} \\
K_s = \frac{[E][S]}{[E][S]} \\
\alpha K_{\text{nc}} = \frac{[E][I_{\text{nc}}][I_{\text{nc}}]}{[E][I_{\text{nc}}][I_{\text{nc}}]}
\]

where \( \alpha \) (the interaction constant) has the same meaning as in Yonetani and Theorell's analysis [7]. It represents the ratio of the dissociation-constant of one inhibitor with an infinite concentration of the other, compared to that in the absence of the second inhibitor.

Also,

\[
[E] = [E] + [ES] + [ESI_{\text{nc}}] + [EI_{\text{nc}}] + [EI_cI_{\text{nc}}]
\]

where \([E]_c\) represents the total enzyme concentration.

\[
[E] = [E] \times \left\{ 1 + \frac{[S]}{K_s} + \frac{[S]}{K_s} \frac{[I_{\text{nc}}]}{K_{\text{nc}}} + \frac{[I_{\text{nc}}]}{K_{\text{nc}}} + \frac{[I_c]}{K_e} + \frac{[I_{\text{nc}}][I_c]}{\alpha K_{\text{nc}} K_e} \right\}
\]

Now, since only ES will react to form product, the reaction velocity will be given by:

\[
v = V_{\text{max}} \times \frac{[ES]}{[E]} = V_{\text{max}} \times \frac{[E]}{[E]} \times \frac{[S]}{K_s}
\]

On substitution and inversion (and factorisation) this gives:

\[
\frac{1}{v} = \frac{K_s}{[S]} \times \frac{1}{v_{\text{max}}} \times \left( 1 + \frac{[S]}{K_s} \right) \times \left( 1 + \frac{[I_{\text{nc}}]}{K_{\text{nc}}} \right) + \frac{[I_c]}{K_e} \times \left( 1 + \frac{[I_{\text{nc}}]}{\alpha K_{\text{nc}}} \right) \]

(1)

Experimentally, if \([I_c]\) is to be varied at different fixed concentrations of \( I_{\text{nc}} \), the intercepts on the abscissa \([I_c]'\) when \(1/v = 0\) and ordinate \((1/v)'\) when \([I_c] = 0\) are calculated thus:

a) \([I_c]'\): When \(1/v = 0\),

\[
1 + \frac{[I]}{K_s} \times \left( 1 + \frac{[I_{\text{nc}}]}{K_{\text{nc}}} \right) = - \frac{[I_c]'}{K_e} \times \left( 1 + \frac{[I_{\text{nc}}]}{\alpha K_{\text{nc}}} \right) \]

b) \((1/v)\): When \([I_c] = 0\),

\[
1 + \frac{[I]}{K_s} \times \left( 1 + \frac{[I_{\text{nc}}]}{K_{\text{nc}}} \right) = (1 + \frac{[I_{\text{nc}}]}{\alpha K_{\text{nc}}} \right) \]

The gradient will be the negative of the ratio of these two,

\[
\text{gradient} = \frac{K_s}{[S]} \times \frac{1}{v_{\text{max}}} \times \frac{1}{K_e} \times \left( 1 + \frac{[I_{\text{nc}}]}{\alpha K_{\text{nc}}} \right).
\]

The intersection of the family of such lines with different fixed concentrations of \( I_{\text{nc}} \) is found from Equation (1) by determining the concentration of \( I_c \) which will give a value of \(1/v\) which is independent of the concentration of \( I_{\text{nc}} \). This \((I_c)''\) is found to be:

\[
[I_c]'' = - \left( 1 + \frac{[S]}{K_s} \right) \times K_e \times \alpha.
\]
This expression is very similar to the corresponding one for two substrate-competitive inhibitors \([7]\), which has no \(1 + \frac{[S]}{K_s}\) term.

In the two special cases of \(\alpha = \infty\) (mutually competitive) and \(\alpha = 1\) (mutually non-competitive), \([I_c]'' = -\infty\) and \((-\frac{[S]}{K_s})\times K_s\), respectively. Therefore, if competitive, a family of parallel lines will be obtained with different concentrations of \(I_{nc}\), as with mutually competitive substrate-competitive inhibitors \([7]\). If non-competitive, however, comparison with Equation (2) shows that the intercept of the lines is the same as the intercept on the abscissa. This is different from two substrate-competitive inhibitors which are mutually non-competitive, which would meet in the second quadrant.

It must be emphasised that the complexes mentioned, and dissociation-constants calculated, concerning inactivation by iodoacetate, are only insofar as they affect alkylation. Other complexes of the enzyme with these ligands (especially, perhaps, chloride) are likely: human carbonic anhydrase B, for example, forms a specific complex with chloride at the active centre but can also bind other chloride ions up to a total of about six, in 0.1 M potassium chloride \([15]\).

**DISCUSSION**

**Binding-Sites of Different Ligands**

In the previous paper \([1]\), all the ligands examined were competitive with iodoacetate, in protecting liver alcohol dehydrogenase from iodoacetate, and there was no evidence for considering the sites to be different (with the possible exception of chloride's). Sigman \([11]\) showed that aliphatic carboxylic acids (with four or more carbon atoms) displaced 2,2'-bipyridyl from the enzyme, and therefore in all probability were bound to zinc. However, acetate and aromatic carboxylic acids (e.g., 4-biphenyl-carboxylic acid) did not displace bipyridyl effectively.

In this study, it has been shown that imidazole is probably competitive with orthophenanthroline (Sigman \([11]\) showed that imidazole displaced 2,2'-bipyridyl), and also with decanoate. The simple conclusion, then, would be that all these ligands bind to the same zinc atom, although there are two different zinc atoms per active subunit \([12,16—18]\). However, there is one difficulty with this explanation: orthophenanthroline does not seem to affect the binding of iodoacetate (see above), and 4-biphenyl-carboxylic acid does not affect the binding of 2,2'-bipyridyl \([11]\). However, as shown above, imidazole does markedly weaken (but not abolish) the binding of these two acids, by five-fold and about seven-fold, respectively. Since one might expect imidazole to occupy one of the two zinc-bonds chelated by orthophenanthroline and bipyridyl, and is of a similar size, it is hard to see, per se, why imidazole should exert an effect which bipyridyl does not. Perhaps the most plausible possibility is that imidazole or orthophenanthroline induces a conformational change in the enzyme. If the enzyme is in different conformations when it binds imidazole and orthophenanthroline, it could explain why imidazole stimulates alkylation of the sulphydryl group by iodoacetate, while orthophenanthroline and bipyridyl protect it.

The pH-dependence of orthophenanthroline \([19]\) and imidazole binding is similar: at pH 10 binding is much (seven-fold) weaker than at around pH 7. Each may displace zinc-bound water molecules \([2,6]\) at neutral pH, but a zinc-bound-hydroxide ion at higher pH \((pK 8.6—9)\); the hydroxide ion may well be bound more firmly than neutral water, which would explain this effect.

![Fig.14. Effect of AMP together with orthophenanthroline on the inactivation of liver alcohol dehydrogenase with iodoacetate. Enzyme, 0.5 μM, at 23.5°, ionic strength 0.1. Phosphate buffer, approximately 40 mM (pH 7.4). Iodoacetate, 3.33 mM C, without orthophenanthroline. Δ, with orthophenanthroline (4.33 μM). •, with orthophenanthroline (8.67 μM)](image-url)
The binding of adenine nucleotides seems to be independent of orthophenanthroline-binding. ADP-ribose does not displace orthophenanthroline [7,19] or 2,2'-bipyridyl [11]; ADP-ribose and orthophenanthroline show mutually non-competitive kinetics [7], and can be co-crystallised as a mosaic complex with the enzyme [20]. However, Theorell and Yonetani [7] found that AMP and ADP were bound tighter in the presence of orthophenanthroline than in its absence; for AMP, the dissociation constant (pH 7) was 140 μM, decreasing to 40 μM in excess orthophenanthroline. In the present study, substrate kinetics gave 69 μM; and inactivation kinetics gave 32 μM in the absence of orthophenanthroline, increasing a little in its presence, to approximately 39 μM. Therefore, although the different methods give different results for the dissociation-constant of AMP from its binary complex with the enzyme, the results converge remarkably in the presence of orthophenanthroline. However, the reasons for the differences in the absence of orthophenanthroline, and for this convergence in its presence, are unknown.

Imidazole (pH 7.4) seems to weaken the binding of AMP and ADP-ribose (Table 4), by about 1.6- to 2-fold. Evans and Rabin [2] claimed that AMP-ribose was bound independently of imidazole: this was probably a coincidence due to the (5-fold) weaker reversible binding of iodoacetate in the presence of imidazole. Imidazole is thought to displace a zinc-bound water molecule [2,6] therefore retaining the positive charge on the zinc at higher pH-values: this makes NAD⁺ binding weaker and independent of pH, indicating charge-repulsion between the positive charges on nicotinamide and zinc: indeed, below pH 8, NAD⁺ is bound more weakly than ADP-ribose or AMP. Imidazole seems to strengthen the binding of AMP and ADP-ribose at pH 10, indicating that, to a small extent, the postulated positive charge on the zinc does play a minor part in attracting the phosphate or phosphates of adenine nucleotides. However the considerably (3-fold) weaker binding of nucleotides at high pH even in the presence of imidazole, indicates that there may be another pH-dependent group involved in the binding of the phosphate of AMP: this is also indicated by the finding that adenosine-binding does not weaken on raising the pH from 7 to 10. The 2.7-fold weakening of AMP-binding, when the ionic strength was raised from 0.1 to 0.6, suggests that electrostatic binding is important. Li and Vallee [21] have also drawn attention to the importance of the AMP group in coenzyme-binding. NMN⁺, on the other hand, is bound very weakly: NAD⁺ displaces 2,2'-bipyridyl from the enzyme but NMN⁺ does not, even at concentrations where it should bind [11]; therefore it seems that, when NMN⁺ does bind, the nicotinamide group may bind where the adenine should normally.

The weaker binding of ADP, and especially ATP, compared with AMP, is interesting; this may have physiological significance, and has been discussed recently [22]. A similar variation occurs in the binding of these adenine nucleotides to glyceraldehyde-3-phosphate dehydrogenase [23].

Iodoacetic acid itself seems to bind in a similar way to aromatic acids (4-biphenyl-carboxylic acid and phenanthroic acid [11], thyroxine and derivatives [24,24a], thyro-acetic acid [24,25] and salicylic acid [26]). These ligands are characterised by being competitive with adenine nucleotides; indeed, salicylic acid seems to be a general inhibitor of nicotinamide nucleotide-linked dehydrogenases [26], competitive with coenzyme. Unlike aliphatic acids [13], they do not form strong ternary complexes with liver alcohol dehydrogenase and NAD⁺. Also, they do not displace orthophenanthroline or bipyridyl, (but tri-iodothyroacetic acid weakens the binding of orthophenanthroline [25]). These results suggest that aromatic acids bind at the coenzyme site. The finding that adenine protects (partially) from iodoacetate is not incompatible with this suggestion.

The carboxylic acid group of aromatic acids could bind where the phosphate of AMP binds, rather than where aliphatic acids bind (probably a zinc atom [6]): the non-interaction with orthophenanthroline also might indicate this. However, the much stronger effect of imidazole in rendering the binding of iodoacetate and 4-biphenyl-carboxylic acid pH-independent, compared with adenine nucleotides, indicates that the carboxylate anion is still attracted to the same "positive centre" as aliphatic acids, rather than that of the phosphate of AMP. The non-competition with orthophenanthroline may be because the acid group is now held further away from the zinc-atom.

Winer and Theorell [13] and Theorell and McKinley-McKee [6] found that aliphatic fatty acids were bound to liver alcohol dehydrogenase competitively with NADH: yet in the presence of fatty-acids, NAD⁺ was bound tighter, which was explained by mutual attraction of the nicotinamide's positive charge and the carboxylate's negative charge. The competition between NADH and decanoate [6,27], and decanoate and ADP-ribose found in this study, are best explained as due to repulsion of the negative charges on the decanoate and phosphate groups. Indeed, the studies reported in this and the earlier paper [1] indicate that, at ionic strength 0.1, usually only one anion (including nucleotides) can be bound at a time, unless a positive charge (as on NAD⁺) is also present. Halide ions are exceptional, in that they can combine with the enzyme-NADH complex (see below).

The effect of ethanol (Fig.7) indicates that an enzyme-alcohol binary complex can form: but its
iodoacetate, when bound in kinetics the 0.1 in indicating oxyquinoline made the presence than the iodide [32] intermediate between that of the enzyme-ligand complex. Indeed, this may be relevant in considering the effect of other ligands, e.g. iodoacetate, 4-biphenyl-carboxylic acid and imidazole; these can all form complexes with some sort of "intermediate" properties; the presence of another ligand alters their affinity for the enzyme, and this could be due to competition for one site, while not interfering with binding at another site.

Chloride-binding is different from decaanoate-binding (a ternary enzyme-imidazole-chloride complex can form, independent of pH); and it is different from iodoacetate-binding (mixed kinetics of inactivation at higher ionic strength); but these two effects may be explained if chloride binds (in the enzyme-imidazole-chloride complex) like iodoacetate, while, partially (at high ionic strength) like imidazole or like the phosphate of AMP. At higher ionic strength, chloride seems to bind more competitively with AMP than does decaanoate, which would favour the latter suggestion.

The experiment with chloride and orthophenanthroline (Fig.13) was designed to test whether they are competitive or not. The results would seem to indicate that they are, at low concentrations of chloride, but that ternary complexes can form at higher concentrations. Chloride [31], bromide [8] and iodide [32] all form ternary complexes with enzyme and NADH or NAD⁺ (the former breaking down faster than the enzyme-coenzyme binary complex, the latter slower). Recent nuclear magnetic resonance studies [33] using 81Br (0.6 M NaBr) have shown that liver alcohol dehydrogenase broadens the peak of 81Br, and that this is not decreased by NAD⁺, NADH, or oxyquinoline; therefore bromide can be bound in the presence of these compounds. Human carbonic anhydrase also broadened the peak of 81Br, and again oxyquinoline made little difference. However, the broadening of the peak of 35Cl was diminished by oxyquinoline, indicating that bromide and chloride-binding may be different. Human carbonic anhydrase B (apo or holo-enzyme) binds about six chloride ions in 0.1 M potassium chloride [15].

Nature and Function of the Reactive Sulphhydryl Group

In the earlier paper [1], it was pointed out that the kinetics of inactivation could be explained equally satisfactorily in two ways: firstly by considering the iodoacetate, when bound reversibly, to protect the reactive sulphhydryl group; or, secondly, by the more obvious mechanism of reversibly-bound iodoacetate then inactivating the enzyme by irreversible alkylation. The present results do not resolve this situation. Recent X-ray studies on lactate dehydrogenase [34] have shown that the essential sulphhydryl group is about 13 Å from the nearest part of the coenzyme molecule (the nicotinamide) on the enzyme-coenzyme complex; yet several lines of evidence [35] indicated that the sulphhydryl group was involved in coenzyme-binding. Since the essential sulphhydryl groups in alcohol and lactate dehydrogenases [4,36] occur in similar sequences, it may be that the "active-centre" sulphhydryl group is some distance away from the active centre of alcohol dehydrogenase as well. NADH shows an extrinsic Cotton-effect when added to carboxymethylated alcohol dehydrogenase, very similar to the one shown by NADH and native enzyme [3]; NADH was bound more weakly by carboxymethylated enzyme, but its environment was considered to be unaltered. Therefore a central function of the sulphhydryl group in coenzyme-binding seems to be ruled out.

Carboxymethylation of the reactive sulphhydryl groups labilises the zinc atoms which do not bind orthophenanthroline or 2,2'-bipyridyl [16]; and (in native enzyme) 0.1 M acetate protects these "non-active-centre" zinc atoms from exchange with 65Zn. A local conformation change was suggested as a possible explanation [16]; it was considered that the acetate must be bound very firmly and not to the "active centre" zinc atom [17,18]. Jornvall [37] found that the enzyme is very difficult to free completely of acetate.

High-resolution X-ray structural studies of liver alcohol dehydrogenase are proceeding in Uppsala [38], and this should help to resolve these ambiguities.

The authors wish to thank the Medical Research Council and the Royal Society for grants aiding this work, the Medical Research Council for personal remuneration for C.H.R. and the Science Research Council for a Studentship for D. I. M.

REFERENCES

26.

Reynolds, D. L. Morris, and J. S. McKinley-McKee: Complexes of Alcohol Dehydrogenase


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Residual Activity in Carboxymethylated Liver Alcohol Dehydrogenase

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(Received 24 July 1970)

Alcohol dehydrogenase (EC 1.1.1.1) from horse liver contains two identical subunits (Jornvall & Harris, 1970); each subunit contains one thiol group that is reactive towards iodoacetate (Li & Vallee, 1963, 1965; Harris, 1964), and alkylation is accompanied by inactivation. We report here that the carboxymethylated enzyme was found to contain 2–2.5%, residual activity, and the possible significance of this is discussed.

Horse liver alcohol dehydrogenase was purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Details of the enzyme, assay and reagents have been described previously (Reynolds & McKinley-McKee, 1969; Reynolds, Morris & McKinley-McKee, 1970). Carboxymethylation of 1ml samples of enzyme in 40mM-sodium phosphate buffer, pH 7.4, was carried out in stopped cellulose acetate tubes. Imidazole was added, because it increases the rate of reaction of the reactive thiol group with iodoacetate (Evans & Rabin, 1968; Reynolds et al. 1970); consequently the enzyme requires a shorter exposure to iodoacetate, and this should minimize the chance of other groups being carboxymethylated. The loss of activity with time is shown in Fig. 1.

It was thought that the residual activity might be due to a minor isoenzyme component. Native enzyme was compared with carboxymethylated enzyme by starch-gel electrophoresis (McKinley-McKee & Moss, 1965) in 10mM-sodium phosphate buffer, pH 7.4; the gel was sliced, and one half stained for alcohol dehydrogenase activity and the other for protein. Native enzyme showed one very intense band of activity, corresponding to the EE isoenzyme (Pietruszko & Thorell, 1969), with one weak basic band (probably ES) and two less basic bands (probably EE’ and EE”). The carboxymethylated enzyme showed just one weak band of activity, corresponding to the main component of native enzyme. The protein staining showed one main band, of the same mobility, for both native and carboxymethylated enzyme, and this corresponded to the main activity bands (EE) described above. The more basic isoenzyme protein (in native enzyme) was also just discernible.

These results show that the residual activity is unlikely to be due to a minor isoenzyme component. The similar mobility of native and carboxymethylated enzyme is not surprising, since they have similar physical properties (Li & Vallee, 1965). The simplest explanation is that it is a genuine catalytic activity of the carboxymethylated enzyme. However, it is also possible that a small fraction of the enzyme had been carboxymethylated at some other amino acid residue, and that this does not abolish activity but does render the protein resistant to alkylation at its essential thiol group.

In the presence of AMP, which protects the enzyme from iodoacetate, the residual activity in this case should be higher, if AMP does not protect this other postulated residue. Since AMP appears not to alter the amount of residual activity (Fig. 1), then this other group would also be protected from carboxymethylation by AMP. However, this result can be considered to strengthen the probability that the residual activity is a genuine property of the carboxymethylated enzyme.

Some creatine kinases, which have a very reactive thiol group, also show considerable residual activity (Kumudavalli, Moreland & Watts, 1970). In lactate dehydrogenase, which contains an essential thiol group in a similar sequence to that in alcohol dehydrogenase (Holbrook et al. 1967), the thiol group is 13Å from the nearest part of the coenzyme on the enzyme surface (Rossmann, 1970). Li & Vallee (1965) showed that carboxymethylated alcohol dehydrogenase has an identical extrinsic Cotton effect with NADH at 327nm, except that the amplitude and breadth are decreased; they interpreted this as showing that coenzyme is bound in an identical asymmetric environment, but is less firmly attached to the enzyme. The finding of residual activity would also support the view that the coenzyme site is not very close to the carboxymethylated residue.

The actual function of the reactive thiol group in the enzyme is still obscure. Carboxymethylation does not prevent the binding of NADH (Li & Vallee, 1965), and it appears now that it is not absolutely essential for catalysis. It may be...
concerned with a subtle stabilization of conformation, or of conformation changes during the reaction cycle. Carboxymethylation does not produce gross changes in conformation (Li & Vallee, 1965). However, we have observed a slow loss of some of the remaining thiol groups (after removal of iodoacetate with Sephadex G-25), which does not occur with native enzyme. [The method of Ellman (1959) was used, in 8M-urea.] It seems that carboxymethylation may make the enzyme more flexible, permitting thiol oxidation, presumably to cystine.

Native liver alcohol dehydrogenase follows the Theorell-Chance mechanism (Theorell & Chance, 1951), in which at maximal velocity the rate-limiting step is the breakdown of the complex between coenzyme-product and enzyme. Therefore (for ethanol oxidation) a ‘burst’, i.e. a rapid production of NADH (stoichiometric with the enzyme active sites present, and bound to enzyme) would be produced. Ethanol (8mM) was added to NAD+ (0.46mM) and carboxymethylated enzyme (0.7 μM) in 62 mM-glycine-NaOH buffer, pH 10, at 23.5°C. The fluorescence at 460 nm (excitation at 340 nm) was observed in a spectrophotofluorimeter (Farrand Optical Co. Inc., New York, N.Y., U.S.A.). No sudden production of NADH was observed, but only the expected steady increase due to residual activity. Therefore, if the residual activity is due to the bulk of the modified enzyme (and not to just a small fraction of it), it does not obey Theorell-Chance kinetics. This could perhaps be a useful method of examining the actual catalytic part of the reaction: with native enzyme, a very small proportion of the enzyme is actually catalysing the interconversion of ternary complexes at any one instant. If the carboxymethylated enzyme is a valid model system, then it may be that the proportion is much higher.

We thank the Medical Research Council for grants supporting this work.