THE FUNCTION AND STRUCTURE OF ALCOHOL DEHYDROGENASES

by

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Ever since the recognition and crystallization of alcohol dehydrogenase from horse liver or yeast, these enzymes have been the subject of very intensive study. However, in spite of all the effort in the past two decades, the theory which attempts to explain the mechanism of enzymatic reaction is still far from complete. Furthermore, from a study of the multitudinous reviews concerning these two enzymes, it is still evident that, to date, relatively little information has been accumulated with regard to the chemistry and functional components of both enzymes. Also, the correlation between function and structure is still lacking.

In view of this deficiency, an attempt has been made to look for enzyme complexes, to identify the binding sites, to determine the number of subunits in these enzymes, and to elucidate further the subunit interactions, the effect of various reagents on dissociation and association phenomena, and the structural function of these subunits in maintaining the enzymatically active structure. This approach may help to bridge the gap between theory and experiment and make possible deductions regarding the mode of enzyme action.

Some of the results in chapters 5, 6 and 8 have been presented at the 4th and 5th Meetings of the Federation of European Biochemical Societies, and at the 480th Meeting of the Biochemical Society of Britain. Subsequently, these reports have been published and reprints are inserted at the end of this thesis.

Grateful acknowledgements are made to Dr. J.S. McKinley-McKee of the Biochemistry Department for his early guidance and useful discussions, and to Dr. C.T. Greenwood for his invaluable advice in the latter stages of this work.
I also wish to express thanks to Professors Sir E.L. Hirst, F.R.S. and C. Kemball, F.R.S. for providing laboratory facilities, and to the University of Edinburgh for the award of a Post-graduate studentship in the period of 1968-69.

My gratitude is also due to Dr. D.J. Hourston for his collaboration in light-scattering measurements.
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>adenosine-5'-phosphate</td>
</tr>
<tr>
<td>2',3'-AMP</td>
<td>adenosine 2',3'-cyclic monophosphate</td>
</tr>
<tr>
<td>3',5'-AMP</td>
<td>adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>ADPR</td>
<td>adenosine-5'-diphospho-ribose</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetate</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>GuHCl</td>
<td>guanidine-hydrochloride</td>
</tr>
<tr>
<td>LADH</td>
<td>alcohol dehydrogenase from horse liver (alcohol: NAD⁺ oxidoreductase, EC 1.1.1.1)</td>
</tr>
<tr>
<td>ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>NAD⁺ (or DPN⁺)</td>
<td>oxidized nicotinamide adenine dinucleotide (or oxidized diphosphopyridine nucleotide)</td>
</tr>
<tr>
<td>NADH (or DPNH)</td>
<td>reduced nicotinamide adenine dinucleotide (or reduced diphosphopyridine nucleotide)</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>oxidized nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal-5'-phosphate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>YADH</td>
<td>alcohol dehydrogenase from yeast (alcohol: NAD⁺ oxidoreductase, EC 1.1.1.1)</td>
</tr>
</tbody>
</table>
SUMMARY

An outline of the general chemical and physical properties of the alcohol dehydrogenases from horse liver (LADH) and yeast (YADH) and their respective coenzymes has been made.

The stability of LADH, YADH, and their complexes has been studied under various conditions. It has been found that the binding of coenzyme, coenzyme moieties, substrate and various inhibitors can either stabilize or destabilize both LADH and YADH against heat or various denaturating agents. A study of the effect of mixtures of such compounds has enabled the possible binding sites of these enzymes to be investigated.

Negative temperature coefficients have been found for the inactivation of LADH and YADH by urea, guanidine-hydrochloride, suggesting that hydrophobic bonds are primarily important in the maintenance of an enzymatically-active structure. However, detailed kinetic studies of the inactivation process have shown that the rate of inactivation is altered by pH, and certain salts. Thus, it has been proposed that several types of chemical forces may play a role in stabilizing the tertiary structure of the protein, through a cooperative phenomenon.

The inactivation rate of LADH, but not YADH, by acid, has also been found to be faster at 0°C than at 23°C, whilst the reverse holds for the inactivation of both enzymes by sodium dodecyl sulfate and alkali. It has been further postulated that on the inactivation of both enzymes by various denaturating agents, an equilibrium between the active and inactive forms of these enzymes is set-up, and certain modifiers can interfere with this equilibrium system.

The loss of enzymatic activity, which occurs at low temperatures, is not reversed by warming to higher temperature. However, under carefully controlled
conditions with the addition of β-mercaptoethanol and removal of the
denaturating agent, it is possible to partially recover the enzymatic
activity. Conditions which are necessary to achieve the optimum reactivation
have been discussed. It has been suggested that the form of these inactive
enzymes which can be reactivated may not involve releasing the zinc moiety
from the proteins. Likewise, the addition of zinc ions does not help the
reversal of inactivation. Loss of zinc ions from both proteins is thus
considered to be part of the subsequent, irreversible process.

By means of ultraviolet absorption spectra, it has been possible to
investigate the environment of the chromophoric residues in LADH and YADH.

Molecular weight studies of the native and inactive forms of LADH and
YADH have been carried out by light-scattering, ultracentrifugation and gel
filtration. The data from light-scattering gave the molecular weights of
YADH and LADH to be 190,000 and 195,000, respectively. Whilst from gel
filtration, the corresponding values were 126,000 and 85,000, and from centri-
fugation, 110,000 and 85,000–88,000. The reasons for these apparent discrepan-
cies have been discussed.

Treatment with urea, guanidine-hydrochloride and acid and alkaline pH
causes both LADH and YADH to dissociate into inactive subunits with molecular
weights of 41,000 and 36,000, respectively; these values are equivalent to
the occurrence of two active sites for LADH and four for YADH. More severe
denaturating agent cause these enzymes to degrade into either smaller fragments,
or result in aggregation, or cause an irreversible loss of structure.

The effect of β-mercaptoethanol and various related compounds on LADH and
YADH has been examined. The differing behaviour of LADH and YADH is of
interest because β-mercaptoethanol reversibly inhibits LADH, whilst it does
not affect YADH. It has been confirmed that $\beta$-mercaptoethanol serves as a substrate of LADH and YADH, because of the presence of an $\text{CH}_2\text{OH}$-group, whilst it behaves as a potent inhibitor of LADH because its $\text{SH}$-group binds to the enzymatic zinc ions.

Fluorescence emission spectra studies have shown that thiol compounds do not affect the fluorescence of free NADH, whilst the fluorescence of the binary LADH–NADH complex is quenched by thiol compounds. This quenching of fluorescence is dependent on both the pH and the concentration of thiol compounds. It has been suggested that ternary complexes composed of LADH, NADH and thiol compounds, may also form. Quenching of fluorescence may also be due to addition reactions which result in breaking up the chromophoric conjugation of NADH, and LADH catalysed these reactions.

Dissociation constants between LADH and various thiol compounds have been measured and these results are discussed.

The partial inactivation of LADH by pyridoxal-5'-phosphate has been shown to be due to the formation of Schiff bases between the $\epsilon$-amino group of lysine residue and the aldehydic group of the inhibitor.

Since pyridoxal-5'-phosphate does not cause a complete loss of LADH activity, it has been concluded that lysine residues of LADH may not play an integral part in the binding between LADH and NADH. In contrast NADH, but not ethanol, specifically protects LADH against this partial inactivation, suggesting that there may be a lysine residue located closely to the coenzyme binding sites and the partial inactivation is due to steric hindrance. Although pyridoxal-5'-phosphate does not affect the activity of YADH, it can also combine with YADH to form Schiff bases.
Sodium borohydrate does not affect LADH-activity but it inactivates YADH. Other compounds, such as zinc ion, has also been found to reversibly inactivate LADH.

It has been suggested that although both LADH and YADH catalyze the reversible oxidation of ethanol to aldehyde in the presence of NAD$^+$, they may not proceed by a similar catalytic mechanism.
CHAPTER 1

GENERAL INTRODUCTION
The first pyridine-nucleotide-dependent dehydrogenase to be successfully crystallized was that alcohol dehydrogenase (Alcohol: NAD oxidoreductase; EC. 1.1.1.1) from brewer's yeast (Negelijn and Wulff, 1937), but the enzyme is now usually prepared from baker's yeast (Racher, 1950). A second crystalline alcohol dehydrogenase was isolated from horse liver by Bonnichsen and Wassin, (1948). Since then, ADH has been found to be widespread in animal and plant tissue. A voluminous literature has appeared in recent years which deals with the physical and chemical properties of these two enzymes and a large number of other pyridine-nucleotide-dependent dehydrogenases (Vallee, 1960; Sund and Theorell, 1963; McKinley-McKee, 1964; Sund, 1965; Theorell, 1967; Colowick, et al., 1966; Sund, 1968). For this reason, only an outline of the general behaviour of these two alcohol dehydrogenases and the more recent work on these enzymes will be given here.

Although both YADH and LADH catalyze the reversible oxidation and reduction of a variety of primary and secondary alcohols to the corresponding aldehydes in the presence of NAD$^+$, i.e.,

$$RCH_2 OH + NAD^+ \xrightarrow{ADH} RCHO + NADH + H^+;$$ (1.1)

their physical and chemical properties (Table 1.1) and amino acid composition (Table 1.2) are quite different.


YADH and LADH also differ in their substrate specificities; YADH reacts more rapidly with lower alcohols than higher alcohols, while LADH has a broader specificity towards carboxyl compounds and acts on long-chain
### TABLE 1.1
Physical and Chemical Properties of YADH and LADH

<table>
<thead>
<tr>
<th>Property</th>
<th>YADH</th>
<th>LADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_{20,w}^o$</td>
<td>$6.72s^a$</td>
<td>$5.11s^b$</td>
</tr>
<tr>
<td>$D_{20,w}^o (cm^2/sec)$</td>
<td>$4.7 \times 10^{-7}^a$</td>
<td>$5.95 \times 10^{-7}^b$</td>
</tr>
<tr>
<td>$V (ml/g)$</td>
<td>$0.769^a$</td>
<td>$0.750^b$</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>$1.1 - 1.5 \times 10^5^a,c$</td>
<td>$7.3 - 8.4 \times 10^4^b,d$</td>
</tr>
<tr>
<td>$f/f_o$</td>
<td>$1.27^a$</td>
<td>$1.23^b$</td>
</tr>
<tr>
<td>Extinction at 280 m (mg/ml)</td>
<td>$1.26^a$</td>
<td>$0.42 - 0.45^b$</td>
</tr>
<tr>
<td>Extinction 280/260</td>
<td>$1.82^a$</td>
<td>$1.73^b$ or $1.30^e$</td>
</tr>
<tr>
<td>Zinc (g./mol.)</td>
<td>$4.5^f,g,*$</td>
<td>$2 - 4^h,i,j,k,l$</td>
</tr>
<tr>
<td>Reactive cysteine residue (g./mol.)</td>
<td>$4^m$</td>
<td>$2^n$</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>pH 5.4$^o$</td>
<td>pH 6.8$q$</td>
</tr>
</tbody>
</table>

References: (a) Hays and Velick (1954); (b) Ehrenberg (1957); (c) Sund (1964); (d) Ehrenberg and Dalziel (1958); (e) Theorell and McKinley-McKee (1961a); (f) Vallee (1960); (g) Wallenfels, et al. (1957); (h) Theorell, et al. (1955); (i) Vallee and Hoch (1957); (j) Åkeson (1964); (k) Oppenheimer, et al. (1967); (l) Kägi, et al. (1969); (m) Harris (1964); (n) Li and Vallee (1963); (o) Keleti (1958); (p) Wallenfels and Arens (1960); (q) Dalziel (1958).
### TABLE 1.2

**Amino Acid Composition of YADH and LADH**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>YADH&lt;sup&gt;a&lt;/sup&gt; (Residues/Molecule)</th>
<th>YADH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LADH&lt;sub&gt;E&lt;/sub&gt;</th>
<th>LADH&lt;sub&gt;S&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>123.5</td>
<td>136</td>
<td>61</td>
<td>62</td>
</tr>
<tr>
<td>Arginine</td>
<td>31.7</td>
<td>32</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>125.4</td>
<td>116</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>Cysteine</td>
<td>36</td>
<td>32</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td>Cystine</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>111.9</td>
<td>128</td>
<td>66</td>
<td>65</td>
</tr>
<tr>
<td>Glycine</td>
<td>150.2</td>
<td>160</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>Histidine</td>
<td>36.6</td>
<td>40</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>189.1</td>
<td>76</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>Leucine</td>
<td>100</td>
<td></td>
<td>54</td>
<td>55</td>
</tr>
<tr>
<td>Lysine</td>
<td>93.0</td>
<td>96</td>
<td>62</td>
<td>63</td>
</tr>
<tr>
<td>Methionine</td>
<td>13.1</td>
<td>24</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>68.2</td>
<td>32</td>
<td>38</td>
<td>37</td>
</tr>
<tr>
<td>Proline</td>
<td>50.6</td>
<td>56</td>
<td>46</td>
<td>45</td>
</tr>
<tr>
<td>Serine</td>
<td>75.4</td>
<td>88</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>Threonine</td>
<td>58.6</td>
<td>64</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>27.1</td>
<td>24</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>51.1</td>
<td>56</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Valine</td>
<td>149.4</td>
<td>128</td>
<td>82</td>
<td>84</td>
</tr>
<tr>
<td>Amide-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>73.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

References: (a) Wallenfels and Arens (1960), (b) Harris (1964), (c) Theorell, et al. (1966).

**LADH<sub>E</sub>** = main fraction (free from the steroid activity).

**LADH<sub>S</sub>** = basic subfraction (solely responsible for the steroid activity).
primary alcohols (Sund and Theorell, 1963). However, in spite of the considerable differences in overall chemical composition and structure between these two dehydrogenases, it is of great interest to note that YADH binds 4 moles of coenzyme per molecule, and contains 4 grams of zinc and reactive sulphydryl groups, whereas LADH is about half of the molecule weight and appears to contain half of the number of binding sites in comparison with YADH.

1.1. THE COENZYME

The metabolic pathways leading to the biosynthesis of pyridine nucleotide coenzymes, their physical and chemical properties, their important role in physiological function and the central part they play in biological oxidations have been most thoroughly reviewed by Mahler, (1957); Kaplan, (1960); Levy, et al., (1962); Sund, et al., (1964); Colowick, et al., (1966); Chaykin, (1967) and Sund, (1968). In combination with specific enzymes, the naturally occurring pyridine nucleotides are involved in the dehydrogenation of more than a hundred different natural substrates. The structure of the oxidized and reduced coenzymes are shown in Fig. 1.1.

Only the nicotinamide moiety of the coenzyme is directly involved in the hydrogen transfer during oxido-reduction reactions catalyzed by pyridine-nucleotide-dependent dehydrogenases. The structure of dihydropyridine ring of NADH was first considered to be a folded-boat configuration (Loewus, et al., 1955), but the currently accepted structure is planar, like the pyridine ring of NAD⁺ (Meyer et al., 1962; Kosower, 1962 a,b). The dihydropyridine ring lies in close juxtaposition to the adenine ring (Weber, 1957).

Owing to the presence of both the adenine and the nicotinamide residues in the coenzyme molecule, the ultra-violet absorption spectrum of NAD⁺ shows
Fig. 1.1. Oxidation-reduction equilibrium of the coenzymes.

a maximum at 259 m\(\mu\), whereas for NADH, in addition to a weaker band at 259 m\(\mu\) due solely to the adenine, a second characteristic "dihydro band" at 340 m\(\mu\) due to the dihydro nicotinamide portion of the molecule is also found (Siegal et al., 1959; Kaplan, 1960). Excitation of \(\beta\)-NADH at 260 m\(\mu\) or 340 m\(\mu\) in aqueous solution at pH 8.3 and 18°C, produces a fluorescence maximum at 457 m\(\mu\) (Weber, 1957; 1958), but not for NAD\(^+\). The fluorescence intensity of \(\beta\)-NADH is strongly temperature- and solvent-dependent (Velick, 1961).

With \(\beta\)-NADH, excitation at 260 m\(\mu\), more than 90% of the light is absorbed by the adenine moiety. This shows the energy-transfer occurs from the excited adenine to the reduced nicotinamide. Since the life-time of the adenine excited state is very short in neutral solution and room temperature, and no adenine fluorescence is observed, it has been suggested that the adenine and the dihydronicotinamide moieties of the coenzyme must be parallel and facing each other in order to permit the energy-transfer. The coenzyme has

Combination with certain dehydrogenases changes the maximum ultraviolet absorption and fluorescence spectrum of β-NADH. In some cases, changes in the ultraviolet and fluorescence spectrum of the enzyme-coenzyme complex also occur when substrate or certain inhibitors are added. These properties provide a useful method for kinetic studies of the enzyme-catalysed reaction and the interaction between enzyme, coenzyme or inhibitors, and also demonstrate the energy-transfer occurring in the reaction (Ehrenberg and Theorell, 1962).

In contrast, the α-coenzyme which has an α-nicotinamide glycosidic linkage, is enzymatically inactive; it has an open structure, not involving any nicotinamide-adenine interaction (Shifrin et al., 1959; 1961; Kaplan, 1960). With this coenzyme, excitation at 260 μm, causes no fluorescence.

1.2. SUBSTRATE SPECIFICITY

Both YADH and LADH possess a low degree of substrate specificity. They react with a great variety of primary alcohols (except methanol) and aldehydes, secondary alcohols and ketones, branched-chain aliphatic and aromatic alcohols and carbonyl compounds. LADH can also oxidize ethyleneglycol (Holzen, et al., 1955), vitamin A, p-nitrobenzyl alcohol (Gillatte, 1959) and cyclic secondary alcohols, but not tertiary alcohols, (i.e. tert.-butanol, tert.-pentanol, and isopropanol) due to steric hindrance and orientation on the enzyme surface. For YADH, the reactivity decreases as the chain-length increases, with the exception of methanol. A linear relation is obtained by graphing the logarithm of the relative velocity of oxidation against the number of carbon atoms in the substrate, indicating the importance of the electronegativity
and the dissociation ability of the carbinol group (van Eys and Kaplan, 1957). Studies of the oxidation rate of methyl-n-alkyl carbinols by YADH, have shown that it goes through a maximum at pentan-2-ol (van Eys and Kaplan, 1957). Recently, it was also reported that in aqueous solution, an equilibrium between acetaldehyde and its hydrate exists, and that the real substrate for YADH is the free, and not the hydrated, acetaldehyde (Müller-Hill and Wallenfels, 1964). Detailed kinetic studies of both enzymes with widely varied substrates and coenzymes have been reported elsewhere (Sund and Theorell, 1963; Dalziel and Dickinson, 1966 a,b).

The physiological function of YADH is undoubtedly that of reducing acetaldehyde to ethanol. In the case of LADH—in spite of it being one of the most extensively studied enzymes—its main physiological function in metabolism is still in conjecture. It is known that LADH is widespread in organisms, but the enzyme never comes into contact with ethanol directly, and so far as is known, ethanol does not form in the animal body. Therefore, the oxidation of ethanol is probably an occasional activity.

Recently, it has been found that LADH catalyzes the NAD⁺-dependent interconversion of certain 3-β-hydroxy- and 3-keto-steroids (Ungar, 1960; Ungar, et al., 1965; Waller, et al., 1965; Graves, et al., 1965 a,b), and also that farnesol is oxidized to farnesal and this might be a normal physiological substrate for LADH (Waller, 1965). These findings have led to the investigation of the possible role of LADH in steroid metabolism; a series of 36 hydroxylated bile acids have been tested as possible substrates (Waller, et al., 1965). The only common feature in the reactive bile acids is the presence of a 3-β-hydroxy- or 3-keto-group. It was also found that LADH could oxidize the primary alcohol group of several sterols, however, in the
presence of a glycol, such as 3-α-, 7-α-, 12-α-, 25-, 26-pentahydroxyprostanol and 26-hydroxy-cholesterol, no oxidation occurred.

Following the discovery that LADH exhibits multiple zones upon starch-gel electrophoresis (McKinley-McKee and Moss, 1965), it has been demonstrated that the steroid activity of LADH is associated with a minor electrophoretically-homogeneous zone (Pietruszko, et al., 1966). But it is still not possible to separate steroid activity from classical LADH-substrate activity. A subfraction from LADH has been isolated and crystallized (Theorell, et al., 1966), but this crystalline steroid-active liver alcohol dehydrogenase still exhibits substantial activity towards alcohol, whereas the main fraction is free from hydroxy-steroid activity. These two enzymes have the same molecular weight, absorbancy at 260 m and contain two coenzyme binding sites per molecule of enzyme. The amino acid composition is indistinguishable, except for possibly threonine. The only marked difference between the physicochemical properties of the hydroxy-steroid and alcohol dehydrogenase is their isoelectric points which are pH 10.0 and 6.8, respectively. This dissimilarity is considered to be due to the difference in the imide-group content.

Kinetic studies have shown that the Theorell-Chance mechanism (see p.28) is not valid for the following catalytic reaction:

\[
3-\alpha\text{-hydroxy-5-\beta\text{-Cholanic acid}} + \text{NAD}^+ \rightleftharpoons 3\text{-Keto-5-\beta\text{-Cholanic acid}} + \text{NADH} + H^+.
\]

(1.2)

3-α-hydroxy-5-β-Cholanic acid is known to be a strong competitive inhibitor with respect to 3-β-hydroxy-5-β-Cholanic acid, but is essentially without effect when ethanol was used as a substrate for both hydroxy-steroid and alcohol dehydrogenase. On the basis of these results, it has been suggested that hydroxy-steroid dehydrogenase has different binding sites for 3-β-hydroxy-
5-β-Cholanic acid and ethanol and that LADH is stereospecific for 3-β-hydroxy-5-β-Cholanic acid.

1.3. THE FUNCTIONAL ROLE OF ZINC IN YADH AND LADH

Recent studies of metallo-enzymes have focused attention upon the role that metals may play in their catalytic function and protein structure. YADH and LADH are zinc-metallo-enzymes. Many speculations on these aspects have been extensively reviewed by Vallee (1960); Vallee and Coleman (1964); and Li (1966). The content of zinc in YADH, prepared from baker's yeast, is found to be 4-5 atoms per molecule of enzyme (Vallee and Hoch, 1955 a,b; Wallenfels, et al., 1957). Since LADH is found to possess half of the number of binding sites and about half of the molecule weight of YADH, it was, therefore, initially considered to contain 2 atoms of zinc per molecule of enzyme (Theorell, et al., 1955; Vallee and Hoch, 1957), but, recently, Åkson (1964); Oppenheimer and McKay, 1966; and Oppenheimer, et al., (1967) have analyzed in more detail the actual zinc-content and have found, in fact, 4 atoms per molecule.

On exposure of YADH and LADH to acid solution, in addition to the loss of catalytic activity and zinc atoms, aggregation results (Vallee, 1958; Hoch and Vallee, 1959; Yonetani, 1963; Åkson, 1964; Oppenheimer, 1967). The tryptophan fluorescence of the protein is also quenched in a time-dependent manner (Brand, et al., 1962; Blomquist, 1967), indicating occurrence of structural changes. This is further supported by the finding that on removal of zinc from LADH, the formerly stable sulfhydryl groups are oxidized to disulfide bonds (Oppenheimer, et al., 1967). Steric restrictions probably prevent this in the native enzyme (Cecil, 1963).
The role of zinc in maintaining the enzymatically active structure of these two enzymes has been studied through the kinetics of inhibition of enzymatic activity by a number of complexing agents capable of combining with zinc. Two types of reaction between the zinc in YADH and 1, 10-orthophenanthroline have been reported by Hoch, et al., (1958) and Vallee and Coombs (1959). The first type is the reversible combination between each zinc atom in the enzyme and one mole of 1, 10-orthophenanthroline. This reaction can be prevented by the presence of a critical excess of metal ions or reversed by the addition of zinc (Vallee and Hoch, 1957). The inhibition is competitive between 1, 10-orthophenanthroline and NAD\textsuperscript{+} or NADH, but uncompetitive between 1, 10-orthophenanthroline and ethanol or acetaldehyde. As a function of time, YADH is irreversibly inhibited by 1, 10-orthophenanthroline. Once the zinc atoms have been removed, the enzyme molecule is dissociated into four subunits with a molecule weight of 36,000 (Käägi, 1959; Käägi and Vallee, 1960). Neither removal of 1, 10-orthophenanthroline through dialysis, nor addition of zinc, brings about reactivation and association. This is also found to be the case for the reaction between zinc in YADH and 8-hydroxyquinine-5-sulfonic acid, but not ethylenediamine tetra-acetic acid. The failure of ethylene diamine tetra-acetic acid to inhibit and to dissociate the enzyme may be due to steric hindrance (Vallee, 1955). On the addition of NADH, the dissociation of the enzyme into subunits by metal chelating agents, does not occur. Thus, the zinc atoms in YADH may act as bridges between subunits, the rupture of these zinc bridges destroying the critical structure required for coenzyme binding. The proper configuration of the subunits, induced by zinc atoms, must be decisive for the ensuing catalytic action. Studies of optical rotatory dispersion have shown that the dissociation of
YADH by 1, 10-orthophenanthroline is not accompanied by changes in the helical conformation of the protein (Ulmer and Vallee, 1960). Thus, it has been concluded that the irreversible transformation of the tetramer of YADH into subunits is attributed to a localized configuration, or a chemical change, at the zinc binding site. Studies of the absorption spectrum obtained by replacing the zinc ions in the YADH molecule by cadmium ions, suggest that zinc is bound to the enzyme through a mercaptide linkage (Druyan and Vallee, 1962).

The binding of 1, 10-orthophenanthroline to LADH, does not dissociate the enzyme molecule into subunits or remove zinc, but forms a mixed protein-zinc-chelate complex (Vallee and Coombs, 1959; Yonetsu, 1963); two moles of 1, 10-orthophenanthroline is found to be bound to two atoms of zinc. Also two of the enzymatic zinc ions can be exchanged with $\textit{Zn}^{65}$ or $\textit{Cd}^{115}$ by equilibrium dialysis in 0.1 M succinic buffer pH 6.0 and the enzymatic activity is retained (Druyan and Vallee, 1962; 1964). The coenzyme, or certain coenzyme inhibitors, in combination with substrates or substrate inhibitors, greatly retards the rate of exchange of the enzymatic zinc ions. Therefore, two of the zinc atoms in LADH are considered to be essential for catalytic function, while the other two zinc atoms are firmly bound to the protein, presumably through zinc-mercaptide linkages (Druyan and Vallee, 1962) and maintain the tertiary structure. The inhibition of LADH by 1, 10-orthophen...
Vallee and Coombs, 1959). These spectrophotometric and kinetic results have suggested that the zinc ions are at, or near, the binding site for the coenzyme (Vallee, 1960). Yonetani (1963 a,b) found that the intensity of the characteristic enzyme-1,10-phenanthroline spectrum is diminished by adding NAD$^+$ or NADH, whereas the potent inhibitors, such as AMP, ADP, ADPR and 4-biphenyl-carboxylic acid, do not. Thus it has been concluded that the zinc ion of the enzyme is bound near the nicotinamide moiety of the coenzyme, and remote from ADPR-binding site. Recently, the formation of the ternary complexes, enzyme-bipyridine-3-pyridine aldehyde adenine dinucleotide and enzyme-bipyridine-3-acetylpyridine adenine dinucleotide, has been reported by Sigman (1967). The formation of these complexes eliminated the enzyme-bipyridine spectrum, and this suggests that the carboxyamide group of the coenzyme is not essential in binding the coenzyme to the enzymatic zinc ion.

Unlike the coenzyme, ethanol and aldehyde do not compete with 1,10-orthophenanthroline in the inhibition of LADH and YADH (Vallee et al., 1959; Mahler et al., 1962), and they are unable to eliminate the enzyme-1,10-orthophenanthroline spectrum. Thus, it has been considered that substrate is not bound to the zinc ion in both enzymes (Hoch et al., 1958; Vallee and Coombs, 1959; Vallee, 1960; Mahler et al., 1962). However, this suggestion has been disputed by Plane and Theorell (1960); Dalziel (1963); and McKinley-McKee (1964). These authors found that imidazole competes with alcohol and aldehyde and forms enzyme-NADH-imidazole and enzyme-NAD$^+$-imidazole complexes. Since imidazole is known to be a zinc complexing agent, therefore, the substrate must also bind to enzymatic zinc ion. This was further supported by a recent finding (Sigman, 1967) that 2,2-bipyridine chelates 2 zinc ions of LADH. Also 2,2-bipyridine is considered to be a useful chelating agent.
with which to study the interaction of substrates, inhibitors and coenzymes with the active site of enzyme, because 2,2-bipyridine binds the enzyme less tightly than 1,10-phenanthroline and because the main absorption maximum of the difference spectrum appears at longer wavelengths where both enzyme and 2,2-bipyridine have low extinction coefficients. The formation of the binary complex, enzyme-2,2-bipyridine, shows an ultra-violet absorption maximum at 308 m\(\mu\). The intensity of the enzyme-2,2-pyridine spectrum is diminished by adding the coenzyme, substrate and substrate inhibitors, such as carboxylic acids, mercaptans, amides and nitrogen bases. However, the competitive inhibitors, such as AMP, ADP, ADPR and \(4\)-biphenylcarboxylic acid, do not have such an effect. These results further confirmed the previous report (Yonetani, 1963 a,b) that the nicotinamide moiety of the coenzyme lies near the enzymatic zinc ion of the active site and that enzymatic zinc ion is also part of the substrate binding site. Since the enzymatic reaction proceeds by direct hydrogen transfer (Vannealand and Westheimer, 1954), the substrate and nicotinamide ring would most likely occupy contiguous position on the enzymic surface.

Interaction of 1,10-phenanthroline or coenzyme with the enzymatic zinc ion causes a strong negative Cotton effect in the region of the absorption band of bound 1,10-phenanthroline or the coenzyme (Ulmer et al., 1961; Li et al., 1962; 1963; 1964; Rosenberg, et al., 1965). The magnitude of the Cotton effect is directly proportional to the number of moles of NADH bound to the enzyme, which permits direct measurement of the stoichiometry of the reaction. Two moles of NADH are found to bind to one mole of protein, in agreement with the previous spectrophotometric studies (Theorell and Bonnichsen, 1951). The addition of NADH to LADH - 1,10-phenanthroline mixture also affects the Cotton effect,
demonstrating the direct competition between NADH and the chelating agent for binding to the zinc site of the enzyme. These Cotton effects have also been used as the evidence for the asymmetric binding of the coenzyme, or 1,10-orthophenanthroline, to the enzymatic zinc ions.

Thus, all current evidence suggests that the role of zinc in YADH and LADH is to serve both in a catalytic function and in maintaining the tertiary structure of the protein. The interaction of the coenzyme and substrate with the enzyme is at, or near, the zinc sites. No doubt, all the findings reported so far can be employed to good advantage to explore further details of the chemical structure of the active role for the zinc ions in the mechanism of action of the enzyme. However, it has not been able to assign the separate function, or structural roles, to any of the zinc atoms of both enzymes. They do not show in what manner the zinc atom participates in the catalytic process, or how NADH and substrates bind to the enzymatic zinc. Furthermore, they do not show how the interaction of zinc with the protein brings about the catalytic activity and the particular specificity-characteristics of each enzyme. The operation of complementary mechanisms cannot be entirely ruled out at present. The nature of the zinc protein bond still remains conjectural.

1.4. THE SULPHHYDYL-GROUPS OF YADH AND LADH

Both YADH and LADH are thiol-dehydrogenases and contain an uncertain number of thiol-groups. The number in YADH even changes with changing the reaction temperature (Hoch and Vallee, 1959), and it lies between 4 and 40 thiol-groups per enzyme molecule under the different methods and experimental conditions (see review by Sund and Theorell, 1963). In general, the most active enzyme gives a value of 36 free thiol-groups (Wallenfels and Arens, 1960).
Inactivation of the enzyme by urea does not result in decrease, or increase, in the number of thiol-groups (Wallenfels and Sund, 1957; Hoch and Vallee, 1959).

In the case of LADH, 8 free thiol-groups per molecule of enzyme are obtained by amperometric titration (Bonnichsen, 1953), whilst spectrophotometric titration with p-mercuribenzoate gives a value of 25-28 (see review by Theorell and Sund, 1963). The reason for this discrepancy is not yet clear, but probably implies that some of the thiol-groups are embedded in the internal structure of the macromolecule, and only on rupture of the tertiary structure of the protein can the maximum number of thiol-groups be obtained.

YADH and LADH are inhibited by a number of thiol-reagents such as p-mercuribenzoate, iodoacetate and iodoacetamide, N(-4-dimethylamino-3,5-dinitrophenylmaleimide (see review by Sund and Theorell, 1963) and 4,5-dibromo-6-pyridazone (Schreiber et al., 1968). The rate of reaction of YADH with iodoacetamide is independent of pH (Whitehead and Rabin, 1964; Rabin et al., 1964), whereas the rate of the irreversible inhibition of YADH by iodoacetate and bromopyruvate, decreases with increasing pH (Rashed and Rabin, 1968). The presence of a positively charged group (a zinc-hydrate) near the reactive, non-ionising, thiol-group may promote the reactivity of negatively charged alkylating agents by electrostatic interaction. The rate of inactivation of LADH by iodoacetamide is independent of pH (Evans and Rabin, 1968). For iodoacetate, the rate of alkylation decreases with increasing pH. A plausible explanation of this behaviour is that the thiol-group is hydrogen-bonded, possibly to an imidazole group.
Thiol-groups have long been known to be essential for the catalytic activity of both alcohol dehydrogenases (Boyer, 1959). The coenzyme protects the enzyme against inactivation by various thiol-reagents. This has led to an investigation of the composition and structure of their active site by means of site-specific and selective reagents. Complete inactivation of YADH and LADH by using [14C] iodoacetic acid has shown that the inactive yeast enzyme derivative contained 3.8 - 4.4 gram atoms of 14C (Whitehead and Rabin, 1964; Rabin et al., 1964) whilst the corresponding derivative of the liver enzyme contained 1.8 - 2.2 gram atoms of 14C (Li and Vallee, 1963; Harris, 1964). Thus, it has been concluded that four of the thiol-groups are essential to the catalytic activity of YADH, whereas only two are necessary for LADH. The product has been isolated and identified as S-carboxymethylcysteine, and the reactive cysteines have been shown to occur in the same unique sequence of amino acids in the primary structure of the enzyme. Digestion of the [14C] iodoacetic acid derivative of both enzymes with trypsin, and isolation of the peptide by gel-filtration and ion-exchange chromatography, has shown that the amino acid sequences around the reactive cysteines in YADH (Harris, 1964) and LADH (Li and Vallee, 1964; Harris, 1964) are as in Fig. (1.2).

This peptide does not bind the functional zinc atoms of the enzyme (Li and Vallee, 1964). A comparison of these two amino acid sequences around the reactive cysteinyl residues in YADH and LADH shows that to be significantly different. Only glycine, aspartic acid, histidine and glycine in the positions 4, 9, 11 and 15, respectively occur in the same relative positions in both enzymes. In the positions 3, 5 and 8, the serine, valine and threonine in the yeast enzyme are replaced by threonine, isoleucine and serine respectively.
### Fig. (1.2). Comparison of the amino acid sequences around the reactive cysteines in alcohol dehydrogenase from yeast (1) and horse liver (2). Following the reaction with $[^{14}\text{C}]$ iodoacetic acid. From (Harris, 1964).

<table>
<thead>
<tr>
<th>Yeast (1)</th>
<th>Horse Liver (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6 7 8 9</td>
<td>10 11 12 13 14 15 16</td>
</tr>
</tbody>
</table>

* $S-[^{14}\text{C}]$ Carboxymethylcysteine.
in the horse liver enzyme. These differences have been explained by Harris (1964) as mutations. The amino acid sequences of the two enzymes in the positions 1 to 2 and 10 to 16, except 11 and 15, are entirely unrelated (Fig. 1.2). It may imply that the sequence in this part of the chain cannot be altered to any appreciable extent without at the same time affecting the tertiary structure which is essential for maintaining the structural integrity of the "active site".

The enzymatic reaction of both dehydrogenases with the coenzyme and the substrate shows strong stereospecificity in the transfer of hydrogen (Levy et al., 1962). Since the thiol-group is involved in the region of the active sites, it may also show stereoselective behaviour against asymmetric reagents. As a result, YADH was investigated by inactivating with antipode stereoisomers of β-iodopropionic acid and its amide (Eisele and Wallenfels, 1968). YADH, like lactate dehydrogenase, is an A-specific dehydrogenase (see review Levy et al., 1962), and is found to be inactivated faster by the D-(+)-antipodes of the reagents, whereas B-specific enzymes (galactose dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase) were found to be inactivated faster by the L-(−)-antipodes of iodopropionic acid and its amide. Thus, the stereoselective alkylation of the essential thiol-groups may indicate that these thiol-groups are situated in a dissymmetric site on the enzyme surface. The coenzyme protects the enzyme against inactivation by thiol-reagents. It is conceivable that the thiol-group is either situated at the coenzyme binding site, or that the binding of the coenzyme to the enzyme, induces a change in the conformation that may lead to a change in the stereoselectivity of the reaction. Studies of the A/B-specific enzyme and coenzyme complexes and the stereoselective reactivity of
functional thiol-groups in active sites of dehydrogenases, may lead to a new understanding of the spatial relationship of coenzyme attaching groups in the active sites of pyridine-nucleotide-dependent dehydrogenases.

1.5. STEREOSELECTIVITY FOR THE HYDROGEN TRANSFER

(A) Stereospecificity with respect to Coenzyme.

Westheimer et al., (1951) and Fisher et al., (1953) first demonstrated that the enzymatic reduction of pyridine nucleotides is stereospecific.

It was found that when reaction (1.3) was conducted with YADH

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{DPN}^+ \xrightleftharpoons{\text{YADH}} \text{CH}_3\text{CHO} + \text{DPNH} + \text{H}^+ \tag{1.3}
\]

in the presence of D_2O, no deuterium was found in the reaction products. \(\text{CH}_3\text{CD}_2\text{OH}\) was then used as the substrate and reaction (1.4) was carried out in H_2O,

\[
\text{CH}_3\text{CD}_2\text{OH} + \text{DPN}^+ \xrightleftharpoons{\text{YADH}} \text{CH}_3\text{CDO} + \text{DPND} + \text{H}^+ \tag{1.4}
\]

when one mole of deuterium per mole of reduced DPN was obtained. These results clearly show that the hydrogen is directly transferred from the \(\alpha\)-carbon atom of ethanol and does not exchange with the protons of the medium.

When enzymatically deuterated DPN from reaction (1.4) was reoxidized with acetaldehyde and the same enzyme, YADH, as shown in reaction (1.5), all the deuterium in the coenzyme was removed again.

\[
\text{CH}_3\text{CHO} + \text{DPND} + \text{H}^+ \xrightleftharpoons{\text{YADH}} \text{CH}_3\text{CHDOH} + \text{DPN}^+ \tag{1.5}
\]

In addition, when chemically deuterated DPN, prepared by the non stereospecific reduction of DPN^+ with Na_2S_2O_4 in D_2O, was used in the reaction (1.5), only about half of the deuterium was lost. These experiments indicate that the reversible transfer of hydrogen ion, catalysed by alcohol
dehydrogenase, involves only one side of the plane of the nicotinamide ring, which is at carbon atom 4 in DPN⁺. (Pullman et al., 1954; Talalay et al., 1955; Loewus et al., 1955; Mauzerall and Westheimer, 1955; Hutton and Westheimer, 1958.) Chemically reduced DPN⁺ consists of a mixture of the two diastereoisomers, designated as A- and B-stereospecificity. From detailed investigations of many other pyridine-nucleotide-dependent dehydrogenases, it has been demonstrated that YADH and LADH show A-stereospecificity and some other dehydrogenases, such as steroid dehydrogenase, use the opposite side of the 4 position of the nicotinamide, showing B-stereospecificity. No non-stereospecific pyridine-nucleotide-dependent dehydrogenases have been found as yet (for reviews see Vennesland and Westheimer, 1954; Vennesland, 1958; Levy et al., 1962; Colowick et al., 1966; Sund, 1968).

Levy and Vennesland (1957) and Levy et al., (1962) have proposed a model (Fig. 1.3) for the action of A- and B-stereospecific pyridine-nucleotide-dependent dehydrogenases. The reduced pyridine ring

![Diagram](image)

**Fig. 1.3.** Proposed conformational changes for the reduced coenzyme (from Levy, et al., 1962). is thought to undergo a change in conformation from one boat form to another. However, recent absorption (Kosower, 1962) and nuclear magnetic resonance spectra studies (Meyer et al., 1962) of NADH and model compounds, such as N-benzyl-1,4-dihydrionicotinamide, do not support such a hypothesis.
They revealed that the two hydrogens at C-4 of the nicotinamide ring are equivalent.

The absolute configurations of DPNH\textsubscript{A} and DPNH\textsubscript{B} have been established by Cornforth et al. (1962), using optical rotatory dispersion, and studying their conversion to deuterated succinic acid of known stereochemistry. The stereochemistry of the hydrogen A and the hydrogen B in DPNH is shown in Fig. (1.4).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1}
\caption{DPNH\textsubscript{A} is (4R) DPNH; DPNH\textsubscript{B} is (4S) DPNH.}
\end{figure}

Cornforth et al. (1962) has proposed the following rule for the A- or B-stereospecificity of the hydrogen transfer of the nicotinamide ring in DPN\textsuperscript{+}:

"When an enzyme of class A transfers hydrogen from a substrate to a pyridine nucleotide, the hydrogen is added to that side of the nicotinamide ring on which the ring atoms 1 to 6 appear in anti-clockwise order."

(B) Stereospecificity with respect to Substrates.

The hydrogen transfer in the catalytic reaction by alcohol dehydrogenase is stereospecific with respect to alcohol. This aspect of the reaction was first established with YADH (Loewus et al., 1953), in the reduction of CH\textsubscript{3}CHO to ethanol in the presence of DPNH (reaction 1.6), or CH\textsubscript{3}CHO in the
presence of DPND\textsubscript{A} (reaction 1-7). Two enantiomorphs of monodeutero ethanol were obtained (Levy and Vennesland, 1957; Streitweiser et al., 1959; Brewster, 1959 a-b; Levy et al., 1962; Lemieux and Howard, 1963).

\[
\begin{align*}
\text{DPND}_\text{A} + \text{DPN}^- + \text{H}^+ \xrightleftharpoons[YADH]{\text{H}} \text{DPNH} + \text{DPN}^+ (1.6) \\
\text{DPND}_\text{A} + \text{DPN}^- + \text{H}^+ \xrightleftharpoons[YADH]{\text{H}} \text{DPNH} + \text{DPN}^+ (1.7)
\end{align*}
\]

(-) (S) ethanol ld.

(+) (R) ethanol ld.

Recently, the steric course of replacement reactions has been investigated with a great number of primary and secondary alcohols, aldehydes and cyclic ketones of known absolute configuration (Prolog, 1963; 1964; Graves et al., 1965; Karabatsos et al., 1966; Dickinson and Dalziel, 1967).

YADH was found to be stereospecific for (+) isomers of butan-2-ol (Dickinson and Dalziel, 1967) and octan-2-ol (van Eys and Kaplan, 1957) and was inactive towards cyclohexanol (Merritt and Tomkins, 1959), hexan-3-ol (van Eys and Kaplan, 1957), pentan-3-ol and heptanol (Dickinson and Dalziel, 1967).

Whereas LADH does not exhibit absolute stereospecificity towards the optically active substrates; it oxidizes cyclohexanol, butan-2-ol, octan-2-ol and hexan-3-ol. These results may indicate a fundamental difference between the active site of the two enzymes. Because of these differences of specificity and stereospecificity, the yeast enzyme cannot catalyse the
oxidation of secondary alcohols in which both alkyl groups are larger than methyl and is considered to be due to steric hindrance, while the liver enzyme is free from this restriction (Dickinson and Dalziel, 1967a). The unusual lack of such stereospecificity by the liver enzyme suggests (a) the presence of an alkyl-binding site that will react with a variety of alkyl groups, and (b) that the enzyme is able to accommodate a fairly large unbound alkyl-group in an active enzyme - NAD⁺ - secondary alcohol ternary complex. Previous stereospecificity studies of the known configurations of \([1^2\text{H}]\) ethanol (Levy et al., 1957; Lemieux and Howard, 1963); and \([1^3\text{H}]\) geraniol (Donninger and Ryback, 1964); and the \(n\)-alkyl-methyl carbinols (Dickinson and Dalziel, 1967a) with both yeast and liver enzyme, provide strong evidence that the alkyl groups of the substrate are bound to the enzyme in the oxidation of \(n\)-alkyl-methyl carbinols. No experimental evidence is yet available to show whether these alcohols, and the (−) isomers of \(n\)-alkyl-methyl carbinols are actually unable to combine with DPN⁺ complex of the yeast enzyme, or whether steric hindrance prevents the conformational change which might follow substrate combination and be essential for reaction.

Although LADH has been generally regarded as lacking stereospecificity, this view is disputed by a recent finding by Hadorn et al., (1963) that glycerol is mainly oxidized to L-glyceraldehyde by LADH and only a small amount of D-glyceraldehyde is formed. This shows that glycerol is stereospecifically oxidized to L-glyceraldehyde by LADH. Waller et al., (1965) also found that 3-\(\alpha\)-hydroxy-5-\(\beta\)-cholanic acid, unlike 3-\(\beta\)-hydroxy-sterol, cannot serve as a substrate for LADH. This presents another example of the stereospecificity of LADH towards a substrate.
1.6. INHIBITION STUDIES

The inhibition of YADH or LADH by a great variety of thiol-reagents, zinc-complexing agents, metal ions, inorganic salts, and some drugs, has been extensively reviewed by Sund and Theorell (1963), and McKinley-McKee (1964). Studies of competitive inhibitors designed to be structural analogues of substrates and coenzymes have provided information regarding the nature of active site of both enzymes. It is these studies which have shown that zinc atoms in both enzymes play an important part in binding the coenzyme and substrate (see p.12); and four thiol-groups for YADH and two for LADH are involved at, or near, the active site (see p.16).

Since the YADH- and LADH-catalysed oxidation of ethanol is pyridine-nucleotide dependent, compounds structurally related to the pyridinium ring moiety, or the purine moiety, of the DPN⁺ molecule, such as adenine derivatives (Wallenfels et al., 1957; Hoch et al., 1960; Anderson and Reynolds, 1965; Anderson et al., 1965a) and pyridinium derivatives (van Eys and Kaplan, 1957; Anderson et al., 1965b), serve as competitive inhibitors with respect to the coenzyme. The inhibition observed with the N₁-alkyl-nicotinamide chlorides increases with increasing chain length of the alkyl substituent due to the enhancement of the binding of the alkyl derivatives to the enzyme through dispersion forces (Anderson and Anderson, 1964; Anderson et al., 1965b). These findings, in conjunction with related studies in which a variety of nitrogen bases (Anderson et al., 1966) and simple hydrocarbons were used as competitive inhibitors (Roger and Candy, 1968), suggested the presence of a hydrophobic area at the "pyridinium ring" region of the DPN⁺-binding site of the enzyme.

Studies of protonated pyridine bases and their corresponding N-methyl
pyridinium derivatives (van Eyss and Kaplan, 1957), and the inhibition of
YADH by alkylammonium chlorides (Anderson and Reynolds, 1965a), have
demonstrated that the positively charged pyridinium ring nitrogen is
important in the binding pyridine nucleotides to YADH. By using the
method of multiple inhibition kinetics (Yonetani and Theorell, 1964), it
has been shown that $N^1$-alkylnicotinamide chlorides can be bound simultane-
ously with adenine derivatives (Fonda and Anderson, 1967), to YADH.

The role of the substituent group in the 3-position of the pyridine ring
in binding to YADH has also been investigated by Heitz and Anderson (1968).
It was found that $N$-benzylpyridinium and its analogs interact with the
"pyridinium ring" region of the DPN$^+$ binding site. The binding properties
of the $N^1$-benzyl derivatives do not alter significantly by varying the sub-
stituent group on the pyridinium ring, unless charged groups are employed.

Studies on inhibition of LADH by fatty acids, fatty acid amides, and
imidazole, using fluorescence methods, have been able to show the formation
of ternary enzyme-coenzyme-inhibitor complexes. Fatty acids form binary
complexes with enzyme, and also form ternary complexes with enzyme-reduced
coenzyme but not with enzyme-oxidized coenzyme. They compete with alcohol,
but not aldehyde, for the same enzyme binding site. In contrast, fatty
acid amides form binary complexes with enzyme and ternary complexes with
coenzyme and oxidized coenzyme, but not with reduced coenzyme, and they do
not compete with alcohol but with aldehyde for the same enzyme binding site
(Theorell, 1959; Winer and Theorell, 1959; 1960; Theorell and McKinley-
McKee, 1961 b,c). The inhibitory effects of both fatty acids, and fatty
acid amides, increase with increasing carbon-chain length, indicating the
significance of lipophilic interactions (Theorell and Bonnichsen, 1951;
Imidazole forms a binary complex with an enzyme, and ternary complexes with both enzyme and reduced or oxidized coenzyme (Theorell and McKinley-McKee, 1961 b,c). Since imidazole is suggested to compete with ethanol and aldehyde to bind at, or near the zinc ion of enzyme, it has been postulated that in the free enzyme, the zinc is bound in an octahedral complex on which zinc is bound by three bonds to the protein (Vallee and Coombs, 1959; Theorell and Plane, 1961; Theorell and McKinley-McKee, 1961 b,c). In neutral or acid solution, the three bonds are usually occupied by water molecules, with one or more of them losing protons at alkaline pH. Kinetic studies show that these three free zinc bonds are not used at random by the coenzymes, substrates and inhibitors.

The presence of enzyme and inhibitors changes the characteristic ultra-violet absorption and fluorescence emission spectra of the reduced coenzyme. These changes provide a useful tool for determining the dissociation constants of binary and ternary complexes (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964). Fatty acids and fatty acid amides stabilize the binding between LADH and DPN⁺, likewise, coenzyme stabilizes the enzyme-inhibitor complexes. Imidazole can either increase or decrease the activity of LADH depending on the concentration of the coenzymes and the substrates. These effects have been explained in terms of the formation of ternary enzyme-coenzyme-imidazole complexes. (Theorell and McKinley-McKee, 1961c).

The formation of binary complexes, or ternary complexes, has been shown to induce changes in protein conformation. The specific Cotton effects have been employed to measure the binding of coenzyme (Ulmer et al., 1961; Li and Vallee, 1964; Rosenberg et al., 1965), and a variety of
Inhibitors, such as 1,10-phenanthroline, p-chloromercuribenzoate and buffer anions and cations (Li et al., 1962; 1963) to LADH and to discern the disymmetry interaction of NADH or inhibitors at the zinc-containing active sites of the enzyme. The crystals of LADH, LADH-NADH and LADH-NADH-inhibitors have been made by Yonetani (1965) and Yonetani and Theorell (1963).

Crystals of the free enzyme are long prisms, whilst the binary complex LADH-NADH, and the ternary complex LADH-NADH-isobutyramide are thick parallel pipeds. X-ray crystallographic data (Brändén, 1965; Brändén et al., 1965; Zeppesauer et al., 1967) have also shown a profound change in the crystal unit cell following complex formation. The free enzyme shows orthorhombic symmetry, whereas the binary complex, LADH-NADH, and the ternary complex, LADH-NADH-isobutyramide, give monoclinic symmetry, but LADH-NAD⁺-Pyrazole gives triclinic symmetry. These results further support the concept that the binding of coenzyme and certain inhibitors to the enzyme, induces the changes of protein conformation.

Recently, it was found that LADH is reversibly inhibited by thyroxine and related compounds (McCarthy et al., 1968). These inhibitors are uncompetitive with respect to the coenzyme. In addition to the inhibitory effect, they also quench the enhanced fluorescence of the bound NADH and greatly reduce the magnitude of the Cotton effect. The results have been explained in terms of thyroxine forming a ternary complex with enzyme and coenzyme which is catalytically inactive, and in which the nicotinamide moiety of the bound NADH shows no enhanced fluorescence. It is, therefore, likely that thyroxine interferes with the binding of the nicotinamide portion of the coenzyme to the enzyme.
1.7. THE MECHANISM OF ENZYME ACTION

(A) Kinetics and Reaction Mechanisms.

YADH and LADH catalyse the same reaction, i.e.,

\[ \text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ \xleftrightarrow{\text{ADH}} \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+ \]

The equilibrium constant, \( K_{eq} \), of this reaction at 20°C in the pH range 7-10 (ionic strength = 0.1) has been found to be (Backlin, 1958)

\[ K_{eq} = \frac{[\text{CH}_3\text{CHO}] [\text{NADH}] [\text{H}^+]}{[\text{CH}_3\text{CH}_2\text{OH}] [\text{NAD}^+]} = 8.01 \pm 0.14 \times 10^{-12} \text{M} \]

The reaction mechanism which was first proposed by Theorell and Chance (1951) is shown below:

\[
\begin{align*}
E + R & \xleftrightarrow{K_1} ER \\
ER + S & \xleftrightarrow{K_3} E' + S' \\
E' + O & \xleftrightarrow{K_2} E + O \\
\end{align*}
\]

where \( E \) represents the enzyme, \( R \), the reduced coenzyme, \( O \), the oxidized coenzyme, \( S' \), the alcohol, and \( S \), the aldehyde. The application of steady-state kinetics treatment of equation (1) to (3), has enabled the forward (4) and reverse (5) initial-rate equations to be obtained, i.e.,

\[
\frac{e}{v} = \frac{1}{K_2} + \frac{1}{K_1[R]} + \frac{1}{K_3[S']} + \frac{K_2}{K_1 K_3[R][S']} \tag{4}
\]
where \( e \) denotes the total concentration of enzyme and \( v \) the reaction rates.

The equations have been simplified by Dalziel (1957) as shown in equation (6):

\[
\frac{e}{v} = \frac{\phi_0}{[S]} + \frac{\phi_1}{[S']} + \frac{\phi_2}{[S][S']}
\]

The reverse reaction obeys the same type of equations but with different constants designed by primes. The following three relationships have been derived by Dalziel (1957):

\[
\phi_0' = \frac{\phi_1' \phi_2'}{\phi_{12}'}
\]

\[
\phi_0'' = \frac{\phi_1'' \phi_2''}{\phi_{12}''}
\]

\[
K_{eq} = \frac{\phi_{12}''}{\phi_{12}'} = \frac{\phi_0'' \phi_1'' \phi_2''}{\phi_0' \phi_1' \phi_2'}
\]

The various rate constants can be obtained by carrying out four types of experiments with submaximal concentrations of the constant reaction partner in each case as shown below:

<table>
<thead>
<tr>
<th>constant</th>
<th>varied</th>
</tr>
</thead>
<tbody>
<tr>
<td>([S])</td>
<td>([R])</td>
</tr>
<tr>
<td>([R])</td>
<td>([S])</td>
</tr>
<tr>
<td>([S'])</td>
<td>([0])</td>
</tr>
<tr>
<td>([0])</td>
<td>([S'])</td>
</tr>
</tbody>
</table>
For case 1, a graph of $\frac{2}{v} \frac{1}{y_A}$ [varied partner] gives a straight line. With several concentrations of constant partner, $[S]$, a family of straight lines, each with its own intercept and slope, are obtained.

A secondary graph of slopes $\frac{1}{y_A}$ [S] is again a straight line, and gives values of $\phi_1$ and $\phi_2$ from intercept and slope, respectively, whilst a graph of intercept $\frac{1}{y_A}$. $[S]$ gives a straight line with a slope of $\phi_2$ and an intercept of $\phi_0$.

Similarly, case 2 gives values of $\phi_0, \phi_1, \phi_2$ and $\phi_{12}$; case 3 gives $\phi_0, \phi_2, \phi_1$ and $\phi_{12}$; and case 4 gives $\phi_0, \phi_1, \phi_2$ and $\phi_{12}$.

Kinetic studies involving a system with two substrates have been described in detail by Dalziel, (1957); Bloomfield et al., (1962); Wong and Hanes, (1962, 1964); and Cleland, (1967, a,b). The necessary conditions to determine various rate constants and dissociation constants between the enzyme and various coenzymes, substrates or inhibitors have been reviewed by Sund and Theorell, (1963), McKinley-McKee, (1964) and Sund, (1968).

Initial rate-data satisfy the requirements of the Theorell-Chance mechanism for all the primary alcohols and aldehydes (McKinley-McKee, 1961 a,b; Dalziel, 1963, Dalziel and Dickinson, 1966a). But the validity of this mechanism depends on the accuracy of the measurements and the purity of the reagents (Dalziel, 1962; 1963). The presence of a small amount of alcohol as an impurity in LADH (Taniguchi, 1967), or the use of commercial enzyme, which can catalyse a definite rate of an ethanol-acetaldehyde exchange reaction even without adding NAD$^+$ or NADH (Wong and Williams, 1968), can cause large unexpected kinetic effects. High concentrations of primary alcohols, inhibit the enzyme due to the formation of an abortive complex of
enzyme, NADH and alcohol, from which NADH dissociates more slowly than from enzyme-NADH complex (Theorell et al., 1955; Theorell and McKinley-McKee, 1961a; Dalziel and Dickinson, 1966a; Shore and Theorell, 1966b). The initial rate-data for cyclohexanol at low concentrations is also consistent with the Theorell-Chance mechanism (Dalziel and Dickinson, 1966b). While with high concentrations of NAD⁺, substrate activation is observed with increasing concentrations of cyclohexanol, attributed to the formation of an abortive complex, E-NADH-ROH, from which NADH dissociates more rapidly than from the normal product complex E-NADH. In the presence of low NAD⁺, substrate inhibition is also observed, and this is attributed to the formation of active complex E-ROH with which NAD⁺ reacts more slowly than with the enzyme. For secondary alcohols, the Theorell-Chance mechanism is not valid because the ternary complex reacts more slowly; its steady-state concentration is greater, and therefore dissociation of coenzyme from it is rate-limiting with non-saturating coenzyme concentrations.

No doubt, the Theorell-Chance mechanism provides an accurate description of the steady-state kinetics, but it does not include the existence of the binary complexes, E-alcohol and E-aldehyde, or of the ternary complexes, E-R-aldehyde and E-O-alcohol, which dissociate and interconvert rapidly and have no influence on the maximal rate. The existence of ternary complexes has been suggested by Winer and Theorell, (1960) and Theorell and McKinley-McKee, (1961a,b), but are difficult to discern with ordinary steady-state kinetics due to their rapid rates of interconversion and dissociation. Direct evidence for the formation of these ternary complexes has been inferred from the production of enzyme-coenzyme-inhibitors using substrate or substrate competitive inhibitors (Winer and Schwert, 1958; Theorell and
In 1958, Albertsy suggested that in the presence of added product, the maximum rate of an enzymatic reaction such as that of LADH would be decreased if ternary complexes existed, but would be unaffected if only binary complexes existed. Wretan and Cleland, (1965, 1965) have provided kinetic evidence for the existence of ternary complexes using product and alternative product inhibition. On this theoretical basis, it has recently been shown that dissociation constants of E-NADH-aldehyde and E-NADH-alcohol can be obtained from the change in maximum rate with the presence of added product (Shore and Theorell, 1966a). Consequently, it now seems well established that enzyme-coenzyme-substrate complexes do exist. The question still remains—and there is no clear agreement about this—as to whether enzyme forms all four binary complexes with NAD\textsuperscript{+}, NADH, Alcohol and Aldehyde. Dalziel, (1963) and Wretan and Cleland, (1963), have shown that the dissociation of oxidized and reduced coenzyme is rate-limiting in LADH catalyzed reaction with excess reactants. Sund and Theorell, (1963) suggested that LADH may bind with NAD\textsuperscript{+}, NADH, alcohol or aldehyde to form binary complexes. However, Boyer and Silverstein, (1963) suggested that the mechanism is best described as kinetically significant but not strictly compulsory order. From detailed studies with pure NAD\textsuperscript{+} and NADH, Dalziel (1963) concluded that there is no definite evidence for kinetically significant binary complexes between LADH and alcohol and aldehyde. On the basis of fluorimetric evaluation, McKinley-McKee, (1963) concluded that alcohol and aldehyde are unable to interact with LADH, without prior binding of the coenzyme. However, measurement of equilibrium reaction rates by means of isotopic techniques (Silverstein and Boyer, 1964) and the direct observation of binary complexes of aldehydes and
alcohols with the enzyme by means of the enzyme-2,2-bipyridine spectrum (Sigman, 1967), are not in agreement with this interpretation. These authors concluded that there is no strictly compulsory order of substrate binding, and thus that all four binary complexes exist. Also, NAD⁺ and NADH dissociation from ternary complexes of enzyme-coenzyme-substrate is slow in contrast to alcohol, or aldehyde, dissociation, whilst ternary complex interconversion is rapid and non-rate-limiting.

For YADH, though product inhibition studies are not in accord with a rate-limiting interconversion of ternary complexes with random dissociation of substrates and coenzymes (Wratten and Cleland, 1963), most kinetic and related studies favour such a mechanism (Alberty, 1953; Hayes and Velick, 1954; Nygaard and Theorell, 1955; Van Eys et al., 1957; Mahler and Douglas, 1957; Thomson, 1963; Silverstein and Boyer, 1964).

(B) Interaction of Enzyme and Coenzyme.

It is well-known that YADH and LADH form a binary complex with the coenzyme. The maximum ultra-violet absorption spectrum of NADH is shifted from 340 mμ to 325 mμ upon the combination with LADH (Theorell and Bonnichsen, 1951), but not YADH (van Eys et al., 1958), and also the fluorescence intensity of NADH increases manyfold in the presence of LADH (Boyer and Theorell, 1955); or YADH (Duysens and Kronenberg, 1957). These properties provide a convenient and accurate method for measuring the various dissociation constants (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964). The effects of fluorescence quenching, fluorescence-emission shifts and fluorescence polarization have been described in detail by Velick (1961).

LADH binds two moles of NAD⁺ per enzyme molecule (Theorell and Bonnichsen, 1951; Theorell and Winer, 1959) and this does not change with pH (McKinley-
McKee, 1962; McKinley-McKee and Donninger, 1962). Each binding site acts independently of the other. NADH is directly competitive for the binding sites of NAD\(^+\), and appears to contain the equivalent number of binding site. But NADH is bound more tightly to the enzyme than NAD\(^+\) (Theorell and Winer, 1959; Theorell and McKinley-McKee, 1961a). While YADH has been shown to bind four moles of NADH per enzyme molecule (Hays and Velick, 1954; van Eys et al., 1957; Wallenfels and Sund, 1957), NAD\(^+\) and NADH bind the same sites to the enzyme, and all the binding sites are equivalent and independent.

A number of coenzyme analogs have been prepared by introducing different groups in place of the 3-carboxamide group of the nicotinamide moiety, or replacement of the adenine ring, or alternation in the pyrophosphate part. The various dissociation constants for enzyme-coenzyme and enzyme-coenzyme analogs have been determined (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964; Colowick et al., 1966; Sund, 1968). A slow rate of oxidation of alcohol by using NADP, NMNPR and NMN as coenzyme with LADH or with YADH is observed. With 3-acetyl-pyridine analogs of NAD\(^+\) and NADH as coenzyme, the activity of LADH is faster than with NAD\(^+\) (Kaplan et al., 1956). The binding of 3-acetyl-pyridine to LADH is accompanied by changes of ultraviolet absorption (Kaplan et al., 1957), and fluorescence emission spectra (Shifrin et al., 1959). It is also reported that the 3-acetyl-pyridine-NADH is less tightly bound to LADH than NADH (Shore and Theorell, 1967; Weiner, 1968), but there is no significant difference between the binding of 3-acetyl-pyridine-NAD\(^+\) and NAD\(^+\) to the enzyme. This shows that the amide group of the pyridine ring of oxidized coenzyme is not bound to the enzyme, perhaps due to the repulsion of the positively charged ring. While the amide group on the pyridine ring of NADH is apparently essential to bind, and might also
explain the much tighter binding of NADH to enzyme as contrasted with NAD$^+$. When the ribose moiety of the coenzyme is substituted by deoxyribose, the resultant molecule can also serve as a coenzyme, but it shows a lower affinity for both enzymes than NAD$^+$. ADP, ATP and ADPR cannot act as coenzyme, but are strong competitive inhibitors of LADH with respect of coenzyme. (See reviews by Sund and Theorell, 1963; McKinley-McKee, 1964; Colowick et al., 1966; Sund, 1968.) Studies of the relative rate of reaction with various coenzyme analogs and inhibitors have shown that all parts of the coenzyme molecule—the carboxamide group, the adenine moiety, the ribose and the 2'-phosphoribose—play a role in the binding between the enzyme and coenzyme. The inhibition studies of LADH by various zinc complexing agents using kinetic, or optical rotatory dispersion measurements, which have been described earlier, suggested that zinc atoms in LADH and YADH play a central part in interacting between enzyme and co-enzyme. It has also been shown by ultraviolet absorption spectra that the carboxamide group of NAD$^+$ is not essential in binding the coenzyme to the enzymatic zinc ion, but the nicotinamide moiety of the coenzyme must be near the enzymatic zinc ion (Yonetani, 1963 a,b; Sigman, 1967).

Recently, the effects of YADH and LADH on the nuclear magnetic resonance spectra of oxidized and reduced nicotinamide dinucleotide have been investigated by Hollis (1967). It was found that the resonances of the adenine C-2 and C-8 protons of NAD$^+$ and NADH are broadened in the presence of YADH, but no effect on the nicotinamide proton resonances of NAD$^+$. For NADH, the C-4 proton peak, but not the C-2 of the nicotinamide moiety is either broadened or decreases in intensity or both when YADH is present. Whilst in the presence
of LADH the intensity of the nuclear magnetic resonances spectra of both the
nicotinamide and adenine moieties of NAD$^+$ and NADH are decreased. Thus, it
has been concluded that only the adenine moiety of NAD$^+$ and NADH is bound to
YADH. But LADH is bound with both the nicotinamide and adenine moieties.
These findings may also explain the shift of 340 m$\mu$ band of NADH to 325 m$\mu$
on binding to LADH, but is unaffected by YADH. However, this report of the
effect of YADH on the nuclear magnetic resonance spectrum of NAD$^+$ is not con-
sistent with the results of a previous study (Jardetzky et al., 1963).

(C) The Binding of Substrate to the Enzyme.

There appears to be no direct evidence to show that the substrate binds
to the free YADH and LADH molecule. The data obtained to date are too
sparse to draw a real conclusion. Kinetic studies give no clear agreement
as to whether the binary complexes - enzyme-alcohol and enzyme-aldehyde - exist.
(See p. 32.) Equilibrium-rate studies (Silverstein and Boyer, 1964; Yonetani
and Theorell, 1964) and inhibition studies with 2,2-bipyridine (Sigman, 1967)
show that YADH and LADH can form binary complexes with ethanol and aldehyde.
Hollis et al.(1966) and Hollis (1967) reported that the nuclear magnetic
resonance spectra of neither ethanol, nor acetaldehyde, are noticeably affected
by YADH or LADH. The addition of NAD$^+$ to the YADH-ethanol and to the LADH-
ethanol system causes the decrease in the intensity of the ethanol spectrum.
These results support a previous suggestion that the binding sites for sub-
strate only exist after the binding of coenzyme to the enzyme. (McKinley-
McKee, 1963.) The possibility of a compulsory reaction sequence in which
acetaldehyde or ethanol must bind to the enzyme first is unlikely. The
binding of coenzyme to the enzyme induces alteration of the protein conformation,
which then generates a binding site for substrate, and permits hydrogen transfer
from ethanol to NAD$^+$. 

It has been reported that ethanol and acetaldehyde are not competitive inhibitors with respect to orthophenantroline for LADH and they do not interfere with the characteristic enzyme-orthophenantroline spectrum (Hoch et al., 1958; Vallee and Coombs, 1959; Vallee, 1960; Mahler et al., 1962). Thus, it has been considered that ethanol and acetaldehyde are not bound to the enzymatic zinc ion. However, studies with other zinc-complexing agents, such as imidazole and 2,2-bipyridine, are not in accord with this suggestion (Theorell and McKinley-McKee, 1961b,c; Plane and Theorell, 1961; Dalziel, 1963a; Sigman, 1967). These studies showed that the enzymatic zinc ion is also an intrinsic part for the binding of substrate.

The existence of a hydrophobic region at the substrate binding site in the enzyme has also been suggested by Winer and Theorell (1960). This is further supported by recent findings (Shore and Theorell, 1966; Sigman, 1967) that the dissociation constants of the enzyme with aliphatic acids, alcohols, amides, aldehydes, dimethylamides or mercaptans decrease with increasing chain-length.

(D) Structure of the Active Site

Current interest in protein structure and its relation to the catalytic process has focussed attention on the active site. Studies have been made on (a) the formation of binary complexes or ternary complexes with enzyme, coenzyme, substrate and various inhibitors by kinetics; (b) the stereochemistry of these complexes using labelled coenzyme, substrates, and various inhibitors; (c) the relative velocity of reaction between the coenzymes and the synthesis of coenzyme model compounds or coenzyme analogs; and (d) the
tertiary structure of the protein and its complexes by high resolution crystallography. All these investigations have drawn us a step nearer to the final goal of determining the entire structure of the active site of both of these enzymes.

On the basis of inhibition studies, a suggestion for the mode of action of alcohol dehydrogenase was first proposed by Wallenfels and Sund (1957). As shown in Fig. 1.5, the sulfhydryl group of the enzyme and the enzymatic zinc ion are considered to play a central role in binding with both coenzyme and substrate. It was suggested that the substrate was bound to the enzyme with two bonds; one as a zinc-oxygen bond and the other as a link between the alkyl group and the enzyme surface. The enzymatic zinc ion is assumed to have a co-ordination-number of six. Three of the co-ordination valencies are to the enzyme and at least one of these is formed as a zinc-sulphur bond. The coenzyme is fixed to the enzyme through the adenine part and pyridinium-nitrogen. The carboxamide group of the nicotinamide moiety is bound to the enzyme surface. The hydrogen at C-1 of ethanol is transferred as hydride ion to the pyridinium ring at C-4 (see reviews by Vennesland, and Westheimer, 1954; Wallenfels, 1959; Westheimer, 1959; Levy, et al., 1962). The suggestion that there is interaction between the adenine part of the coenzyme and the enzymatic zinc ion has been further supported by optical rotatory dispersion studies (Li, et al., 1963; Li and Vallee, 1964a). These authors have found that AMP-, ADP- and ADPR-moiety of the coenzyme all reversibly reduce the magnitude of the cotton effect of the enzyme-NADH complex. Moreover, AMP markedly alters the Cotton effect of LADH-1,10-phenanthroline complex, suggesting that the adenine portion of the ribonucleotide moiety is responsible, in large measure, for interacting at the
vicinity of, or at, the zinc-containing active center of the enzyme. Similar conclusions have also been reached from observations of the effect of coenzyme moieties in preventing zinc exchange (Druyan and Vallee, 1964). However, this suggested reaction mechanism is disputed by Yonetani and Theorell (1963; 1964) who found that ADPR can still be bound to LADH even when the enzymatic zinc atom is blocked by 1,10-phenanthroline. Also AMP, ADP and ADPR do not interfere with the enzyme-2,2-bipyridine spectrum (Sigman, 1967). Thus it is now thought that the zinc atom in LADH is unlikely bound to the adenine portion of the coenzyme. A modified mechanism of action of LADH has been proposed by Theorell and Yonetani (1963). As shown in Fig. 1.6, the hydrogen is assumed to be transferred very rapidly from the enzyme-coenzyme substrate complex (Theorell and McKinley-McKee, 1961a). Under the influence of Zn^{2+}, the hydroxyl-H is expelled to form an alcololate ion, from which the hydrogen at C-1 is transferred, as hydride ion, to the pyridine ring at C-4. The evidence in support of a hydrophobic region at the active site of the enzyme is the discovery of complex formation between long-chain fatty acid amides, NADH and enzyme as well as long-chain fatty acids, NAD^+ and enzyme (Winer and Theorell, 1960; Shore and Theorell, 1966a), and kinetic studies with various substrates (Sigman, 1967).

Based on kinetic studies of the interaction between NAD^+ and LADH (Theorell and McKinley-McKee, 1961a; Taniguchi et al., 1967), Taniguchi proposed a schematic representation of repulsion effects in the operation between the enzymatic Zn^{42} ion and NAD^+ (Fig. 1.7). LADH is known to have two independent and equivalent binding sites for NAD^+ per molecule of enzyme throughout the pH range 6 - 10 (Theorell and Bonnichsen, 1951; McKinley-McKee and Donninger, 1962). OH is considered as an unidentate ligand, which
Various Models Proposed for the Mechanism of Action of Alcohol Dehydrogenases

Fig. 1.5. From Wallenfels and Sund (1957).

Fig. 1.6. From Theorell and Yonetani (1963).

Fig. 1.7. From Taniguchi, et al. (1967).
Fig. 1.8. From (A) Theorell and McKinley-McKee (1961c); (B) Winer and Theorell (1960); and (C) Theorell and McKinley-McKee (1961b,c).

Fig. 1.9. From Theorell and McKinley-McKee (1961c).

Fig. 1.10. From van Eys, et al. (1958b).
Fig. 1.11. From Evans and Rabin (1968).

Fig. 1.12. From Kosower (1962a).
plays an essential role in effecting a coulombic interaction with a positively-charged pyridinium ring of NAD$^+$. The acidic (410 M) and alkaline (5.2 M) asymptotes are found closely to the pH-insensitive values of $K_{EI,0} = 440$ M and $K_{EFa,0} = 5\mu$M, where I is imidazole and Fa is fatty acid such as caprate. This supports a previous assumption (Theorell and McKinley-McKee, 1961b,c) that LADH-imidazole-NAD$^+$ and LADH-fatty acid-NAD$^+$ complexes are analogous to the acid and alkaline extremums of the LADH-NAD$^+$ complex. These analogies are explained by assuming that the Zn$^{2+}$-bound water is replaced by imidazole, and OH is replaced by fatty acid (Fig. 1.8). However, the question remains to be solved as to whether Zinc has a coordination number of 4 or 6 (Theorell and McKinley-McKee, 1961c; McKinley-McKee, 1964; Theorell, 1967). If a co-ordination of six is assumed, Fig. 1.9 depicts the formation of binary and ternary complexes with tridentate LADH, bidentate adenine or monodentate water, substrate ligand or inhibitor (Theorell and McKinley-McKee, 1961c).

Another general theory of the reaction mechanism of alcohol dehydrogenase has been proposed by van Eys et al., (1958b). This theory assumed that each active site contains two or more coenzyme molecules (Fig. 1.10). In native enzyme, the essential sulphydryl group are linked through the enzymatic zinc atom. The addition of coenzyme results in the breaking-down of the zinc-sulphur bonds which make the Zn$^{2+}$ ion available for the attackment with pyrophosphate group of the coenzyme. The substrate binds through the C-4 position of the NAD$^+$ molecule, with the release of a hydride ion which is accepted by the second molecule of NAD$^+$, thereby being converted to the reduced form. The transfer of a hydride ion is considered to be a single electron shift from the nitrogen of one NAD$^+$ molecule to the nitrogen of the
second molecule. When the product is released, the first NAD$^+$ molecule is regenerated. However, this suggestion is not inconsistent with the studies of the coupling between NAD$^+$ and the enzyme, as it shows that the coenzyme acts independently from each other in LADH (Theorell and Winer, 1959) and also YADH (Wallenfels and Sund, 1957). Furthermore, the attachment of zinc atom to coenzyme pyrophosphate is unlikely as the activity of the enzyme is not affected by pyrophosphate (Wallenfels and Sund, 1957; Wallenfels et al., 1957; Plane and Theorell, 1961). In addition, zinc is found to be tightly bound to the enzyme (Plane and Theorell, 1961). From the study of the stability of the chelation between zinc ion and the sulfur bonds (Agren and Schwarzenback, 1955) it seems unlikely that the zinc-sulfur bonds can break apart for the coenzyme attaching, through zinc-pyrophosphate bonds, and the linkage of the pyridinium nitrogen and the adenine amino with sulphydryl groups. Thus, the theory shown in Fig. 1.10 has been modified by both Fawcett and Kaplan (1962) and Burton and Kaplan (1963), who have concluded that adenine is not bound through a sulphydryl group but at N-1 or N-3 positions, or double bond $\pi$ electrons, either directly or through metal ligands: The substrate does not attach itself, but forms as a charge-transfer complex.

On the basis of the inactivation of both YADH and LADH by iodoacetamide, followed by the finding that at each active site a thiol reactive group is essential for the enzyme action (Li and Vallee, 1963; 1964b; 1965; Harris, 1964; Whitehead et al., 1964), a tentative mode of action of alcohol dehydrogenases (Fig. 1.11), which is a modification and extension of a previous study (Rabin and Whitehead, 1962) has been proposed by Evans and Rabin (1968). A binary complex is first formed by the interaction of the enzyme with ADPR-
moiety of \( \text{NAD}^+ \) (Shore and Theorell, 1967), followed by the protonation of the adenine ring (Fisher et al., 1967). The substrate is linked to the enzyme with two bonds, one as alkoxide-zinc bond (Wallenfels and Sund, 1957; Theorell and McKinley-McKee, 1961c; and Taniguchi, et al., 1967) and the other as a link through the hydrophobic environment in the enzyme (Winer and Theorell, 1960). The reactive sulphydryl group functions catalytically by forming a covalent adduct at the C-2 of the pyridine ring which gives an activated \( \text{E-NAD}^+ \)-alcohol complex. The hydrogen is transferred as a modified Meerwein-Ponndorf reaction process, with zinc participating in a cyclic flow of electron pairs to produce an activated \( \text{E-NADH-aldehyde} \) complex. Release of the activating sulphydryl group is followed by the sequential dissociation of the products and the reaction is then completed.

However, there are the arguments against whether the excited fluorescence would remain if there was a thiol adduct at C-2 of the nicotinamide ring breaking up the chromophore conjugation (McKinley-McKee, 1964). Also, kinetic studies with coenzyme and coenzyme analogs (Li and Vallee, 1964a; Sigman, 1967) do not agree with the suggestion that the carboxamide group of the coenzyme is attached to the enzymatic-zinc. If zinc-amide bonds do exist, the formation of \( \text{NADH-ADH} \) complex should exhibit a shift of maximum to longer wavelength, since the zinc ion would favour the charge distribution of the excited state (Kosower, 1962 a,b). But the observed absorption band is shifted to a shorter one (Theorell and Bonnichsen, 1951). The evidence obtained from the investigations of the enzyme and zinc-complexing agents (Vallee, 1960), the competition between zinc and cadmium (Witter, 1960), reveal that zinc is more likely to participate in the interaction between the
enzyme and the coenzyme rather than in that involving the oxidation-reduction process. Thus, the zinc ion is unlikely to be associated with the stereospecificity of the acetaldehyde reduction (Ulmer and Vallee, 1961; Kosower, 1962a,b). Kosower (1962a,b) proposed that an ε-amino group of the lysine residue is a key component of the active site of alcohol dehydrogenase (Fig. 1.12). A positively charged nitrogen in the form of an ammonium ion is located 3 Å from the nitrogen of the dihydropyridine ring and produces the shift by repulsion of the increased charge produced on the dihydropyridine nitrogen by light absorption. One hydrogen bond is combined to the oxygen in the ribose ring to which the dihydronicotinamide is attached. Another hydrogen bond is designed to be linked to the oxygen of an acetaldehyde molecule, thereby placing the carbonyl group above the C-4 position of the dihydropyridine ring, permitting the direct hydrogen transfer from NADH to the aldehyde (Vennesland and Westheimer, 1954).

Since the classical demonstration that the transfer of the enzymatic hydrogen, catalysed by YADH, the enzyme becomes labeled with tritium when it is incubated with tritiated ethanol and the coenzyme (see reviews by Vennesland and Westheimer, 1954; Levy et al., 1962). Recently, it has further shown that the tritium of the enzyme is located in the methylene group of a tryptophane residue (Schellenberg, 1965, 1966, 1967; Schellenberg and McLean, 1966; Chan and Schellenberg, 1967). The detailed working on the chemistry of indolenine (3H-indolylidene) cations has led to the hypothesis that a specific tryptophane residue of the enzyme also plays a part in the active site of the enzyme by participating in the reaction by means of a reversible dehydrogenation to a cationic indolenine.

In spite of the great effort which has been expended in elucidating
detailed physical and chemical properties of these enzymes in the last two decades, no truly successful model for the active sites of these enzymes has been postulated. The full identification of the basic structural and compositional unit in the coenzyme which governs its specific reactivity toward enzymes are important. In ascertaining the nature of the entire active sites of the enzyme, both the chemistry of the reactive groups in the enzyme for the interaction with coenzyme and substrate and their arrangement in three dimensional space are also critical.
CHAPTER 2

STUDIES ON THERMAL STABILITY OF LDH AND THE EFFECTS OF MODIFIERS
2. INTRODUCTION

LADH has an unusual substrate specificity (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964). It catalyses the reversible oxidation of a variety of primary and secondary alcohols to the corresponding aldehydes in the presence of NAD⁺. Winer (1958) and Merrit and Tomkins (1959) found that cyclic alcohols are also good substrates for this enzyme. On the other hand, aliphatic secondary alcohols and ketones are poor substrates (Merrit and Tomkins, 1959; Dalziel and Dickinson, 1966). Witter (1960) observed activity at high concentrations of propan-2-ol. Van Eys (1961) also showed that LADH can serve as an isomerase in the conversion of lactaldehyde to acetol, and of glyceraldehyde-3-phosphate to dehydroxyacetone phosphate with the equilibrium in favour of ketose formation. Recently, it has been further demonstrated that LADH can oxidize certain bile acids and the primary alcohol group of several sterols (Ungar et al., 1965; Waller et al., 1965; Graves et al., 1965). The mechanism of enzyme reaction has been the subject of most extensive study (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964). On the basis of detailed kinetic studies using several alcohols, aldehydes and ketones as substrates, a unified mechanism has been proposed for the reversible oxidation of primary, secondary and cyclic alcohols, and the dismutation of aldehydes catalysed by LADH (Dalziel, 1963b; Dalziel and Dickinson, 1966a,b). To a certain degree, this mechanism is consistent with the Theorell-Chance mechanism (Theorell and Chance, 1951), except with high concentrations of substrates or secondary alcohols, attributed essentially to the fact that the rate of intramolecular oxidation-reduction within the ternary complex of alcohol, NAD⁺ and enzyme is faster with primary alcohols than with secondary alcohols.

Several lines of evidence for the formation of ternary enzyme-coenzyme-
substrate complex have been obtained by inhibition studies, using substrate competitive inhibitors (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961), or using product inhibition (Wratten and Cleland, 1963), and alternate product inhibition (Wratten and Cleland, 1965). Direct evidence for the existence of ternary complexes has also been provided by using spectrophotometric methods (Theorell and Yonetani, 1962) and kinetic studies (Shore and Theorell, 1966a; Dickinson and Dalziel, 1967b). Consequently, it now seems that this ternary complex does exist, but it is difficult to discern with ordinary steady state kinetics, due to the rapid rates of interconversion and dissociation.

However, it is well-established that the enzyme forms binary complexes with reduced and oxidized coenzyme (Theorell and Bonnichsen, 1951). But the suggestion that the enzyme can also form binary complexes with alcohol and aldehyde is a matter of controversy. On the basis of fluorimetric evaluation, McKinley-McKee (1963) concluded that the alcohol and aldehyde are unable to interact with the enzyme, unless the enzyme is in a binary complex form with the coenzyme. Dalziel (1963b) also found no definite evidence for kinetically-significant binary complexes with alcohol and aldehyde. In contrast, Sund and Theorell (1963) suggested that binary complexes between the enzyme and alcohol or aldehyde may form, but that these are kinetically insignificant because of their rapid interconversion and dissociation. From equilibrium rates studies by means of isotopic techniques, Silverstein and Boyer (1964) concluded that all four binary complexes between the enzyme and alcohol, aldehyde, reduced and oxidized coenzyme are in existence. A similar conclusion has also been reached by Yonetani and Theorell (1964), Shore and Theorell (1966b), and Sigman (1967).
Investigations of the thermal stability of LADH and the effect of modifiers might provide an convenient method to measure the enzyme complexes. The stability of the enzyme in the presence of other substances may cause effects which reflect the binding. This, in turn, might permit delineating further the nature of active enzymatic sites that exist.

Materials

Crystalline LADH was purchased from C. F. Boehringer and Soehne (Mannheim, Germany). NAD+ and NADH were obtained from Sigma Chemical Co. (St. Louis, Missouri). Analar ethanol was used without further purification. Other reagents were commercial reagent grade. All solutions were made in quartz, double distilled water.

Methods

Fresh solutions of LADH were prepared by centrifuging the enzyme suspension (≈ 10 mg/ml) at 15,000 r.p.m. and at -14 °C for 15 minutes. The supernatant was discarded and the crystals were dissolved in the same volume of 0.1 M glycine-NaOH buffer, pH 10.0. The solution was then centrifuged at 15,000 r.p.m. at 2 °C for 15 minutes before being introduced into dialysis tubing which was pretreated with distilled water for 24 hours at room temperature, and being dialysed against four changes of two litres 0.1 M phosphate buffer, pH 7.3 at 4 °C over a period of three days. The buffer was changed each 12 or 24 hours. Finally, the enzyme solution was centrifuged at 15,000 r.p.m. and 2 °C. The stock solutions were kept at 2 °C and examined after not longer than one week's storage at this temperature. During this storage, no change of enzymatic activity was observed.

The concentration of LADH was determined spectrophotometrically with the use of a Hilger Uvispec spectrophotometer. A temperature of 23.5 °C was
maintained by water circulated from an ultrathermostat to the cuvette compartment. Measurements of LADH concentration were either based on an absorbency index of 0.455 mg$^{-1}$ cm$^2$ (Taniguchi et al., 1967) or according to the method of Dalziel (1957), as modified by McKinley-McKee (1962). 10-50 µl of the diluted enzyme solution (1-2.0 µM) were withdrawn with a Cargille micropipette and introduced into a 3.0 ml standard assay mixture in a silica 1.0 cm cuvette by means of a glass-spatula. The 3.0 ml standard assay mixtures were made of 0.062 M glycine-NaOH buffer, pH 10.0, 0.15 ml ethanol (1.0%) and 1 ml NAD$^+$ (mg/ml). The rate of NADH formation followed at 340 m$m$ until the optical density of 0.2 units was attained. On the basis of a molecular weight of 84,000 (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964) the activity LADH concentration, $E_a$ in moles/litre is calculated as:

$$E_a = 1.13/(V)(t_{0.2})(84,000)$$

where 1.13 depends on the number of mg of LADH protein per ml., $V$ is the volume of the enzyme solution pipetted into the standard assay solution, and $t_{0.2}$ is time in seconds required to attain the increased absorbancy of 0.2 units at 340 m$m$. The ratio between the extinction coefficient of LADH at 280 m$m$ and 260 m$m$ was found to be 1.31 - 1.36.

NAD$^+$ was dissolved in double distilled water and the concentration was assayed spectrophotometrically, based on an absorbancy index at 260 m$m$ of 18.0 mM$^{-1}$ cm$^{-1}$ (Kornberg and Fricer, 1953; Siegal et al., 1959). The purity of NAD$^+$ was about 90-91% by weight and 99-100% from the absorbancy at 260 m$m$. NADH is unstable in water or below pH 7.5. Thus, its stock solution was made in 0.01 M glycine-NaOH buffer, pH 10.0, and kept at 2°C for a period of not longer than 40 hours before use. The concentration was
determined at 340 mμ, on the basis of a specific absorbancy index of 6.22 mM⁻¹ cm⁻¹ (Rafter and Colowick, 1957). The purity of NADH was about 70-71% by weight and 95% by absorbancy at 340 mμ.

The activity of the enzyme was assayed under the standard assay conditions each day before any experiment was done. The above procedures for determining the concentration of LADH, NAD⁺ and NADH were used throughout this thesis, unless otherwise stated.

Thermal studies of LADH were carried out by heating a total volume of 1.02 - 2.04 ml of the enzyme alone (1.0 - 2.0 μM), or in the presence of various effectors at various temperatures with the use of a water-bath of which the temperature was maintained by an ultrathermostat. Aliquots (10 - 50 μl) were withdrawn and introduced into assay mixtures and its residual activity was measured at 23.5°C. All experiments were conducted with a stoppered glass vessel, unless specifically stated. The conditions employed in individual experiments are described in detail either in the text, or as legends of tables or figures.

RESULTS

Effect of Different Containers on LADH Activity during Storage

The effect of the nature of the container surface on the stability of LADH was examined. An identical concentration of 2.0 μM LADH in 0.1 μ phosphate buffer, pH 7.3 was kept in containers made of different materials, i.e. glass, cellulose nitrate, polyethylene and tetrafluoroethylene. The enzymatic activity of these solutions was first determined, and then they were left standing for various lengths of time at room temperature. As shown in Fig. 2.1, the different containers have an effect on the enzymatic activity. The enzyme seems best to be kept in a cellulose nitrate container, from which
Fig. 2.1. Effect of container surface on the stability of LADH. An identical concentration of 2.0 µM LADH in 0.1 M sodium phosphate buffer, pH 7.3, was kept at room temperature in vessels made of: (○) cellulose nitrate, (△) glass, (×) tetrafluoroethylene, (◇) polyethylene. Aliquots of each sample were withdrawn and assayed for the residual activity.
the loss of enzymatic activity is minimized. Whilst, maximum loss of enzymatic activity was found when the enzyme was kept in polyethylene.

Similar experiments were carried out by heating the enzyme at various temperatures with the use of different materials of containers. Residual activity was assayed by pipetting 50 μl of aliquots to assay mixtures as previously described at various time intervals for at least up to one hour. A graph of the initial rate of the inactivation process against temperature is shown in Fig. 2.2. Again, there was more extensive loss of LADH activity when the enzyme was incubated in polyethylene container. Minimum loss of enzymatic activity was with the cellulose container. The rate of inactivation of LADH in various containers used is in the order of polyethylene > tetrafluoroethylene > glass > cellulose nitrate.

This result may suggest that under conditions typical of some proteins purification procedures, there is evidence that adsorption of certain amount of protein onto the dialysis membrane or the container surface during storage of the protein. Thus, purification methods which are carried out in glass vessels, or which involve dialysis, and storage of the protein in container could, if not carefully controlled, danger or destroy biological potency inadvertently.

Effect of Dilution on LADH Activity

The effect of LADH concentration on its catalytic activity was tested. A concentration of 3.0 μM LADH was incubated in 0.1 M phosphate buffer, pH 7.3 at 25°C for 20 minutes. Then, 20 μl of aliquots were withdrawn and introduced into assay mixtures for measuring its activity at 25°C. Successive dilution was made by adding 0.1 M phosphate buffer, pH 7.3, to the enzyme solution. Under the same conditions, the enzymatic activity was assayed again. Fig. 2.3 shows that over the range of concentration of enzyme tested
Fig. 2.2. A graph of the initial rate of thermal inactivation of LADH in different materials of container against temperature. 1.7 μM LADH in 1.02 ml 0.1 M phosphate buffer, pH 7.3, were kept in vessels made of: (○) polystyrene, (✓) tetrafluoroethylene, (△) glass, (◦) cellulose nitrate, and incubated at various temperatures.
Fig. 2.3. Effect of dilution on LADH activity. 3.0 μM LADH in 0.1 μ phosphate buffer, pH 7.3, at 23°C in a glass vessel was first assayed and successive dilution was made with 0.1 μ phosphate buffer, pH 7.3.
(3.0 \mu M to 0.09 \mu M), the activity is almost independent on the enzyme concentration. This result indicates the nature of the stability of LADH. With a concentration as low as 0.09 \mu M, there is neither dissociation of the enzyme nor an effect on its catalytic activity.

Effect of Substrate Concentration on Assay LADH Activity

The reaction was initiated by introducing a final concentration of 2.8 \times 10^{-8} M LADH to a 3.0 ml assay mixture containing 0.062 M glycine-NaOH, pH 10.0, 0.42 mM NAD\(^+\) and various concentrations of ethanol. Rates of NADH formation were followed at 340 \text{ m\mu} till the absorbancy of 0.2 units was recorded. The result of the substrate effect on assaying LADH activity is shown in Fig. 2.4. The data clearly show that the optimum activity is achieved when about 21 mM ethanol is present in assay mixtures. Ethanol in concentrations below or above this amount, results in low catalytic activity. From fluorimetric measurements, it has also been shown that the presence of an ethanol concentration higher than 10 mM in assay mixtures, results in low activity (Theorell et al. 1955; Theorell and McKinley-McKee, 1961). This effect has been explained as being due to substrate inhibition. The value obtained here is nearly two times higher than that of previous reports. It is not clear whether this result is attributed to pH and coenzyme concentration effect for these earlier workers assayed enzymatic activity by using 242 \mu M NAD\(^+\) and pH 7.0. A higher value for ethanol inhibition has also been found by Shore and Theorell (1966b) with the use of 672 \mu M NAD\(^+\) in assay mixtures at pH 7.0.

Effect of pH on the Thermal Inactivation of LADH

LADH is known to be one of the most exceptionally stable enzymes. Exposure of this enzyme to a pH range of 6.7 - 10.0 for several hours at 20 -23^\circ C,
Fig. 2.4. Effect of the substrate concentration on LADH activity. 50 µl of 1.7 µM LADH were added to a 3.0 ml assay solution at 23.5 °C containing 0.062 M glycine-NaOH, pH 10.0, 0.42 mM NAD⁺ and various concentrations of ethanol. $t_{0.2}$ is the time in seconds required to attain an absorbancy of 0.2 units at 340 nm.
results in no appreciable loss of catalytic activity. It is, therefore, of interest to examine whether to same extent pH stability is applied to thermal inactivation of LADH. The effect of pH on thermal inactivation of LADH was investigated in the range pH 6.1 - 10.0. As shown in Table 2.1, after incubation of 1.6 µM LADH in pH 6.1, 9.0 and 10.0 at 70°C for about 60 minutes, no activity remained. Whilst at pH 7.3 and 8.0, there was a loss of 79% and 87% initial activity, respectively. This result indicates that LADH has maximum stability against heat at neutral pH. Away from neutrality, the rate of heat inactivation is faster.

**Effect of Substrate and Coenzyme on the Inactivation of LADH by Heat**

Table 2.2 shows the effect of the substrate and coenzyme on the thermal inactivation of LADH. LADH is well-known to form a binary complex with coenzyme (Theorell and Bonnichsen, 1951). Thermal studies show that the presence of 140 µM NADH profoundly protects the enzyme against heat inactivation. In agreement with kinetic studies, the oxidized coenzyme is less tightly bound to the enzyme at neutral pH. The reverse result is true for the reduced coenzyme (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964) and thermal measurements also show that the reduced coenzyme more effectively protects the enzyme from heat inactivation than oxidized coenzyme at pH 7.3. Thus, incubation of 1.7 µM LADH alone at pH 7.3 and 70°C for 60 minutes gives 79% loss of the initial activity (Table 2.2). Under the same conditions, except in the presence of 140 µM of NAD⁺ or NADH, the activity lost is 51% and 26%, respectively. The protection of LADH against heat inactivation by NAD⁺ and NADH has also been reported by Yonetani and Theorell (1962).

LADH has been demonstrated to form a binary complex with substrate (Sund and Theorell, 1963; Silverstain and Boyer, 1964; Shore and Theorell, 1966b;
### Table 2.1

**Effect of pH on heat inactivation of LADH**

<table>
<thead>
<tr>
<th>pH</th>
<th>5 min.</th>
<th>15 min.</th>
<th>60 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>36</td>
<td>69</td>
<td>100</td>
</tr>
<tr>
<td>7.3</td>
<td>19</td>
<td>31</td>
<td>79</td>
</tr>
<tr>
<td>8.0</td>
<td>27</td>
<td>38</td>
<td>87</td>
</tr>
<tr>
<td>9.0</td>
<td>38</td>
<td>62</td>
<td>100</td>
</tr>
<tr>
<td>10.0</td>
<td>47</td>
<td>76</td>
<td>100</td>
</tr>
</tbody>
</table>

*6 μM LADH were incubated in different pH as indicated in the table at 70°C. Buffers of 6.1 - 8.0 were made of 0.1 M phosphate, whilst buffers of 9.0 - 10.0, 0.1 M glycine-NaOH were used. 50 μl aliquots were withdrawn and introduced to 3.0 ml assay mixtures for measuring the residual activity.*
Table 2.2

Thermal stability of LADH complexes with substrate and coenzymes

<table>
<thead>
<tr>
<th>Additions</th>
<th>Loss of Initial Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>19</td>
</tr>
<tr>
<td><strong>140μM NAD⁺</strong></td>
<td>17</td>
</tr>
<tr>
<td><strong>140μM NADH</strong></td>
<td>2</td>
</tr>
<tr>
<td><strong>0.2 M ethanol</strong></td>
<td>15</td>
</tr>
<tr>
<td><strong>0.9 M ethanol</strong></td>
<td>23</td>
</tr>
<tr>
<td><strong>140μM NADH + 0.2 M ethanol</strong></td>
<td>3</td>
</tr>
<tr>
<td><strong>140μM NADH + 0.9 M ethanol</strong></td>
<td>3</td>
</tr>
<tr>
<td><strong>140μM NADH + 1.8 M ethanol</strong></td>
<td>14</td>
</tr>
</tbody>
</table>

* A total volume of 2.04 ml containing 1.7 μM LADH, 0.1 μM phosphate buffer, pH 7.3 and substrate and coenzymes as indicated in the table was incubated at 70°C.
Sigman, 1967) and a ternary complex with coenzyme and substrate (Sund and Theorell, 1963; McKinley-McKee, 1964; Wratten, 1963, 1965; Shore and Theorell, 1966a; Dickinson and Dalziel, 1967b). From heat inactivation measurements, it was found that the inactivation of LADH by heating at 70°C and pH 7.3 in the presence of 0.2 M ethanol, only reduced the rate of inactivation a little (4–7%), whilst the presence of 0.9 M ethanol accelerates the inactivation of LADH by heat (Table 2.2).

Incubation of a total volume of 2.04 ml reaction mixtures containing 0.1 μl phosphate buffer, pH 7.3, 140 μM NADH, 1.7 μM LADH and 0.2 M - 0.9 M ethanol at 70°C for 60 minutes followed by enzymatic assays showed that the loss of initial activity is 19-21%. By contrast, when the heat inactivation of the LADH and NADH mixture was studied, the inactivation was 26%. The thermal inactivation of LADH either in the presence of NADH or a mixture of NADH and ethanol, shows no significant difference in thermal properties. However, when 140 μM NADH and high concentration (1.8 M) of ethanol are present in the enzyme mixture, the rate of thermal inactivation is faster than when only 140 μM NADH is used alone (Table 2.2).

Effect of Coenzyme Moieties on Thermal Inactivation of LADH

The resulting effect of various nucleotides on thermal inactivation of LADH is shown in Table 2.3. After treatment of 1.4 μM LADH in 0.1 μl phosphate buffer, pH 7.3 at 75°C for 20 minutes, no activity remained, and after about 30 minutes, a white precipitate was visible. Whilst after heating the enzyme in pH 7.3 at 70°C for about 40 minutes, the loss of initial activity is 56%. The presence of various nucleotides profoundly affects the thermal stability of LADH. When the inactivation was carried out by heating 1.4 μM LADH at pH 7.3 and in the presence of 5 x 10⁻² M ADP at 70°C for 15 minutes, assay
Table 2.3

Effect of various nucleotides on thermal inactivation of LADH

<table>
<thead>
<tr>
<th>Additions</th>
<th>Loss of Initial Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min.</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
</tr>
<tr>
<td>5 x 10^{-2} M AMP</td>
<td>24</td>
</tr>
<tr>
<td>5 x 10^{-2} M ADP</td>
<td>94</td>
</tr>
<tr>
<td>5 x 10^{-2} M ATP</td>
<td>40</td>
</tr>
<tr>
<td>1.5 x 10^{-2} M GDP</td>
<td>60</td>
</tr>
<tr>
<td>1.5 x 10^{-2} M GTP</td>
<td>35</td>
</tr>
<tr>
<td>1.5 x 10^{-2} M 2' 3'-AMP</td>
<td>17</td>
</tr>
<tr>
<td>1.5 x 10^{-2} M 3' 5'-AMP</td>
<td>15</td>
</tr>
</tbody>
</table>

*A total volume of 2.04 ml reaction mixtures containing 1.4 μM LADH, 0.1 μ phosphate buffer, pH 7.3 and various nucleotides as indicated in the table was incubated at 70°C.*
showed that the enzyme is completely inactivated. Under the same conditions, except in the presence of $5 \times 10^{-2}$ M ATP instead of ADP, the loss of initial activity is 62%, and in the corresponding concentration of AMP, it is 36%.

In order to delineate further the chemical detail of the individual moiety of coenzyme which may be critical for interaction with the enzyme, the effect of other nucleotides were studied in analogous manner. It was found that the effect of GDP and GTP on heat inactivation of LADH is similar to those of ADP and ATP. Thus, the inactivation rate of LADH by heat in the presence of GDP is faster than in GTP. Whilst $2'3'\text{AMP}$ and $3'5'\text{AMP}$ has no effect on heat inactivation of LADH (Table 2.3).

Summarizing the above results, it may be suggested that the pyrophosphate group of the coenzyme plays a role in coenzyme binding to the enzyme, and the adenine amino group is not essential for this purpose.

**Thermal Stability of LADH Complexes with Imidazole and the pH-effect**

The effect of imidazole on the thermal inactivation of LADH was investigated. The addition of 0.1 M imidazole to 1.3 uM LADH in 0.1 \text{M} phosphate buffer, pH 7.3 at 23°C was first studied. Assay showed that the enzymatic activity is increased by about 19 - 22%. Furthermore, when this solution is allowed to stand at room temperature over a period of 24 hours, there is no loss of the stimulating effect of LADH activity. Enzymatic activity stimulated by imidazole has also been reported by Theorell and McKinley-McKee (1961b, c).

Incubation of the corresponding solution at 70°C shows that the loss of enzymatic activity is faster than without the presence of imidazole. As shown in Table 2.4, after heating a mixture of 1.3 \text{uM} LADH and 0.1 M imidazole at pH 7.3 and 70°C for 40 minutes, no activity is retained. In the absence of imidazole, the loss of initial activity is 56%. The thermal inactivation
Table 2.4

Effect of pH on thermal stability of LADH complexes with imidazole and NADH

<table>
<thead>
<tr>
<th>Medium *</th>
<th>pH</th>
<th>Loss of Initial Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 min.</td>
</tr>
<tr>
<td>EI</td>
<td>6.1</td>
<td>59</td>
</tr>
<tr>
<td>EI</td>
<td>7.3</td>
<td>38</td>
</tr>
<tr>
<td>EI</td>
<td>9.0</td>
<td>27</td>
</tr>
<tr>
<td>EI</td>
<td>10.0</td>
<td>22</td>
</tr>
<tr>
<td>ERI</td>
<td>7.3</td>
<td>14</td>
</tr>
<tr>
<td>ERI</td>
<td>9.0</td>
<td>6</td>
</tr>
</tbody>
</table>

*2.0 ml reaction mixtures were composed of:

EI = 1.3 μM LADH + 0.1 M imidazole and

ERI = 1.3 μM LADH + 0.1 M imidazole + 140 μM NADH in different buffers. Buffers of pH 6.1 and 7.3 were made of 0.1 M phosphate, whilst pH 9.0 and 10.0, 0.1 M glycine - NaOH were used. The incubation temperature was 70°C.
of LADH by imidazole is also pH-dependent. The results are shown in Table 2.4. The data indicate that the rate of thermal inactivation of LADH by heat in the presence of 0.1 M imidazole decreases with increasing pH.

It has been demonstrated that imidazole forms a ternary complex with LADH and NADH (Theorell and McKinley-McKee, 1961). The formation of this ternary complex against thermal inactivation was tested. In this experiment a total volume of 2.04 ml reaction mixture containing 1.3 μM LADH, 14.0 μM NADH and 0.1 M imidazole at pH 7.3 was heated at 70°C. Aliquots were withdrawn and introduced into 3.0 ml assay mixtures as previously described for measuring the residual activity at 23°C. It was found that the ternary LADE-NADH-Imidazole complex is more stable against heat than the binary LADH-Imidazole complex, (Table 2.4), but is less stable than the binary LADH-NADH complex. Under the same conditions, except that the thermal inactivation was carried out at pH 9.0 instead of pH 7.3, the rate of heat inactivation was also reduced. It was found that the binary LADH-NADH is slightly less stable against heat at pH 9.0 than at pH 7.3.

Both imidazole and ADP have been demonstrated to inhibit LADH (Theorell and McKinley-McKee, 1961; Li and Vallee, 1964b; Sigma, 1967). Thermal studies show that in the presence of 0.05 M ADP in 1.5μM LADH at pH 7.3, the inactivation rate at 70°C is faster than that in the presence of 0.1 M imidazole (Fig. 2.5). A further experiment was carried out by using a mixture of 0.025 M ADP and 0.05 M imidazole in 1.5μM LADH. Fig. 2.5 shows that the inactivation curve lies in between imidazole and ADP, indicating that both ADP and imidazole bind to the enzyme independently.
Fig. 2.5. The action of various compounds on the thermal inactivation of LADH. A total volume 1.02 ml reaction mixture in a glass vessel containing 1.5 μM LADH, 0.1 μM phosphate buffer, pH 7.3 and (Θ) 0.1 M imidazole, (O) 0.05 M imidazole + 0.025 M ADP, (Δ) 0.05 M ADP was incubated at 70°C.
Effect of Neutral Salts on the Heat Inactivation of LADH

The effect of various neutral salts on the thermal inactivation of LADH is shown in Table 2.5. The presence of an identical concentration of 0.1 M sodium sulfate, ammonium sulfate, sodium citrate and sodium acetate in 1.3 μM LADH at pH 7.3 and 25°C, activates the catalytic activity by about 21-24%. In the presence of 0.1 M of sodium chloride or sodium nitrate, under the standard assay conditions, no significant change in catalytic activity was observed. The stimulation of LADH activity by certain salts has also been reported by Theorell et al. (1955).

By heating 1.3 μM LADH in pH 7.3 buffer at 70°C for 60 minutes, the loss of initial activity is 79%, but the presence of 0.1 M of sodium sulfate, ammonium sulfate, sodium acetate and sodium citrate strongly protects the enzyme from heat inactivation (Table 2.5). Whereas the corresponding concentration of sodium chloride and sodium nitrate fails to protect the enzyme, and makes it more unstable. Since the identical concentration and the same valence type of the neutral salts has a different effect on heat inactivation of LADH, it is difficult to establish a common feature of the mechanism of how these neutral salts affect the conformation of the enzyme molecules.

DISCUSSION

A variety of conditions can alter the stability of LADH. If, as is generally maintained, the loss of enzymatic activity on heating is a result of a secondary change in the enzyme conformation, then alterations in pH, and the presence of certain metabolites or inhibitors may cause effects by either stabilizing or weakening the native conformation of the enzyme molecules.

Normally, the purification procedure and the storage of the enzyme are carried out in glass or plastic vessels, which may damage the enzymatic
Table 2.5

Effect of neutral salts on thermal inactivation of LADH

<table>
<thead>
<tr>
<th>Salts (0.1 M)*</th>
<th>Loss of Initial Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min.</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>8</td>
</tr>
<tr>
<td>sodium sulfate</td>
<td>9</td>
</tr>
<tr>
<td>sodium acetate</td>
<td>11</td>
</tr>
<tr>
<td>sodium citrate</td>
<td>4</td>
</tr>
<tr>
<td>sodium chloride</td>
<td>22</td>
</tr>
<tr>
<td>sodium nitrate</td>
<td>26</td>
</tr>
</tbody>
</table>

*Inactivation was carried out by heating a final concentration of 1.3 μM LADH and 0.1 M various salts as indicated in the table in 2.04 ml 0.1 M phosphate buffer, pH 7.3 at 70°C.
activity. As shown in Fig. 2.1, when LADH was kept in containers of different materials over a period of three days, different degrees of loss of enzymatic activity resulted. This may be due to adsorption of protein onto the container surface as has been found in the purification of several enzymes (James and Augenstein, 1966). The potential hazards of adsorption of LADH is in the order of polyethylene > tetrafluoroethylene > glass > cellulose nitrate.

LADH is a stable enzyme. Dilution of the enzyme concentration from 3.0 μM to 0.09 μM does not affect the catalytic activity. On exposure of the enzyme to a pH-range 6.7 - 10.0 at 23°C over a period of 2-3 hours, there is no significant loss of enzymatic activity. However, on inactivation of LADH by heat, a maximum stability occurs at neutral pH; away from neutrality, acceleration of the rate of thermal inactivation results.

From kinetic studies, it has been shown that the formation of binary enzyme-substrate complex (Sund and Theorell, 1963; Silverstein and Boyer, 1964; Shore and Theorell, 1966b; Sigman, 1967), or the formation of ternary enzyme-coenzyme-substrate complex (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961; Wratten and Cleland, 1963, 1965; Shore and Theorell, 1966a; Dickinson and Dalziel, 1967b) is difficult to discern, due to their rapid interconversion and dissociation. However, when the rate of thermal inactivation, between the enzyme alone and a mixture of the enzyme and ethanol, at pH 7.3 is compared, there is no appreciable difference. There is also no conclusive evidence for the existence of ternary enzyme-coenzyme-substrate complex from thermal investigations.

LADH is inhibited by high concentrations (10-21mM) of ethanol. It is not clear whether the inhibitory effect is dependent upon pH and concentration
Several lines of evidence favor the formation of a ternary LADH-NADH-CH\(_3\)OH complex being responsible for the substrate inhibition (Theorell et al., 1955; Theorell and McKinley-McKee, 1961; Shore and Theorell, 1966a), because the tendency for LADH-NADH to break down is strong enough to prevent the formation of an abortive LADH-NADH-CH\(_3\)OH complex unless high concentrations of ethanol are present. No, or little, effect against thermal inactivation of LADH is afforded by low concentrations (0.2M) of ethanol, but inactivation occurs at high concentration (0.9M) of ethanol. This result may reflect occupation of other sites of the substrate to the enzyme. A second binding site for the substrate has also been postulated by Wratten and Cleland (1963); Grisolia (1964); and Dalziel and Dickinson (1966).

Certain neutral salts, such as ammonium sulfate, sodium sulfate, sodium acetate and sodium citrate, stimulate the catalytic activity of LADH, and they also protect the enzyme from heat destruction. Whilst under the same conditions, sodium chloride and sodium nitrate do not show such a stimulating effect, but to some extent, they accelerate the thermal inactivation of LADH. The mechanisms by which these neutral salts act have been inferred from kinetic studies (Theorell et al., 1966; Theorell and McKinley-McKee, 1961; Plane and Theorell, 1961) and by optical rotatory dispersion studies (Li, et al., 1963). These approaches have shown that the interaction of anions is at, or near, the zinc-containing active sites of the enzyme. The present data also indicate that the stimulation of LADH activity by certain neutral salts as a result of imidazole may be due to the formation of the ternary LADH-NADH-Anion complex, in which the rate of the dissociation of NADH from the LADH-NADH-Anion complex is faster than it is from the binary LADH-NADH complex.
The effect of these neutral salts on the thermal inactivation of LADH in the presence of salts may be a consequence of the formation of a binary LADH-Anion complex. The binding of these neutral salts to LADH can either serve as a stabilizer or a destabilizer. The common mechanism of the effect of these neutral salts on LADH cannot be unequivocally established, as identical concentration and equivalent valence type has such different effects.

The activity of LADH is stimulated by imidazole. This stimulating effect has been explained in terms of the formation of a ternary complex composed of LADH, NADH and imidazole, in which the rate of dissociation of the coenzyme from the ternary LADH-NADH-imidazole is faster than the rate of dissociation of the coenzyme from the binary complex composed of LADH and NADH only. However, the data from thermal studies show that LADH is destabilized by imidazole, and the stability of these complexes against heat is in the order of $\text{LADH-NADH > LADH-NADH-imidazole > LADH-imidazole}$. The stability of the binary LADH-imidazole complex against heat is also pH-dependent. The rate of thermal inactivation of the binary LADH-imidazole complex decreases with increasing pH. The locus of imidazole-binding is the enzymatic zinc ion. ADP and imidazole have been shown to inhibit LADH (Theorell and McKinley-McKee, 1961; Li and Vallee, 1964a). Measurements from thermal stability show that the binary complex of LADH-ADP is less stable than that for LADH-imidazole or LADH alone. Since ADP and imidazole do not interfere with each other on the rate of thermal inactivation, they seem to bind at a different site of LADH.

$\text{NAD}^+$ and NADH protect LADH from the thermal destruction. In this respect, NADH shows a stronger protective effect than $\text{NAD}^+$, which may be attributed to the fact that NADH is more tightly bound to LADH than $\text{NAD}^+$ at
neutral pH (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964). The protection of LADH by the coenzymes has also been reported by Yonetani and Theorell (1962), and it has long been established that LADH forms a binary complex with the coenzyme (Theorell and Bonnichsen, 1951). The binary LADH-NADH complex induces the changes in conformation of the enzyme molecule (Ulmer et al., 1961; Li and Vallee, 1964a; Rosenberg et al., 1965). The protection of LADH by NADH against thermal destruction may also suggest that the new configuration of the binary LADH-NADH complex has a more rigid structure, which is less accessible to thermal destruction.

Unlike the whole coenzyme molecule, the individual components of the coenzyme, such as ADP, greatly accelerated the inactivation rate of LADH by heat, indicating that ADP is bound to the enzyme; AMP, ADP and ATP show a different degree in the rate of thermal inactivation of LADH, as do GDP and GTP (Table 2.3). This may indicate that whilst the pyrophosphate part of the coenzyme molecule plays a role, the adenine amino group is not essential in binding to the enzyme. Since 2'3' AMP and 3'5' AMP do not affect the heat inactivation of LADH, this may suggest that the steric orientation of pyrophosphate groups in the coenzyme molecules are also critical.

However, the nature of the interaction between the coenzyme and LADH is difficult to establish yet. Indeed, the enzymatic zinc ions play an integral role in coenzyme binding (see reviews by Vallee, 1960; Sund and Theorell, 1963; McKinley-McKee, 1964; Li, 1966). The multiple attachment of the NADH molecule to the enzyme has also been proposed by Wallenfels and Sund (1957); Kosower (1962a,b); Yonetani (1963a); Yonetani and Theorell (1964); Evans and Rabin (1968); and Weiner (1969). It has been suggested further that the pyrophosphate groups of the coenzyme molecule are bound to the enzymatic
zinc ion (Van Eys, et al., 1957; van Eys, et al., 1958b). Further studies of AMP and zinc ions by a variety of physico-chemical techniques (Taqui Khan and Martell, 1962; Druyan and Vallee, 1964) and by optical rotatory dispersion (Li and Vallee, 1964a), have also come to the same conclusion. Whilst, from kinetic and spectral studies, a reverse conclusion is reached (Yonetani, 1963a; Yonetani and Theorell, 1964; Sigman, 1967). These latter authors concluded that ADFR moiety of the coenzyme molecule played no part in binding with the enzymatic zinc, and that the nicotinamide part of the coenzyme lies at or near the proximity of the zinc. In accord with this circumstance, there is no clear agreement about the nature of the interaction between the coenzyme and the enzyme. Common mechanistic denominators to reconcile these apparent discrepancies remains to be sought. The full identification of the basic structural and compositional units in the coenzyme, which governs its specific reactivity toward the enzyme are important. Above all, the absolute configuration of the individual groups in the coenzyme molecule and the location in the three dimensional array of the functional groups in the enzyme molecule must be also critical.
CHAPTER 3

EFFECT OF PYRIDOXAL-5'-PHOSPHATE ON LADH AND YADH
3. INTRODUCTION

Recent studies of LADH and YADH have been devoted toward determining which of the functional groups in these enzymes are directly involved in the catalytic mechanism. The binding of pyridine nucleotide coenzymes and substrates to LADH and YADH has been extensively reviewed by Kaplan (1960); Sund and Theorell (1963); McKinley-McKee (1964); and Sund (1968). The interaction of NADH with LADH changes the maximum absorption spectrum of NADH (Theorell and Bonnichsen, 1951) and of NAD$^+$ (Taniguchi, et al., 1967), and results in Cotton effect (Ulmer et al., 1961; Li, et al., 1962; Rosenberg, et al., 1965). From kinetic studies, it has been shown also that LADH (Theorell and Chance, 1951) and YADH (Hayes and Velick, 1954) have two and four coenzyme-binding sites per molecule, respectively. NADH is more tightly bound to LADH than is NAD$^+$ (Winer and Theorell, 1959; Theorell and McKinley-McKee, 1961).

The chelating agent, ortho-phenanthroline, competes with the coenzyme for the binding to the enzyme (Vallee et al., 1959; Plane and Theorell, 1961; Mahler, et al., 1962), but it does not remove the enzymatic zinc ions, and it forms a dissociable mixed protein-zinc-chelate complex with the enzyme (Vallee and Coombs, 1959). Utilizing these findings, and subsequent studies of the coupling between the enzyme and coenzyme analogs and zinc-chelating agents, by optical rotatory dispersion, it has been concluded that the ADPR moiety of the coenzyme molecule is responsible in large measure for binding in the vicinity of the enzymatic zinc ion (Li, et al., 1962, 1963; Li and Vallee, 1964a; Druyan and Vallee, 1964; Ulmer and Vallee, 1965; Li, 1966). Indeed, the binding of pyrophosphate groups of the coenzyme to the enzymatic zinc ion has been previously suggested by van Eys, et al., (1958b), and
Kosower (1962a,b). On the other hand, based on kinetic studies, Wallenfels et al., (1957) and Theorell and McKinley-McKee (1961) concluded that the amino group and the nitrogen atom in position 9 of the adenine ring of the coenzyme formed a bidentate chelate with the zinc atom at the active site of the enzyme. However, data obtained by spectral measurements, using coenzyme inhibitor or the zinc chelating agents (Windmueller and Kaplan, 1961; Yonetani 1963a; Yonetani and Theorell, 1964; Sigman, 1967) invalidate this suggestion. Yonetani (1963a) and Yonetani and Theorell (1964) have demonstrated that an ortho-phenanthroline-LADH-ADPR complex exists. This finding indicates that ADPR and ortho-phenanthroline can be simultaneously bound to the enzyme. Thus, it has been concluded that the competitive inhibitions by ADPR and ortho-phenanthroline are additive, and the ADPR part of the coenzyme molecule plays little or no part in the coenzyme-binding to LADH. But since ortho-phenanthroline is a competitive inhibitor with regard to NADH and NAD$^+$ (Vallee et al., 1959; Plane and Theorell, 1961; Mahler, et al., 1962), it is concluded that the nicotinamide part of the coenzyme molecule is responsible for the binding in the proximity of the enzymatic zinc. Detailed studies of the complexes between metals and amide, have shown that the carboxamide group of the coenzyme complexes the zinc atom of the enzyme and the site of interaction is with the amide oxygen atom (Rabin, 1960; Rabin and Whitehead, 1962).

However, as pointed out by Kosower (1962a,b) the suggestion of the interaction between the enzymatic zinc ion and carboxamide group of the coenzyme is contradicted by the properties of the spectral shift. If a positively charged species like the zinc ion were near the carboxamide group as required in the binding of the coenzyme to the enzyme, it would exhibit a shift of the maximum absorption band of NADH to a longer wavelength, not a
shorter one, for zinc ion favors the charge distribution of the excited state. More recent data obtained from the study of the modification of the carboxamide groups of the coenzyme molecule, illustrates that the modified coenzyme is still able to bind to the enzyme (Li, et al., 1962; Shore and Theorell, 1967; Sigman, 1967; Shore, 1969). This finding indicates the non-essentiality of the carboxamide group in binding of the coenzyme to the enzyme. Thus, it is postulated that the shift of the NADH spectrum to shorter wavelengths is due to the proximity of a positive charge to the nitrogen of the dehydropyridine ring, and that an ε-amino group of lysine residue of the enzyme plays a key component in the coenzyme binding (Kosower, 1962a,b). This suggestion, in fact, has been re-emphasised by Fisher, et al. (1967).

In contrast to LADH, YADH appears to be more sensitive to changes in the purine moiety since these dinucleotide analogues show much lower activity with YADH than with the natural coenzyme (Fawcet and Kaplan, 1962). It has been postulated also that there is a hydrophobic area at the "pyridinium ring" region of the coenzyme binding site of YADH (Anderson and Anderson, 1964; Anderson, et al., 1965b, 1966; Royer and Candy, 1968).

It is apparent that the nature of the groups and moieties of the coenzyme molecule which are involved in binding to these enzymes have not been fully settled. A study of site-specific and selective reagents, which affect catalytic activity through the interaction of a specific chemical modification of the enzyme molecule, may constitute a useful means of elucidating the chemical nature of active enzymatic sites.

Materials

LADH and YADH were obtained from C.F. Boehringer and Soehne (Mannheim,
Germany). NAD⁺, NADH, pyridoxal-5-phosphate and sodium borohydride were purchased from Sigma Chemical Co. (St. Louis, Missouri) with the best reagents grade. All other chemicals used were obtained commercially and were of the highest purity available. These chemicals were stored in a desiccator in the dark at 2°C.

Methods

LADH was freshly prepared and the concentration of LADH, NAD⁺ and NADH and the enzymatic activity was determined spectrophotometrically as described in Chapter 2. The molecular weight of LADH was taken as 84,000 and the absorbancy as £ = 0.455 at 280 m. (Taniguchi, et al., 1967). YADH was prepared by centrifuging the crystalline suspension at 15,000 r.p.m. and 2°C for 15 minutes. The supernatant was discarded and the precipitate was dissolved with 0.1M phosphate buffer, pH 8.0. The undissolved protein was removed by centrifuging at 15,000 r.p.m. and 2°C for 15 minutes. This solution was then introduced into dialysis tubing which had been pretreated with distilled water for about 24 hours at room temperature. The enzyme solution was dialyzed with three changes of 2 litres 0.1M phosphate buffer, pH 7.3 over a period of 48 hours. The dialysed solution was then centrifuged again at 15,000 r.p.m. and 2°C. The stock solutions were kept at 2°C for not longer than 3 days before use.

A temperature controlled cell compartment of an Unicam SP 500 spectrophotometer fixed with a Gilford (Model 2000) recorder was used to measure the concentration and activity of YADH. The concentration was determined at 280 m, based on an extinction coefficient of $1.89 \times 10^5 \text{ cm}^2 \text{ mole}^{-1}$, and a molecular weight of 150,000 (Hayes and Velick, 1954). The enzymatic activity was assayed at 23.5°C in duplicate by introducing 5 - 20 μl of an
appropriate enzyme concentration (3 - 5 M) to a 3.0 ml assay mixture in a 1.0 cm silica cell. The assay mixtures contained 0.1 M ethanol, 0.42 mM NAD⁺ and 0.1 M glycine-NaOH buffer, pH 8.6. The rate of the reduction of NAD⁺ was recorded at 340 m. The progress curves were linear for a few minutes and the initial velocity easily measured. These procedures for assaying the concentration and activity were used throughout this thesis, unless specifically stated.

The concentration of pyridoxal-5-phosphate was determined spectrophotometrically according to the method of Peterson and Sober (1954), on the basis of a molar extinction coefficient of 4900 at 388 mμ or of 2500 at 330 mμ.

LADH or YADH, effectors and buffer to a total volume of 1.0 - 3.0 ml were incubated in small stoppered glass-vessels under the various conditions stated in the text. At the required times, 5 - 50 μl of this solution were withdrawn with a Carlsberg micropipette and introduced into the assay mixtures by means of a glass spatula. Controls were carried out by measuring the enzymatic activity without the presence of effectors before each experiment was done. The absorbancy of the assay mixtures at 340 mμ were appropriately corrected for the presence of pyridoxal-5-phosphate.

Reduction with sodium borohydride: The enzymatic activity was first assayed in duplicate. The modified LADH and YADH were prepared by following the procedures as described by Fischer, et al. (1958). A total volume of 3.0 ml solution containing 0.1 μ sodium phosphate buffer, pH 7.3, various concentrations of pyridoxal-5-phosphate and 21 μM LADH or 4.8 μM YADH was incubated at 23°C for 30 minutes. The enzymatic activity of this solution was then assayed. This enzyme-pyridoxal-5-phosphate mixture was cooled to 0°C and
adjusted to pH 6.5 by the addition of 1.0 M ortho-phosphoric acid. A drop of octyl alcohol was added in order to avoid foaming. Sodium borohydride was then added slowly in 50-fold excess with respect to pyridoxal-5'-phosphate, and this solution was left at 0°C for 30 minutes. The enzymatic activity was assayed again before centrifuging at 15,000 r.p.m. and 2°C for 10 minutes. This solution was then introduced into dialysis tubing, and dialysed against 3 changes of 2 litres 0.1 M sodium phosphate buffer, pH 7.3 over a period of about 30 hours, in order to remove the excess pyridoxal-5'-phosphate and sodium borohydride. The dialysed solution was centrifuged at 15,000 r.p.m. and 2°C for 15 minutes. The enzymatic activity again measured.

Ultraviolet absorption spectra were recorded with a Beckman (Model DK-2) ratio recording spectrophotometer, using two well-matched silica cells; the reference cell was filled with the dialysed buffer.

Fluorescence emission spectra were measured at 23°C with a Farand spectrofluorimeter fitted with an automatic recorder and a 1.0 cm silica cell was used. NADH or a plexiglass was used as the standard. The light source was equipped with a 150 watt DC Xenon Arc.

Measurements of pH were made with a Cambridge "Bench type" pH meter.

**RESULTS**

**Effect of Pyridoxal-5'-phosphate on LADH**

The effect of pyridoxal-5'-phosphate on the activity of LADH was investigated by incubating a total volume of 1.02 ml reaction mixture containing 1.65 μM LADH, 0.1 μM sodium phosphate buffer, pH 7.3 and different concentration of pyridoxal-5'-phosphate at 23°C (Fig. 3.1). At various time intervals, 50 μl aliquots were withdrawn and introduced into a 3.0 ml assay mixture as described in Chapter 2, for determining the residual activity at
Fig. 3.1. Inactivation of LADH by Pyridoxal-5'-phosphate. Incubation was carried out at 23°C by adding a final concentration of 1.65 μM LADH to 1.02 ml 0.1 M phosphate buffer, pH 7.3, containing pyridoxal-5'-phosphate of (O) 0.5 mM (△) 1.0 mM (●) 2.5 mM (△) 5.0 mM (x) 10.0 mM.
23.5°C. An appropriate correction for the absorbancy at 340 mm was made during the assay of enzymatic activity, due to the absorption of pyridoxal-5'-phosphate. When LADH is incubated alone at pH 7.3 and 23°C, there is no appreciable loss of enzymatic activity over a period of 60 minutes. The presence of pyridoxal-5'-phosphate causes an extensive loss of enzymatic activity, which as shown in Fig. 3.1, is a time-dependent process. The enzymatic activity was initially lost rapidly, followed by a much slower process which is complete in about 15 minutes. Thereafter, there is no further loss of enzymatic activity over a period of 3 hours. Thus on incubation of 1.5μM LADH and 5 mM pyridoxal-5'-phosphate at pH 7.3 for 15 minutes, the loss of initial enzymic activity is about 28%.

Fig. 3.1 also shows that the degree of loss of enzymatic activity increases with increasing the concentration of pyridoxal-5'-phosphate. However, if a concentration over 5 mM of pyridoxal-5'-phosphate is used, the loss of enzymatic activity is substantially reduced. Thus, after inactivating LADH by 10 mM pyridoxal-5'-phosphate at pH 7.3 and 23°C, the loss of initial activity is 32%.

The inactivation of LADH by pyridoxal-5'-phosphate can be completely recovered by removal of pyridoxal-5'-phosphate through dialysis against three changes of 2 litres 0.1 sodium phosphate buffer pH 7.3 at 4°C over a period of about 30 hours.

The effect of temperature on the inactivation of LADH by pyridoxal-5'-phosphate was also examined. Incubation of 1.7μM LADH at 70°C in 0.1μM sodium phosphate buffer, pH 7.3 for about 34 minutes, results in 50% loss of initial activity, and precipitation is visible. Under the same conditions,
except the addition of 1.0 mM pyridoxal-5'-phosphate to the enzyme solution, loss of enzymatic activity occurred rapidly. No catalytic activity remained after heating at 70°C for 34 minutes (Fig. 3.2), but the reaction mixture of LADH and pyridoxal-5'-phosphate at this stage is still clear, and no precipitation occurs. When this solution was cooled to 2°C and dialysed at 4°C with three changes of 0.1M sodium phosphate buffer, pH 7.3 over a period of about 30 hours, no activity was recovered.

**Effect of pH on Inactivation of LADH by Pyridoxal-5'-phosphate**

The effect of pH on inactivation of LADH by pyridoxal-5'-phosphate was examined over the pH range of 6.1 - 10.3. It was found that pH affects the inactivation rate. Fig. 3.3 shows that the rate of inactivation is decreased when the pH is lower than 7.3. The maximum loss of enzymatic activity is at pH 8.0, whilst above pH 8.0, the loss of enzymatic activity is again reduced. If a pH range of from 8.9 to 10.3 is used, pyridoxal-5'-phosphate is unable to inactivate LADH. Thus, the incubation of 2.0 μM enzyme LADH in a pH range 8.9 - 10.3 at 25°C over a period of 50 minutes, with or without the presence of 2.5 mM pyridoxal-5'-phosphate, results in no loss of initial activity.

**Effect of Coenzyme and Ethanol on Inactivation of LADH by Pyridoxal-5'-phosphate**

NADH is known to form a binary complex with LADH (Theorell and Bonnichsen, 1951). This binary complex is more stable than LADH, and protects the enzyme against inactivation by heat, pH and other denaturating agents (see Chapter 2). NADH also exercises a significant protection against the inactivation of LADH by pyridoxal-5'-phosphate (Fig. 3.4). Incubation of 1.4 μM LADH and 5.0 mM pyridoxal-5'-phosphate at pH 7.3 and 25°C, causes a loss of initial activity of 28%, whilst the presence of 140 μM NADH results
Fig. 3.2. Effect of pyridoxal-5'-phosphate on the thermal inactivation of LADH. Incubation was carried out at 70°C with a reaction mixture containing 0.1 M phosphate buffer, pH 7.3, 1.7 μM LADH and (○) control (•) 1.0 mM pyridoxal-5'-phosphate.
Fig. 3.3. Effect of pH on inactivation of LADH by pyridoxal-5'-phosphate. 2.0μM LADH were incubated at 23°C with 2.5 mM pyridoxal-5'-phosphate at pH (o) 6.5, (e) 8.0 (Δ) 8.3, (x) 8.9 - 10.3, (Δ) 7.3. Buffers of pH 6.5 - 8.0 were made of 0.1 M phosphate, whilst pH 8.3 was made of 0.1 M phosphate pH 7.3 titrated with 0.1 M glycine - NaOH, pH 10.0, and for pH 8.9 - 10.3, 0.1 M glycine - NaOH was used.
Fig. 3.4. Effect of NADH and ethanol on the inactivation of LADH by pyridoxal-5'-phosphate. 1.4 μM LADH were incubated at 23°C with 0.1 μM phosphate, pH 7.3, containing 5.0 mM pyridoxal-5'-phosphate (○) and either 140 μM NADH (●) or 0.1 M ethanol (△).
in no loss of enzymatic activity.

Ethanol is known to protect the enzyme against urea inactivation (see chapter 5). Whilst the addition of 0.1 M ethanol to a mixture of 1.11 M LADH and 5.0 mM pyridoxal-5′-phosphate at pH 7.3 and 25°C, does not prevent the inactivation of LADH by pyridoxal-5′-phosphate.

Identification of LADH-Pyridoxal-5′-phosphate Complex

(a) Reduction of LADH-Pyridoxal-5′-phosphate Complex: In order to find out whether the partial inactivation of LADH by pyridoxal-5′-phosphate is due to the formation of Schiff bases, a study was carried out by stabilizing the LADH-Pyridoxal-5′-phosphate complex through the reduction with sodium borohydride. The activity of native enzyme was first assayed. Four samples were prepared each with a total volume of 3.0 ml in a stoppered-glass vessel containing 0.1 M sodium phosphate buffer, pH 7.3; and samples (1) and (2) contained in addition 21.0 μM LADH, while samples (3) and (4) contained 21.0 μM LADH and 3.0 mM pyridoxal-5′-phosphate. After 30 minutes, the enzymatic activity of these samples was determined, and then cooled to 0°C and adjusted to pH 6.5 by the addition of 1.0 M pyrophosphoric acid. Assays showed that there was no effect on the activity of the native enzyme under these conditions over a period of 30 minutes. A drop of octyl alcohol was then added to each of these four samples, and a final concentration of 0.1 M NaBH₄ each was gradually added to sample (1) in which was contained LADH alone and sample (3) in which was contained LADH and pyridoxal-5′-phosphate. These samples were kept in the dark at 0°C for 30 minutes. An appropriate correction was applied for the dilution of the enzyme solution due to the addition of NaBH₄. The enzymatic activity of these samples was assayed before centrifuging at 15,000 and 2°C for 15 minutes, followed by dialysing
against three changes of 2 litres 0.1 M sodium phosphate buffer, pH 7.3 at 4°C over a period of 30 hours. Then these solutions were again centrifuged and the enzymatic activity of these four samples was assayed. Table 3.1 summarises the results. After these manipulations, loss of initial activity of LADH alone is 9%. The loss of enzymatic activity due to the presence of pyridoxal-5'-phosphate is completely recovered after the removal of pyridoxal-5'-phosphate through dialysis. NaBH₄ has no effect on the activity of LADH. Whilst the reduction of LADH-Pyridoxal-5'-phosphate complex with NaBH₄ results in a loss of initial activity of 31%.

(b) Ultraviolet Absorption Spectra: The above four samples after dialysing, centrifuging and assaying the enzymatic activity, were also subjected to the measurement of the ultraviolet absorption spectra. As shown in Fig. 3.5, the native LADH in 0.1 M sodium phosphate buffer, pH 7.3 exhibits a maximum peak at 280 mμ and there are several clear maxima and minima in the region between 270 and 250 mμ. By contrast, pyridoxal-5'-phosphate in 0.1 M sodium phosphate, pH 7.3 has a maximum absorption band at 388 mμ and a shoulder at 330 mμ. The peak at 388 mμ has been assigned to the -CHO group in the pyridoxal-5'-phosphate molecule (Peterson and Sober, 1954). The spectrum of the reduced LADH-Pyridoxal-5'-phosphate complex shows that the original protein absorption band is shifted from 280 mμ to 283 mμ. There is also a distinct peak at 325 mμ which is absent in the native protein. The absorption band in the region of 270 mμ to 250 mμ is increased compared to that for the original protein spectrum. The absorption band at 325 mμ is known to be the characteristic of pyridoxamine derivatives (Fischer et al., 1958, 1963; Dempsey and Christensen, 1962; Snell, 1963). By using a molar extinction coefficient of 10,150 for ε-pyridoxalysine (Fischer et al., 1963), it can be
Table 3.1

Reduction of Pyridoxal-5'-phosphate - LADH or YADH Complex with NaBH₄

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative Activity</th>
<th>30 min.</th>
<th>After dialysing for 30 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LADH</td>
<td>100</td>
<td></td>
<td>91</td>
</tr>
<tr>
<td>LADH + 3 mM PLP*</td>
<td>75</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>LADH + 0.15 M NaBH₄</td>
<td></td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>LADH + 3 mM PLP + 0.15 M NaBH₄</td>
<td></td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>YADH</td>
<td>92</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>YADH + 1.5 mM PLP</td>
<td>91</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>YADH + 0.1 M NaBH₄</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>YADH + 2 mM PLP + 0.1 M NaBH₄</td>
<td>-</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

A final concentration of 21 μM LADH, or 4.8 μM YADH, was incubated for 30 min. in 3.0 ml of reaction mixture containing 0.1 μM phosphate buffer, pH 7.3, and the additions indicated in the Table. After assaying the enzymatic activity, reduction of enzyme-PLP complex with NaBH₄ was carried out as described in the text.

*PLP = pyridoxal-5'-phosphate.
Fig. 3.5. Ultraviolet absorption spectra of (a) 21 μM LADH in 0.1 μ phosphate buffer, pH 7.3 or after dialysis for 30 hours (b) 21 μM LADH + 3.0 mM pyridoxal-5′-phosphate, (c) 21 μM LADH + 0.1 M NaBH₄, and (d) 21 μM LADH + 3.0 mM PLP + 0.1 M NaBH₄. Spectra were measured at room temperature.
Fig. 3.6. Ultraviolet absorption spectrum of $1.3 \times 10^{-4} \text{M}$ pyridoxal-5′-phosphate in 0.1 μ phosphate buffer, pH 7.3 at 20°C.
calculated from Fig. 3.5 that approximately 3.9 moles of pyridoxal-5'-phosphate are bound per mole of LADH when 3.0 mM pyridoxal-5'-phosphate was used.

The combination of pyridoxal-5'-phosphate to LADH is concentration-dependent. If a similar experiment was done by using 1.0 mM pyridoxal-5'-phosphate, and 21.0 μM LADH after reducing this LADH-pyridoxal-5'-phosphate complex with 50-fold excess of NaBH₄, 2.3 moles pyridoxal-5'-phosphate are bound per mole of enzyme. Table 3.2 summarizes the results.

(a) Fluorescence Emission Spectra: The reduced LADH-pyridoxal-5'-phosphate complex was also measured by means of fluorescence spectroscopy. As illustrated in Fig. 3.7, excitation of the reduced LADH-pyridoxal-5'-phosphate in 0.1 M sodium phosphate buffer, pH 7.3 at 325 μm, results in one major fluorescence band at 395 μm, which is characteristic of pyridoxamine-5'-phosphate residues (Churchill, 1965b). Whilst the fluorescence emission spectrum of the reduced LADH-Pyridoxal-5'-phosphate complex greatly differs from that of the native enzyme. When the excitation of the native enzyme in 0.1 M sodium phosphate buffer, pH 7.3, was done at 280 μm, a maximum fluorescence peak appears at 338 μm, attributed to tryptophan chromophore. The reduced LADH-Pyridoxal-5'-phosphate complex is characterized by three fluorescence bands at 320 μm, 355 μm and 390 μm. The new band at 390 μm has been explained solely to be due to energy transfer from the aromatic residues to the bound pyridoxyl groups (Churchich, 1965a), and this interpretation is consistent with the finding that the presence of the pyridoxyl group causes a dramatic quenching of the native protein fluorescence.

The modified enzyme is more stable against aggregation than the native enzyme. When the modified enzyme and the native enzyme were kept under the
Table 3.2
Combination of Pyridoxal-5'-phosphate with LADH and YADH

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>PLP used (mM)</th>
<th>PLP incorporated* (equiv./mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 μM LADH</td>
<td>0.5</td>
<td>2.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.0</td>
<td>2.3</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.0</td>
<td>3.9</td>
</tr>
<tr>
<td>4.8 μM YADH</td>
<td>2.0</td>
<td>4.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>2.5</td>
<td>5.4</td>
</tr>
<tr>
<td>&quot;</td>
<td>60.0</td>
<td>29.6</td>
</tr>
</tbody>
</table>

* 3.0 ml of reaction mixture containing 0.1 μ phosphate buffer, pH 7.3, 21 μM LADH and various concentrations of PLP, was incubated at 23°C for 30 min., and then cooled to 0°C for 10 min. The pH was then adjusted to 6.5 and sodium borohydrate was added in 50-fold excess over the amount of PLP as described in the text. Reduced samples were then centrifuged and dialysed exhaustively against 0.1 μ phosphate buffer, pH 7.3, for about 30 hrs. The values for incorporation of PLP to the enzyme was calculated directly from the absorbance at 325 μm using the value of 10,150 (Fischer et al., 1963) for the molar absorptivity.

Similar methods were applied to determine the YADH-PLP complex.
Fig. 3.7. Fluorescence emission spectra of the reduced LADH-PLP complex (---) and YADH-PLP complex (..........) in 0.1 μ phosphate buffer, pH 7.3 at 23°C. Excitation = 325 μm. Other conditions were as shown in Fig. 3.5 and 3.9.
Fig. 3.8. The fluorescence emission spectra of (a) LADH, and (b) LADH derivatives. Spectra were obtained in 0.1μ phosphate buffer, pH 7.3 at 23°C with excitation at 280 μ. The optical density for both samples was 0.802 at 280 μ. The reduced LADH-PLP complex with NaBH₄ was prepared as described in the text.
same conditions at pH 7.3 and room temperature for about 3 days, the native enzyme solution undergoes coagulation, but the modified enzyme solution still remains clear.

There is no change in the enzymatic activity and ultraviolet absorption spectrum, or the fluorescence emission spectrum, when NaBH₄ was added to LADH for 30 minutes, followed by dialysing away NaBH₄ over a period of about 30 hours. The same holds true if pyridoxal-5'-phosphate was added to LADH, without the NaBH₄ reduction.

**Effect of Pyridoxal-5'-phosphate on YADH**

The effect of pyridoxal-5'-phosphate on YADH was also investigated in an analogous manner as for LADH. Incubation of a total volume of 3.0 ml reaction mixture containing 0.1 μ sodium phosphate, pH 7.3, 4.8μM YADH and 1.0 - 5.0 mM pyridoxal-5'-phosphate at 25°C for 30 minutes, showed that there is no change in enzymatic activity in comparison with the enzyme in the absence of pyridoxal-5'-phosphate (Table 3.1).

The methods used for the reduction of LADH-pyridoxal-5'-phosphate complex with NaBH₄, were also employed to modify the molecule of YADH. As shown in Fig. 3.9, the native YADH in 0.1 μ sodium phosphate buffer, pH 7.3, exhibits a maximum peak at 278 μ which is 2 μ shorter than for LADH (Fig. 3.5). Unlike LADH, there is also no clear peak below 275 μ for YADH. The ultraviolet absorption spectrum and fluorescence emission spectrum of the YADH-pyridoxal-5'-phosphate mixture after the reduction with NaBH₄, are also entirely different from that of the native enzyme. The modified YADH in 0.1 μ sodium phosphate buffer, pH 7.3 yields a new peak at 325 μ. Excitation of the native YADH in the same buffer at 280 μ gives a fluorescence band at 340 μ, whilst the modified enzyme results in quenching the native protein fluores-
Fig. 3.9. Ultraviolet absorption spectra of YADH and the reduced YADH-PLP complex. After dialysis for 30 hours (a) 4.8 µM YADH, (b) 4.8 µM YADH + 2.0 mM PLP, (c) 4.8 µM YADH + 0.1 M NaBH₄, and (d) 4.8 µM YADH + 2.0 mM PLP + 0.1 M NaBH₄. Spectra were measured at 20°C.
cence, and a new fluorescence peak arises at 390 m\(\mu\). Likewise, excitation of the modified YADH at 325 m\(\mu\), results in one major fluorescence emission band at 395 m\(\mu\) (Fig. 3.7).

The quantity of pyridoxal-5'-phosphate bound to YADH can be estimated spectrophotometrically, assuming a value of 10,150 for the molar extinction coefficient of \(\epsilon\)-pyridoxyllysine at 325 m\(\mu\) (Fischer et al., 1963). The addition of 2.0 mM pyridoxal-5'-phosphate to 4.8 \(\mu\)M YADH at pH 6.5 and 0\(^0\)C, followed by reduction with 0.1 M NaBH\(_4\), shows that approximately 4.8 moles of pyridoxal-5'-phosphate are combined with the enzyme per 150,000 molecular weight. Incorporation of pyridoxal-5'-phosphate into YADH increases with increasing concentration of pyridoxal-5'-phosphate (Table 3.2). At a concentration of 60 mM, approximately 29.6 M of pyridoxal-5'-phosphate are bound per mole of enzyme.

It was also noted that the addition of 0.1 M NaBH\(_4\) to 4.8 \(\mu\)M YADH at pH 6.5 and 0\(^0\)C for 30 minutes, followed by dialysis against 3 changes of 2 litres of 0.1 \(\mu\) sodium phosphate over a period of about 30 hours, results in nearly complete loss of catalytic activity (Table 3.1). Likewise, after the reduction of YADH-Pyridoxal-5'-phosphate with NaBH\(_4\), and dialysis over a period of about 30 hours, no enzymatic activity can be detected. However, treatment of 4.8 \(\mu\)M YADH either with 0.1 M NaBH\(_4\) or 2.0 mM pyridoxal-5'-phosphate at pH 6.5 and 0\(^0\)C for 30 minutes, and followed by dialysis, does not cause a change in absorption or fluorescence emission spectrum in comparison with the native enzyme.

**DISCUSSION**

Generally, the active sites of many enzymes contain some chemically unique, or unusually reactive, amino acid residue, or group of residues,
Fig. 3.10. Fluorescence emission spectra of (a) YADH and (b) modified YADH. The wavelength of the exciting light was 280 m\(\mu\). Both samples had the same absorbancy of 0.905 at 280 m\(\mu\). For other experimental conditions, see Fig. 3.9.
important to the catalytic function. A study of site-specific and selective reagents which affect catalytic activity through the induction of a specific, chemical modification of the enzyme molecule may provide an additional evidence for the identification of the chemical nature of active enzymatic sites. Pyridoxal-5'-phosphate has been subsequently employed for this purpose, and of all the amino acid side-chains, it reacts only with ε-amino group of lysine residue. Because of its highly specific reactivity (Fischer et al., 1958; Guirard and Snell, 1964), it has also been employed to demonstrate that the incorporation of pyridoxal-5'-phosphate into several enzymes which do not require this compound as a coenzyme for catalytic activity, such as bovine glutamic dehydrogenase (Anderson et al., 1964), AMP deaminase (Kaldor and Weingach, 1966), 6-phosphogluconic dehydrogenase (Rippa et al., 1967), rabbit muscle aldolase (Shapiro et al., 1968) and rabbit liver and kidney fructose 1,6-diphosphatase (Krulwich et al., 1969), causes a complete loss of catalytic activity, attributed to the formation of a Schiff base between the aldehyde group of pyridoxal-5'-phosphate and an ε-amino group of lysine residue of these enzymes. Thus, it has been further concluded that a reactive lysine residue is involved in the active sites among these enzymes.

LADH is shown to be partially inactivated by pyridoxal-5'-phosphate. The degree of inactivation by pyridoxal-5'-phosphate during a given period of incubation increases with increasing the concentration of pyridoxal-5'-phosphate. However, this partial inactivation is pH-dependent and the enzymatic activity can be retained by dialysis, suggesting a more covalent like interaction between the enzyme and pyridoxal-5'-phosphate.

Evidence for the partial inactivation process being due to the formation of Schiff bases is afforded by stabilizing the unstable LADH-pyridoxal-5'-
phosphate complex by NaBH₄ reduction. After NaBH₄ reduction, dialysis does not result in recovery of this partial inactivation by pyridoxal-5'-phosphate. The spectrum of the reduced LADH-pyridoxal-5'-phosphate complex is characteristic of pyridoxamine derivatives (Fischer et al., 1958, 1963; Dempsey and Christensen, 1962; Snell, 1963). Excitation of this modified enzyme at 325 mμ, results in a fluorescence band at 395 mμ which is likewise characteristic of pyridoxamine derivatives (Churchich, 1965b). Using the molar extinction coefficient of 10,150 for the LADH-pyridoxal-5'-phosphate complex at 325 mμ (Fischer et al., 1963), a concentration ratio of pyridoxal-5'-phosphate:LADH of 143 produced results which showed that 3.9 moles of pyridoxal-5'-phosphate are bound per 84,000 molecular weight of LADH. The incorporation of pyridoxal-5'-phosphate into LADH depends upon the amount of pyridoxal-5'-phosphate added to a certain concentration of the enzyme solution (Table 3.2).

By contrast, pyridoxal-5'-phosphate does not interfere with the activity of YADH. Unlike LADH, either YADH alone or a mixture with pyridoxal-5'-phosphate, is inactivated by NaBH₄, and the inactivation cannot be restored by dialysis (Table 3.1). It is also found that YADH can form Schiff bases with pyridoxal-5'-phosphate and the incorporation of pyridoxal-5'-phosphate into YADH increases by increasing the concentration of the former substance, (Table 3.2). It has been reported that the content of lysine residue of YADH (Wallenfels and Arens, 1960; Harris, 1964) and LADH (Theorell et al., 1960) is 93-96 and 61 groups, respectively. These values show that YADH has a higher lysine content than that of LADH. More pyridoxal-5'-phosphate has also been found to be incorporated into YADH than LADH, suggesting that more lysine residues of YADH are exposed on the outside surface of the protein molecule.
Treatment of both YADH and LADH with either NaBH$_4$ or pyridoxal-5'-phosphate, followed by dialysis, does not arise a new absorption band at 325 m$m\mu$, and there is no fluorescence emission band at 395 m$m\mu$ when excitation was done at 325 m$m\mu$.

Although the involvement of an $\varepsilon$-amino group of lysine residue in the mechanism of action of both dehydrogenases has been proposed by Kosower (1962a, b) and Fisher et al. (1967), the evidence presented here does not corroborate such a suggestion. It has been clearly shown that after blockage of the lysine residue with pyridoxal-5'-phosphate, these enzymes are still capable of reducing NAD$^+$ to NADH in the presence of substrate. For a group which is involved in the catalytic function, such as the zinc ion or sulphydryl group, the blocking of such a group with zinc-chelating agents (see reviews by Vallee, 1960; Sund and Theorell, 1963; McKinley-McKee, 1964; Li, 1966) or thiol-reagents (Witter, 1960; Yonetani and Theorell, 1962; Li, and Vallee, 1965) results in a 100% loss of catalytic activity. But this does not occur in the case of the inactivation of LADH and YADH by pyridoxal-5'-phosphate. Since LADH is partially inactivated by pyridoxal-5'-phosphate and, NADH prevents this partial inactivation, the results seem to be best interpreted by the suggestion that there is a lysine residue which is located near the region of the coenzyme binding site of LADH, and that the partial inactivation is due to steric hindrance.

Pyridoxal-5'-phosphate does not interfere with the activity of YADH, although after the reduction of YADH-pyridoxal-5'-phosphate with NaBH$_4$, only a small amount of residue activity can be detected. This loss of enzymatic activity may be due to the effect of NaBH$_4$ itself, because NaBH$_4$ is found to inactivate YADH directly. Thus the $\varepsilon$-amino group of the lysine residue of YADH may play no part in coenzyme binding.
CHAPTER 4

INHIBITION OF LADH AND YADH BY THIOL COMPOUNDS
4. INTRODUCTION

Both LADH and YADH contain zinc atoms and sulphydryl groups which are essential for the catalytic function of both enzymes. Thus, a variety of compounds which are capable of binding to the zinc atom and sulphydryl group act as inhibitors of both dehydrogenases. The inhibitory effect is due either to the formation of binary complexes consisting of enzyme and inhibitors, or ternary complexes composed of enzyme, coenzyme and inhibitors. The way by which the formation of these complexes take place has been extensively reviewed by Vallee (1960); Sund and Theorell, (1963); McKinley-McKee (1964); Colowick, et al., (1966); and Suná (1968).

It has also been demonstrated that the fluorescence intensity of NADH is increased many-fold in the presence of LADH or YADH (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964). This increased fluorescence is further enhanced by the addition of acetamide (Winer and Theorell, 1960), imidazole or isobutyramide (Theorell and McKinley-McKee, 1961 b,c; Yonetani and Theorell, 1962). The amplitude of the Cotton effect for LADH-NADH complex is also increased by the addition of acetamide (Ulmer, et al., 1961).

The inhibition of LADH and YADH by thyroxine and L-3,5,3',5-triiodothyronine has also been reported (Wolff and Wolff, 1957; McCarthy, et al., 1968; Gilleland and Shore, 1969). In addition to the inhibitory effect, these compounds also quench the fluorescence intensity of both LADH and LADH-NADH.

In this section, investigations were made of the effect of thiol compounds on LADH and YADH. Thiol compounds are of interest in the light of the previous reports of van Eys, et al. (1957, 1958a); and Lambe and Williams, (1965). It has been found that several mercaptans react non-enzymically with pyridine nucleotides to form addition products. In the presence of a suitable
dehydrogenase, this reaction between coenzyme and mercaptan becomes much more favourable. However, NAD$^+$ shows little reaction with YADH and ethyl-mercaptan, whilst LADH forms a ternary complex with the thiol compound and NAD$^+$ (van Eys, et al., 1957; Sigman, 1967). Lambe and Williams (1965) also reported that β-mercaptoethanol strongly inhibits LADH, but has no effect on YADH.

**Materials**

The sources of LADH, YADH, NAD$^+$ and NADH had been described in Chapters 2 and 3. All thiol compounds were purchased from Sigma Chemical Co. (St. Louis, Missouri), and kept at 2°C, and used without further purification. Other chemicals were of "Analar" grade.

**Methods**

The spectrophotometric assay for the activity and concentration of LADH, YADH and NADH were carried out according to the methods described in Chapters 2 and 3. All the solutions - except the enzymes - were prepared daily in fluorescence-free quartz double distilled water.

Inactivation experiments were carried out by incubating a total volume of 1.02 ml enzyme, inhibitor and buffer in a stoppered glass vessel at 23°C. 10-50 µl of these reaction mixtures were withdrawn and introduced into the assay solution (see Chapters 2 and 3) for determining the residual activity.

Fluorescence emission spectra and intensity measurements were made with a Farrand Spectrophotofluorimeter fixed with an automatic recorder. Light source was a 150 watt DC xenon arc. A slit combination of 10, 5; 10 and 5 mµ was used in sequence with the light source. Excitation was with light of 350 mµ, using a Corning glass filter CS7-54, and the emitted light passed through a Corning glass filter CS 7-53. Both filters were supplied by
Measurements of quenching fluorescence were made by directly pipetting thiol compounds into a 3.0 ml solution containing LADH, NADH and buffer in a silica curette (1-cm light path). All solutions were withdrawn by means of Carsberg pipettes and stirred with care with a small glass spatula. Before and after each measurement, a standard, made of 10-11 µM NADH in 0.05 M glycine-NaOH buffer, pH 10.0, was immediately measured in order to ensure there had been no apparatus drift. The temperature was maintained at 23.5°C by rapidly circulating water from a thermostat.

All pH values were determined before and at the end of each experiment at room temperature with either a Cambridge bench type pH meter, or a radiometer (model 26), Copenhagen, Denmark, with microelectrodes. The pH was adjusted by the addition of 2.0 N NaOH or 1.0 M orthophosphoric acid, when required.

RESULTS

(A) Kinetic Studies

β-Mercaptoethanol as a Substrate of LADH and YADH

β-Mercaptoethanol contains a CH₂OH group which is similar to that of ethanol. The possibility of that β-mercaptoethanol will act as a substrate of LADH was tested by substituting β-mercaptoethanol for ethanol in the assay solution, i.e. 3.0 ml assay solution were prepared containing 0.062 M glycine-NaOH buffer, pH 10.0, 0.42 mM NAD⁺ and 9.4 x 10⁻⁵ M β-mercaptoethanol.

Without the presence of LADH, no change of optical density at 340 m.µ was observed. When 1.2 x 10⁻⁷ M LADH were added to this assay solution, changes in the optical density at 340 m.µ occurred with time which show that NAD⁺ is slowly reduced to NADH (Fig. 4.1). At higher concentration of LADH (6.0 x 10⁻⁷ M)
Fig. 4.1. β-Mercaptoethanol as a substrate of both LADH and YADH. Each reaction mixture (3.0 ml) contained 0.062 M glycine-NaOH, pH 10, 0.42 mM NAD⁺ and (a) $6 \times 10^{-7}$ M LADH + $9.4 \times 10^{-5}$ M β-mercaptoethanol, (b) $1.2 \times 10^{-7}$ M LADH + $9.4 \times 10^{-5}$ M β-mercaptoethanol, (c) $8 \times 10^{-8}$ M YADH + $9.4 \times 10^{-5}$ M β-mercaptoethanol, (d) $6 \times 10^{-7}$ M LADH + $9 \times 10^{-5}$ M ethanethiol, (e) $6 \times 10^{-7}$ M LADH + $3.8 \times 10^{-3}$ M 3-mercaptopyruvate, and (f) $6 \times 10^{-7}$ M LADH + $4.8 \times 10^{-3}$ M mercaptoacetic acid.
the reduction rate of NAD$^+$ to NADH is faster.

A similar test was also conducted with YADH. A concentration of $8 \times 10^{-3}$ M was added to a 3.0 ml assay solution containing 0.062 M glycine-NaOH, pH 8.6, 0.42 mM NAD$^+$ and $9.4 \times 10^{-5}$ M β-mercaptoethanol. Likewise, β-mercaptoethanol was also found to be a poor substrate for YADH (Fig. 4.1).

In order to investigate further whether the reduction of NAD$^+$ to NADH is due to accepting the hydrogen atom from the OH-group or SH-group of β-mercaptoethanol, $9.0 \times 10^{-5}$ M ethanethiol, $4.8 \times 10^{-3}$ M mercaptoacetic acid or $3.8 \times 10^{-3}$ M 3-mercaptopropionic acid was added separately to assay solution instead of β-mercaptoethanol. Then a final concentration of either 0.60 μM LAD!! or 0.08 μM TAD!! was added to this assay solution. It was observed that under these conditions the absorbancy at 340 m$\mu$ did not change over a period of 2 – 3 hours. Thus, it may be concluded that the donation of the hydrogen atom to NAD$^+$ is due to OH-group rather than to SH-group of β-mercaptoethanol. It is to be noted that Lambe and Williams (1965) have also reported that β-mercaptoethanol can act as a substrate of LADH or YADH.

**Effect of β-Mercaptoethanol on the Activity of LAD!! and TAD!!**

The effect of β-mercaptoethanol on LAD!! was examined spectrophotometrically by incubating a total volume of 1.02 ml reaction mixtures containing 1.57 μM LAD!!, 0.1 μM phosphate buffer, pH 7.3 and various concentrations of β-mercaptoethanol at 23°C. 50 μl aliquots were then withdrawn immediately and introduced into assay solution (see chapter 2) for measuring the residual activity. It was found that inactivation was complete within a few seconds and thereafter there was no further loss of enzymatic activity over a period of about 24 hours. And the degree of loss of enzymatic activity depended upon the concentration of β-mercaptoethanol (Fig. 4.2). Under the conditions examined, a graph of
Inhibition of LADH by β-mercaptoethanol. 1.57 μM LADH were incubated at 23°C with various concentrations of β-mercaptoethanol in 1.02 ml 0.1 μM phosphate buffer, pH 7.3. Aliquots were then withdrawn for determining the residual activity. $E_i$ and $E_a$ are the enzymatic activity with and without the presence of inhibitor, respectively.
$E_a / E_i$ (where $E_a$ is the enzymatic activity without the presence of $\beta$-mercaptoethanol and $E_i$ with $\beta$-mercaptoethanol) against concentration of $\beta$-mercaptoethanol yields a linear relationship (Fig. 4.2), from which a dissociation constant of $2.2 \mu M$ is obtained for the LADH-$\beta$-mercaptoethanol complex.

Reversal of the inactivation of LADH by $\beta$-mercaptoethanol was also examined by subjecting a final concentration of $3.6 \mu M$ LADH to $0.1 M \beta$-mercaptoethanol in $2.0 ml 0.1 \mu$ phosphate buffer, pH 7.3 at $23^\circ C$. A second sample was prepared by adding a corresponding concentration of LADH to the same buffer, except without the presence of $\beta$-mercaptoethanol. The enzymatic activity was assayed in duplicate for both samples. The sample which contained $\beta$-mercaptoethanol showed that no enzymatic activity remained. Dialysis of these two samples against 2 changes of $2 l. of 0.1 \mu$ phosphate buffer, pH 7.3 at $2^\circ C$ over a period of about 24 hours, removed the inhibitor, and resulted in 100% recovery of enzymatic activity. No appreciable loss of catalytic activity with the sample which contained no $\beta$-mercaptoethanol was also observed after dialysing.

Similar experimental conditions were also employed to study the effect of $\beta$-mercaptoethanol on YADH. Concentrations of up to $0.15 M \beta$-mercaptoethanol were added to $2.1 \mu M$ YADH in $0.1 \mu$ phosphate buffer, pH 7.3 at $23^\circ C$. Aliquots (10 $\mu l$) were withdrawn and introduced into the assay solution (see chapter 3) for determining the activity. No change of catalytic activity was observed in comparison with the YADH activity in the absence of $\beta$-mercaptoethanol. These experiments show that, unlike LADH, $\beta$-mercaptoethanol does not affect the activity of YADH. Thus, further experiments presented in this section were mainly carried out with LADH.
Effect of Imidazole on the Inhibition of LADH by β-Mercaptoethanol

Imidazole is known to stimulate the catalytic activity of LADH due to the formation of a ternary LADH-NADH-imidazole complex, and it competes with substrate for binding to the enzymatic zinc ions (Theorell and McKinley-McKee, 1961 b,c). As shown in Fig. 4.3, an experiment was set-up in which LADH to a final concentration of 1.5 M was added to each of 1.02 ml 0.1 M phosphate buffer, pH 7.3 containing (a) none, (b) 0.1 M imidazole, (c) 1.5 mM β-mercaptoethanol, and (d) 0.1 M imidazole and 1.5 mM β-mercaptoethanol, at 23°C. Assay shows that the presence of 0.1 M imidazole (sample b) stimulates the enzymatic activity by about 20%, whilst with 1.5 mM β-mercaptoethanol (sample c), the loss of initial activity is 42%, and for the mixture of imidazole and β-mercaptoethanol (sample d), the loss of initial activity is 21%. These results suggest that imidazole partially prevents the inactivation of LADH by β-mercaptoethanol.

Further studies of the stability of these four samples against heat were carried out by incubating them at 70°C. The results are shown in Fig. 4.3. By comparison of the relative loss of initial activity of each sample, it can be seen that imidazole accelerates the heat inactivation of LADH, whilst β-mercaptoethanol protects the enzyme from heat inactivation. The accelerated rate of heat inactivation of LADH by imidazole is slowed down when β-mercaptoethanol is present. It, therefore, may be concluded that β-mercaptoethanol and imidazole bind to the same site of LADH.

Effect of Mercaptoacetic Acid and 3-mercaptopropionic Acid

The strong observed inhibition of LADH by β-mercaptoethanol prompted a study of the effect of other thiol compounds. Fig. 4.4 and 4.5 illustrate the inhibition of LADH by mercaptoacetic acid, and Fig. 4.6 by 3-mercaptopro-
Fig. 4.3. Effect of imidazole on the inhibition of LADH by β-mercaptoethanol. 1.54 μM LADH were incubated at 70°C in a 1.02 ml solution containing 0.1 μ phosphate buffer, pH 7.3 and (O) control, (X) 0.1 M imidazole, (△) 1.5 mM β-mercaptoethanol, (Θ) 1.5 mM β-mercaptoethanol + 0.1 M imidazole. The enzymatic activity of each sample at 20°C was first determined before heating to 70°C.
Fig. 4.4. Inhibition of LADH by mercapto-acetic acid. Experiments were carried out at 23°C by adding a final concentration of 1.57 μM LADH to 1.02 ml 0.1 M phosphate buffer, pH 7.3 containing mercapto-acetic acid of: (a) 14.4 mM, (b) 28.8 mM, (c) 72.0 mM and (d) 144 mM.
Effect of mercaptoacetic acid concentration on inactivation of LADH. Data were obtained from Fig. 4.4 after incubating LADH with various concentrations of mercaptoacetic acid at 23°C and pH 7.3 for 25 minutes.
Fig. 4.6. Inhibition of LADH by 3-mercaptopropionic acid. Conditions were similar to that of Fig. 4.2, except that 3-mercaptopropionic acid was used instead of β-mercaptoethanol.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_{E,I}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Mercaptoethanol</td>
<td>2.2</td>
</tr>
<tr>
<td>Mercaptoacetic acid</td>
<td>107.0**</td>
</tr>
<tr>
<td>3-mercapto-propionic acid</td>
<td>44.5</td>
</tr>
</tbody>
</table>

* The concentration of LADH used was 1.57 μM.

** The value was obtained after incubating LADH in mercaptoacetic acid for 25 min.
pionic acid. By contrast, the results show that although both compounds inactivate LADH, mercaptoacetic acid gives a different degree of inhibition.

The inactivation rate of LADH by 3-mercaptopropionic acid is rapid and no further loss of activity occurs over a period of about 24 hours. In contrast, the inactivation rate of LADH by mercaptoacetic acid is time-dependent; the loss of enzymatic activity is complete after about 20 minutes and thereafter no further loss of activity is observed (Fig. 4.4). The dissociation constants for the binary complexes of LADH with either mercaptoacetic acid or 3-mercaptopropionic acid under the experimental conditions are shown in Table (4.1).

(B) Fluorescence Studies

Effect of β-Mercaptoethanol and 3-Mercaptopropionic Acid on LADH-NADH Fluorescence Emission Spectra

On irradiation by light at 350 mμ, NADH in 0.1 μ phosphate buffer, pH 7.3 at 23.5°C exhibits fluorescence with an emission at 450 mμ. When NADH is bound to LADH, the intensity of NADH fluorescence is enhanced and the emission maximum is shifted to 445 mμ (Fig. 4.7).

Addition of either β-mercaptoethanol, or 3-mercaptopropionic acid, to the solution of LADH and NADH in 0.1 μ phosphate buffer, pH 7.3 at 23.5°C, rapidly quenches the fluorescence of LADH-NADH, whereas the presence of either 20 mM β-mercaptoethanol or 0.15 M 3-mercaptopropionic acid exerted no effect on the fluorescence of free NADH (6.8 μM) over a period of about 30 minutes.

Quenching of LADH-NADH fluorescence by β-mercaptoethanol is shown in Fig. 4.7, and by 3-mercaptopropionic acid, in Fig. 4.8.

Quenching of LADH-NADH fluorescence by either β-mercaptoethanol, or 3-mercaptopropionic acid, was found to be pH-dependent. Although the enhanced
Fig. 4.7. Fluorescence emission spectra for NADH, LADH and β-mercaptoethanol at pH 7.3. 3.0 ml solution at 23.5°C containing 0.1 μM phosphate buffer, pH 7.3 and (a) 0.92 μM LADH + 6.8 μM NADH; (b) 6.8 μM NADH; (c) 6.8 μM NADH + 20 mM β-mercaptoethanol; (d) 6.8 μM NADH + 0.92 M LADH + 17.9 mM β-mercaptoethanol; (e) none; (f) 1.38 μM LADH; (g) 1.38 μM LADH + 20 mM β-mercaptoethanol.

Excitation = 350 μm.
Fig. 4.8. Fluorescence emission spectra for LADH, NADH and 3-mercaptopyruvic acid in 0.1 M phosphate buffer, pH 7.3 and 23.5°C. (a) 1.72 μM LADH + 6.3 μM NADH; (b) 6.3 μM NADH; (c) 6.3 μM NADH + 0.15 M 3-mercaptopyruvic acid; (d) 1.72 μM LADH + 6.3 μM NADH + 0.15 M /-propionic acid; (e) 0.15 M 3-mercaptopyruvic acid; (f) 1.72 μM LADH + 0.15 M 3-mercaptopyruvic acid.

Excitation = 350 mμ
fluorescence of LADH-NADH in 0.05 M glycine-NaOH buffer with a pH over 9.2 is still quenched by β-mercaptoethanol, the fluorescence emission spectrum shifts to a longer wavelength which is characteristic of free NADH fluorescence spectrum (Fig. 4.9), suggesting that the binding of NADH to LADH is replaced by β-mercaptoethanol. The enhanced fluorescence of LADH-NADH at pH 9.2 is also quenched by the addition of 3-mercaptopropionic acid, unlike β-mercaptoethanol, and the fluorescence is still lowered than that of free NADH (Fig. 4.10).

Quenching of the LADH-NADH fluorescence caused by either β-mercaptoethanol or 3-mercaptopropionic acid can be studied quantitatively. Typical quenching curves resulting from titration of LADH-NADH at 23.5°C with β-mercaptoethanol at pH 7.3 are shown in Fig. 4.11, and pH 9.2 and 9.7 in Fig. 4.12. The titration curves with 3-mercaptopropionic acid at pH 7.3 and 9.2, and at 23.5°C, are shown in Figs. 4.13 and 4.14, respectively.

It should be noted that the titration curves with thiol compounds of either LADH in the excess of NADH, or NADH in the excess of LADH, do not show a significant difference; once the maximum quenching is attained, further additions of thiol compounds do not cause any other significant quenching of the fluorescence of LADH-NADH. However, the data obtained/shown a discrepancy in stoichiometry with the number of coenzyme molecules bound per mole of enzyme.

DISCUSSION

The characteristic optical properties of the binding between LADH and NADH have proved to be very fruitful in the study of complex formation. The inhibition of LADH by β-mercaptoethanol presents an interesting model compound for further study. Although both LADH and YADH catalyse the oxidation of alcohol to aldehyde in the presence of NAD⁺, a strong inhibitory effect on LADH
Fig. 4.9. Fluorescence emission spectra for LADH, NADH and β-mercaptoethanol in 0.05 M glycine-NaOH buffer, pH 9.2 and 9.7. (a) 0.92 μM LADH + 6.8 μM NADH at pH 9.2; (b) same as (a) except at pH 9.7; (c) 0.92 μM LADH + 6.8 μM NADH + 2.15 mM β-mercaptoethanol at pH either 9.2 or 9.7; (d) 6.8 μM NADH at both pHs; (e) 6.8 μM NADH + 3.22 mM β-mercaptoethanol at both pHs; (f) 0.92 μM LADH at both pHs; (g) 0.05 M glycine-NaOH at both pHs. Excitation = 350 μm.
Fig. 4.10. Fluorescence emission spectra for LADH, NADH and 3-mercapto-
propionic acid in 0.05 M glycine-NaOH, pH 9.2 at 23.5°C.
(a) 6.3 μM NADH + 1.7 μM LADH; (b) 6.3 μM NADH; (c) 6.3 μM
NADH + 3-mercaptopropionic acid; (d) 1.7 μM LADH + 6.3 μM NADH
+ 74.1 mM 3-mercaptopropionic acid.
Excitation = 350 mμ
Fig. 4.11. Effect of \( \beta \)-mercaptoethanol concentration on the quenching of the fluorescence of the binary NADH-LADH complex at pH 7.3, and 23.5°C. Aliquots of \( \beta \)-mercaptoethanol were added to a 1 cm cuvette containing 3.0 ml of 0.1\( \mu \) phosphate buffer, pH 7.3, 6.8\( \mu \)M NADH and 0.92\( \mu \)M LADH.

Excitation = 350 m\( \mu \)
Fig. 4.12. Titration curve for the quenching of the fluorescence of NADH-LDH by β-mercaptoethanol at pH either 9.2 or 9.7. Conditions were similar to that of Fig. 4.11 except buffer of 0.05 M glycine-NaOH, pH 9.2 (ο) or 9.7 (φ) was used instead of 0.1μ phosphate buffer.
Excitation = 350 mμ
Fig. 4.13. Titration curve for the quenching of the fluorescence of LADH-NADH by 3-mercaptopropionic acid at pH 7.3 and 23.5°C. 6.3 μM NADH + 1.7 μM LADH in 0.1 M phosphate buffer, pH 7.3 were used.

Excitation = 350 μm
Fig. 4.14. Titration curve for the quenching of the fluorescence of LADH-NADH by 3-mercaptopropionic acid. 6.3 \mu M NADH + 1.7 \mu M LADH in 0.05 M glycine-NaOH pH 9.2 at 23.5°C was titrated with 3-mercaptopropionic acid. \textit{Excitation} = 350 \text{ nm}.
is shown by β-mercaptoethanol, but this compound has no effect on YADH, indicating that the mechanism of catalysis may not be the same for both enzymes.

β-Mercaptoethanol, but not mercaptoacetic acid or 3-mercaptopropionic acid, can serve as a substrate for both LADH and YADH, thus suggesting that β-mercaptoethanol exhibits two different reactions toward LADH. Action as a substrate must be due to the presence of the CH$_2$/OH-group in the β-mercaptoethanol, whilst the inhibitory effect may be due to the binding of the SH-group to LADH which hinders the catalytic function. The locus of imidazole binding has been suggested to be enzymatic zinc atom (Theorell and McKinley-McKee, 1961). Imidazole protects LADH from β-mercaptoethanol inactivation, indicating that the SH-group of β-mercaptoethanol may be bound to the enzymatic zinc ions. The sulphhydryl group favours the linkage with zinc atom as has also been reported by Agren and Schwarzenbach (1955). Other evidence which adds to the strength of this suggestion is that the irreversibility shown upon the dilution of LADH-β-mercaptoethanol mixture for the assay solution for determining the residual activity, does not result in free dissociation, but inactivation is reversed by dialysis, indicating a more covalent-like interaction between thiol compounds and LADH.

The dissociation constants obtained for binary complexes of LADH with thiol compounds show that the order of tightness of binding to LADH is: β-mercaptoethanol > 3-mercaptopropionic acid > mercaptoacetic acid. The preferential substrate binding to a hydrophobic slot of LADH has been reported by Winer and Theorell (1960); Shore and Theorell (1966); and Sigma (1967). The present results show that there is a more than 2-fold difference in the dissociation constants for 3-mercaptopropionic acid and mercaptoacetic acid.
Thus, the binding of thiol compounds to LADH is also probably stabilized by the same hydrophobic binding site of the enzyme. Also the presence of an OH-group in thiol compounds causes a greater degree of inhibition for LADH than the presence of a COOH-group.

Several mercaptans have been found to react non-enzymatically with NAD⁺ to form addition products (van Eys, et al., 1957, 1958a; Colowick, et al., 1966). The presence of certain dehydrogenases catalyses these addition reactions. In view of these findings, two possibilities may be responsible for quenching of LADH-NADH fluorescence by thiol-compounds: (a) the formation of ternary LADH-NADH-thiol compounds complexes; and (b) breaking up the chromophore conjugation of NADH by thiol compounds. A mixture of 6.8 μM NADH with either 0.02 M β-mercaptoethanol, or 0.15 M 3-mercaptopropionic acid, does not significantly change the fluorescence of free NADH. However, in the presence of LADH, quenching of LADH-NADH fluorescence by thiol compounds is rapid. This result strongly suggests that LADH catalyses the formation of addition products between NADH and thiol compounds, and results in the breaking-up of the chromophore conjugation of nicotinamide moiety of NADH.

Quenching of LADH-NADH fluorescence by thiol compounds is also found to be pH-dependent. On the addition of β-mercaptoethanol at a pH higher than 9.0, the enhanced fluorescence of LADH-NADH is quenched to give a spectrum which is characteristic of free NADH. This result suggests that the coenzyme bound to LADH is replaced by β-mercaptoethanol.

LADH does not undergo inhibition by all thiol compounds as it is found that 0.05 - 0.1 M thioacetamide does not significantly affect the activity of LADH.
CHAPTER 5

COLD SENSITIVE INACTIVATION OF LADH AND YADH
5. INTRODUCTION

Native protein molecules are known to be folded into well-defined, usually essentially-rigid, three-dimensional structures. Increasing evidence has also shown that many proteins are composed of more than one polypeptide chain (Kauzmann, 1959; Koshland et al., 1960; Schachman, 1963; Reithel, 1963; Laaks et al., 1964; Sund and Weber, 1966; Joly, 1965; Tanford, 1968). As a result, there is much current interest in the nature of the intra- and inter-molecular bonds which stabilize the protein interactions and maintain it in an enzymatically-active form, and also about the possible role of the individual subunits in biological control.

From kinetic studies of coenzyme binding, and the formation of ternary complexes of enzyme, coenzymes and inhibitors, it is well-established that LADH and YADH contain two and four active sites (Sund and Theorell, 1963; McKinley-McKee, 1964; Sund, 1968). More evidence for the existence of two polypeptide chains for LADH, and four for YADH, have been accumulated from the peptide mapping and sequencing of labelled peptides (Li and Vallee, 1964b; Harris, 1964), end-group analysis (Jörvall, 1965, 1967) and x-ray crystallography (Bräden, 1965; Bräden et al., 1965).

YADH and LADH have also been shown to contain 4 gram atoms of zinc each per molecule of protein (Vallee and Hoch, 1955b; Wallenfels et al., 1957; Akeson, 1964; Oppenheimer et al., 1967; Drum et al., 1969; Sandler and McKay, 1969). Zinc atoms in both enzymes are essential for structural stabilization and catalytic function (Snodgrass, et al., 1960; Kägi and Vallee, 1960; Akeson, 1964; Drum et al., 1967). Kinetic studies by means of zinc-chelating agents have shown that ortho-phenanthroline and 2,2'-bipyridine have two binding sites per molecule of LADH (Vallee and Coombs,
1959; Yonetani, 1963a; Sigman, 1967). These chelating agents competitively inhibit the enzyme by interfering with coenzyme binding (Vallee et al., 1959). Treatment of both dehydrogenases with low pH results in loss of enzymatic activity, removal of enzyme-bound zinc and quenching of the protein fluorescence, but loss of enzymatic activity is faster than the removal of zinc atoms (Vallee, 1960; Akeson, 1964; Oppenheimer et al., 1967; Drum et al., 1969). Moreover, two zinc atoms per molecule of LADH can be more easily exchanged with \( {\text{\textsuperscript{65}Zn}}^{2+} \) than the other two zinc atoms (Druyan and Vallee, 1964; Drum et al., 1969).

Non-competitive inhibition of YADH and LADH by low concentrations of urea, without concomitant change of protein structure has also been demonstrated by Rajagopalan et al. (1961) and Brand et al. (1962), whilst on treatment with high concentrations of urea, in addition to the loss of enzymatic activity, YADH (Sund, 1960; Ohta and Ogura, 1965) and LADH (Drum et al., 1967; Pietruszko et al., 1969a) are dissociated into subunits. YADH and LADH have also been found to be dissociated into inactive subunits by sodium dodecyl sulfate (Hersh, 1962; Blomquist et al., 1967) and guanidine-hydrochloride (Castellino and Barker, 1968; Butler et al., 1969; Green and McKay, 1969).

In an attempt to establish the nature of the chemical bonds which are responsible for the subunit interactions, investigations are described in this thesis of the effect of temperature and various effectors on the rate of inactivation of both LADH and YADH by various denaturating agents.

**Materials**

LADH and YADH were obtained from C.F. Boehringer and Son (Mannheim, Germany), NAD\(^+\), NADH and sodium dodecyl sulfate from Sigma Chemical Co. (U.S.A.); Urea was obtained from B.D.H. Chemicals and guanidine-hydrochloride from Eastman
Kodak Chemicals (U.S.A.). All other reagents were the best grades available.

Methods

Procedures for preparing the enzymes and assaying their concentration and activity are described in Chapters 2 and 3. Stock solutions of LADH were used within five days and stock solutions of YADH within three days. The enzymatic activity was assayed each day before carrying out any experiments. An appropriate concentration of the enzyme was added to the denaturing agent which had already been equilibrated to a preset temperature. The concentration of the enzyme and denaturant used are described in detail in the text, legends of figures or tables. Rates of inactivation of LADH were assayed by withdrawing aliquots (10-50 μl) at various time intervals up to at least one hour, and introduced to a 3.0 ml assay mixture which was made of 1.85 ml glycine-NaOH (0.1 M), 0.15 ml ethanol (1%) and 1.00 ml NAD⁺ (mg/ml) without containing any denaturating agent, and the enzymatic activity was assayed at 23.5°C.

Rates of inactivation of YADH were assayed under the same conditions for LADH, except that the assay mixture was made of 0.05 M glycine-NaOH buffer pH 8.6, 0.1 M ethanol and 0.42mM NAD⁺ in a total volume of 3.0 ml.

The enzyme was incubated in various denaturating agents at different temperatures, and the rates of inactivation were compared.

RESULTS

Effect of Temperature on the Inactivation of LADH by Urea, Guanidine-hydrochloride and Sodium Dodecyl Sulfate

The effect of temperature on inactivation of LADH by urea is illustrated in Fig. 5.1. The results show that the addition of 1.4 μM LADH to 0.1 μ phosphate buffer, pH 7.3 at 0°C, 11°C or 23°C over a period of 80 minutes caused
no appreciable loss of enzymatic activity. In contrast, incubation of the same enzyme concentration in 4.0 M urea and 0.1 μM phosphate buffer, pH 7.3 at 0°C, 11°C or 23°C shows that in most cases, the enzyme undergoes a rapid initial loss of activity which is followed by a prolonged and much more gradual inactivation (Fig. 5.1). The slowest inactivation rate occurs at 23°C and increases with decreasing the temperature.

The inactivation of the enzyme by urea is also found to be pH-dependent (Table 5.1). Incubating 1.4 μM LADH in 4.0 M urea at 0°C and at a pH range between 7.3 – 9.0, causes a loss of enzymatic activity which decreases with increasing pH. Over, or below, this pH range, the inactivation rate is faster again. By contrast, 72% of the initial activity is retained when 1.4 μM LADH was incubated in 4.0 M urea at pH 7.3 and 23°C for one hour (Fig. 5.1), and 54% is retained at pH 10.0 (Fig. 5.2). At 11°C, 66% of the activity is retained at pH 7.3 and 65% at pH 10.0. The temperature effect on inactivation rate of LADH by 4.0 M urea at pH 10.0 is found to be in the order of 0°C > 23°C > 11°C (Fig. 5.2). Here the maximum stability occurs at 11°C.

The effect of temperature on the inactivation of LADH by guanidine-hydrochloride and sodium dodecyl sulfate was also investigated. Fig. 5.3 shows that when LADH is incubated in 1.5 M guanidine-hydrochloride and 0.1 μM phosphate buffer, pH 7.3, the rate of inactivation is found to be increased at low temperatures. Unlike urea and guanidine-hydrochloride, the rate of inactivation of LADH by 7.0 x 10⁻⁴ M sodium dodecyl sulfate at pH 7.3 increases as the temperature gets higher (Fig. 5.4).

Sodium dodecyl sulfate has been shown to be the strongest denaturing agent for LADH, for it inactivates the enzyme with a very low concentration. The second strongest denaturing agent is guanidine-hydrochloride, and then
Fig. 5.1. Effect of temperature on the urea inactivation of LADH at pH 7.3.
A 1.02 ml reaction mixture containing 0.1 μM phosphate buffer, 4M urea, and 1.4 μM LADH was incubated at: (O) 23°C; (X) 11°C; (Δ) 0°C; (□) control at 23°, 11° and 0° C.
Fig. 5.2. Effect of temperature on the urea inactivation of LADH at pH 10.0. Incubation was carried out at: (○) 23°C; (X) 11°C; (△) 0°C; (□) control at 23°C, 11°C and 0°C. Other conditions were similar to those for Fig. 4.1, except that buffer of 0.1 M glycine-NaOH, pH 10.0 was used instead of 0.1 μM phosphate, pH 7.3.
### TABLE 5.1

Effect of pH on inactivation of LADH by urea

<table>
<thead>
<tr>
<th>pH</th>
<th>Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>6.5</td>
<td>57</td>
</tr>
<tr>
<td>7.3</td>
<td>68</td>
</tr>
<tr>
<td>8.0</td>
<td>69</td>
</tr>
<tr>
<td>9.0</td>
<td>73</td>
</tr>
<tr>
<td>10.0</td>
<td>64</td>
</tr>
</tbody>
</table>

*Inactivation was carried out by adding 1.40 μM LADH to 4.0 M urea at 0°C in the different buffers. Buffers pH 6.5 - 8.0 were made of 0.1 M phosphate, whilst for pH 9.0 - 10.0, 0.1 M glycine-NaOH was used. 50 μl of aliquots were withdrawn and introduced into the assay mixtures for measuring the activity.
Fig. 5.3. Effect of temperature on the inactivation of LADH by guanidine-hydrochloride. A final concentration of 1.3 μM LADH was incubated in 1.02 ml 0.1 M phosphate buffer, pH 7.3 containing 1.5 M guanidine-hydrochloride at (o) 23°C; (X) 11°C; (△) 0°C.
Fig. 5.4. Effect of temperature on the inactivation of LADH by sodium dodecyl sulfate. Inactivation was carried out by adding a final concentration of 1.4 μM LADH to 1.02 ml phosphate buffer, pH 7.3, containing 7 x 10^{-4} M sodium dodecyl sulfate at (O) 23°C; (X) 11°C; (A) 0°C.
comes urea. However, in all cases, the inactivation rate proceeds with an initial rapid loss of the activity and then follows a much slower process. LADH is known to be non-competitively inhibited by a concentration of urea below 4.0 M (Rajagopalan et al., 1961; Brand et al., 1962). With a concentration of 8.0 M urea, LADH is dissociated into subunits (Drum et al., 1967; Pietruszko et al., 1969a). It has also been demonstrated that LADH is dissociated into subunits by 5.0 - 6.0 M guanidine-hydrochloride (Castellino and Barker, 1968; Butler et al., 1969; Green and McKay, 1969), and by 5m M sodium dodecyl sulfate (Blomquist et al., 1967). When guanidine-hydrochloride concentration is lower than 1.0 M, the enzyme is also found to be competitively inhibited by the guanidium ion with NAD\textsuperscript{+}, and mixed inhibition occurs with ethanol (Green and McKay, 1969). In light of these results and since the inactivation process by these denaturating agents is found to be time-dependent, the rapid loss of the initial activity may involve both an inhibitory effect and a dissociation process. With urea and guanidine-hydrochloride, the dissociation is faster at low temperature. Upon inactivation of LADH by urea or guanidine-hydrochloride at 0\textdegree C for 30 minutes, the activity loss cannot be recovered either by warming up to 23\textdegree C or through dilution. Also, the activity cannot be recovered after the enzyme is inactivated by sodium dodecyl sulfate or guanidine-hydrochloride under the conditions examined.

**pH Stability of LADH**

The stability of LADH was investigated by treating the enzyme with various pHs at 23\textdegree C. Aliquots were withdrawn for assaying the enzymatic activity at various time intervals up to a period of at least one hour. A graph of the initial rate of inactivation against pH is shown in Fig. 5.5. The enzyme is found to be stable in a pH range between 6.7 and 10.0. On exposure of LADH
Fig. 5.5. A graph of the initial rate of inactivation of LADH as a function of pH. 1.6 μM LADH were incubated with 1.02 ml buffer solution of different pHs at 25°C. pHs 5.3 - 8.0 were made of 0.1 μM sodium phosphate, whilst pH below 5, 0.05 M glycine-H₃PO₄ was used and pH above 8.5, 0.1 M glycine-NaOH.
to this pH-range at 23°C over a period of three hours, there is negligible loss of activity, whilst below, or above, this pH-range, the rate of inactivation is markedly increased. On exposure of the enzyme to pH lower than 4.0 or higher than 12.0, the loss of enzymatic activity is so rapid that no activity can be detected after a few minutes. In all cases, the rate of inactivation by low or high pH is also found to be rapid at the first 20 minutes, and this is then followed by a much slower loss of activity.

**Effect of Temperature on the Inactivation of LADH and YADH by Extremes of pH**

Table 5.2 shows that the inactivation rate of LADH by extremes of pH is also temperature-dependent. The loss of LADH activity by low pH increases with a lowering of the temperature. Thus, on exposure of 1.50 μM LADH to pH 4.3 at 0°C for 20 minutes, only 18% of the initial activity is retained; at 23°C, this value is 32%. With high pH inactivation, the opposite effect is found with changes in the temperature, and the rate of inactivation is found to be higher with increase in the temperature. When 1.50 μM LADH is treated with 0.1 M glycine-NaOH pH 11.7 at 23°C for 20 minutes, the enzymatic activity is completely lost, but under the same conditions, at 0°C, 62% of the initial activity is still retained.

The effect of temperature on inactivation of YADH by acid and alkaline pH was also investigated. Table 5.3 shows the results. Unlike LADH, the rate of inactivation by high or low pH is found to be increased with increasing temperature. High concentrations of YADH protect the enzyme against alkaline inactivation either at 0°C or 23°C a little (4-8%).

In contrast, YADH is less stable at alkaline pH than LADH but is more stable at acid pH. The loss of YADH activity is complete and rapid by exposing to pH above 11.0 or below 3.5. For LADH, the range of instability is
TABLE 5.2

Effect of temperature on the inactivation of 1.5 μM LADH by extremes of pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>0.1 M phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.3</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>98</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 11.3</td>
<td>0</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>54</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 11.7</td>
<td>0</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>0.1 M Glycine-H$_3$PO$_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 4.6</td>
<td>0</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>87</td>
</tr>
<tr>
<td>0.1 M Glycine-H$_3$PO$_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 4.3</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>4.1</td>
</tr>
</tbody>
</table>
TABLE 5.3

Effect of temperature on the inactivation of YADH by extremes of pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>0.1 μ phosphate</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>pH 7.3</td>
<td>23</td>
<td>95</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>pH 10.0</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>pH 10.0</td>
<td>23</td>
<td>34</td>
</tr>
<tr>
<td>0.1 M Glycine-H₃PO₄</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>pH 4.3</td>
<td>23</td>
<td>69</td>
</tr>
<tr>
<td>0.1 M Glycine-H₃PO₄</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>pH 3.6</td>
<td>23</td>
<td>16</td>
</tr>
</tbody>
</table>

* 0.16 mg/ml of YADH was inactivated by various pH.

10 μl of aliquots were withdrawn and introduced to assay mixtures for determining the activity.

** 0.32 mg/ml of YADH was used.
above pH 12.0 or below pH 4.0. These results may indicate that the stability of both enzymes against pH is correlated to their isoelectric points, as YADH is known to have a lower isoelectric point, pH 5.4-5.8 (Hayes and Velick, 1954; Keleti, 1958; Wallenfels and Arens, 1960) than LADH, pH 6.8 (Dalziel, 1958). Loss of enzymatic activity by low pH has been demonstrated to be associated with the removal of enzyme-bound zinc (Vallee, 1957; Kägi and Vallee, 1960; Akeson, 1964; Drayan and Vallee, 1964; Oppenheimer, 1967; Drum et al., 1967, 1969). The inactivation of LADH and YADH by extremes of pH appear to involve complex structural changes, including probably changes in conformation as well as dissociation and aggregation (Chapter 8).

**Effect of Glycerol on the Urea Inactivation of LADH and YADH**

Glycerol is found to be a poor substrate of both LADH and YADH. In the presence of 0.05 M glycerol in the assay mixtures (without ethanol), NAD\(^+\) is reduced to NADH at a slow rate when LADH or YADH is added.

Adding 1.4 μM LADH to 0.1 μ phosphate buffer, pH 7.3 containing 4.0 M urea at 0°C, or 6.0 M urea at 23°C, the rate of inactivation in both cases is greatly hindered by the presence of 25% glycerol (v/v). The results are summarized in Table 5.4. The greater protective effect against urea inactivation by glycerol is found when a higher glycerol concentration is used. However, the initial rapid loss of enzymatic activity is not completely arrested by glycerol, but after incubating for a period of about 10 minutes, thereafter very little loss of enzymatic activity occurs when 25% of glycerol (v/v) is present.

YADH in 0.1 μ phosphate buffer, pH 7.3 at 2°C is found to be stable over a period of two or three days, and usually less than 15% of the initial
TABLE 5.4

Effect of glycerol on the inactivation of LADH by urea

<table>
<thead>
<tr>
<th>Medium*</th>
<th>Temperature (°C)</th>
<th>Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>6.0 M urea</td>
<td>23</td>
<td>58</td>
</tr>
<tr>
<td>6.0 M urea + 10% glycerol (v/v)</td>
<td>23</td>
<td>65</td>
</tr>
<tr>
<td>6.0 M urea + 25% glycerol (v/v)</td>
<td>23</td>
<td>67</td>
</tr>
<tr>
<td>4.0 M urea</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>4.0 M urea + 10% glycerol (v/v)</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>4.0 M urea + 25% glycerol (v/v)</td>
<td>0</td>
<td>78</td>
</tr>
</tbody>
</table>

*1.4 μM LADH was inactivated by 0.1μ phosphate buffer pH 7.3 containing various media.
activity is lost after standing at 23°C for 60 minutes. The effect of temperature on urea inactivation of YADH is shown in Fig. 5.6. The enzyme (0.58 mg/ml) was incubated in 0.1 M phosphate buffer, pH 7.3 containing 3.0 M urea either at 23°C or 0°C for various lengths of time. Assays of the activity were performed at 23.5°C. The inactivation rate is also found to be faster at 0°C than 23°C (Fig. 5.6). After incubating the enzyme in 3.0 M urea at pH 7.3 and 23°C for 50 minutes, 34% of the initial activity is retained, while this value is 21% at 0°C. As shown in Fig. 5.6, glycerol protects YADH against urea inactivation at both temperatures. In the presence of 10% glycerol (v/v), after 50 minutes of incubating the enzyme in 3.0 M urea at pH 7.3, the initial activity retained is 68% at 23°C and 57% at 0°C. The inactivation process is also found to be rapid for the first 20 minutes and is then followed by a slower process.

Early investigations have shown that the loss of enzymatic activity by a urea concentration lower than 2.0 M is due to reversible, non-competitive inhibition (Rajagopalan et al., 1961; Brand et al., 1962; Ohta and Ogura, 1965). If the urea concentration exceeds 2.0 M, the enzyme is dissociated into subunits (Sund, 1960; Ohta and Ogura, 1965; Butler et al., 1969). After exposing the enzyme to 3.0 M urea at 0°C for 30 minutes neither warming up the temperature from 0°C to 23°C, nor diluting the inactivated enzyme, results in regaining the enzymatic activity under the conditions examined. The inactivation process suggests that the loss of enzymatic activity by 3.0 M urea, is not only due to inhibitory effect, but also the dissociation is occurred as a time- and temperature-dependent manner.

In contrast, YADH is less stable than the corresponding enzyme from horse liver, becoming inactivated at lower urea concentrations. The greater
Fig. 5.6. Effect of temperature and glycerol on the usea inactivation of YADH. YADH (0.58 mg/ml) was incubated in 0.1 M phosphate buffer, pH 7.3, containing (○) 3 M urea, at 23°C; (△) same as (○) except at 0°C; (△) 3 M urea + 10% (v/v) glycerol, at 23°C; (△) same as (○) except at 0°C; (○) control.
lability of yeast enzyme is reasonable in view of its higher molecular weight and greater substrate and coenzyme specificity (see reviews by Sund and Theorell, 1963; McKinley-McKee, 1964).

**Effect of LADH Concentration on the Urea Inactivation**

Examination of the effect of LADH concentration on the rate of inactivation by 6.0 M urea in 0.1M phosphate buffer, pH 7.5 at 23°C, reveals that the loss of enzymatic activity is greatly retarded by high concentrations of LADH. The time course of inactivation of LADH with several concentrations of 6.0 M urea inactivation is shown in Fig. 5.7. The treatment of 1.6 μM LADH with 6.0 M urea for 60 minutes, results in only 28% retention of the initial activity. If 16.0 μM was used, this value would be 75%. The data show that the enzyme concentration influence not only the rate but also the extent of inactivation of the enzyme. This dependence of the enzyme concentration may suggest that high concentrations facilitate protein-protein interactions which can, however, prevent the action of urea.

**Effect of Coenzyme and Ethanol on the Inactivation of LADH by Urea**

The rate of inactivation of LADH by urea at 0°C, 11°C and 23°C is also profoundly affected by the presence of coenzyme and ethanol. As shown in Table 5.5, the addition of 140 μM to the different inactivating media, causes markedly less inactivation than occurs when the urea was used alone. NADH is well-known to form a binary complex with enzyme, (Bonnichsen and Theorell, 1951). This binary complex not only induces the change in absorption spectra (Boyer and Theorell, 1956), but also the conformation of the protein molecule (Ulmer et al., 1961; Li and Vallee, 1964a; Rosenberg, et al., 1965). It also exercises its protective effect against inactivation by heat and acid (Yonetani and Theorell, 1962; Drum, et al., 1969) and thiol-reagents (Li and Vallee, 1965). It is reasonable that tighter binding of the coenzyme to LADH prevents
Fig. 5.7. Effect of the concentration of LADH on the urea inactivation. A 1.02 ml reaction mixture containing 0.1 M phosphate buffer, pH 7.3, 6 M urea and LADH of (△) 1.6 mM; (○) 4.0 mM; (Δ) 8.0 mM; and (□) 16 mM was incubated at 23°C.
TABLE 5.5

Effect of coenzyme on the inactivation of LADH by urea

<table>
<thead>
<tr>
<th>Medium*</th>
<th>Temperature (°C)</th>
<th>Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>4.0 M urea</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>4.0 M urea + 1.4 μM NADH</td>
<td>0</td>
<td>89</td>
</tr>
<tr>
<td>4.8 M urea</td>
<td>11</td>
<td>65</td>
</tr>
<tr>
<td>4.8 M urea + 1.4 μM NADH</td>
<td>11</td>
<td>83</td>
</tr>
<tr>
<td>6.0 M urea</td>
<td>23</td>
<td>58</td>
</tr>
<tr>
<td>6.0 M urea + 1.4 μM NADH</td>
<td>23</td>
<td>79</td>
</tr>
</tbody>
</table>

*Inactivation was carried out by adding 1.4 μM LADH to different media at pH 7.3.
### TABLE 5.6

Effect of substrate on the inactivation of LADH by urea

<table>
<thead>
<tr>
<th>Medium*</th>
<th>Temperature (°C)</th>
<th>Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>4.0 M urea</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>4.0 M urea + 0.42 M ethanol</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>4.0 M urea + 0.85 M ethanol</td>
<td>0</td>
<td>84</td>
</tr>
<tr>
<td>4.0 M urea + 1.70 M ethanol</td>
<td>0</td>
<td>79</td>
</tr>
<tr>
<td>4.8 M urea</td>
<td>11</td>
<td>65</td>
</tr>
<tr>
<td>4.8 M urea + 0.42 M ethanol</td>
<td>11</td>
<td>73</td>
</tr>
<tr>
<td>4.8 M urea + 0.85 M ethanol</td>
<td>11</td>
<td>79</td>
</tr>
<tr>
<td>4.8 M urea + 1.70 M ethanol</td>
<td>11</td>
<td>72</td>
</tr>
<tr>
<td>6.0 M urea</td>
<td>23</td>
<td>58</td>
</tr>
<tr>
<td>6.0 M urea + 0.42 M ethanol</td>
<td>23</td>
<td>58</td>
</tr>
<tr>
<td>6.0 M urea + 0.85 M ethanol</td>
<td>23</td>
<td>64</td>
</tr>
<tr>
<td>6.0 M urea + 1.70 M ethanol</td>
<td>23</td>
<td>38</td>
</tr>
</tbody>
</table>

*1.4 μM LADH was added to 0.1 μ phosphate buffer pH 7.3 containing different medium.
dissociation by urea.

The urea inactivation of LADH is also protected by certain concentrations of ethanol (Table 5.6). The strongest protection is found with the addition of 0.85 M ethanol in the inactivating medium at the three different temperatures tested. However, the inactivation by urea is stimulated by the presence of 1.70 M ethanol. The addition of LADH to an ethanol concentration over 1.70 M also causes the enzyme to show a loss of activity. These results may suggest that the enzyme is stabilized by a certain concentration of ethanol, while high concentrations of ethanol accelerate the inactivation.

The protective effect of ethanol is interesting as it has been suggested that the enzyme is unable to bind the substrate prior to the formation of the binary LADH-NADH complex (McKinley-McKee, 1963; Dalziel, 1963b). Ethanol has also been found to influence the enzyme stability against heat inactivation (Grisolia and Joyce, 1959, 1960). It is not clear whether the stabilizing effect of the substrate is similar to that of the coenzyme, and can be attributed to the formation of an enzyme-substrate complex which stabilizes the configuration at the active site.

**Effect of Imidazole and ZnCl₂ on the Urea Inactivation of LADH**

Addition of 0.1 M imidazole to 1.3 μM LADH at pH 7.3 shows that the enzymatic activity is increased by about 19-22%, and no further change of this activity occurs over a period of 24 hours. Activation of LADH by imidazole has also been reported by Theorell and McKinley-McKee (1962). This effect has been explained by the properties of the formation of ternary enzyme-coenzyme-imidazole complexes. The locus of imidazole-binding is the enzyme-bound zinc.

The inactivation of LADH by urea is effectively arrested by 0.1 M imidazole. The data show that when 1.3 μM LADH in 6.0 M urea at 23°C (Fig. 5.8) and 4.0 M
urea at 0°C (Fig. 5.9) are incubated for 60 minutes, the loss of the initial activity is found to be 72% and 83%, respectively. Addition of 1.3 μM LADH to the incubating medium containing 0.1 M imidazole and either 6.0 M urea at 23°C or 4.0 M urea at 0°C, still results in about 14% activation in both cases, and thereafter, less than 15% of the initial activity is lost over a period of 60 minutes.

Zn^{2+} ion is found to be a strong inhibitor of LADH. The addition of $1.0 \times 10^{-4}$ M ZnCl$_2$ to 1.3 μM LADH causes complete loss of enzymatic activity. But the activity can be recovered by dialyzing away the ZnCl$_2$, thus indicating that the ZnCl$_2$ does not induce the pronounced disruption of protein structure and the inhibitory effect is reversible. The inactivation of LADH by ZnCl$_2$ can also be prevented by the presence of 0.1 M imidazole. As shown in Fig. 5.8, the incubation of 1.3 μM LADH in 0.1 M imidazole and $1.0 \times 10^{-4}$ ZnCl$_2$ at pH 7.3, over a period of 60 minutes, causes only slight loss of the initial activity. The presence of $5.0 \times 10^{-5}$ M ZnCl$_2$ stimulates the inactivation of LADH by 6.0 M urea.

The inactivation of LADH by Zn$^{42+}$ ion has been suggested to be due to the interaction with thiol groups of the enzyme (Wallenfels et al., 1959; Witter, 1960). The protective effect of imidazole against inactivation of LADH by ZnCl$_2$ is easily understood, as imidazole is a well-known zinc complexing agent. Since the activation of LADH by imidazole is due to its interaction with enzymatic zinc ions, its protection against urea inactivation may also suggest that the action of urea is initiated from the enzymatic zinc. Furthermore, it might be assumed that the reactive zinc ion and thiol group are situated close to each other in the enzyme surface.

Effect of Salts on the Urea Inactivation of LADH

The influence of salts on the urea inactivation of LADH was studied.
Fig. 5.8. Effect of imidazole and ZnCl₂ on the urea inactivation of LADH at 23°C. Incubation was carried out at 23°C by adding a final concentration of 1.5 μM LADH to 1.02 ml 0.1 M phosphate buffer, pH 7.3, containing (Δ) 0.1 M imidazole; (■) 0.1 M imidazole + 1 x 10⁻⁴ M ZnCl₂; (△) 0.1 M imidazole + 6 M urea; (□) control; (×) 5 x 10⁻⁵ M ZnCl₂; (○) 6 M urea; (●) 6 M urea + 5 x 10⁻⁵ M ZnCl₂; (⊙) 1 x 10⁻⁴ M ZnCl₂.
Fig. 5.9. Effect of imidazole on the urea inactivation of LADH at 0°C. 1.3 μM LADH was incubated at 0°C in 0.1 M phosphate buffer, pH 7.3, containing (△) 0.1 M imidazole; (△) 0.1 M imidazole + 4 M urea; (□) control; (○) 4 M urea.
Table 5.7 shows that the presence of 0.1 M of ammonium sulfate, sodium sulfate, sodium citrate or sodium acetate protects the enzyme against 4.0 M urea inactivation at pH 7.3 and 0°C. Under the same conditions, the same concentration of sodium chloride and sodium nitrate strongly stimulates the rate of inactivation by urea. There are several indications that anions bind to active-site zinc atoms (Theorell et al., 1955; Plane and Theorell, 1961; Li et al., 1963; Sigman, 1967; Drum et al., 1969). The protection of certain neutral salts may indicate that the inactivation of LADH by urea is initially occurring at the enzymatic zinc atoms. Different neutral salts may have different effect on the inactivation of this enzyme by urea.

**Effect of Temperature on the Inactivation of LADH by Thiol-reagents**

Several investigations have shown that the content of thiol groups in LADH varies from 20 - 28 (Witter, 1960; Li et al., 1962; Yonetani and Theorell, 1962). Thiol reagents such as heavy atoms, $p$-chloromercuri-benzoate or sulphonate, iodoacetamide or iodoacetic acid inhibit the enzymatic activity. Substrates or coenzymes, ADP and ADPR provide little if any protection against the inhibition (Witter, 1960; Li et al., 1962; Yonetani and Theorell, 1962; Evans and Rabin, 1968). Iodoacetamide has also been shown to be a competitive inhibitor of LADH; a kinetic manifestation of the affinity of this agent for the active centre sites (Woronick, 1961). Followed by this finding two of the thiol groups have been further shown to be essential for catalytic function (Li and Vallee, 1963; 1964b; 1965; Harris, 1964; Evans and Rabin, 1968). Peptides containing those active centre residues have been isolated and their sequences characterized (Li and Vallee, 1964b; Harris, 1964).

The resulting effect of temperature on the inactivation of LADH by $p$-chloromercuri-benzoate, iodoacetamide and iodoacetate are given in Table 5.8.
**TABLE 5.7**

Effect of neutral salts on the inactivation of LADH by urea

<table>
<thead>
<tr>
<th>Addition*</th>
<th>Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>4.0 M urea</td>
<td>68</td>
</tr>
<tr>
<td>4.0 M urea + 0.1 M NaCl</td>
<td>48</td>
</tr>
<tr>
<td>4.0 M urea + 0.1 M NaNO₃</td>
<td>36</td>
</tr>
<tr>
<td>4.0 M urea + 0.1 M Na₂SO₄</td>
<td>86</td>
</tr>
<tr>
<td>4.0 M urea + (NH₄)₂SO₄</td>
<td>86</td>
</tr>
<tr>
<td>4.0 M urea + 0.1 M sodium citrate</td>
<td>87</td>
</tr>
<tr>
<td>4.0 M urea + 0.1 M sodium acetate</td>
<td>82</td>
</tr>
</tbody>
</table>

*1.7 µM LADH was inactivated at 0°C by 0.1 µ phosphate buffer pH 7.3 containing 4.0 M urea and various neutral salts.*
<table>
<thead>
<tr>
<th>Medium</th>
<th>Temperature (°C)</th>
<th>Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 min.</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>98</td>
</tr>
<tr>
<td>0.3 p-chloromercuribenzoate</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>56</td>
</tr>
<tr>
<td>2.5 Iodoacetate</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>54</td>
</tr>
<tr>
<td>2.5 Iodoacetamide</td>
<td>0</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>71</td>
</tr>
</tbody>
</table>

*The reaction mixtures contained 1.3 \(\mu\)M LADH, 0.1\(\mu\) phosphate buffer pH 7.3 and various thiol-reagents.*
\( \rho \)-Chloromercuri-benzoate inactivates the enzyme at a very rapid rate. After incubating 1.3 \( \mu \)M LADH in 3.0 x 10^{-4} \text{ M} \( \rho \)-chloromercuri-benzoate at pH 7.3 and 23°C or 0°C, measurements of enzymatic activity were immediately carried out, and the initial activity retained is 55% at 23°C and 63% at 0°C, and thereafter only increase the loss of enzymatic activity a little (Table 5.8). In contrast, the loss of activity at 23°C is only faster marginally (6 - 8%) than at 0°C. Although the inactivation of LADH by iodoacetamide and iodoacetate is found to be profoundly time-dependent, it is also greatly affected by temperature. As shown in Table 5.8, incubation of 1.3 \( \mu \)M LADH in 2.5 x 10^{-5} \text{ M} iodoacetamide at pH 7.3 and 0°C for 60 minutes, the initial activity retained is 82%, whilst it is 56% at 23°C. Under the same conditions for iodoacetamide, the inactivation of LADH by iodoacetate was carried out at 0°C and 23°C for 60 minutes, and the initial activity retained was 60% and 5% respectively.

**DISCUSSION**

The rate of inactivation of LADH by sodium dodecyl sulfate and various thiol reagents, such as \( \rho \)-chloromercuri-benzoate, iodoacetate acid and iodoacetamide, is faster at 23°C than at 0°C. However, this does not appear to be the case for all inactivating agents, for example, inactivation experiments of LADH and YADH by urea and guanidine-hydrochloride show that a greater stability of the enzyme occurs near room temperature than at 0°C. Using 4.0 M urea at pH 7.3, the rates of inactivation of LADH are: 0°C > 11°C > 23°C, and at pH 10.0, the order is: 0°C > 23°C > 11°C. After inactivating LADH or YADH by urea for 30 minutes, either at 0°C or 23°C, the loss of enzymatic activity cannot be recorded either by warming from 0°C to 23°C, or through dilution under the conditions examined.
The urea inactivation of LADH is also pH-dependent. In a pH range between 7.0 - 9.5, the loss of enzymatic activity decreases with increasing pH, while at pHs below or above this range, loss of enzymatic activity is again faster. It has been demonstrated that the inactivation of YADH and LADH by urea (Sund, 1960; Ohta and Ogura, 1965; Drum et al., 1967; Pietruszko et al., 1969a; Butler et al., 1969), and guanidine-hydrochloride (Castellino and Barker, 1968; Green and McKay, 1969) is associated with the dissociation of the enzymes into subunits. In light of these results, the fact that the rate of inactivation increases at low temperature is interesting. Precedents for this behaviour are cited by Kauzmann (1959); Némethy and Scheraga (1962) and Brandt (1964), who have suggested that of the different bonds postulated to maintain the protein structure, only hydrophobic bonds have properties of consistent with a decrease in stability at low temperature. Thus, the dissociation of LADH and YADH by urea or guanidine-hydrochloride is most likely to be occurring through the breakdown of hydrophobic bonds. Though sodium dodecyl sulfate is also found to dissociate LADH and YADH into subunits (Hersh, 1962; Blomquist, 1967), it does not show the similar temperature effect. This may indicate that sodium dodecyl sulfate does not proceed by the same dissociation process of these enzymes as urea and guanidine-hydrochloride.

Kinetic studies of the inactivation process of both YADH and LADH also suggest that a subunit equilibria is in existence. The inactivation process is time-dependent, and there is much effect of both enzyme and denaturant concentration on the extent of inactivation. Furthermore, the cold sensitivity to urea inactivation which occurs with threonine deaminase, has also been used by Changeux (1964) as indirect evidence for enzyme-subunit equilibria.
NADH and ethanol strongly protect the enzyme against urea inactivation. The protective effect of NADH is due to the fact that it forms a binary complex with the enzyme (Bonnichsen and Theorell, 1951) which induces the changes in protein conformation (Ulmer et al., 1961; Li and Vallee, 1964; Rosenberg et al., 1965), and this in turn makes the enzyme less sensitive to the inactivation by various denaturing agents. Whilst the protection shown by substrate is unusual, it has been suggested by McKinley-McKee (1963, 1964) that the substrate is unable to interact with the enzyme prior to the attachment of the coenzyme. Dalziel (1963b) also pointed out that there is no clear kinetically-significant evidence for the formation of binary enzyme-substrate complex. However, there are arguments against this suggestion (Sund and Theorell, 1963; Silverstein and Boyer, 1964; Yonetani and Theorell, 1964; Sigman, 1967). These authors showed that the mechanism did not proceed with a strictly compulsory order and LADH could form binary complex with ethanol and aldehyde. The influence of substrate against the inactivation rate by heat has also been reported by Grisolia and Joyce (1959, 1960) and Grisolia (1964). It is not clear whether this effect is parallel to the coenzyme protection by forming enzyme complexes, or other factors may involve.

The protection of the enzyme against inactivation is not restricted to the coenzyme and substrate, but is also shared by imidazole and certain salts, such as ammonium sulfate, sodium sulfate, sodium citrate and sodium acetate. The extent to which the inactivation rate is retarded by these compounds is striking; low concentrations (0.1 M) can stabilize the entire enzyme molecule against the inactivation by very high concentrations (4.0 - 6.0 M) of urea. There are several reports that the effect of imidazole and anions on LADH is
related to the interaction with the enzymatic zinc ions, i.e. largely in
the vicinity of the coenzyme binding site (Theorell et al., 1955; Winer
and Theorell, 1960; Theorell and Mc Kinley-McKee, 1961b,c; Li et al., 1963;
Sigman, 1967; Drum et al., 1969). These results may suggest that the
action of these denaturating agents is initiated at a specific region in
the enzyme molecule, namely, the enzymatic zinc ion. The interaction of
these compounds with the enzyme, shields this region which makes it in-
sensitive to the inactivation by various denaturating agents. ZnCl₂ is a
strong inhibitor of both enzymes. The inactivation of LADH by ZnCl₂ has been
suggested to be due to the interaction with sulphhydril residues of the enzymes
(Wallenfels et al., 1959; Witter, 1960). It has also been suggested that
the reactive sulphhydril groups are situated close to enzyme-bound zinc (Evans
and Rabin, 1968). The presence of ZnCl₂ accelerates the urea inactivation.
Thus, all available evidence indicates that the reactive sulphhydril residues
and the enzymatic zinc ion are correlated to each other, and that they both
may be located at, or near to, the hydrophobic regions of the enzyme molecule.

However, the mechanism by which these neutral salts alter the stability
of macromolecular conformations cannot be unequivocally defined at present.
Indeed, identical concentrations of various neutral salts can either serve as
structural stabilizers or destabilizers. And the identical concentrations of
different neutral salts of the same valence type have also different effects
on urea inactivation of LADH.

Glycerol is a poor substrate for LADH and YADH. It also affords pro-
found protection against the urea inactivation. The reason for its protective
effect is not clear. However, urea induces low temperature instability in
these stable enzymes. The protection of glycerol may parallel to its well-known effect in stabilizing labile and cold sensitive enzymes, such as carbamyl phosphate synthetase (Novoa and Grisolia, 1964), pyruvate carboxylase, (Utter et al., 1964), phosphorylase (Graves et al., 1965), and 17β-
hydroxysteroid dehydrogenase (Jarabak et al., 1966).

Besides the action of urea, guanidine-hydrochloride/sodium dodecyl
sulfate, causing the dissociation of the apoenzyme into inactive subunit forms, this effect has also been successfully brought about by extremes of pH in recent years (Hass and Lewis, 1963; Schlesinger and Barrett, 1965; Schwartz and Norecker, 1966; Sine and Hass, 1967; Jaenicke et al., 1968; Sia and Norecker, 1968). LADH is stable over a large pH range (6.7 - 10.0). The immediate and complete loss of enzymatic activity only occurs after exposing the enzyme to pH over 12.0, or below 4.0. YADH has a lower isoelectric point, pH 5.4 - 5.8 (Hayes and Velick, 1954; Keleti, 1958; Wallenfels and Arens, 1960) than LADH, pH 6.8 (Dalziel, 1958). Thus, YADH is more stable at low pH and less stable at high pH, than LADH, and there is a rapid loss of the YADH activity which occurs at pHs below 3.5 or above 11.0. The acid inactivation of both enzymes has been shown to be due to removal of enzymatic zinc ions (Vallee, 1957; Klüti and Vallee, 1960; Druyan and Vallee, 1964; Oppenheimer, 1967; Drum et al., 1967, 1969). Here it is found that both pH 10 and 3.9 - 4.3, YADH is inactivated faster at 23°C than at 0°C. With LADH, the same holds true at alkaline pH, but not at pH 4.3 - 4.6, where, as in the case of urea inactivation, enzymatic activity falls off more rapidly at 0°C than at 23°C.

The order of effectiveness in inactivating the enzyme is: sodium dodecyl
sulfate > guanidine-hydrochloride > urea. By comparison, the influence of
temperature on the inactivation rate of LADH and YADH by sodium dodecyl sulfate, guanidine-hydrochloride, urea or extremes of pH, suggests that their action towards the destabilisation of the protein molecules may not proceed all the same. It may also be noteworthy that certain substances such as imidazole and glycerol, protect the enzyme against urea inactivation, but do not show the same effect against heat inactivation.
CHAPTER 6

REVERSIBLE INACTIVATION OF LADH AND YADH
INTRODUCTION

In recent years, proteins have been considered to exist in their native form as highly ordered aggregates of polypeptide chains. The inactivation of a number of enzymes with urea, lauryl sulphate, guanidine-HCl and extremes of pH have been shown to involve dissociation into subunits (Kauzmann, 1959; Koshland et al., 1960; Schachman, 1963; Reithel, 1963; Lacks et al., 1964; Sund, 1964; Joly, 1965; Tanford, 1963). Freezing as a cause of dissociation has also been reported (Kirkman and Hendrickson, 1962). In some cases, p-chloromercuribenzoate or chelating agents like 1,10-phenanthroline or high salt concentrations can also dissociate enzymes into subunits (Kägi and Vallee, 1960; Antonini et al., 1962; Kirshner and Tanford, 1964). Studies on reversible dissociation have provided considerable information about the interaction of subunits and their role in maintaining the structure and function of the enzymatically active form. Generally, the conditions required for association are neutral pH, the presence of β-mercaptoethanol and dilution of the dissociated protein solution with buffer. Reversible dissociation has also been carried out by freezing and thawing at high ionic strength (Van Eys, 1964). Lactate dehydrogenase has also been reported to be dissociated by urea or guanidine-HCl and then reactivated by dialysis against saturated sodium chloride (Markert, 1963; Chilson et al., 1966).

The mechanism of action of LADH and YADH has been extensively discussed by Sund and Theorell (1963); McKinley-McKee (1964); and Sund (1968). The native forms of LADH and YADH have been reported to be composed of two and four active centres, respectively (Theorell and Bonnichsen, 1951; Hayes and Velick, 1954). LADH contains four gram atoms of zinc and YADH contains four or five gram atoms of zinc per molecule of enzymes (Vallee and Hoch, 1955a; Wallenfels and Sund, 1957; Åkeson, 1964; Oppenheimer et al., 1967). It is of particular
interest to investigate the structural changes of the active and inactive forms of these zinc metallo enzymes. Recently, the inactivation of LADH to be by various methods has been considered/due to loss of the zinc-moiety resulting in the enzyme being unable to be reconstituted (Chilson et al., 1966). However, reactivation of LADH and YADH is more critical than for many other enzymes. With careful control, it is possible to reactivate these enzymes under suitable conditions after inactivating by urea, lithium chloride and extremes of pH.

Materials

LADH and YADH were freshly prepared and assayed as described in Chapters 2 and 3. Analar urea and guanidine-hydrochloride from Eastman Chemicals were used. Other reagents were purchased from commercial sources with "AnalaR grade"; they were used without further purification. All solutions were made in quartz double distilled water. pH was measured with a Cambridge (Bench Type) pH meter with micro-electrodes. All manipulations were done in glass containers, unless where stated.

Methods

Inactivation and Reactivation of LADH and YADH by high or low pH: -

Stock solutions of enzymes were diluted with 0.1 M phosphate buffer pH 7.3 where required. The activity was assayed in duplicate and this solution was then titrated at room temperature, with phosphoric acid to pH 4.0 or below, or with sodium hydroxide to pH 12.0. Assays showed that no activity remained. Immediately the inactive enzyme solution was back-titrated to pH 7.5 by sodium hydroxide or phosphoric acid, β-Mercaptoethanol was then added and the solution incubated in a water bath. The temperature and other conditions are as described in the legends of the tables or figures. At various time-intervals,
solutions were placed in a dialyzing tubing to dialyse away the β-mercaptoethanol over a period of about 20 hours, using two changes of 0.1 M phosphate buffer pH 7.3 at 4°C. Enzymatic activity from this solution was again assayed in duplicate.

Inactivation and reactivation of LADH and YADH with urea or lithium chloride: - Inactivation was initiated routinely by mixing enzyme with urea or lithium chloride of appropriate concentration in 0.1 M phosphate pH 7.3. Assays showed that no enzymatic activity remained after exposure of the enzyme to 8 M urea or 6 M lithium chloride at 0°C. Reactivation was immediately carried out by dilution of the inactive enzyme solution either in the presence or absence of β-mercaptoethanol. This was followed by dialysing away the urea or lithium chloride first at room temperature with two litres 0.1 M phosphate buffer pH 7.3 containing the same concentration of β-mercaptoethanol as added to the inactive enzyme solution. At various time intervals, the dialysing was transferred to 4°C as described previously. Urea solutions were prepared fresh each time when in 0.1 M phosphate to reduce the risk of contamination by cyanate (Marier and Rose, 1964). High concentrations of lithium chloride do not dissolve in phosphate buffer, so 0.1 M HCl-Tris pH 7.1 was used. All pHs were adjusted either by phosphoric acid or sodium hydroxide to 7.1 - 7.3.

β-Mercaptoethanol is a potent inhibitor of LADH, but it does not inhibit YADH (Lamb and Williams, 1965). Thus, in assaying the enzymatic activity after reactivation, it is important to first remove all the β-mercaptoethanol from LADH. Also, in order to ascertain the loss of enzymatic activity is not due to inhibition by β-mercaptoethanol, this was added only after LADH was inactivated by extremes of pH, urea or lithium chloride.
RESULTS

Effect of time course on reversible inactivation of LADH with Acid pH

Enzymatic activity is lost as a result of titrating LADH solution from pH 7.3 to pH 4.0 at room temperature, and the effect is reversed by back-titrating to neutral pH. Maximum recovery of the initial activity is obtained by carrying out the reactivation in the presence of β-mercaptoethanol at 23°C and neutral pH. Kinetics studies (Fig. 6.1) of the reactivation process shows that there is a slow partial recovery of the enzymatic activity followed by a slower process. The reactivation is nearly completed in about two hours. If the enzyme was inactivated to a pH lower than 4.0, or reactivation was carried out at pH 9.0, less activity was recovered. Apparently, exposure of enzyme to a lower pH, results in secondary loss of structure. While with reactivation at higher pH the activity recovered proved more difficult. These inactivations and reactivations were done in a glass container. If a cellulose nitrate container was used, as much as 85% of the initial activity was recovered.

Reversible Inactivation of YADH with Low pH

The methods for inactivation and reactivation described for LADH were followed. 6.0 ml of 0.1 M phosphate buffer, pH 7.3 containing 0.75 mg YADH in a cellulose nitrate tube were used. Titration at 0°C to pH 3.4 resulted in complete inactivation. Immediately the solution was back-titrated to pH 7.5. Reactivation was carried out at 23°C in the presence of 0.15 M mercaptoethanol. Table 6.1 shows that 13% and 17% of the initial activity is recovered after incubating for two and four hours respectively in the presence of β-mercaptoethanol and then removing it. Without β-mercaptoethanol, it is 9% and 14%. β-Mercaptoethanol does not inhibit YADH, but it is a poor substrate.
Fig. 6.1. Reversible inactivation of LADH by acid. 1.1 μM LADH in 3.0 ml 0.1 μM phosphate buffer in a glass vessel was inactivated by titrating with concentrated H₃PO₄ to (a) pH 4.0; (b) pH 2.5; (c) pH 4.0; (d) pH 4.0, but for this experiment 10 ml sample in a cellulose nitrate vessel was used.

Reactivation was carried out at 23°C by back-titrating with 2N NaOH to pH 7.3 for samples (a), (b) and (d), and sample (c) to pH 9.0. And immediately, 0.1 M β-mercaptoethanol was added to each sample.
TABLE 6.1.
Reversible Inactivation of YADH with acid pH

<table>
<thead>
<tr>
<th>Inactivation</th>
<th>β-Mercaptoethanol</th>
<th>% Activity Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3.4</td>
<td>0.15</td>
<td>13</td>
</tr>
<tr>
<td>pH 3.4</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>pH 2.5</td>
<td>0.15</td>
<td>7</td>
</tr>
</tbody>
</table>

* 0.75 mg YADH in 6 ml 0.1Μ phosphate buffer pH 7.3 was inactivated by titrating with concentrated H₃PO₄ to pH 3.4 or 2.5 at room temperature. Reactivation was carried out at 23°C by back-titrating to pH 7.5 with 2 N NaOH, with or without the addition of β-mercaptoethanol as indicated in the table.

TABLE 6.2.
Effect of the concentration of β-mercaptoethanol on the reversible inactivation of LADH

<table>
<thead>
<tr>
<th>β-Mercaptoethanol (M)</th>
<th>% Activity Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>34</td>
</tr>
<tr>
<td>0.05</td>
<td>51</td>
</tr>
<tr>
<td>0.10</td>
<td>56</td>
</tr>
<tr>
<td>0.23</td>
<td>62</td>
</tr>
</tbody>
</table>

A final concentration of 1.3 μM LADH in 3 ml 0.1Μ phosphate buffer was inactivated at 20°C with pH 4.0. Reactivation was carried out at pH 7.5 and 23°C in the presence of β-mercaptoethanol.
The presence of 0.3 M β-mercaptoethanol in 0.3 mg/ml YADH solution at pH 7.3 shows no appreciable changes of the catalytic activity, while reactivation of YADH after acid inactivation shows that, under the conditions used, the β-mercaptoethanol is also not a very effective reagent for catalyzing the reactivation of YADH. If YADH is exposed to pH 2.5, less activity (7 - 12%) is also obtained.

**Effect of β-Mercaptoethanol on Reactivation of LADH**

β-Mercaptoethanol is a strong inhibitor of LADH. A final concentration of 0.072 M β-mercaptoethanol was added to 1 ml 0.1 M phosphate buffer pH 7.3 containing 5.6 μM LADH at 23°C. This nearly caused the complete loss of enzymatic activity. Thorough dialysis over a period of about 20 hours with two changes of two litres 0.1 M phosphate, removes the inhibitor and inhibitory effect. Beside its inhibitory effect, β-mercaptoethanol also catalyses the association of the dissociated forms of the enzyme presumably through preventing disulphide bridging among dissociated chains. Under the conditions used, the optimal concentration of β-mercaptoethanol required for recovering the maximum activity is found to be 0.1 M. Table 6.2 summarizes the results. Without mercaptoethanol, or if lower concentrations are used, less activity is achieved.

**Effect of Temperature on Reactivation of LADH**

The native enzyme is stable in the temperature range 0 - 33°C. When reactivation was carried out at 0°C after acid inactivation at 23°C, the activity recovered was substantially less (Table 6.3). Optimal activity is achieved at 23°C. At higher temperatures, less activity is obtained. This result indicates that reactivation leads to a form which is enzymatically active, but labile at higher temperature. Using 0°C, the lower temperature prevented the association of the dissociated enzyme to the active form. The
TABLE 6.3.
Effect of temperature on the reversible inactivation of LADH

<table>
<thead>
<tr>
<th>Reactivation at °C</th>
<th>β-mercaptoethanol (M)</th>
<th>1 hr.</th>
<th>2 hr.</th>
<th>4 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>-</td>
<td>37</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>-</td>
<td>24</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.1</td>
<td>26</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>11</td>
<td>0.1</td>
<td>52</td>
<td>58</td>
<td>61</td>
</tr>
<tr>
<td>23</td>
<td>0.1</td>
<td>56</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>33</td>
<td>0.1</td>
<td>66</td>
<td>61</td>
<td>57</td>
</tr>
</tbody>
</table>

Conditions were those described in Table 6.2.

TABLE 6.4.
Effect of volume of sample used on reactivation

<table>
<thead>
<tr>
<th>Volume of Sample (ml)</th>
<th>1 hr.</th>
<th>2 hr.</th>
<th>4 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.02</td>
<td>37</td>
<td>41</td>
<td>46</td>
</tr>
<tr>
<td>3.06</td>
<td>44</td>
<td>53</td>
<td>59</td>
</tr>
<tr>
<td>5.10</td>
<td>51</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>10.20</td>
<td>56</td>
<td>63</td>
<td>69</td>
</tr>
</tbody>
</table>

Each sample contained an identical concentration of 1.7 μM LADH and was
inactivated at 20°C with pH 4.0. Reactivation was carried out at pH 7.5 and
11°C in the presence of 0.1M β-mercaptoethanol.
same result is obtained without the presence of β-mercaptoethanol.

Effect of Volume of Sample Used for Reactivation

If inactivation was done at 0°C and reactivation at 23°C, more activity was recovered than with inactivation at room temperature. This suggests that 0°C promotes the dissociation of the enzyme, while minimizing irreversible aggregation. Room temperature promotes the association of the subunits into active form. Various amounts of sample were used to examine the activity recovered after acid inactivation. Reactivation was carried out at 11°C with the addition of 0.1 M mercaptoethanol. Table 6.4 shows that with different volumes of buffer used, each one containing the same concentration of enzyme, more activity is obtained after acid inactivation by using a larger amount of sample. Presumably, use of a larger amount of sample minimizes loss of enzymatic activity during reactivation through minimizing absorption onto the glass surface.

Effect of NADH on Reactivation

NADH is known to form a binary complex with LADH. In this complex the protein structure is more compact and protection against inactivation results. After acid inactivation, the addition of NADH (72 μM) only increases the activity recovered marginally (Table 6.5). With β-mercaptoethanol (0.1 M), the presence of NADH results in less activity being recovered than if mercaptoethanol is used alone. Perhaps with protection more drastic conditions are needed to inactivate and therefore to attack subunits when produced.

Effect of Concentration of LADH on Reactivation

The concentration of the enzyme is one of the important factors involved in the recovery of enzymatic activity after inactivation. Fig. 6.2 shows the results of various concentrations of LADH used for reactivation after acid
### TABLE 6.5.

**Effect of NADH on the reactivation of LADH**

<table>
<thead>
<tr>
<th>Addition</th>
<th>% Activity Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hr.</td>
</tr>
<tr>
<td></td>
<td>22</td>
</tr>
<tr>
<td>72 μM NADH</td>
<td>27</td>
</tr>
<tr>
<td>0.1 M β-mercaptoethanol</td>
<td>76</td>
</tr>
<tr>
<td>0.1 M β-mercaptoethanol + 72 μM NADH</td>
<td>59</td>
</tr>
</tbody>
</table>

A sample containing 3.0 ml 0.1 μ phosphate buffer and 1.7 μM LADH in a cellulose nitrate vessel was inactivated at 0°C with pH 4.0 and reactivation was carried out at 23°C and pH 7.5.
Fig. 6.2. Effect of LADH concentration on the reversible inactivation of LADH by acid. A 3.0 ml reaction mixture in a glass vessel containing 0.1 M phosphate buffer, pH 7.3, and LADH of $(\Delta)$ 0.57 $\mu$M; (o) 1.4 $\mu$M; (X) 2.8 $\mu$M, was inactivated at 20°C by titrating to pH 4.0. Reactivation was carried out at pH 7.3 and 23°C in the presence of 0.1 M $\beta$-mercaptoethanol.
inactivation. With very low concentrations of LADH, low activity is recovered. This is presumably due to protein absorption into the glass surface. When the enzyme concentration is higher than 3 μM, after about one hour of reactivating the inactive enzyme at 23°C, precipitation is visible, even if β-mercaptoethanol is added, and less activity is recovered. This seems due to aggregation or coagulation of the inactive protein.

Reversible Inactivation of LADH with High pH

LADH was inactivated by titrating the enzyme solution from pH 7.3 to pH 12.0 with 2N sodium hydroxide. This inactivation was reversible by back-titrating to neutral pH with phosphoric acid. Fig. 6.3 shows the recovery of enzymatic activity, after inactivation by high pH. The reactivation process is similar to acid inactivation, where this is firstly slow partial recovery of the enzymatic activity, followed by a slower process. The maximum recovery of the enzymatic activity is 28% in the presence of 0.1 M mercaptoethanol and 17% without β-mercaptoethanol. With high concentrations of LADH, turbidity resulting from aggregation is visible after about one hour of reactivating the inactive enzyme at neutral pH and 23°C. It has been reported that high pH favours the formation of disulfide bonds (Cecil, 1963) which would account for ready aggregation of the subunits. While it is not entirely clear whether structural changes induced in the enzyme by high pH lead to aggregation or whether dissociation, followed by subunit aggregation, occurs.

Reversible Inactivation of LADH with Urea

Urea has been suggested as a useful reagent for the rupture of hydrophobic bonds (Kauzmann, 1959; Brandt, 1964). This is shown by the fact that the inactivation of LADH is faster at low temperature than at
Fig. 6.3. Reversible inactivation of LADH by alkaline pH. A final concentration of 1.3 μM LADH in 10 ml 0.1 M phosphate buffer, pH 7.3 at 20°C was inactivated by titrating with 2 N NaOH to pH 12.0. Reactivation was carried out at 25°C and pH 7.3 in the presence (○) and absence (□) of 0.1 M β-mercaptoethanol.
room temperature. Urea at low concentrations has been reported to be a non-competitive inhibitor of both LADH and YADH (Brand et al., 1962).

Substrate, coenzyme, glycerol and neutral salts have been found to protect the enzyme against the urea inactivation. In 8M urea, in addition to the loss of enzymatic activity, LADH is also dissociated into subunits whether β-mercaptoethanol is present or not. It is possible to reactivate the enzyme, but this is difficult after urea inactivation (Table 6.6). The period required to produce complete inactivation has to be restricted to a minimum. Otherwise unfolding with irreversible loss of structure may occur. A suitable concentration of enzyme and urea is necessary and a dilution such that the final concentration of urea is not too high to prevent reactivation, or of enzyme to give aggregation. After urea inactivation, the solution was immediately diluted with 0.1 μ phosphate buffer, pH 7.3 followed by the addition of β-mercaptoethanol. In order to remove the urea rapidly, it was immediately dialysed at 20°C for 2-4 hours against 0.1 μ phosphate buffer, pH 7.3 which contained the same concentration of β-mercaptoethanol as had been added to the inactive enzyme solution. This was followed by similarly dialyzing away the β-mercaptoethanol at 4°C over a period of 20 hours, and residual urea. 17% of the original activity is recovered. It is only 8% if β-mercaptoethanol is not used. The presence of glycerol has no effect on the reactivation. Zinc ion is found to inactivate LADH. The present of 5 x 10^{-5}M zinc ion in urea stimulated urea inactivation. Similarly, the recovery of enzymatic activity after urea inactivation was not promoted by zinc ions.

Recently, Drum et al. (1967) reported that in the reversible inactivation of LADH by urea, 30% of the original activity is recovered. However,
### TABLE 6.6
Reversible inactivation of LADH with urea

<table>
<thead>
<tr>
<th>Addition</th>
<th>% Activity Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>0.1 M β-Mercaptoethanol</td>
<td>17</td>
</tr>
<tr>
<td>0.1 M β-Mercaptoethanol + 5% (v/v) glycerol</td>
<td>16</td>
</tr>
<tr>
<td>5% (v/v) glycerol</td>
<td>9</td>
</tr>
<tr>
<td>-</td>
<td>6*</td>
</tr>
<tr>
<td>5% (v/v) glycerol</td>
<td>7*</td>
</tr>
</tbody>
</table>

Inactivation was carried out at 0°C by adding a final concentration of 6.6μM LADH to 0.5 ml 0.1 M phosphate buffer, pH 7.3 containing 8 M urea. Reactivation was carried out by 10-fold dilution of this inactivated enzyme with 0.1 M phosphate buffer, pH 7.3 with or without the addition of compounds as indicated in the table. This sample was first dialysed at 20°C for 2-4 hours, followed by dialysis at 4°C for 20 hours.

*without dialysis.*
the β-mercaptoethanol is added before urea inactivation and it is a potent inhibitor for LADH. Thermal denaturation studies have shown that β-mercaptoethanol protects LADH against heat inactivation. Thus, during incubation of LADH at 70°C for about 34 minutes, 50% of enzymatic activity is lost. In the presence of 1.5mM β-mercaptoethanol this is only 28%. The protection involves the formation of an enzyme-inhibitor complex which also protects LADH against urea inactivation. Thus, with inactivation in the presence of β-mercaptoethanol, it was not clear whether reactivation is being measured, or residual activity detected after the removal of β-mercaptoethanol by dialysis.

**Reversible Inactivation of LADH and YADH with Lithium Chloride**

LADH and YADH were inactivated at neutral pH by high concentrations of lithium chloride and this inactivation was also reversible by dilution and the present of β-mercaptoethanol (Table 6.7). 8 μM of LADH were added to 1.0 ml 6M lithium chloride in 0.1 M HCl-Tris buffer pH 7.3 at 0°C; this resulted in complete loss of enzymatic activity. Immediately 5 ml .01 M HCl-Tris pH 7.3 and a final concentration of 0.1 M β-mercaptoethanol were added. The lithium chloride was removed rapidly by dialysis for 2 - 4 hours at 20°C against 0.01 M HCl-Tris buffer pH 7.3 containing 0.1 M β-mercaptoethanol. This was followed by dialysing away the β-mercaptoethanol and lithium chloride at 4°C for about 20 hours using the 0.01 M HCl-Tris buffer. Aliquots were withdrawn for assay; 72% of the original activity was recovered. Without β-mercaptoethanol this was 11%.

Likewise, 0.75 mg/ml YADH was added to 1 ml 6M HCl in 0.1 M HCl-Tris at 0°C. Assay showed no activity remained. This solution was immediately diluted 6-fold with 0.01 M HCl-Tris, pH 7.3. Reactivation was carried out
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>β-Mercaptoethanol (M)</th>
<th>% Activity Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>LADH</td>
<td>0.1</td>
<td>72</td>
</tr>
<tr>
<td>LADH</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>YADH</td>
<td>0.1</td>
<td>19</td>
</tr>
<tr>
<td>YADH</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>YADH</td>
<td>0.1</td>
<td>21</td>
</tr>
</tbody>
</table>

Conditions for inactivation and reactivation were as described in the text.
at $23^\circ C$ for 2 - 4 hours. The recovery of the enzymatic activity was not stimulated by the addition of $\beta$-mercaptoethanol (0.1 M). Also, whether the $\beta$-mercaptoethanol was removed or not, no great change in the recovery of activity resulted. After removing the $\beta$-mercaptoethanol and lithium chloride, 21% of the initial activity was achieved. If the $\beta$-mercaptoethanol and lithium chloride was not dialysed away, then 19% was recovered.
DISCUSSION

LADH and YADH are reversibly inactivated by extremes of pH and high concentrations of urea and lithium chloride. The recovery of the enzymatic activity is dependent both on the conditions under which inactivation has occurred, and on the conditions for reactivation which is carried out. Optimum reactivation depends on the pH, the temperature, the concentration of enzyme, the time course of reactivation, the nature of the container and the presence of β-mercaptoethanol. More activity can be achieved if inactivation is carried out at 0°C, and reactivation at 23°C and neutral pH with the presence of β-mercaptoethanol. With high concentrations of enzyme, precipitation occurs during reactivation. Therefore, a suitable concentration of enzyme, and a dilution such that the final concentration of urea is not too high to prevent reactivation, is necessary. With very low enzyme concentration, low enzymatic activity is recovered. This seems due to adsorption onto the container surface. Irretrievable activity depends on time course of exposure of the enzyme to the denaturating agent and its concentration. The period required to produce complete inactivation has to be restricted to a minimum, so that a secondary irreversible loss of structure is minimized.

The effect of β-mercaptoethanol in facilitating reactivation is particularly interesting, as it is a potent inhibitor of LADH, and the complex formed with LADH protects the enzyme against inactivation. This protection may stabilize the enzyme-thiols, and therefore improve reactivation by preventing irreversible aggregation. Otherwise, β-mercaptoethanol may directly maintain thiol-groups through exchange. Too high a concentration of β-mercaptoethanol is found to inactivate and precipitate LADH at neutral
pH, perhaps by reaction with enzymatic zinc ions.

Reversible inactivation occurs best at neutral pH, while away from neutrality less activity is recovered. Activity that cannot be regained is considered to be due to irreversible structural changes. This enzymatically inactive form may then aggregate or give further breakdown. Loss of enzymatic zinc ions is considered as a part of irreversible process.

It is known that the acid inactivation of YADH (Hoch and Vallee, 1959; Druyan and Vallee, 1964) and LADH (Akeson, 1964; Oppenheimer and McKay, 1966; Oppenheimer et al., 1967; Drum et al., 1969) is associated with removal of enzymatic zinc. Zinc ions are found to be a potent inhibitor of both enzymes. The addition of zinc does not promote reactivation. This is considered as evidence that loss of enzymatic zinc ions are not part of the reversible inactivation process. Further evidence for this is the lag of zinc removal behind loss of activity (Akeson, 1964; Oppenheimer et al., 1967; Drum et al., 1969), suggesting that loss of enzymatic zinc ions is not to be regarded as evidence against the possibility of reactivation (Kägi and Vallee, 1960; Chilson et al., 1966; Blomquist, 1967), for actual inactivation occurs prior to any zinc loss. In this connection, ultra-violet difference absorption spectra on low-zinc LADH preparations (Oppenheimer and McKay, 1966; Oppenheimer et al., 1967), seem similar to urea or acid inactivation difference spectra, and while interpreted to indicate conformational change due to loss of zinc, must also appear to reflect the dissociation of the enzyme into subunits.

Because inactivation of LADH and YADH was considered to be irreversible (Kägi and Vallee, 1960; Chilson et al., 1966; Blomquist, 1967), zinc atoms were considered to be essential for maintaining the quarternary structure of
the enzymes. However, reversible inactivation also indicates that zinc is essential for maintaining the enzymatically active form. If it is lost from the enzyme, then it cannot revert to active enzyme. It is thought, therefore, that the reactivation must be occurring prior to loss of the metal.

The fact that inactivation with urea, or lithium chloride, occurs more readily at 0°C than 23°C is already an indication of dissociation of LADH or YADH into subunits, which the molecular weight measurements substantiate. The temperature-dependence of reversible inactivation supports this, and indicates that hydrophobic bonds which are more stable at room temperature than at lower temperature are important in the reversible and irreversible processes involved. Thus, room temperature rather than 0°C promotes formation of active enzyme, while at 0°C inactivation is promoted and irreversible aggregation during inactivation by urea or lithium chloride is minimized. Whilst specific subunit association can regenerate an enzymatically active form, irreversible aggregation must occur through the random joining of the non-polar parts of subunits. That non-polar bonds predominate in holding the subunits together in the apoenzyme is already suggested by the negative temperature coefficients found for LADH and YADH inactivation by urea and guanidine-hydrochloride, and by the dissociation of these molecules into subunits (Sund, 1960; Ohta and Ogura, 1965; Drum et al., 1967; Castellino and Barker, 1968; Butler et al., 1969; Pietruszko et al., 1969a; Green and McKay, 1969).

However, the molecular weight of protein is usually very big. The inactivation of the enzyme by various denaturating agents can either by affected directly or indirectly through the active sites. The inactivation
process of both enzymes appear to be complex. At least, two main processes are involved: (1) a reversible effect that occurs under relatively mild conditions, and (2) an irreversible inactivation that results from prolonged exposure of the enzyme to too high a concentration of urea, guanidine-hydrochloride, and lithium chloride, or more severe acid or alkali conditions. This is supported by the fact that it is impossible to obtain total regain of the activity after the enzyme is inactivated by various denaturating agents under the normal conditions. The inactivation may first result in a conformational change of the protein molecule with the result that the enzyme is no longer enzymatically active. The new configuration is more susceptible to denaturation. On removal of denaturating agent, there is a return to the enzymatically active form, and this leads to an equilibrium, whereas the other leads to irreversible inactivation, due to structural changes induced by the denaturating agent, which enable side-reactions to take place. These mechanisms would account for the occurrence of precipitation. Optimum reactivation is achieved only with high dilution during the first stage of the reactivation process, suggesting that the intra- and inter-molecular interactions of irreversible aggregation are minimized. Further evidence is given by the finding that the loss of the zinc ions from LADH resulted in oxidizing the former stable sulphydryl groups to form disulfide bonds (Oppenheimer et al., 1967), while in the apoenzyme, steric hindrance probably prevents them.

Difficulties in recovering the enzymatic activity after inactivating LADH with acid (Chilson et al., 1966; Blomquist, 1967), seem due to the conditions necessary being more critical than for many other dehydrogenases.
Precipitation of enzyme on adjustment to neutral pH after acid inactivation and consequent lack of reversibility, result from the critical nature of concentration in these processes.

Recently, Drum et al., (1967) reported that 30% of the activity of LADH can be recovered after urea inactivation, and 60-70% with the presence of zinc ions and coenzyme. However, zinc ions and β-mercaptoethanol are potent inhibitors of LADH. The inactivation was carried out with the presence of β-mercaptoethanol. It is not clear whether the enzyme is completely inactivated by urea or due to inhibitory effect. The formation of enzyme-inhibitor complex protects against urea inactivation. Thus, the reactivation detected after urea inactivation in the presence of β-mercaptoethanol may also reflect a certain amount of residual activity which can be detected only after the removal of urea and inhibitor by dialysis. The addition of zinc ions do not promote reactivation, but are deleterious. This is further supported by recent reports (Blomquist, 1967; Pietruszko and Theorell, 1969).
CHAPTER 7

STUDIES ON ABSORPTION SPECTRA OF LADH AND YADH
Previous investigations have shown that both YADH and LADH are reversibly inactivated by extremes of pH, urea, and high concentrations of salt. The rate of inactivation increases with decreasing temperature. Moreover, on exposure of these enzymes to low pH, the loss of enzymatic activity is accompanied by the removal of enzymatic zinc atoms. The loss of enzymatic activity is more rapid than is the loss of zinc, (Drum et al., 1967). Zinc atoms in native LADH can be replaced by Co$^{2+}$, the resultant cobalt-LADH is enzymatically active and exhibits the same characteristic absorption properties as zinc-LADH. Removal of zinc atoms with 1,10-phenanthroline and 8-hydroxyquinoline sulfonic acid (Snodgrass et al., 1960; Kagi and Vallee, 1960), dissociates YADH to four subunits with a molecular weight of 36,000, while LADH remains unchanged. YADH is also reported to be dissociated to four subunits by urea, or sodium dodecyl sulfate (Sund, 1966; Hersh, 1962; Ohta and Ogura, 1965). Quenching of fluorescence has also been found when these enzymes are exposed to urea (Brand et al., 1962) and acid solution (Blomquist, 1967). These changes have been explained on the basis of an uncoiling of the protein molecule.

In recent years, considerable attention has been centered on the location of the residues of tyrosine, tryptophane and phenylalanine and their possible involvement in the protein structure. It is well known that treatment of the proteins with various denaturating agents has an appreciable effect on the degree of resolution of the fine-structure and on the wavelength of electronic absorption bands. These spectral changes are related to changes in bonding or other factors in the environment of the side chains of certain chromophoric residues (Beaven and Holiday, 1952; Wetlaufer, 1962; Scheraga and Rupley,
1962; Edsall, 1963; Herskovits, 1967). A study of the effect of various denaturating agents on the ultraviolet absorption spectra of aromatic amino acids in a protein gives information, which in conjunction with evidence from other sources, may throw some light on the internal configurations of both YADH and LADH.

Materials

YADH and LADH were freshly prepared as previously described (Chapters 2 and 3). All other solvents and reagents were of 'AnalaR' grade.

Methods

Ultraviolet difference absorption spectra produced from a comparison between the native enzyme in 0.1 M phosphate buffer, pH 7.3 and in various denaturating agents were recorded with a Beckman (Model DK-2) ratio recording spectrophotometer. Two pairs of well-matched, spectrosil, silica cells of 1.0 cm pathlength were obtained from Thermal Syndicate Ltd. (Wallsend, Northumberland). The width of the slit was fixed before each measurement. To obtain a solvent-corrected absorbance difference spectra in a single measurement, the cells were arranged as shown in Fig. (7.1)

![Cell Arrangement Diagram](image)

Fig. 7.1. Arrangement of the cells for the measurement of difference absorption spectra between native and denatured protein.
In the sample beam, the first cell contained the reference solvent and the second cell contained absorber in the test solvent. In the reference beam, the first cell contained the absorber in the reference solvent and the second cell contained the test solvent. The filled cells are positioned in the compartment with the blank facing the source of radiation, so that the light beams enter blank-containing sections of the cell first. Before each set of measurements, the spectrophotometer was checked to give a satisfactorily straight base-line in the region of the wavelength of interest. This was done with each set of cells filled with solvent and test solvent without protein, and with the cells arranged as shown in Fig. (7.1).

A total amount of 3.0 ml solution was introduced to each cell. The same Carlsberg pipette was used throughout to reduce pipetting errors. Solutions of pH 12 were made by adding an appropriate amount of 2 N NaOH to 0.1M phosphate (pH 7.3), and solutions of pH 2.2 were made by adding phosphoric acid. The pH was measured before and after each experiment with a 'Cambridge' (Bench Type) pH. meter.

RESULTS

Ultraviolet Absorption Spectra of YADH and LADH

The typical ultraviolet absorption spectra between the regions of 250 m\(\mu\) and 330 m\(\mu\) of YADH and LADH are illustrated in Fig. 7.2. Measurements were carried out with YADH, or LADH, in 0.1M phosphate buffer (pH 7.3 at 23°C). The spectrum of LADH shows that in addition to a maximum absorption at 280 m\(\mu\), due solely to tyrosine and tryptophane residues, a few remarkable peaks at 270, 265, 260, 253 m\(\mu\) and a sharp increase absorption below 250 m\(\mu\) are also obtained. The peaks below 270 m\(\mu\) may be attributed
Fig. 7.2. Ultraviolet absorption spectra of LADH (-----) and YADH (---). The concentration of LADH was 2.16 mg/ml and that of YADH 0.71 mg/ml.
to phenyl-alanine residues. In contrast to LADH, the absorption spectrum of YADH gives only a characteristic tyrosine and tryptophane peak at 278 mμ and a sharp increase absorption below 250 mμ. The wavelength of the tyrosine and tryptophane peak of YADH is 2 mμ shorter than LADH.

Effect of Acid, Urea and Guanidine-hydrochloride on the Ultraviolet Absorption Spectra of LADH

Measurements of ultraviolet difference absorption spectra between native and inactive enzyme were performed immediately after exposure of LADH to pH 2.2, 8 M urea, or 4 M guanidine-hydrochloride. Fig. 7.3 shows that the absorption spectra are in all cases shifted to a shorter wavelength; a blue shift. The similarity of the three spectra is noticeable. In each case, there are two prominent troughs, one at 286-7 mμ, and one at 294 mμ with a shoulder at 300 mμ. The trough at 286-7 mμ is assigned to alterations in the environment of tyrosine, and the peak at 294 mμ to tryptophane residues. In addition to these, on exposure of the enzyme to 8 M urea in 0.1 mμ phosphate buffer pH 7.3, or to pH 2.2, there is a decreased absorbancy below 245-250 mμ and in the region between 245 and 280 mμ, a general increase in absorbancy with several small but easily distinguished maxima and minima. However, with 4 M guanidine-hydrochloride at neutral pH, these effects are not seen. No doubt, this must be due to the presence of absorbing materials in the guanidine-hydrochloride. On treatment of the enzyme with 8 M urea, 4 M guanidine-hydrochloride, or at pH 2.2, no enzymatic activity can be detected and the spectral changes appear to be rapid; the initial measurements are complete after a few minutes, and no further change is observed even over a period of 24 hours. A similar result to that for
Fig. 7.3. Difference spectra of LADH resulting from denaturation with (---) 8 M urea; (- - - -) pH 2.2; (-----) 4 M guanidine-hydrochloride. In all cases, the concentration of LADH used was 2.5 mg/ml, and the conditions of measurement those in Fig. 7.1.
pH 2.2 has been found at pH 2.7 and pH 4.5 (Blomquist, 1967). At pH 4.5, the difference spectra is time-dependent with the tryptophane band at 294 m\(\mu\), still changing after the tyrosine band at 287 m\(\mu\) has been stabilized.

**Effect of Acid and Guanidine-hydrochloride on the Ultraviolet Absorption Spectra of YADH**

Fig. 7.4 shows that the ultraviolet difference absorption spectra due to pH 2.2 or 3 M guanidine-hydrochloride are similar to that produced by 8 M urea (Ohta and Ogura, 1965). A blue shift occurs and there are two troughs at 280 and 286 m\(\mu\) and a shoulder at 292 m\(\mu\). The troughs are assigned to changes in the environment of tyrosine, and the shoulder to tryptophane residues. These changes are considered to be due to the dissociation and unfolding of the YADH molecule. Again, although the spectra are recorded immediately after mixing the enzyme with denaturant, no further difference spectra changes occur over a period of 24 hours. The conditions are such that parallel experiments showed that the enzyme is inactive and dissociated into subunits. A contrast to LADH is that the spectra are displaced some 3 m\(\mu\) towards the shorter wavelength, and a broad peak in the region between 245 and 270 m\(\mu\) does not show fine structure.

Summarizing, it is found that acid, urea and guanidine-hydrochloride give similar ultraviolet difference absorption spectra for LADH or for YADH. Also the difference absorption spectra obtained for LADH and YADH are similar to each other and to those obtained for glyceraldehyde-3-phosphate dehydrogenase and lactic dehydrogenase (Mór and Elödi, 1968). For the two alcohol dehydrogenases as Mór and Elödi, (1968), have shown for glyceraldehyde-3-phosphate dehydrogenase and lactic dehydrogenase, urea, acid pH and sodium dodecyl sulfate give similar ultraviolet difference
Fig. 7.4. Difference spectra of YADH resulting from denaturation in 0.1 μ phosphate, pH 7.3 with (−−−−) 3 M guanidine-hydrochloride; (−−) pH 2.2. In each case, the concentration of YADH used was 1.1 mg/ml.
absorption spectra with maxima at 292-3 m\(\mu\) due to tryptophane residues and at 286-7 m\(\mu\) due to tyrosine residues.

**Effect of Alkali pH on the Ultraviolet Absorption Spectra of YADH and LADH**

Fig. 7.5 shows the difference absorption spectra in the region between 260 and 350 m\(\mu\) resulting from a comparison of the enzymes in 0.1 \(\mu\) phosphate buffer, pH 7.3 and pH 12.0. The spectra are measured immediately after adding enzyme to denaturant. The changes are rapid and do not change with time thereafter. For both enzymes, the difference absorption spectra show a distinct peak at 295 m\(\mu\) and a trough at 276 m\(\mu\). The peak is considered to be due to the phenolic dissociation of tyrosine side chains, which intensify the ultraviolet absorption spectra and cause a red shift (to a longer wavelength). The tyrosine groups are considered to be buried in the native enzyme and exposed to the surface and freely contact with solvent only after the treatment with alkali pH. A sharply increased absorbancy below 270 m\(\mu\) is also observed.

**DISCUSSION**

In the compact, highly-ordered structure of native protein, it is possible that the chromophoric side-chains are situated in four different ways: (1) exposed on the outside surface of the enzyme molecule and surrounded by solvent medium; (2) embedded in the internal parts of the macromolecule and surrounded by the other side-chains; (3) the chromophoric side chains are partly exposed to the surface and the remainder of them are bound in the interior of the protein molecule; and (4) partly projected to the surface and partly buried but near the surface of the molecule. Any disruption of the native structure of the protein by various denaturating agents may cause the change in the environment of these chromo-
Fig. 7.5. Difference spectra of LADH (—–) and YADH (—) resulting from treatment of both proteins with pH 12.0. The concentration of LADH used was 3.1 mg/ml, and YADH, 1.1 mg/ml.
phoric residues and change in light absorption spectrum (Beaver and Holiday, 1952; Wetlaufer, 1962; Scheraga, 1962; Edsall, 1963). These changes may result either from that particular chromophoric residue itself or the whole chromophoric side chains of the protein. The changes of the absorption spectrum in the region between 250-300 m\(\mu\) have customarily been interpreted on the basis of the transfer of chromophores from the interior of the protein molecule to aqueous environment. Thus, if the spectrum shifts to a shorter wavelength (blue shift), it is thought that the aromatic amino acids are exposed to the solvent medium. If the spectrum shifts to a longer wavelength (red shift), the aromatic amino acids are incorporated into the protein molecule. The manner by which the various denaturing agents induce the structural changes of the protein molecule is not yet entirely understood. But there is a common feature that the essential protein structure must be disrupted.

Previous studies on the basis of the temperature effect on inactivation of YADH and LADH by urea and guanidine-hydrochloride indicate that hydrophobic bonds are important in the maintenance of the native structure. The disruption of hydrophobic bonds may result in dissociating both enzymes into inactive subunit forms. Ultraviolet difference spectrum measurements show that in all cases, a shift to a shorter wavelength occurs after adding both enzymes to pH 2.2, urea, or guanidine-hydrochloride. A nearly similar pattern of absorbancy at the peak of 286-7 m\(\mu\) and 294 m\(\mu\) for LADH and 280 m\(\mu\) and 286 m\(\mu\) for YADH. On exposure to high pH, the protein absorption spectra show an intensification at 295 m\(\mu\) for both enzymes. The intensification of the light absorption may result from the release and ionization of bound tyrosine groups (Beaver and Holiday, 1952; Wetlaufer, 1962). In each
case, the loss of enzymatic activity is rapid, no further change in absorbancy occurring over a period of 24 hours, suggesting that the structural changes are complete under these conditions.

It has also been shown by Ohta and Ogura, (1965), and Blomquist, (1967), that the tyrosine environment of both LADH and YADH changes much more rapidly than that of tryptophane, also seen best interpreted as resulting from dissociation and size as well as shape changes, with the tyrosine groups intimately involved in the non-polar regions holding the subunits together. Similar difference absorption spectra on low-zinc LADH preparations, considered to represent conformation change due to zinc loss, also appear to represent dissociation (Oppenheimer, et al., 1967). However, structural change in the subunits can still be involved where the tyrosine groups are in non-helical regions quickly unfolding, and the tryptophane groups stacked in helical regions slowly unfolding.

Clearly, ultraviolet spectral studies can provide useful information about the location of chromophoric side chains of the protein molecules. Some, or all, of the chromophoric residues may become freely into contact with solvent, or undergo protonation, after the destruction of the native protein structure. Difference spectra of model chromophores have been extensively studied with solvent perturbation (Yanari and Bovey, 1960; Wetlaufer, 1962; Herskovits and Sorensen, 1968). Attempts to interpret quantitatively the individual chromophores of various contributions of protein difference absorption spectra have also been made. However, owing to the uncertainty of these aspects, the shape of the difference absorption spectra by itself is not enough to give detailed information regarding the interpretation of the nature of the changes in environment of the protein structure.

There-
fore, the results obtained should be judged in conjunction with other
criteria. Further studies on the ionization of the tyrosine group, or
the acid and base binding groups liberated during the denaturation process
of the enzyme, could help to explain how tyrosine hydrogen bonds are being
broken in various denaturating agents.
CHAPTER 8

MOLECULAR WEIGHT OF LADH AND YADH AND THEIR PROTOMERS
5.1. INTRODUCTION.

A great deal of evidence has accumulated in recent years which indicates that LADH and YADH contain two and four active sites respectively, (Sund and Theorell, 1963; McKinley-McKee, 1964; Sund, 1968).

Studies of peptide-mapping with tryptic and chymotryptic digestion of both enzymes (Li and Vallee, 1964; Harris, 1964) have shown that each enzyme contains one reactive cysteine residue in a unique amino acid sequence. Kinetic studies (Sund and Theorell, 1963; McKinley-McKee, 1964), and X-ray crystallographic studies (Branden, 1965; Branden et al., 1965) of the free LADH and binary and ternary complexes, have indicated that LADH has two, and YADH has four, identical polypeptide chains. Evidence has also been obtained that LADH contains two C-terminal and two acetylated N-terminal amino acids (Jörvall, 1965; 1967).

However, the molecular weights of the native YADH and LADH reported in the literature differ quite widely. For LADH, determinations by sedimentation velocity and diffusion yielded values of 67,500 - 84,400 (Theorell and Bonnichsen, 1951; Ehrenberg, 1957; Ehrenberg and Dalziel, 1958). Using the Archibald method, Ehrenberg (1957) found a molecular weight of 84,400 for this enzyme. The molecular weight of YADH has been reported to be 110,000 - 157,000 (Hayes and Vallick, 1954; Sund, 1964). Moreover, a recent investigation of the molecular weight of the subunit of LADH has raised the question of the number of subunits in the molecule. Drum et al. (1967) reported that in 8 M urea, LADH is dissociated to two subunits with a molecular weight of 40,000, and that further treatment with β-mercaptoethanol, or ethylene diamine tetra-acetate, results in dissociation into four subunits with molecular
weight of 20,000. But a report by Blomquist et al. (1967) of denaturation studies with sodium dodecyl sulphate by density gradient ultracentrifuge experiment using glycerol gradients, indicated that the subunits had a molecular weight of 39,000. Number-average molecular weight determined by membrane osmometry for the guanidine hydrochloride dissociated LADH demonstrated the subunit of LADH is 40,790 (Castellino and Barker, 1968). Butler et al. (1969) has also reported that after treating LADH and YADH with 8 M urea, or 6 M guanidine hydrochloride, and then maleylating these inactivated enzymes, molecular weights of 42,000 for YADH and 39,000 for LADH are obtained.

In order to ascertain the molecular weight of both active and inactive forms of LADH and YADH, and to delineate further the nature of the structural changes which accompany the inactivation of both enzymes by extremes of pH, urea and guanidine hydrochloride, molecular weight studies have been carried out by light scattering, ultracentrifugation, and gel filtration.

8.2. ULTRACENTRIFUGATION.

In the past decades, the development of the theory, as well as the practice, for the use of the ultracentrifuge as a tool in biochemical research has developed in a dramatic fashion. (For details see Williams et al, 1958; van Holde and Baldwin, 1958; Schachman, 1959; Fujita, 1962; Yphantis, 1960; 1964; Adams and Fujita, 1963; Osterhoudt and Williams, 1965; Donnelly, 1966; Fujita and Williams, 1966; Richards et al, 1968.) To date, the most common methods employed are (1) the sedimentation velocity method, and (2) the sedimentation equilibrium method.

8.2.3. Sedimentation Velocity

Under the influence of a centrifugal field caused by the high speed, the
initially uniformly-distributed solute throughout the solution in the ultracentrifuge cell is caused to migrate towards the periphery of the cell. The use of Schlieren optical systems enables the movement of the boundary of solute molecules in the cell to be measured from the photographs taken at various time intervals.

**THEORY**

When the solute molecules are moving with constant velocity, the centrifugal force is being balanced by the frictional resistance of the medium. The sedimentation coefficient $S$ is usually expressed as the sedimentation rate of solute per unit centrifugal field of force and has dimensions of time. Thus

$$S = \frac{dx}{dt} \left(\frac{1}{w^2 x}\right) \text{ or } \frac{-2}{w^2} \left[\ln (1/w^2) \frac{dx}{dt}\right]$$

where $x$ in centimeters is the distance of the boundary from the centre of rotation at time $t$ in seconds and $w$ is angular velocity in radians per second.

For most macromolecules, the sedimentation coefficient is concentration-dependence because the frictional coefficient of the polymer varies with concentration. Usually a plot of $S$ versus concentration, and extrapolation to zero concentration enables a more accurate value of sedimentation coefficient denoted as $S^0$ to be obtained. There is no general formula for the concentration-dependence of $S$. As pointed out by Gralén (1954), for less compact or less symmetric particles, a better empirically linear relation can be obtained by equation (8.2).

$$\frac{1}{S} = \frac{1}{S^0} + kc$$

Whilst for more compact solute particles, the sedimentation coefficient may usually be taken as varying linearly with concentration and has the form

$$S = S^0 - kc$$
where \( c \) is the concentration and \( k \) is constant.

**Molecular Weights from Sedimentation and Diffusion**

If a non-electrolyte macromolecular component is subjected to a centrifugal field, it sediments with a centrifugal force per gram-mole, \( M(1-\bar{V}\rho)w^2x \), which is counterbalanced by the frictional force per gram-mole, \( F \frac{dx}{dt} \), i.e.

\[
M(1-\bar{V}\rho)w^2x = F\frac{dx}{dt} \tag{8.4}
\]

where \( M \) is the molecular weight of the solute, \( \bar{V} \) is the partial specific volume of the solute, \( \rho \) is the density of solvent. The molar frictional coefficient \( F \) can be expressed in terms of the kinetic energy and diffusion coefficient \( D \) by the relation

\[
F = \frac{RT}{D} \tag{8.5}
\]

where \( R \) is the gas constant in ergs per mole per degree and \( T \) the absolute temperature. A combination of equations (8.1), (8.4) and (8.5) gives the Svedberg equation

\[
M = \frac{RTS^0}{D^0(1-\bar{V}\rho)} \tag{8.6}
\]

This equation requires the knowledge of \( \bar{V} \) and \( D^0 \). \( \bar{V} \) can be easily measured by means of pyknometry, whereas the measurement of \( D^0 \) usually presents considerable obstacles in order to obtain high accuracy. To overcome this, Scheraga and Mandelkern (1953) combined the relation between the viscosity and sedimentation coefficient, and derived the following equation

\[
M = \left[ N\eta S^0/\beta(1-\bar{V}\rho) \right]^{3/2}[\eta]^2 \tag{8.7}
\]

where \( N \) is Avagadro number, \( \eta \) is viscosity of solvent, \( \eta^0 \) is intrinsic viscosity of solute, and \( \beta \) is a constant related to the axial ratio of the molecules (Ogston, 1953). This equation has been proved to be more strictly valid for proteins.

**Materials**

LADH and YADH were freshly prepared and assayed as described in Chapters
The stock solution was diluted with the desired solvent. All reagents were 'AnalaR' grade. Solutions were made up with double quartz-distilled water. pHs were measured with a Beckman 'Zeromatic' pH meter. The various buffer used was described in the text. pH above 10 was made by adding 2N NaOH to 0.1 M Glycine NaOH pH 10.0. pH below 4.0 was made by adding concentrated orthophosphoric acid to 0.1 M phosphate buffer, pH 7.3 to the desired pH.

Methods

Sedimentation experiments were performed in a Spinco Model E analytical ultracentrifuge, equipped with schlieren optics. Most of the sedimentation velocity studies were made at a temperature of 20° by means of a Rotor Temperature Indicator and Control (RTIC) unit. Photographs of sedimentation boundaries were taken at various time intervals. The absolute distance of the boundaries from the axis of rotation (x) were measured to the nearest 0.01 mm by means of a microcomparator. The value of S was calculated from the gradient of the line of least square from the plot of log x versus time. The maximum ordinate of the schlieren diagram was taken as the boundary position. Although this is true only for symmetrical boundaries (Goldberg, 1953), the error involved is usually small (Williams et al, 1953).

RESULTS

Sedimentation Coefficient of YADH and LADH

The freshly prepared LADH and YADH in 0.1 M phosphate buffer, pH 7.3 at 20° migrate as a single and very sharp, well-defined peak. There was no evidence of any aggregates or dissociation products. The schlieren pattern of YADH and LADH are presented in Fig. 8.1. After extrapolating to zero
Fig. 8.1. Schlieren pattern of: (a) LADH 6.8 mg/ml (upper pattern) and 5.0 mg/ml (lower pattern) after 38 minutes at 50,740 r.p.m. and 20°C; (b) YADH 7.8 mg/ml (upper pattern) and 11.1 mg/ml (lower pattern) after 30 minutes at 50,740 r.p.m. and 20°C. Both proteins were in 0.1 M phosphate buffer, pH 7.3. The pictures were taken at bar angles of 65° for YADH, and 60° for LADH.
concentration the sedimentation coefficient of YADH has been reported to be in a range of 6.72 - 7.67 S (Hayes and Velick, 1954; Sund, 1968). This discrepancy has been investigated in this work in more detail over a wide range of concentrations with two different field-forces. The results are shown in Fig. 8.2. It was found that the sedimentation coefficients of YADH are apparently dependent on the rotor speed of the centrifuge at which they were measured. The observed sedimentation coefficients increase with increasing rotor speed. At a speed of 50,740 r.p.m., the sedimentation coefficient obtained was plotted against various concentrations. The value extrapolated to zero concentration is found to be 6.85 in Svedberg units. The best fitting line has been calculated by means of the least square method. Its equation is

\[ S = 6.85 - 0.0308c \]  

where \( c \) is concentration of YADH in mg/ml. If the sedimentation velocity measurements were carried out at 59,780 r.p.m. The following equation is obtained

\[ S = 7.51 - 0.0336c \]

where 7.51 is the sedimentation coefficient extrapolated to infinite dilution. The explanation of this effect of force-field is not known, but the effect appeared to be reproducible.

Sedimentation constants of LADH at protein concentrations ranging from 2.2 mg/ml to 11 mg/ml were determined at rotor speed of 50,740 r.p.m. and 59,780 r.p.m. The concentration-dependence of the sedimentation coefficient is shown in Fig. 8.3. The equation calculated from the least square method gives

\[ S = 4.96 - 0.036c \]
Fig. 8.2. Concentration dependence of the sedimentation coefficient of YADH. Measurements were conducted in 0.1 \( \mu \) phosphate buffer, pH 7.3 at 20\(^\circ\)C and a rotor speed of either 50,740 r.p.m. (o) or 59,780 r.p.m. (o).
Fig. 8.3. Concentration dependence of the sedimentation coefficient of LADH. Experiments were carried out in 0.1M phosphate buffer, pH 7.3 at 20°C and at two rotor speeds: (○) 50,740 r.p.m., and (○) 59,780 r.p.m.
By extrapolation to zero concentration, the sedimentation coefficient \( (s_{20,w}^0) \) of LADH is 4.96. This value agrees well with those (5.11S) reported in the literature (Ehrenberg and Dalziel, 1958). Unlike YADH, there is no significant change of the sedimentation coefficient with the two different field-forces at which employed.

**Effect of Alkaline pH on the Sedimentation Coefficient of LADH and YADH**

The effect of pH on both LADH and YADH was investigated by sedimentation analysis in order to determine whether the inactivation of both enzymes by alkali and acid pH led to an observable dissociation process. The results of sedimentation velocity studies at various pHs are given in Table 8.1. The materials sediment as a single symmetrical peak without significant difference in sedimentation coefficient over a pH range of 5.0 - 8.0 for YADH, and from pH 6 to pH 10 for LADH. The isoelectric point of YADH is known to be pH 5.4 - 5.8 (Hays and Velick, 1954; Keleti, 1958; Wallenfels and Arens, 1960) and for LADH is pH 6.8 (Dalziel, 1958). The results indicate that YADH is more stable at low pH and less stable at high pH; the converse effects are true for LADH. This may show that the pH-effect is correlated to the isoelectric points of these enzymes. Table 8.1 also shows that there is no significant charge effect for the pH range examined, for no changes of the sedimentation coefficient occurred when the enzyme was subjected to either 0.1M phosphate buffer at pH 7.0, or 0.1 M sodium acetate - acetic acid buffer at pH 5.8 for both enzymes.

On exposure of YADH to 0.1 M Tris-H\(_3\)PO\(_4\) buffer pH 8.8, sedimentation analysis was carried out at 50,740 r.p.m. and, in addition to the original peak of 6.73 S, a second more slowly moving peak of 2.67 S was observed. These changes were independent of the concentration of YADH used (Fig. 8.4).
<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>$S_{20,w}$</th>
<th>No. of Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N NaOH</td>
<td>13</td>
<td>1.31a,b</td>
<td>1</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>13+</td>
<td>1.30a,b</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>12</td>
<td>1.67a,b</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>12+</td>
<td>1.70a,b</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>11</td>
<td>2.50a,b</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>10.2</td>
<td>2.67a</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>9.2</td>
<td>2.67, 6.62a</td>
<td>2</td>
</tr>
<tr>
<td>0.1 M Tris-H$_3$PO$_4$</td>
<td>8.8</td>
<td>2.67, 6.73a</td>
<td>2</td>
</tr>
<tr>
<td>0.1 μ Phosphate</td>
<td>8.0 - 5.3</td>
<td>6.8a, 7.4b</td>
<td>1</td>
</tr>
<tr>
<td>0.1 μ Phosphate</td>
<td>3.5</td>
<td>9.21a</td>
<td>1</td>
</tr>
<tr>
<td>0.1 μ Phosphate</td>
<td>3.3</td>
<td>7.89a</td>
<td>1</td>
</tr>
<tr>
<td>0.1 μ Phosphate</td>
<td>3.0</td>
<td>6.84a</td>
<td>1</td>
</tr>
<tr>
<td>0.1 μ Phosphate</td>
<td>2.5</td>
<td>3.48a</td>
<td>1</td>
</tr>
<tr>
<td>0.1 μ Phosphate</td>
<td>2.2</td>
<td>2.66b</td>
<td>1</td>
</tr>
</tbody>
</table>

The protein (3.6 - 4.4 mg/ml) was exposed to the medium for 10 minutes before carrying out measurements at 20°C with a rotor speed of (a) 50,740 r.p.m. or (b) 59,780 r.p.m. (c) after exposing the protein into the medium for 24 hours.
Fig. 8.4. Sedimentation pattern of YADH (3.6 mg/ml) after exposing to 0.1 M Tris-H$_3$PO$_4$, pH 8.8 (upper pattern) and pH 8.0 (bottom pattern) for 10 minutes.

The pictures were taken at bar angle 55° after 15 minutes (a); and 60 minutes (b); at 50,740 r.p.m. and 20°C.
On increasing pH from 8.8 to 9.2, the amount of the 2.67 S-component increases with concomitant decreases in the amount of the 6.73 S-component (Fig. 8.5). Both boundaries are symmetrical, indicating two homogeneously sedimenting species. The 6.73 S-component can be completely transformed into a single symmetrical peak of 2.67 S if YADH was exposed to 0.1 M Glycine-NaOH buffer pH 10.2. Furthermore, at pH 11 a value of 2.5 S is obtained. When YADH was added to pH 12.0, or in 0.1 N NaOH, there is a further marked reduction in the sedimentation coefficients to 1.67 S and 1.31 S, respectively (Fig. 8.6). The time effect on exposure of YADH to high pH was also examined. When 0.4% of YADH was added to pH 11.0 and allowed to stand at room temperature for a period of 24 hours, the sedimentation velocity analysis shows that the single boundary of 2.5 S is changed to two boundaries with S values of 2.09 and 6.11 (Fig. 8.7). If similar experiments were carried out by exposure of YADH to pH 12 and 0.1 N NaOH, the sedimentation boundary remains virtually unchanged over a period of 24 hours with a value of 1.70 S and 1.30 S, respectively. These further changes in sedimentation coefficient at pH 11 but not at pH 12 and 0.1 N NaOH, may suggest that the enzyme has not been completely dissociated, and that it is only the entire dissociation which produces stable subunits.

β-Mercaptoethanol is widely used as a stabilising reagent for protein subunit. The effect of β-Mercaptoethanol on YADH in high pH is shown in Table 8.2. In the presence of 0.1 M β-Mercaptoethanol the sedimentation patterns of YADH at pH 10.2 still consist of two peaks of 2.70 S and 7.10 S, and in pH 11.0 with two peaks of 2.51 S and 6.81 S (Fig. 8.8). These solutions were allowed to stand at room temperature for a period of 24 hours, and were then subjected to sedimentation analysis again. At pH 10.2, a
Fig. 8.5. Sedimentation pattern of (a) 3.6 mg/ml YADH in 0.1 M glycine-NaOH buffer, pH 9.2 (upper pattern) and pH 10.2 (bottom pattern); (b) 7.2 mg/ml at pH 9.2 (upper pattern) and at pH 12.0 (bottom pattern); for 10 minutes before being centrifuged at 50,740 r.p.m. and 20°C.

The pictures were taken after 60 minutes of attaining the maximum speed at bar angle 55° for (a), and 60° for (b).
Fig. 8.6. Sedimentation pattern of YADH (4.1 mg/ml) after exposing to 0.1 N NaOH (upper pattern) and 0.1 M glycine-NaOH pH 11.0 (lower pattern) for 10 minutes before being centrifuged at 50,740 r.p.m. and 20°C. The picture was taken after 50 minutes of attaining the maximum speed at bar angle 60°.
Fig. 8.7. Schlieren pattern of (4.7 mg/ml) YADH, after exposing to 0.1 M glycine-NaOH, pH 11.0 in the presence (upper pattern) and absence (lower pattern) of 0.1 M β-mercaptoethanol for 24 hours at room temperature. The picture was taken at bar angle 60° after 12 minutes at 59,780 r.p.m. and 20°C.
<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>$S_{20,w}$</th>
<th>No. of Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N NaOH</td>
<td>13</td>
<td>1.31</td>
<td>1</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>13$^a$</td>
<td>1.31</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>11</td>
<td>2.51, 6.81</td>
<td>2</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>11$^a$</td>
<td>2.09, 13.1</td>
<td>2</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>10.2</td>
<td>2.70, 7.10</td>
<td>2</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>10.2$^a$</td>
<td>15.6</td>
<td>1</td>
</tr>
<tr>
<td>0.1 μ Phosphate</td>
<td>3.5</td>
<td>9.21$^b$</td>
<td>1</td>
</tr>
<tr>
<td>0.1 μ Phosphate</td>
<td>2.5</td>
<td>3.43$^b$</td>
<td>1</td>
</tr>
</tbody>
</table>

Measurements were carried out at 20°C and a rotor speed of 59,780 r.p.m., using protein concentration in the range 3.6 - 4.5 mg/ml. The medium also contained 0.1 M β-mercaptoethanol.

(a) On exposure of the protein into the medium at 20°C for 24 hours.
(b) Rotor speed of 50,740 was used.
Fig. 8.8. Effect of β-mercaptoethanol on sedimentation coefficient of YADH at alkaline pH. After adding the protein to solution of: (a) pH 10.2 + ME (upper pattern) and pH 12.0 (lower pattern); (b) pH 11.0 + ME (upper pattern) and pH 11.0 (lower pattern); (c) 0.1 N NaOH + ME (upper pattern) and 0.1 N NaOH (lower pattern); for 10 minutes before being centrifuged at 59,780 r.p.m. and 20°C. (d) and (e) were similar to (a) and (c), respectively, except the protein was exposed to the alkaline pH for 24 hours.

The concentration of the protein used in each sample was 4.7 mg/ml. The schlieren patterns were photographed at 40 minutes for (a) and (b), 43 minutes for (c), 25 minutes for (d), and 45 minutes for (e), after attaining the maximum speed.

ME = 0.1 M β-mercaptoethanol. Bar angle = 60°.
single peak of 15.60 S was obtained. While at pH 11.0, the component of 2.51 S is degraded further to 2.09 S, and the fast moving peak is changed from 7.10 S to 13.10 S. On exposure of YADH to 0.1 N NaOH, whether \( \beta \)-Mercaptoethanol is present or not, a single symmetrical peak of 1.31 S is obtained. There is no further change over a period of 24 hours. These results may indicate that \( \beta \)-Mercaptoethanol only protects YADH against dissociation in a certain pH range, whilst in the range of 8.8 - 11, it promotes aggregation as a function of time. It was also observed that schlieren patterns with higher sedimentation coefficients are more diffuse than those at lower ones.

The same method for YADH was applied to study the effect of alkaline pH on sedimentation coefficient of LADH. Table 8.3 lists the sedimentation coefficients of LADH at various pHs. LADH is known to be very stable in a pH range of 6 - 10. It was also found that in this pH range a single sharp boundary was obtained indicating a single component with sedimentation coefficient of about 4.7 S. At pH 11 and 11.7 the sedimentation coefficient is 4.46 S and 3.35 S, respectively. When 0.4% LADH was added to pH 12 for 10 minutes before sedimentation, two peaks with sedimentation coefficients of 1.72 S and 3.69 S are obtained (Fig. 8.9). In the presence of 0.1 M \( \beta \)-Mercaptoethanol, the corresponding peaks have S-values of 1.75 and 3.72. In 0.1 N NaOH, with or without the addition of 0.1 M \( \beta \)-Mercaptoethanol, values of the sedimentation coefficient 1.74 S and 2.54 S are obtained (Fig. 8.10). Solutions of both pH 12 and 0.1 N NaOH were kept for about 24 hours at room temperature, and then subjected to sedimentation analysis. At pH 12, in the presence of 0.1 M \( \beta \)-Mercaptoethanol gives a single broad schlieren pattern of 7.23 S; without \( \beta \)-Mercaptoethanol, it is 6.74 S. Over a period of
<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>$S_{20, w}$</th>
<th>No. of Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N NaOH</td>
<td>13.0</td>
<td>1.74, 2.54</td>
<td>2</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>13.0</td>
<td>1.33</td>
<td>1</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>13.0</td>
<td>1.14</td>
<td>1</td>
</tr>
<tr>
<td>0.1 N NaOH + ME</td>
<td>13.0</td>
<td>1.74, 2.54</td>
<td>2</td>
</tr>
<tr>
<td>0.1 N NaOH + ME</td>
<td>13.0</td>
<td>1.29, 6.21</td>
<td>2</td>
</tr>
<tr>
<td>0.1 N NaOH + ME</td>
<td>13.0</td>
<td>1.16, 6.60</td>
<td>2</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>12.0</td>
<td>1.72, 3.69</td>
<td>2</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>12.0</td>
<td>6.74</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH + ME</td>
<td>12.0</td>
<td>1.75, 3.72</td>
<td>2</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH + ME</td>
<td>12.0</td>
<td>7.23</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>11.7</td>
<td>3.35</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH or</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M Phosphate</td>
<td>10 - 6.0</td>
<td>4.70</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Phosphate</td>
<td>4.0</td>
<td>5.75</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Phosphate</td>
<td>3.2</td>
<td>4.80</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Phosphate</td>
<td>3.2</td>
<td>6.96</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M Phosphate + ME</td>
<td>2.4</td>
<td>3.20</td>
<td>1</td>
</tr>
</tbody>
</table>

The protein (3.3 - 4.6 mg/ml) was exposed to the medium for 10 minutes at 20°C before being centrifuged at 59,780 r.p.m. and 20°C.

(a) and (b) are after exposure of the protein to the medium for 24 and 48 hours, respectively.

ME = 0.1 M β-mercaptoethanol.
Fig. 8.9. Sedimentation pattern of LADH (4.3 mg/ml) after exposing to 0.1 M glycine-NaOH pH 12 in the presence (upper pattern) and absence (lower pattern) of 0.1 M β-mercaptoethanol. The picture was taken at bar angle 60° after 50 minutes of attaining the maximum speed of 59,780 r.p.m. at 20°C.

Fig. 8.10. Sedimentation pattern of LADH (4.6 mg/ml) after exposing to 0.1 N NaOH in the presence (upper pattern) and absence (lower pattern) of 0.1 M β-mercaptoethanol. The picture was taken after 70 minutes at 59,780 r.p.m. and 20°C. The bar angle was 60°.
48 hours in the presence of mercaptoethanol, the sedimentation coefficient is 7.28 S, and without mercaptoethanol, a broad unsymmetrical peak is obtained from which it is impossible to evaluate the sedimentation coefficient. At 0.1 N NaOH for 24 hours in the presence of mercaptoethanol gives two shoulder peaks with sedimentation coefficients of 1.29 S and 6.21 S. Again in the absence of mercaptoethanol, only a single peak with a sedimentation coefficient of 1.33 S is obtained. After a prolonged period of exposure of LADH to 0.1 N NaOH (for 48 hours) in the absence of β-Mercaptoethanol, the enzyme was further degraded to material with a sedimentation coefficient of 1.14 S. In contrast with β-Mercaptoethanol, two peaks with sedimentation of 1.16 S and 6.65 S are obtained (Fig. 8.11), indicating one component is further degraded to smaller units while the other component is aggregated to larger molecules as a function of time.

Effect of Acid pH on Sedimentation Coefficient of LADH and YADH

LADH and YADH are inactivated by low pH. The effect of acid pH on LADH and YADH has also been investigated at 20° with sedimentation analysis in order to ascertain whether the acidification process had involved the dissociation of the proteins into subunits. The sedimentation velocity studies of LADH in different acidic media are given in Table 8.3 and YADH in Table 8.1. At acid pH, sedimentation velocity studies of both enzymes are complicated by the fact that aggregation occurs. These aggregation processes depend upon time, pH, temperature and concentration of the protein used. Aggregated species occur more readily at pH 3.5 for YADH, and pH 4.0 for LADH, and the sedimentation coefficients are found to be 9.21 S and 5.75 S, respectively. As shown in Tables 8.1 and 8.3 above pH 5.0, no gross changes in sedimentation coefficient occur for both enzymes in comparison with the effects at pH 7.0.
Fig. 8.11. Sedimentation pattern of LADH (4.6 mg/ml) after treatment with 0.1 N NaOH in the presence (upper pattern) and absence (lower pattern) of 0.1 M β-mercaptoethanol for 24 hours (a); and 48 hours (b).

The pictures were taken at bar angle 55° for (a), and 55° for (b), after 36 minutes at 59,780 r.p.m. and 20°C.
Treatment of LADH with pH 3.2, gives a sedimentation coefficient of 4.80 S, while after a further period of 24 hours, the sedimentation coefficient is found to be 6.96 S. At pH 2.4, the value of 3.2 S is found.

For YADH, at pH 2.5 and 2.2, the sedimentation coefficients obtained are 3.48 S and 2.66 S, respectively. With or without the presence of 0.1 M β-Mercaptoethanol, no alteration of sedimentation coefficient occurs when both YADH and LADH are exposed to pH 3.5 and pH 2.4, respectively, indicating that mercaptoethanol does not affect the sedimentation coefficient of these enzymes at low pH.

Sedimentation studies were also conducted at the isoelectric point of each enzyme at a buffer concentration of 0.1 M. No significant difference in sedimentation coefficient is found. No doubt, the molecule is highly charged at low pH, and the ionic strength of the supporting medium is low. Thus, the sedimentation velocities reported must be treated with caution as they represent hydrodynamic properties obtained under conditions where intermolecular interactions are not minimized.

8.2.b. Sedimentation Equilibrium

Equilibrium ultracentrifugation has several advantages for the characterization of macromolecular systems; not only is it one of the more attractive methods of determining molecular weights of large and small molecules, but also the more common weight-average molecular weights in pauci-disperse or polydisperse solute systems can be determined. In addition, it may also provide activity, or second virial coefficient, data. If the sedimentation equilibrium experiment is carried out at or near the isoelectric point, in the presence of adequate amounts of supporting electrolyte in the medium, the interactions effects of solute-solute or solute-solvent can be minimized and many
theoretical complexities can be removed.

Recently, the sedimentation equilibrium method, or the approach to sedimentation equilibrium method, has been employed to study the proteins which undergo dissociation or association reactions and to evaluate the weight-average molecular weights and equilibrium constants (Adams, 1967).

Theory

When the ultracentrifuge is operated at a relatively low speed, the migration of solute in a centrifugal direction due to sedimentation is sufficiently slow as to be counter-balanced by diffusion resulting from the concentration gradient created by the partial sedimentation of the macromolecules, when equilibrium is attained, i.e.

$$cS_w^2 x = D(dc/dx)$$  (8.11)

where $c$ is the concentration of macromolecule. Application of the Svedberg equation gives the relation

$$\mu_w = \left[ \frac{2RT}{(1-\bar{v})w^2} \right] \left[ \frac{\partial \ln c}{\partial (x^2)} \right]$$  (8.12)

The usual differential equation for sedimentation equilibrium of a two-component system, composed of a solvent and a homogeneous solute, is

$$\mu_w = \left[ \frac{RT}{(1-\bar{v})w^2} \right] \left( \frac{1}{\mu_x} \right) (dc/dx) \left[ 1 + c(\partial \ln y/\partial c) \right]$$  (8.13)

where $y$ is the activity coefficient of the solute on $c$ scale. For ideal systems, as is the case for many proteins, the term in parentheses in equation (8.13) is equal to unity; thus

$$\mu_w = \left[ \frac{RT}{(1-\bar{v})w^2} \right] \left( \frac{1}{\mu_x} \right) (dc/dx)$$  (8.14)

The molecular weight can be obtained by several empirical methods of graphing the data. When $\ln c$ is plotted against $x^2$, the slope of the resulting line is a measure of the molecular weight. If this graph shows an upward concave curvature, polydispersity is indicated, whilst a downward
concave curvature is an index of the non-ideality of the solution.

Lamm (1929) pointed out that if solute is homogeneous, the data can be plotted as \( \ln(1/x)(dc/dx) \) versus \( x^2 \). This method requires very little effort with regard to calculations, but it proves to be an insensitive way of handling the data, and it is difficult to judge precisely when equilibrium is attained. A more critical method of handling the data is to graph \( (1/xo)(dc/dx) \) versus \( x \); a method suggested by Archibald (1947). This graph provides a precise index of the attainment of equilibrium, it also involves the use of all the data and therefore gives a reliable index of the molecular weight. Plots with a positive slope, indicate polydispersity, whilst a negative slope indicates non-ideality.

However, the major disadvantage of employing the sedimentation equilibrium technique to study the molecular weight has been the long time required to attain equilibrium; sometimes it may take as long as 4 days for a protein of molecular weight about 60,000. Thus, many unstable proteins are not suitable to be studied by this technique. Since the time required to reach equilibrium is proportional to the square of the depth of solution, van Holde and Baldwin (1958) pointed out that equilibrium time can be drastically reduced when a short column (1 - 3 mm) is used. If the position in the column where the concentration is equal to the original concentration, \( C_0 \), is known, the concentration gradient \( (dc/dx) \) at that point can be determined, and thus equation (8.14) can be applied to evaluate the molecular weight. Van Holde and Baldwin (1958) showed that at the point

\[
x' = \left[ \frac{(a^2 + b^2)}{2} \right]^\frac{1}{2}
\]

(8.15)

which for a 1 mm column is within 0.002 mm of the midpoint, the concentration is given by the equation (8.16)
\[
\frac{c_0}{c(x')} = \frac{\sinh H}{H} = 1 + \frac{H^2}{6} + \ldots \quad (8.16)
\]
and derived the equation (8.17)
\[
H = \frac{w^2M(1-V)}{4RT} \left(\frac{b^2-a^2}{a^2}\right) \quad (8.17)
\]
where \(a\) and \(b\) are the distance from the center of rotation to the meniscus and base of the solution. The concentration at the midpoint of a short column is within \(1\%\) of the initial concentration of \(H \leq 0.25\). This technique has been applied to study the weight-average molecular weight of ribonuclease using either 3 mm or 1 mm column at rotor speed of 20,410 r.p.m. The result gives a molecular weight of \(1.382 \times 10^6\) and is in good agreement with the known molecular weight (van Holde and Baldwin, 1958).

Even shorter column (0.7 mm) with multi-channel cell has been designed by Yphantis (1960), and applied to study the weight-average molecular weight of several proteins, and have given consistent results.

A Z-average molecular weight, \(\bar{M}_z\), may be obtained from the slope of a plot of \(dc/dx\) versus \(x\), but the result is rather unsatisfactory.

However, owing to the small volume of solution used, several experimental errors may result, e.g., evaporation which can lead to an erroneous value for \(C\), absorption onto the walls of cells if low concentration of the protein is used. More precise results can be achieved only with the use of longer column and longer equilibrium times.

As a consequence of the renewed interest in the sedimentation equilibrium method, many extensions to the theory have resulted (Fujita, 1962; Yphantis, 1964; Hermans, 1964; Lutze, 1964; Osterhoudt and Williams, 1965; Fujita and Williams, 1966; Donnelly, 1966; Richards et al, 1968). For a paucidisperse system, the following equations have been derived:
\[
\bar{M}_{w,x} = \frac{[RT/(1-V)p]w^2}{(dc/dx)x(1/xc_x)} \quad (8.18)
\]
\[ \overline{M}_w = \frac{RT}{(1-V/0)w^2} \left[ C_b \int_a^b C_x \, dx \right] \]  
(8.19)

\[ \overline{M}_n = \frac{RT}{(1-V/0)w^2} \left( \int_a^b C_x \, dx \right) / \left( \int_a^b a x \, dx \right) \]  
(8.20)

where \( \overline{M}_{w,x} \) is weight-average molecular weight, \( \overline{M}_w \) is weight molecular weight, \( \overline{M}_n \) is number-average molecular weight and the subscript \( x \) refers to the level in cell at \( x \) cm from the axis of rotation.

For a nonideal system, at low concentration, equation (8.21) can be applied

\[ \frac{1}{\overline{M}_w} \text{app} = \frac{1}{\overline{M}_w} + B'' C + \ldots \]  
(8.21)

where

\[ B'' = (1 + \lambda^2_{M} 2^L/12) B_{LS} \]  
(8.22)

The intercept from a plot of \( 1/\overline{M}_w \text{app} \) versus \( C \) which permits the evaluation of the weight-average molecular weight of the solute and the limiting slope is correlated with the second virial coefficient, \( B'' \) obtained from light scattering measurement, \( B_{LS} \) (Kegelos et al., 1957).

**Methods**

The short column method of Yphantis (1960) was used to determine the weight-average molecular weight throughout this work. The low speeds were attained by means of a 6:4:1 step-down gear. A standard 12 mm double sector epoxy cell with quartz windows was used. For some experiments, in order to obtain a precise base-line, a drop of silicone oil was introduced into one sector before adding 0.05 ml solution by means of an "Alga" micrometer syringe which gave the height of the liquid column of approximately 1.5 mm. As a precaution, the syringe was rinsed with solution before use. An appropriate speed was chosen according to equation 8.17. Prior to the equilibrium experiments, the initial concentration \( C \) was determined by means of a synthetic
boundary centre-piece. The equilibrium experiments were performed at 20°C by using schlieren optics and a phase-plate angle between 60° and 80°. To equilibrate the temperature of the cell and rotor, a period of about 10 minutes was allowed before evacuating the chamber. The specific refractive increment is known to be temperature dependence, therefore, synthetic boundary and equilibrium experiments were performed at the same temperature and same bar angle. In order to ascertain that the equilibrium time had been attained in the short column experiments, photographs were taken at one-hour intervals; equilibrium was established when successive photographs showed no further changes. A micro-comparator was used to analyse the photographic records. C was determined by measuring the area beneath the synthetic boundary peak and correcting for radial magnification. Using the solvent pattern as a base line, value of the magnified dc/dx at the midpoint of the solution column, and its absolute distance x from the axis of rotation, were determined. The weight-average molecular weights of LADH and YADH were obtained by substituting these data in equation 8.14.

RESULTS

Weight-average Molecular Weight of YADH

The weight-average molecular weight of YADH was determined by short-column, low-speed equilibrium technique of Yphantis (1960). The effect of force-field on the molecular weight of YADH was investigated by employing a series of rotor speeds ranging from 5,560 r.p.m. to 12,590 r.p.m. Table 8.4 lists the results. These results were calculated by using a value of partial specific volume 0.769 (Hays and Velick, 1954). Each run was performed at 20°C at a concentration of 0.27% of YADH in 0.1 M phosphate buffer, pH 7.3. Photographs were taken at one hour intervals over a period of 8 hours after
TABLE 8.4
Effect of force-field on the molecular weight of YADH

<table>
<thead>
<tr>
<th>Rotor Speed (r.p.m.)</th>
<th>Molecular Weight $\times 10^{-5}$</th>
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</thead>
<tbody>
<tr>
<td>5,560</td>
<td>1.10</td>
</tr>
<tr>
<td>6,570</td>
<td>1.10</td>
</tr>
<tr>
<td>7,450</td>
<td>1.08</td>
</tr>
<tr>
<td>8,225</td>
<td>1.08</td>
</tr>
<tr>
<td>9,340</td>
<td>0.824</td>
</tr>
<tr>
<td>12,590</td>
<td>0.470</td>
</tr>
</tbody>
</table>

$0.27\%$ of YADH in $0.1\mu$ phosphate buffer, pH 7.3 was used for determining the molecular weight at $20^\circ C$.

TABLE 8.5
The molecular weight of YADH obtained from determinations at various concentrations

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Molecular Weight $\times 10^{-5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
<td>1.10</td>
</tr>
<tr>
<td>4.5</td>
<td>1.09</td>
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</tr>
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<td>6.5</td>
<td>1.11</td>
</tr>
<tr>
<td>8.0</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Measurements were performed at 7,450 r.p.m. and $20^\circ C$. YADH was dissolved in $0.1\mu$ phosphate buffer, pH 7.3.

* $0.1\ M$ acetic-acetate buffer, pH 5.8 was used.
attaining the maximum speed; no further change was observed after 5 hours. The molecular weight was found to decrease as the rotor speed employed was higher than 9,340 r.p.m. At the rotor speeds of 9,340 r.p.m. and 12,590 r.p.m. the apparent values for the molecular weight of YADH of 82,400 and 47,000, respectively were obtained, whilst a rotor speed range between 8,225 r.p.m. and 5,560 r.p.m. gives values of 108,000 - 112,000.

Obviously these molecular weights are lower than those of 150,000 reported by Hays and Velick (1954). These authors evaluated the molecular weight from the diffusion and sedimentation constants.

More experiments were carried out in order to find out whether this discrepancy is due to (1) the protein concentration-dependence, (2) the time required to attain equilibrium, (3) pH-effects and (4) the presence of the silicone oil.

The sedimentation equilibrium were performed at a concentration range of 0.27% - 0.30% in 0.1 M phosphate buffer pH 7.3 at a rotor speed of 7,450 r.p.m. and 20°C. Table 8.5 lists the results. These values were again found to be equivalent to that of the molecular weight of 110,000. Significantly, there is no anomalous dependence on protein concentration in the average molecular weight of YADH.

The molecular weight determination was performed at a concentration of 0.45% in 0.1 M phosphate buffer at 7,450 r.p.m. at 20°C over a period of 24 hours. The results for apparent molecular weight as a function of the time are shown in Fig. 8.12 and the typical equilibrium patterns in Fig. 8.13.

A constant molecular weight of 110,000 is obtained between 5 and 15 hours after reaching the maximum speed. This constant value implies that
Fig. 8.12. Sedimentation equilibrium experiments with YADH. A graph of apparent molecular weight as a function of time. Experiments were performed at 7,450 r.p.m. and 20°C with the use of (4.5 mg/ml) YADH in 0.1 M phosphate buffer, pH 7.3
Fig. 3.13. Typical equilibrium pattern of YADH 4.5 mg/ml in 0.1 M phosphate buffer, pH 7.3.

The pictures were taken at bar angle (a) 60°, and (b) 70°, after 8 hours at 7,450 r.p.m. and 20°C.
true equilibrium is reached. Over the period of 15 hours, the molecular weight is to decrease very little.

Experiments were also performed with 0.46% YADH at the isoelectric point of YADH in 0.1 M sodium acetic-acetate buffer pH 5.8 at 7,450 r.p.m. and 20°C. Again a similar result of 110,000 for the molecular weight of YADH was obtained either with, or without, the presence of silicone oil.

**Molecular Weight of Ovalbumin**

In view of these results, a check was made in order to find out whether there was a systematic error in the technique employed. Egg ovalbumin of known molecular weight was examined by the same technique and under the same conditions used for YADH. A solution of 1% egg ovalbumin in 0.1 M NaCl was examined at 12,590 r.p.m. The molecular weight obtained is 44,700 based on a partial specific volume 0.75 (Warner, 1954). Whereas the molecular weights from literature are 44,000 - 46,000 (Warner, 1954; Andrew, 1965). This result shows that the molecular weight obtained by the technique used is satisfactory.

**Molecular Weight of LADH**

Table 8.6 shows the weight-average molecular weight of LADH under the same conditions for YADH at three different rotor speeds employed and with a solution of 0.4% LADH in 0.1 M phosphate buffer, pH 7.3. The typical sedimentation equilibrium pattern is shown in Fig. 8.14. A partial specific volume 0.75 (Ehrenberg, 1957) was used for the calculation of the molecular weight. The molecular weight of LADH was found to be 84,500 - 88,000 in good agreement with the value 84,000 (see review, Sund and Theorell, 1963).

**Comments on the Results for YADH**

Low molecular weights for YADH (126,000 - 129,000) have also been reported
### TABLE 8.6

Molecular Weight of LADH

<table>
<thead>
<tr>
<th>Rotor Speed</th>
<th>Molecular Weight x 10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,450</td>
<td>8.45</td>
</tr>
<tr>
<td>9,340</td>
<td>8.50</td>
</tr>
<tr>
<td>12,590</td>
<td>8.80</td>
</tr>
</tbody>
</table>

Measurements were carried out at 20°C with 0.33 - 0.40% LADH in 0.1 M phosphate buffer, pH 7.3.

### TABLE 8.7

Molecular Weight of YADH at alkaline pH

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>Molecular Weight x 10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N NaOH</td>
<td>13.0</td>
<td>2.0 - 2.3*</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>12.0</td>
<td>3.6</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>11.0</td>
<td>7.2</td>
</tr>
<tr>
<td>0.1 M Glycine-NaOH</td>
<td>10.2</td>
<td>7.2</td>
</tr>
</tbody>
</table>

0.32 - 0.4% YADH was exposed to the alkaline pH for 10 minutes at 20°C before being employed for determining the molecular weight at 7,450 r.p.m. and 20°C.

* Rotor speeds of 7,450, 9,340, or 12,590 r.p.m. were used.
Fig. 8.14. Typical equilibrium pattern of LADH in 0.1M phosphate buffer, pH 7.3.

(a) Protein = 4.0 mg/ml, speed = 12,590 r.p.m.
   bar angle = 70°

(b) Protein = 3.3 mg/ml, speed = 9,340 r.p.m.
   bar angle = 70°

(c) as in (b) but at 7,450 r.p.m.

Each experiment was performed at 20°C and each picture was taken after 8 hours of attaining the maximum speed.
by Armstrong et al. (1963) and Andrews (1964), using approach sedimentation equilibrium technique and gel filtration. However, the reason for this discrepancy is uncertain, although the sedimentation equilibrium experiments were performed without the presence of supporting electrolyte and the ionic strength used was low. It is unlikely that these are the main reasons responsible for the low molecular weight which resulted, in view of the results obtained from sedimentation analysis and when the experiments were performed at the isoelectric point of YADH.

**Molecular Weight of YADH at High pH.**

YADH is reversibly inactivated by alkaline pH. Sedimentation velocity experiments have shown that two separate boundaries exist when the enzyme was exposed to 0.1 M Tris- \( \text{H}_3 \text{PO}_4 \) buffer pH 8.8 - 9.2 (see p. 209), whilst at 0.1 M glycine-NaOH, pH 10.2, or pH 11.0 without the presence of \( \beta \)-mercaptoethanol, a symmetrical schlieren pattern with sedimentation coefficient of 2.67 S and 2.50 S, respectively, are obtained. At pH 12 and 0.1 N NaOH, whether \( \beta \)-mercaptoethanol is added or not, it gives 1.69 S and 1.30 S, respectively.

In light of these results, the evaluation of the molecular weights were carried out at 20°C by means of the short-column, low-speed technique (Yphantis, 1960). The molecular weight calculated from these measurements are shown in Table 8.7, based on a value of partial specific volume 0.769 ml/g. (Hayes and Velick, 1954). Experiments were carried out by adding 0.4% YADH to pH 10.2 or 11 for 10 minutes, and sedimentation equilibrium measurements were performed at 7,450 r.p.m. and 20°C. Fig. 8.15 shows the typical equilibrium pattern. The molecular weight obtained from both pH is 72,000. YADH was also added to pH 12.0 and experiments were carried out.
Fig. 8.15. Sedimentation equilibrium pattern of YADH (a) 3.7 mg/ml at pH 10.2, and (b) 4.1 mg/ml at pH 11.0. The pictures for both experiments were taken at bar angle 60° after 7 hours at 7,450 r.p.m. and 20°C.

Fig. 8.16. Sedimentation equilibrium pattern of YADH (3.6 mg/ml) in 0.1 M glycine-NaOH pH 12.0. The picture was taken at bar angle 60° after 3 hours at 7,450 r.p.m. and 20°C.
at 7,450 r.p.m. and 20°C. The typical equilibrium pattern is shown in Fig. 8.16. A smaller molecular weight of 36,000 is obtained. Whilst at 0.1 N NaOH the speeds of 7,450 r.p.m. and 12,590 r.p.m. were employed; both experiments give molecular weights of 20,000 - 23,000. The sedimentation equilibrium pattern of YADH at 0.1 N NaOH is shown in Fig. 8.17. However, previous reports have shown that YADH is dissociated to four subunits with a sedimentation coefficient of 2.5 - 2.8 S and a molecular weight of 36,000 after the treatment of the protein with 1,10-phenanthroline and 8-hydroxy-quinoline-5-sulfonic acid (Kagi and Vallee, 1960; Snodgrass, et al., 1960), urea (Sund, 1960; Ohta and Ogura, 1965) and sodium dodecyl sulphate (Hersh, 1962). The molecular weight obtained in 1,10-phenanthroline and urea is evaluated from sedimentation coefficient and diffusion and the weight-average molecular weight of the mixture (subunit and native enzyme). And also these results have not been corrected for absolute viscosity. A recent report by Butler et al. (1969) who showed that YADH is dissociated in to four subunits after the enzyme is activated by urea or guanidino hydrochloride followed by reaction with maleic anhydride. The maleyl protein has a sedimentation coefficient of 1.7 S and a molecular weight of 42,000. The sedimentation coefficient which was obtained here after the treatment of YADH with pH 12 favours this report. But it seems that the native enzyme can be further degraded into smaller fragments at 0.1 N NaOH and only that a complete dissociation of the native enzyme which produces stable subunits.

However, in view of the apparent time-dependent aggregation effects shown by sedimentation velocity studies when YADH and LADH are exposed to acidic media, and also for LADH at high pH (see p. 218), no attempt was made to determine the molecular weight of both enzymes under those conditions. These
Fig. 8.17. Sedimentation equilibrium pattern of YADH (3.2 mg/ml) in 0.1 N NaOH. Experiments were performed at 20°C and either (a) 9,340 r.p.m., or (b) 12,590 r.p.m. The pictures for both experiments were taken at bar angle 60° after 8 hours of attaining the maximum speeds.
phenomena are the subject of further investigations.

8.3. REFRACTIVE INDEX INCREMENTS

The specific refractive index increment, $dn/dc$, is an optical constant required in the light-scattering method of evaluating the molecular weight of macromolecules. Its precise measurement is important as it enters equation 8.27 as a square term. A small error can lead to inaccuracies in the determination of molecular weight. The specific refractive index increment depends upon temperature and wavelength of the light employed as well as the nature of the solvent (O’Mara and McIntyre, 1959).

Methods

The determinations were performed at $23^\circ C$ with a differential refractometer designed by Brice and Halwer (1951) and manufactured by the Brice-Phoenix Precision Instrument Corporation (Philadelphia, U.S.A.). The instrument was equipped with an AH-3 mercury lamp and with mounted monochromatic filters for isolating blue and green light of wavelengths 4360A and 5461A, respectively. An optical sinter-fused cell with two diagonally-arranged compartments is mounted by means of set screws bearing against plastic sheets on a rotatable holder in a jacketed housing. The cell can be rotated through $180^\circ$ by means of a handle stopped by Allen-head screws. When in use, the cell was covered by plastic plugs to prevent evaporation. The solvent was clarified with a MSE Model Superspeed 50 preparative ultracentrifuge at a rotor speed of 20,000 - 30,000 r.p.m. for 2 - 3 hours and solution at 15,000 r.p.m. for $\frac{1}{2}$ hour.

To determine $c_1 - c_2$ (the displacement of the image of the cell) for solvent, each compartment was filled with 1 ml solvent, and the readings were taken after waiting about 10 minutes for temperature equilibrium. To determine $c_1 - c_2$ for solution, the solvent was emptied from one compartment labelled
"solution" by a syringe. All traces of solvent were removed with strips of filter paper, the compartment was then rinsed several times with distilled water and acetone and finally with portion of the solution before the final solution to be measured was introduced. The instrument was calibrated by Hourston (1967) with the use of standard solutions of sucrose and polystyrene. The calibration constant was found to be $1.021 \times 10^{-3}$ at 5461A and $1.025 \times 10^{-3}$ at 4358A.

RESULTS

The refractive index increments of YADH and LADH in various solvents are listed in Table 8.8. The measurements were carried out at $23^\circ$C and at a wavelength of 5461A. In 0.1 M phosphate buffer, pH 7.3, $dn/dc$ for both YADH and LADH was found to be 0.200. There is no gross changes for both enzymes in 0.1 phosphate buffer or pH 4.0. However, at pH 2.5 a lower value of 0.167 was obtained.

The values of $dn/dc$ for LADH in 0.1 M phosphate buffer pH 7.3 containing 8 M urea or 4 M guanidine-hydrochloride are 0.140 and 0.060, respectively. While in 8 M with the presence of 0.1 M $\beta$-mercaptoethanol gives a value of 0.110 for LADH. On exposure both enzymes to pH 12.0, $dn/dc$ were found to be 0.057 for LADH and 0.060 for YADH.

When a final concentration of 14.0 $\mu$M NADH was added to LADH in 0.1 M phosphate buffer pH 7.3, there was an increase in $dn/dc$ from 0.200 to 0.210.

3.4. LIGHT-SCATTERING

The fundamental laws of the scattering of light, by calculating the polarizability of individual gases molecules occurring in the oscillating electromagnetic field of a light beam, were first formulated by Lord Rayleigh (1871). But only two decades ago, after Debye (1947) pointed out that the
<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>$n_0$</th>
<th>dn/dc</th>
<th>LADH</th>
<th>YADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 μM Phosphate</td>
<td>7.3</td>
<td>1.334</td>
<td>0.200</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td>$+$ 140 μM NADH</td>
<td></td>
<td></td>
<td>0.210</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.1 μM Phosphate</td>
<td>4.0</td>
<td>1.334</td>
<td>0.200</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.1 μM Phosphate</td>
<td>2.5</td>
<td>1.334</td>
<td>0.167</td>
<td>0.167</td>
<td></td>
</tr>
<tr>
<td>0.1 μM Phosphate</td>
<td>2.2</td>
<td>1.334</td>
<td>0.167</td>
<td>0.167</td>
<td></td>
</tr>
<tr>
<td>0.1 μM Phosphate + 8 M Urea</td>
<td>7.1</td>
<td>1.407</td>
<td>0.140</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.1 μM Phosphate + 8 M Urea + 0.1 M ME</td>
<td>7.1</td>
<td>1.407</td>
<td>0.110</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.1 μM Phosphate + 4 M GuHCl</td>
<td>7.1</td>
<td>1.389</td>
<td>0.060</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.1 μM Phosphate + 3 M GuHCl</td>
<td>7.1</td>
<td>1.381</td>
<td>-</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>0.1 μM Phosphate + 2 N NaOH</td>
<td>12.0</td>
<td>1.334</td>
<td>0.057</td>
<td>0.060</td>
<td></td>
</tr>
</tbody>
</table>

Measurements were performed at 23°C using light of wavelength of 5461 Å.

GuHCl = guanidine-hydrochloride

ME = β-mercaptoethanol.
intensity of the scattered light depends on a number of measurable quantities, and can be expressed as a function of the number of centres of scattering (i.e., molecules) per unit volume, has the technique come into rapid use and acquired its position as one of the most important means of studying macromolecular solutions.

In studies of the physical chemistry of the biological macromolecules, particularly of proteins, light scattering has proved extremely useful. The determination of the molecular weight of several proteins has been confirmed, and the method has been of particular value in providing information about the molecular radius of gyration, about the molecular shape, about solute-solute and solute-solvent interactions, and about dissociation and association of the protein which involve changes in molecular weight. It has the great advantage in convenience, since the measurements are rapid and instantaneous, a fact which favours studies of reaction kinetics.

Theory

The theory of light scattering and the interpretation of the data have been extensively discussed elsewhere by Debye (1947); Stacey (1956); Bier (1957); Geiduschek and Holfzer (1958); Tanford (1961); and Nichol (1964). For this reason, only the equations of immediate use in the evaluation of light-scattering data are presented here.

The scattering of light is defined in terms of the turbidity, $\tau$, i.e.

$$ I = I_0 e^{-\tau l} \quad (8.23) $$

where $I_0$ and $I$ are the intensities of the incident and transmitted light, respectively; $l$ is the pathlength of light through the non-absorbing scattered medium.

The reduced intensity of the scattered light in the transmitted beam
which is generally known as Rayleigh ratio, \( R_\theta \) is given by

\[
R_\theta = \frac{i_\theta r^2}{I_o (1 + \cos^2 \theta)} \tag{8.24}
\]

where \( i_\theta \) is the light scattered by a unit volume of solution at an angle \( \theta \) to the incident beam, \( r \) is the distance from the scattering volume to the detector, and \( I_o \) is the incident intensity of unpolarized light. It has been shown that the relation between \( \tau \) and \( R_\theta \) is

\[
\tau = (16\pi/3)R_{90} = (8\pi/3)R_0 \tag{8.25}
\]

where \( R_{90} \) and \( R_0 \) are the Rayleigh ratios at 90° and 0°, respectively.

According to Einstein (1910), Debye (1947) and Zimm (1948), the molecular weight is related to the turbidity, or Rayleigh ratio, by the expression

\[
(\frac{K_o}{R_\theta}) (1 + \cos^2 \theta) = \frac{1}{M} + 2Bc + 3C^2c + \ldots \tag{8.26}
\]

or

\[
\frac{K_o}{R_{90}} = \frac{H_c}{\gamma} = \frac{1}{M} + 2Bc + 3C^2c + \ldots \tag{8.27}
\]

where \( M \) is the molecular weight of the solute, \( B \) and \( C \) are the second and third virial coefficients, respectively, \( c \) is the concentration of the solute in gm/ml and \( H \) and \( K \) are optical constants for the solute-solvent system at a particular wavelength of the incident beam.

\[
K = 2\gamma^2n_o^2(\frac{dn}{dc})^2/N\lambda^4 \quad \text{and} \quad H = (16\pi/3)K \tag{8.28}
\]

where \( n_o \) is the refractive index of the solvent, \( \frac{dn}{dc} \) is the specific refractive index increment, \( N \) is the Avogadro number and \( \lambda \) is the wavelength of incident light. The equation 8.27 applies only to systems of identical, isotropic particles where the largest dimensions are less than \( \lambda/20 \). This means that there is no long range interaction between the molecules and is always true for many proteins since the scattering intensity is independent of the concentration. A plot of \( K_o/R_{90} \) against \( c \) and extrapolation to
infinite dilution, gives the reciprocal of the molecular weight from the
value of the intercept. If the particles carry a net charge, a positive
value for \( B \) can be obtained from the slope of the resulting graph, and this
is generally interpreted as being due to solvent-solute interaction, whereas
a negative slope indicates attractions between solute particles (Edsall et al.,
1950). Under isoelectric conditions, the converse relation holds (Dandliker,
1954; Timshelf and Coleman, 1960).

For particles of larger dimensions, internal interference effects become
significant, and equation 8.27 must be modified in order to give the correct
angular distribution of the intensity of scattered light, i.e.

\[
\frac{K_0}{R_0} = \frac{1}{MP(\theta)} = 2Bc + 3c^2 + \ldots
\]

(8.29)

where \( P(\theta) \), the particle scattering factor, is a function of the radius of
gyration of the particle. The value of the function \( P(\theta) \) usually depends
upon particle shapes, namely spherical molecules, rod-like particles or
randomly coiled linear polymers. They are frequently written in the forms:

**Spheres:**

\[
P(\theta) = \left[ \frac{3}{\pi} \right] \left( \sin x - x \cos x \right)^2
\]

(8.30)

**Rods:**

\[
P(\theta) = \left( \frac{1}{u} \right) \log \left[ \frac{\sin w}{w} \right] \left( \sin \frac{w}{u} \right)
\]

(8.31)

**Random Coils:**

\[
P(\theta) = \left( \frac{2}{y^2} \right) \left[ \exp(-y) - (1-y) \right]
\]

(8.32)

where \( x = kSD/2 \), \( u = kSL/2 \), \( k = \frac{2\pi}{\lambda} \), \( S = 2 \sin z/2 \), \( y = k^2S^2D^2/6 \), \( D \) is
the sphere diameter, \( R \) is the root mean square end-to-end length between the
random coils, \( L \) is the length of the rod and \( \lambda \) is the wavelength/in the
solution and is equal to \( \lambda/n_0 \) where \( n_0 \) is the refractive index of the solvent.

It is fortunate that the characteristic of this function, \( P(\theta) \), can be
obtained by measuring the intensity of the scattering at any two angles for
the calculation of the particle size. The two symmetrical angles of 45° and
135° are usually chosen, and the ratio of scattering intensities at two angles
is referred to as the dissymmetry of scattering, Z, but is only valid after extrapolating to infinite dilution.

The shortcoming of the above method is that it depends on the proper selection of the macromolecular model, and for larger particles the method fails to give any indication of the actual shape of the particles. Zimm (1948) pointed out that the observed angular intensities of the scattering can be simultaneously graphically extrapolated to zero angle and zero concentration whereby the effect of interference disappears, as $P(0) = 0$ is always unity. This method of treating the data is usually referred to as a "Zimm plot", i.e., a graph of $Kc/R_0$ versus $\sin^2 \theta/2 + Kc$, where $K$ is an arbitrary constant selected to spread the data conveniently. Equation 8.29 permits the molecular weight, $P(0)$ and $B$ to be determined as

$$1/M = (Kc/R_0)_{\theta=0}$$

$$1/P(0) = (Kc/R_0)_{c=0}^M$$

$$2B = [d(Kc/R_0)/dc]_{c=0}$$

As $P(0)$ becomes independent of particle shape as $\theta$ approaches zero, the limiting conditions can be used to determine the radius of gyration of the scattering particle $P_g$

Initial slope/Intercept = $$(16\pi^2/3)(P_g^2/\lambda^2)$$

Equation 8.33 is related to the dimensions of the three particle shapes considered above by the relations:

For a sphere $P_g^2 = 3D^2/20$

For a rod $P_g^2 = L^2/12$

For a random coil $P_g^2 = r^2/6$
The dimensions determined thus are the Z-average values and the ratio \( M_L/M_w \) must be known before the weight-average dimensions can be calculated. This method can also provide additional information. A rapid change in slope at low angles is a very sensible indicator of the presence of dust, or a very wide spread of molecular weight distribution and some guide to the stiffness of the chain or the molecular weight distribution may also be obtained from the shape of the curve at the higher angle.

**Methods**

Light scattering measurements were performed with a Brice-Phoenix light-scattering Photometer (Model 1000-D) manufactured by the Phoenix Precision Instrument Company (Philadelphia, U.S.A.). This instrument is based on the design of Brice et al. (1950). In order to give a higher transmission and to allow the instrument to be operated at a low photomultiplier potential resulting in steadier galvanometer readings, the supplied blue and green monochromatic filters were replaced by mercury green (5461A) and mercury blue (4358A) transmission filters (Grubb Parsons, Newcastle, England). The collimating system was also modified to reduce stray reflections from the light-trap by fitting a 1 cm slit in front of the cell table diaphragm.

**Clarification of solution and solvent**

The light scattered from a particle is largely affected by the presence of dust, or microgel, in the solution. Quartz double-distilled water was used in all experiments. Filtration, or ultracentrifugation, was used, prior to the introduction of the solvent or solution into the light-scattering cell. Filtration was normally carried out by means of various porosities of Millipore filters (Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.). Ultracentrifugation is more convenient and proved to be more successful a method of
clarification. A preparative MSE (Model Super Speed 50) centrifuge was used. Solvents were spun at 20,000 - 30,000 r.p.m. for 2 - 3 hours. To avoid the sedimentation of solute from solution, the solution (about 2.0 ml) was spun at 15,000 r.p.m. for 0.5 hour. The solvent and the solution were transferred to light-scattering cell by an apparatus designed by Schneider (1958). The light-scattering cell and other glassware were cleaned up in hot detergent solution and rinsed several times with distilled water before being put into a stream of condensing acetone in an apparatus similar to that of Thurmond (1952).

Optical Arrangement for Measuring the Scattering Ratio

The experiments were performed in a constant room temperature of 23°C. A 50 ml cylindrical cell (C-101) was used throughout this work. With the cell in position, and all the neutral filters in the primary beam, set the disc to read 0° and open the photographic shutter. The sensitivity was adjusted with the control knob to give a large deflection (70 - 100 mm) on the galvanometer. The readings at disc set at 0° and various other angles were recorded. The large deflection can be obtained at non-zero angle by withdrawing a neutral filter or combination of neutral filters. The actual observed values of scattering ratio \( \frac{G_\theta}{G_0} \) are

\[
\frac{G_\theta}{G_0} = \left( \frac{\varepsilon_\theta}{\varepsilon_0} \right) F_\theta
\]

where \( F_\theta \) is the product of transmittances of the neutral filter or filter combination withdrawn in measuring the deflection. This scattering ratio was determined for both solvent and several concentrations of solution.

Thus the actual scattering ratio of solute is given by

\[
\left( \frac{G_\theta}{G_0} \right)_{\text{solute}} = \left( \frac{G_\theta}{G_0} \right)_{\text{solution}} - \left( \frac{G_\theta}{G_0} \right)_{\text{solvent}}
\]
This method has the advantage for it includes extraneous reflected light as well as light scattered from the solvent, if the presence of a minor defect in cell geometry or dust is automatically subtracted out.

**Absolute calibration and working standard constants**

\[
\frac{G_0}{G_o} \text{ solute} \quad \text{is related to Rayleigh ratio if the precise knowledge of the geometry of the instrument is known or by applying a working standard constant.}
\]

An equation for the residual reflection correction \( R_w/R_c \) and of \( K \) has been derived from the geometry of the optical system

\[
R_{90} = K \left( n_o^2 \frac{R_w}{R_c} \right) aF_\theta (G_0/G_o)
\]

where \( a \) is a constant relating the working standard to the opal glass reference standard, \( k \) is the experimentally determined product of the diffuse transmittance of the opal glass reference standard and a diffusor correction factor, \( n_o \) is the refractive index of the solvent and \( R_w/R_c \) is an experimentally determined correction for incomplete compensation of refraction effects.

But in this work, standard calibrants had been used by Hourston (1967). Hourston used Ludox, Syton 2X and Brewers Yeast Glycogen as calibrants because (1) their dimensions are small if compared with wavelength of light, (2) no absorption in the wavelength studied, (3) the depolarisation of the scattered light can be negligible, and (4) the materials have a high scattering-power and the solutions are easily clarified. The relation of turbidity to the scattering intensity at \( 90^\circ \), neutral filter transmittance and reflection corrections of the instrument were carried out by Hourston (1967). The angular uniformity of scatter of the cylindrical cell was performed with a dilute fluorescent solution in 0.05 \( M \) NaCl at 4358A. The photo tube was screened with a yellow filter which permits only green fluorescence to pass through. As fluorescence is radiated equally in all directions
$1/(1 - \cos^2 \theta)$ is negligible, only $\sin \theta$ is required to correct the change in the scattering volume seen by the photomultiplier. The light-scattering cell is symmetrical as shown below.

<table>
<thead>
<tr>
<th>Angle:</th>
<th>30</th>
<th>35</th>
<th>40</th>
<th>45</th>
<th>50</th>
<th>55</th>
<th>60</th>
<th>65</th>
<th>70</th>
<th>80</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G \sin \theta$:</td>
<td>55.6</td>
<td>54.8</td>
<td>55.3</td>
<td>55.8</td>
<td>55.5</td>
<td>55.7</td>
<td>55.5</td>
<td>55.0</td>
<td>55.2</td>
<td>55.4</td>
<td></td>
</tr>
</tbody>
</table>

Angle: 100 110 115 120 125 130 135

| G $\sin \theta$: | 55.9 | 55.9 | 55.5 | 55.9 | 55.9 | 55.9 | 55.9 |

RESULTS

Light-scattering measurements were performed at 23°C and light of mercury green line ($\lambda = 5461\text{Å}$) was used. The concentration range of 0.14% - 0.06% LADH in 0.1 M phosphate pH 7.3 was clarified and examined in the light-scattering cell. The scattering intensities were determined at angles of observation ranging from 35° to 135°. Using Zimm plot, the results represent the weight-average of the molecular weight ($\overline{M}_w$). The resulting limited value gives a molecular weight for LADH of 95,000 (Fig. 8.18). LADH is known to form a binary complex with NADH (Sund and Theorell, 1963). Fig. 8.19 shows the Zimm plot obtained on the addition of 140 μM NADH to LADH in 0.1 M phosphate buffer, pH 7.3. The intercept at zero concentration and zero angle corresponds to a molecular weight of 94,000, indicating that whether LADH is free, or mainly as a binary complex with NADH, there is no aggregation nor dissociation.

The molecular weight of YADH was evaluated by dissymmetry method with a concentration between 0.24% and 0.11% in 0.1 M phosphate buffer, pH 7.3. By extrapolating to zero concentration, gives a molecular weight value of 190,000 (Table 8.9). Apparently, the molecular weight of YADH and LADH obtained by this technique are higher than those found from centrifugation (see p.227). However, these differences may be due to the traces of aggregates formed in the protein solutions.
Fig. 8.18. Zimm-plot for determining the molecular weight of LADH by light-scattering. Measurements were carried out at 23°C, using light of wavelength of 5461 Å. The concentration range of LADH used was 1.4 - 0.6 mg/ml in 0.1 M phosphate buffer, pH 7.3.
Fig. 8.19. Zimm-plot for determining the molecular weight of LADH in the presence of 140 μM NADH. Conditions were as described in Fig. 8.18.
### TABLE 8.9

**Molecular Weight of LADH and YADH from light-scattering**

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>LADH $\times 10^{-4}$</th>
<th>YADH $\times 10^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1μ Phosphate</td>
<td>7.3</td>
<td>9.5</td>
<td>19.0</td>
</tr>
<tr>
<td>0.1μ Phosphate + 140μM NADH</td>
<td>7.3</td>
<td>9.4</td>
<td>-</td>
</tr>
<tr>
<td>0.1μ Phosphate</td>
<td>2.2</td>
<td>4.1 - 5.0</td>
<td>4.6</td>
</tr>
<tr>
<td>0.1μ Phosphate</td>
<td>4.0</td>
<td>32.0*</td>
<td>-</td>
</tr>
<tr>
<td>3 M Guanidine-HCl</td>
<td>7.1</td>
<td>-</td>
<td>3.9</td>
</tr>
<tr>
<td>4 M Guanidine-HCl</td>
<td>7.1</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>8 M Urea</td>
<td>7.1</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td>8 M Urea + 0.1 M</td>
<td>7.1</td>
<td>1.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Measurements were carried out at 23°C, using a concentration range of 0.5 - 2.0 mg/ml for both enzymes.

Wavelength of light = 5461 Å.

*After spinning the solution for three hours in a preparative ultracentrifuge at 70,000 x g.*
The effect of urea, guanidine-hydrochloride, and extremes of pH upon the molecular weight of LADH and YADH are shown in Table 8.9. The light scattering measurements were evaluated with dissymmetry method by plotting \( \frac{Kc}{R_90} \) against \( c \). The molecular weight of YADH (2.4 - 1.6 mg/ml) in 4 M guanidine-hydrochloride and 0.1 M phosphate buffer, pH 7.3, was found to be 39,000, whilst in 0.1 M phosphate buffer, pH 2.5, it was 46,000, suggesting that the enzyme is dissociated into 4 subunits. On exposure (1.4 - 0.9 mg/ml) of LADH to pH 2.2, the light scattering data were evaluated by plotting \( \frac{Kc}{R_90} \) versus \( \sin^2 \theta/2 + Kc \) (Zimm plot). The intercept from this plot gives a molecular weight of 41,000 (Fig. 8.20). Aggregation from the species measured occurred readily at pH 4.0 which gives the molecular weight of 320,000, indicating that aggregation resulted. Table 8.9 also shows that after the treatment of LADH with 8 M urea or 4 M guanidine in 0.1 M phosphate pH 7.3, the enzyme is further degraded to smaller fragments. The reason for this is not yet clear, it may be due to the presence of a small amount of protease impurity, which breaks down an unspecific peptide bond. The dissociation of LADH by urea is independent of whether \( \beta \)-mercaptoethanol was added or not for the turbidity of LADH and YADH solution does not change over a period of 24 hours when 0.1 M \( \beta \)-mercaptoethanol was added.

8.5. GEL-FILTRATION

When gel filtration was first introduced to the study of polymers, interest was focussed mainly on the isolation and purification. Since a mixture of molecules pass through a volume of porous gel granules, the flow rate of the molecules is related inversely to their size. Therefore, gel filtration can be used as a comparative method to give useful estimations of the molecular weight of macromolecules (Andrew, 1965; Determann and Michel, 1966).
Fig. 8.20. Zimm plot for determining the molecular weight of LADH in 0.1 M phosphate, pH 2.2 at 23°C. The concentration range of LADH used was 1.4 - 0.9 mg/ml.
Materials

The following proteins of known molecular weights were used: pure cytochrome C (horse heart; Koch-light Ltd.) in 0.9% NaCl; chicken-egg ovalbumin (crystallized and lyophilized salt free, Grade V, electrophoretic purity approximately 99%), trypsin inhibitor (soya bean, 2 times crystallized and lyophilized), and haemoglobin (bovine, 2 times crystallized) from Sigma Chemical Co.; and serum albumin (Fraction V, bovine plasma) from Armour Pharmaceutical Co. Sephadex G-200 gel (particle 40 - 120 μ) was obtained from Pharmacia, Uppsala, Sweden.

Methods

Preparation of Gel Column

About 7 mg of Sephadex G-200 gel was suspended in 0.1M phosphate buffer, pH 7.3 and allowed to swell for 4 - 5 days at room temperature before use. The small particles were removed by decantation. The column (3 x 55 cm) was formed with a sintered-glass to support the gel and was first partly filled with phosphate buffer. The gels were deaerated under reduced pressure before use. A slurry of gel particles was poured into the column and gradually filled up until a bed height of 51-52 cm was obtained; whilst at the same time excess of liquid was allowed to percolate through the growing gel bed. A solvent reservoir was connected to the top of the column and the column was equilibrated with buffer at a flow rate of approximately 20 ml/hr. for at least 24 hours. The bed height was adjusted to 50 cm by the addition or removal of buffer equilibrated gel by stirring up the top 2 - 3 cm and allowing the gel particles to settle again. A piece of Whatman filter paper was carefully placed to cover the top of the gel. Before loading any sample, the top buffer at the top of the gel was carefully removed by means of a medical dropper. The void volume of the column was obtained with blue dex-
The proteins were dissolved in the equilibration buffer (0.5 - 1 ml) and the solutions were applied to the top of the column by layering the buffer already present. The column effluent was collected with a fraction collector (Aimer Products Ltd., London) filled with a 3 ml siphon.

**Estimation of Column Effluents**

Proteins were estimated spectrophotometrically with a Hilger Spectrophotometer in silica cuvette with 1 cm light-path. The appropriate wavelength was selected according to the quantity of protein used (Table 8.10). The column effluent without containing protein was used as a reference beam.

**Table 8.10**

Proteins used in gel-filtration experiments

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amounts used (mg in 0.5-1.0 ml)</th>
<th>Molecular weight (x 10^{-4})</th>
<th>Extinction at wavelength (m,μ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>2</td>
<td>1.24</td>
<td>412</td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>2</td>
<td>21.5</td>
<td>230</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>3</td>
<td>4.5</td>
<td>280</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>4</td>
<td>6.45</td>
<td>410</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>6</td>
<td>6.70</td>
<td>230 or 280</td>
</tr>
<tr>
<td>Blue dextran</td>
<td>3</td>
<td>20.0</td>
<td>230</td>
</tr>
</tbody>
</table>
RESULTS

Cytochrome C, trypsin inhibitor, ovalbumin, serum albumin, and haemoglobin of known molecular weight (Table 8.10) were used as calibrants of the column. The void volume \( (V_0) \) of the column was taken as the elution volume for "Blue Dextran, 2,000". The ratio of \( \frac{\text{elution volume of protein}}{V_0} \) was graphed against the \( \log_{10} \) (molecular weight). Fig. 8.21 shows that a linear relationship was obtained. This curve was used to estimate the molecular weight of YADH and LADH. A value of 126,000 was found for YADH and 85,000 for LADH.

Both LADH and YADH were inactivated by extremes of pH, urea and guanidine-hydrochloride. The inactivated forms of these enzymes were applied to the column and flow with 0.1 \( \mu \) phosphate buffer pH 7.3, precipitation was observed. When these enzymes were brought back to pH 7.3 by the addition of concentrated phosphoric acid, or when the urea and guanidine-hydrochloride were dialysed away, precipitation of protein still occurred. However, no attempt was made to study the molecular weight of the inactivated enzymes by equilibrating the gels with extremes of pH or the presence of urea or guanidine-hydrochloride.
Fig. 8.21. Estimation of the molecular weight of LADH (X), and YADH (O) by gel filtration. Conditions were described in the text.
8.6 DISCUSSION

Early investigations have shown the sedimentation coefficient of YADH varying from 6.72 - 7.61 S (Hayes and Velick, 1954; Snodgrass et al., 1960; Kägi and Vallee, 1960; Hersh, 1962; Ohta and Ogura, 1965; Bühner and Sund, 1969). This discrepancy has been examined in detail in this work. Several concentrations of YADH in 0.1 M phosphate buffer, pH 7.3 were subjected to sedimentation analysis at two different rotor-speeds and 20°C. The sedimentation coefficients after extrapolating to zero concentration, yield a value of 6.85 S at 50,740 r.p.m. and 7.51 S at 59,780 r.p.m. (Fig. 8.2). These results suggest that the variation in sedimentation coefficients are mainly due to the force-field employed. The reason for the force-field dependence of sedimentation coefficient is not known, but the effect appears to be reproducible. It is unlikely that this discrepancy is attributed to analytical methodology, but appears to be intrinsic to the sample analysed, as some experiments were carried out with another Spinco (Model E) analytical ultracentrifuge, and the same field-force dependence of the sedimentation coefficient has been obtained. Several proteins, e.g. T4R DNA (Hearst and Vinegrad, 1961), bacterial DNA (Rosenbloom and Schumaker, 1963) and calf thymus DNA (Schumaker et al., 1965) have also been reported to show a similar effect, and this has been explained in terms of either a pressure dependent aggregation-process, alignment of the solute molecules in the direction of sedimentation or due to a change in molecular shape.

Under the same conditions for YADH, the measurements of the sedimentation coefficient of LADH give an identical S-value of 4.96 after extrapolating to infinite concentration either at 50,740 r.p.m. or 59,780 r.p.m. (Fig. 8.3). This value is in good agreement with other reports (Ehrenberg and Dalziel,
1958; Drum et al., 1967). It is known LADH is more stable than YADH. In comparison the results obtained for YADH and for LADH, it may also suggest that the molecule of YADH is less rigid than LADH.

The molecular weight of YADH appearing in several reports is found to be in a range of 110,000 - 151,000 (Hayes and Velick, 1954; Kagi and Vallee, 1960; Hershey, 1962; Armstrong et al., 1963; Andrews, 1964, 1965; Ohta and Ogura, 1965; Bühner and Sund, 1969), and LADH is 67,500 - 84,400 (Theorell and Bonnichsen, 1951; Ehrenberg, 1957; Ehrenberg and Dalziel, 1958; Drum et al., 1967). For this reason, the molecular weight of LADH and YADH has been redetermined by low-speed, short column sedimentation equilibrium of Yphantis technique, (1960), and by light-scattering. The value obtained by sedimentation equilibrium for YADH is found to be 108,000 - 112,000. Apparently, these values are lower than those of 151,000. In view of this result, it has been investigated in a more careful manner by examining (1) various protein concentrations (2) pH effect (3) rotor-speed dependence, and, (4) effect of silicone oil. However, under all conditions examined, a value of about 110,000 is obtained. With a rotor-speed higher than 9,000 r.p.m., a lower molecular weight is obtained; 9,340 r.p.m. and 12,590 r.p.m. yielded an apparent molecular weight of 82,400 and 47,000, respectively. It is likely that when YADH is subjected to sedimentation equilibrium studies with a speed higher than 9,000 r.p.m., the concentration of heaviest components will decrease at the midpoint of the cell thus lowering the apparent molecular weight. In addition, when YADH is used for sedimentation equilibrium studies at 20° and pH 7.3 over a period of 8 hours, more than 30% of the enzymatic activity is lost. It is not clear whether the loss of enzymatic activity is a result of dissociation, and the molecular weight measured is a mixture of
subunit and its apoenzyme. Moreover, the experiments were carried out with low ionic strength and without the addition of electrolyte supporting medium. But, if it is due to intermolecular interaction, higher, and not lower, molecular weight would result. In this connection, when the molecular weight of LADH is determined under the same conditions as for YADH, values of 84,500 - 88,000 are obtained. These are in good agreement with most of the accepted values in the literature (Ehrenberg, 1957; Ehrenberg and Dalziel, 1958; Drum et al., 1967, 1969). In addition, a stable protein of known molecular weight, egg ovalbumin, was employed as a standard to determine the molecular weight with the same technique. The resultant molecular weight is 44,700 which is consistent with other reports (Warner, 1954; Andrew, 1965). This suggests that the low molecular weight obtained for YADH is best explained in terms of the behaviour of protein structure. Low molecular weight obtained for YADH has also been reported by Armstrong et al., (1963); Andrew, (1964) and Sund (1964).

Whilst from light-scattering measurements, the molecular weights obtained for YADH and LADH are 190,000 and 95,000, respectively. Apparently, these values are higher than those of 150,000 reported for YADH and 84,000 for LADH. High molecular weights obtained for both enzymes may be due to the presence of a small amount of dust or aggregate protein in the measured solution. LADH is known to form/binary complex with NADH (Theorell and Bonnichsen, 1951). Light-scattering measurements show that the presence of the coenzyme neither dissociates nor aggregates the enzyme. A comparison of the molecular weights of YADH and LADH obtained from different methods are listed in Table 8.11. There is no direct evidence to show which factors may have contributed to the variation of the molecular weights for both YADH and
TABLE 8.11
Molecular weight of LADH and YADH from the literature

<table>
<thead>
<tr>
<th>Method</th>
<th>Molecular Weight x 10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LADH</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH titration</td>
<td></td>
</tr>
<tr>
<td>Sedimentation velocity</td>
<td>14.0a</td>
</tr>
<tr>
<td>Sedimentation velocity</td>
<td>8.8c</td>
</tr>
<tr>
<td>Sedimentation velocity</td>
<td>8.33d</td>
</tr>
<tr>
<td>Sedimentation velocity</td>
<td>8.0g</td>
</tr>
<tr>
<td>Sedimentation equilibrium</td>
<td>8.44i</td>
</tr>
<tr>
<td>Sedimentation equilibrium</td>
<td>8.25k</td>
</tr>
<tr>
<td>Sedimentation equilibrium</td>
<td>7.82l</td>
</tr>
<tr>
<td>Approach-to-equilibrium</td>
<td>12.9m</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>12.6n</td>
</tr>
<tr>
<td>Osmometry</td>
<td>8.6k</td>
</tr>
</tbody>
</table>

References: (a) Bonnichsen (1953); (b) Sund (1964); (c) Theorell and Bonnichsen (1951); (d) Ehrenberg and Dalziel (1958); (e) Hays and Velick (1954); (f) Kägi and Vallee (1960); (g) Drum, et al. (1967); (h) Bühner and Sund (1969); (i) Ehrenberg (1957); (j) Hersh (1962); (k) Castellino and Barker (1968); (l) Green and McKay (1969); (m) Armstrong, et al. (1963); (n) Andrews (1964).
LADH among the different methods applied.

Kinetic studies have shown that LADH binds two moles of coenzyme per molecule of protein (Theorell and Bonnichsen, 1951) and four for YADH (Hayes and Velick, 1954). Carboxymethylation of these two enzymes, followed by peptide mapping and sequencing of the labelled peptides also indicates that two and four cysteiny1 residues are essential for the enzymatic activity of LADH and YADH, respectively (Li and Vallee, 1963, 1964b; Harris, 1964). X-ray crystallographic studies further demonstrate that LADH consists of two subunits (Bräden, 1965; Bräden et al., 1965). This suggestion is supported by end-groups analysis which shows two identical carboxy-terminal groups and covalently linked acetyl groups per mole of LADH (Jörvall, 1965, 1967). Thus, all these cases reveal that LADH has two and YADH has four very similar, if not identical, polypeptide chains.

On treatment of YADH with 3.0 M guanidine-hydrochloride in 0.1 M phosphate, pH 7.3 at 20°C, in addition to the loss of enzymatic activity, the enzyme is dissociated to four subunits with a molecular weight of 39,000. This result is in agreement with the findings of dissociating YADH into four subunits by urea (Sund, 1960; Ohta and Ogura, 1965), removal of the enzymatic zinc ions by chelating agents (Snodgrass et al., 1960; Kägi and Vallee, 1960), or detergents (Hersh, 1962), (Table 8.12).

LADH has also been reported to be dissociated into two subunits by 5 M sodium dodecyl sulfate (Blomquist et al., 1967), 6.0 M guanidine-hydrochloride in the presence of 0.5 M β-mercaptoethanol (Castellino and Barker, 1968), and by maleylation of the urea-inactivated or the guanidine-hydrochloride-inactivated enzyme (Butler, et al., 1969). Evidence for this dissociation has also been obtained from electrophoresis studies of urea-inactivated LADH.
TABLE 8.12
Molecular weight of protomer of LADH and YADH from the literature

<table>
<thead>
<tr>
<th>Dissociating agent</th>
<th>Orthophenanthroline or 8-hydroxyquinoline-5-sulfonic acid</th>
<th>Storage</th>
<th>Sodium dodecyl sulfate</th>
<th>Urea</th>
<th>Urea + β-Mercaptoethanol or EDTA</th>
<th>Guanidine-HCl</th>
<th>Guanidine-HCl + β-mercaptopethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>-</td>
<td>2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>2.3&lt;sup&gt;s&lt;/sup&gt;, 2.4&lt;sup&gt;s&lt;/sup&gt;, h</td>
<td>1.7&lt;sup&gt;f&lt;/sup&gt;, 2.4&lt;sup&gt;s&lt;/sup&gt;, h</td>
<td>-</td>
<td>1.98&lt;sup&gt;s&lt;/sup&gt;</td>
</tr>
<tr>
<td>LADH</td>
<td>-</td>
<td>2.8&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>3.5&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>2.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.6-3.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>YADH</td>
<td></td>
<td>3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.0&lt;sup&gt;g,h&lt;/sup&gt;</td>
<td>4.1&lt;sup&gt;k&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

References: (a) Kagi and Ville (1960); (b) Sund (1960); (c) Bühner and Sund (1969); (d) Hersh (1962); (e) Blomquist, et al. (1967); (f) Butler, et al. (1969); (g) Drum, et al. (1967); (h) Pietruszko, et al. (1969a); (i) Ohta and Ogura (1965); (j) Castellino and Barker (1968); (k) Green and McKay (1969).
However, measurements from light-scattering are not consistent with these findings. It seems that LADH can be further degraded to smaller fragments, by urea and guanidine-hydrochloride. Dissociation of LADH into two or four subunits by urea has also been reported by Drum et al., (1967) and Pietruszko et al., (1969a). These authors found that in 8.0 M urea, the sedimentation coefficient of LADH decreased from 4.83 to 2.43, and a molecular weight of 40,000 was obtained. Further, the treatment of the enzyme with 8.0 M urea in the presence of 0.1 M β-mercaptoethanol or 0.01 M EDTA, yielded a product with a sedimentation coefficient of 1.5 S and molecular weight of 20,000 (Table 8.12).

Further evidence of LADH degrading to smaller fragments is given by ultracentrifugation studies of the enzyme after inactivating by alkaline pH. On exposure of LADH to pH 12.0, with or without the presence of β-mercaptoethanol, bimodal patterns with sedimentation coefficients of 1.72 S and 3.69 S are obtained (Fig. 8.9), and 1.74 S and 2.54 S in 0.1 N NaOH (Fig. 8.10). The slower moving peak indicates that the enzyme is dissociated to the subunit component, and the faster moving component may attribute to apoenzyme or a mixture of partially dissociated enzyme and its apoenzyme. When the enzyme is treated at pH 12.0 for 24 hours aggregation results, whilst in 0.1 N NaOH for 24 hours one peak is degraded to 1.33 S in the absence of β-mercaptoethanol and with β-mercaptoethanol two peaks with 1.29 S and 6.21 S are observed. Furthermore, after 48 hours, without β-mercaptoethanol, a peak of 1.14 S is obtained, and 1.16 S and 6.60 S with the presence of β-mercaptoethanol. These results may suggest that the dissociation of the enzyme into subunits which can be further degraded to smaller fragments, as a function of time, but the incompletely dissociated enzyme is aggregated to larger molecules. The presence of β-mercaptoethanol promotes the aggregation.
On exposure of YADH to pH 8.8, without β-mercaptoethanol, two distinct sedimentation peaks with 2.67 S and 6.73 S are found. On increasing the pH to 9.2, the 2.67 S-component increases with concomitant decrease in that of the 6.73 S-component. pH 10.2 and 11.0 yield a peak with S-value of 2.67 and 2.51, respectively. In these cases, both the 2.67 S- or 2.51 S-component give an identical apparent molecular weight of 72,000. Previous investigations (p.139) have shown that the immediate loss of the activity of YADH is achieved only after treating the enzyme with a pH higher than 11. A molecular weight of 72,000 may suggest that this molecular weight being measured is a weight-average molecular weight of the mixture between the subunit and its apoenzyme. As pointed out by Field and Ogston (1955) and discussed in detail by Gilbert (1959), when a substance is subjected to sedimentation experiments, no resolution of boundaries will occur in moving boundary if it is associated with dissociation and association system, because in a rapid equilibrium, the boundary which moves with the weight-mean velocity of its components spreads more rapidly than is accounted for by diffusion; and only a single discrete boundary would be produced. In contrast, for those proteins which do undergo partial resolution of the boundary into two peaks during the sedimentation must be due to the rate of interconversion of the migrating species not being sufficient to maintain equilibrium in the boundary to a solution; it therefore, tends to behave as if it contains a number of mutually independent species. Further evidence for the existence of subunit-equilibria is given by kinetic studies which show that the inactivating process (Chapter 5) is dependent on the concentration of both the enzyme and the dissociating medium, and also by the finding of sedimentation patterns which can change from one component to another with more severe conditions.
β-mercaptoethanol is found to protect YADH against dissociation by alkaline pH. The presence of 0.1 M β-mercaptoethanol, the bimodal patterns of 2.51 S and 6.81 S are still retained at pH 11.0 and 2.70 S and 7.10 S at pH 10.2. The sharp decrease of the sedimentation coefficient of YADH with a single peak of 1.67 S and 1.31 S at pH 12.0 and 0.1 N NaOH, respectively are also observed. The magnitude of the sedimentation constant for the 1.67 S-component is consistent with the report that there is a subunit of molecular weight of 36,000. While the molecular weight of 1.31 S-component yields values of 20,000 - 23,000, neither the addition of β-mercaptoethanol nor prolonged exposure of the enzyme to pH 12.0 and 0.1 N NaOH over a period of 24 hours, causes further changes of the sedimentation coefficients. Whilst those species produced by pH below 12.0, with or without the presence of β-mercaptoethanol, result in further degradation or aggregation. These results suggest that only the complete dissociation of the enzyme produces the stable subunit. It also seems that YADH can be further degraded into small fragments with the treatment of more severe alkaline medium.

YADH has been shown to contain 4 - 5 gram atoms of zinc per mole of enzyme (Vallee and Hoch, 1955a,b; Wallenfels et al., 1957) and the zinc-contents of LADH vary from 2 - 4.3 gram atoms per mole of enzyme (Theorell and Bonnichsen, 1951; Theorell et al., 1955; Vallee and Hoch, 1957; Akeson, 1964; Oppenheimer et al., 1967; Sandler and McKay, 1969; Drum et al., 1969). The zinc atoms of LADH (Vallee and Hoch, 1957; Plane and Theorell, 1961; Yonetani, 1963b; Drum et al., 1967, 1969) and YADH (Wallenfels et al., 1957; Snodgrass et al., 1960; Kägi and Vallee, 1960) are directly involved in both catalytic function and structural stabilization. On treatment of both enzymes with low pH or chelating agents (Snodgrass et al., 1960; Kägi and Vallee, 1960; Akeson,
1964; Oppenheimer et al., 1967; Drum et al., 1969) in addition to the loss of enzymatic activity, the zinc contents are found to be progressively decreased (Akesson, 1964; Oppenheimer et al., 1967; Drum et al., 1969) but loss of enzymatic activity is faster than is the corresponding loss of zinc atoms.

However, it is found that the loss of activity is rapid on exposure of YADH and LADH to pH-values below 3.5 and 4.0, respectively. Sedimentation velocity measurements show that aggregation occurs more readily at pHs between 3.3 and 3.5 with the formation of material with sedimentation coefficients of 7.89 S and 19.21 S, respectively for YADH and with 5.75 S for LADH at pH 4.0. Aggregation resulting from complete removal of zinc atoms from both enzymes by low pH has been found by Kügi and Vallee (1960), Oppenheimer et al., (1967) and Drum et al., (1969). However, it is not clear whether the enzyme is dissociated into subunits by removing the enzymatic zinc atoms and then immediately aggregating, or whether aggregation occurs through a conformational change which enables the side-reaction to take place. On treatment of YADH either with pH 3.0 or 7.3, an identical sedimentation coefficient (6.84 S) results, and for LADH, an identical sedimentation coefficient (4.80 S) is obtained when the enzyme is exposed to pH 7.3 or 3.2. A similar result for LADH has also been reported by Chilson et al., (1966) and Blomquist et al., (1967). Thus the inactivation of these enzymes by low pH has been explained solely to be due to conformational change without altering the molecular size. On further treatment of LADH with pH 2.4, a lower sedimentation coefficient of 3.20 S is obtained, and for YADH the values are 3.48 S and 2.66 S at pH 2.6 and 2.2 respectively. These results indicate that the inactive form can also be degraded into smaller fragments on treatment of more severe acidic conditions. There is no appreciable effect on sedimentation coefficient when 0.1 M β-
mercaptoethanol is present to both acid inactivated enzymes, and with or without the presence of \( \beta \)-mercaptoethanol, aggregation is formed with time. By contrast, the inactivation of both enzymes by acid and alkaline pH, may suggest that the inactivating process is probably not identical.

Inactivation of both enzymes by various denaturating agents appears to be followed by dissociation and aggregation which are pH and time-dependent. These phenomena cause difficulty in permitting an accurate determination of the molecular weight of subunit and its apoenzyme. It may be safe to suggest that the inactivation of both enzymes involve at least three processes: (1) conformational change - loss of enzymatic activity without altering the molecular weight, (2) dissociation, and (3) aggregation. The stable subunit may be obtained only after the dissociation is complete. Molecular measurements of the inactive species do show that both YADH and LADH dissociate into subunits. It also leaves little doubt that the subunit form of both enzymes is enzymatically inactive. The temperature nature of reversible inactivation makes subunit-equilibria likely, and it is suggested that hydrophobic bonds are of primary importance in holding subunits together. Conformational changes involving non-polar regions of the enzyme molecule may also occur. Measurements of the subunit molecular weight are not necessarily those of the actual forms from which reactivation after inactivation occurs. This is indicated by the reduced recovery of activity with standing or higher concentrations of urea, guanidine-hydrochloride and sodium dodecyl sulfate or more severe acid and alkaline conditions. High concentrations of salts may also dissociate the enzyme into inactive subunits. Molecular weight measurements of LADH after inactivation by lithium chloride are difficult, owing to precipitation. Optimum activity regained is probably from the species prior
to the complete removal of the enzymatic zinc ions or complete dissociation. No direct evidence has yet been given to show whether the inactivation of the enzyme is first due to conformational change followed by dissociation and aggregation, or through direct dissociation, though conformational change prior to alteration of the molecular weight has been suggested by Oppenheimer et al. (1967), Blomquist (1967) and Bühner and Sund (1969).

A definition of the ultimate number of subunits of a protein with multiple chains presents a problem for analysing the molecular weight. Many of the proteins seem to consist of an equivalence between the number of binding sites and the number of subunits. However, this does not appear always to be the case. If a protein is an aggregate form of polypeptide chains, there is no reason why further breakdown of the subunit to smaller fragments through an unspecific bond should not occur. Several proteins have been found to contain non-identity of subunits, chains and active sites, e.g. rabbit muscle aldolase (Hass and Lewis, 1963; Sine and Hass, 1967; Sia and Horecker, 1968), certain malic dehydrogenase (Munkres, 1965; Siegel, 1967), lactic dehydrogenases (Appella, 1964; Jaenicke and Knof, 1968; Miller et al., 1969), glyceraldehyde-3-phosphate dehydrogenases from yeast and rabbit muscle (Jaenicke, 1969) and pyridoxamine pyruvate transaminase (Kolb et al., 1968).

Recently, LADH has been shown to exhibit multiple bands on electrophoresis (McKinley-McKee, 1965), and to exist in several isoenzyme forms. The two leading most cathodal bands not only possess alcohol acitivity, but also steroid dehydrogenase activity (Pietruszko et al., 1966; Theorell et al., 1966; Pietruszko and Ringold, 1968; Pietruszko et al., 1969b). Non-identical subunits have been identified and their hybridization accounts for the forma-
tion of at least three of the nine isoenzymes of LADH identified thus far (Pietruszko et al., 1969a; Pietruszko and Theorell, 1969; Jörvall, 1969). Whether variations in isoenzyme composition significantly affect the subunit structure remains unresolved until these fundamental relationships can be discerned.
**Function of Alcohol Dehydrogenase:**

A detailed insight into the chemical nature of the active sites of an enzyme is a fundamental and essential foundation for elucidating the dynamic mechanism of enzyme catalysis. Numerous criteria are available to define the state of active sites of both LADH and YADH; not only their structures and catalytic activities, but also their capacities to bind co-enzymes, coenzyme analogues, substrates, substrate homologues and inhibitors can serve as an indication of their active sites.

Previous data on differential activities and the different complexed states reveal that LADH and YADH bind two and four moles of coenzyme per molecule of protein, respectively (see Chapter 1). The enzymatic zinc ion and the sulphydryl group are essential for catalytic function. The coenzyme binding site, the enzymatic zinc atom and the reactive sulphydryl residue, all three loci are situated in close proximity to one another in the three-dimensional array of the enzyme surface. There are also several indications that the coenzyme binding induces a conformational change of the enzyme molecule, which makes the protein structure more rigid. Careful and detailed kinetic studies have led to the conclusion that a ternary enzyme-coenzyme-substrate complex exists. The interaction of the coenzyme and substrate with the enzyme is in close juxtaposition which permits the transfer of hydrogen from a meso carbon atom of the substrate to A-face of the nicotinamide ring (at C-4) in the coenzyme. Thus, the reaction catalysed by LADH or YADH is sterically specific with regard to both the coenzyme and the substrate. The substrate also undergoes a high degree of favourable hydrophobic binding with the enzyme. It is also evident that the preferential binding of the coenzyme to YADH occurs at a hydrophobic slot (see Chapter 1).
However, the manner in which the substrate and the groups and moieties of the coenzyme molecule are involved in the interaction with the enzyme is still a matter of controversy. There is also no consistent evidence regarding the nature of the zinc-protein bond, or the catalytic function of the zinc atom and the reactive sulphhydryl residue.

The known characteristics of the enzyme together with isotope-exchange studies have led to the conclusion that the dominant roles of the four zinc atoms per molecule of YADH are essential for catalytic function and structural stabilization (see Chapters 1 and 8). In contrast, the four gram atoms of zinc per molecule of LADH have been differentiated into two chemically distinct classes (Drum, et al., 1969). Two of these zinc atoms are considered to be exposed to the ambient environment and function as catalysis sites where they are accessible to zinc-chelating agents and isotope-exchange. The other two zinc atoms are buried in the interior of the protein molecule, and they appear to play a role in stabilizing the protein structure (Drum, et al., 1967), although there is argument against this suggestion (Green and McKay, 1969). However, the exact manner by which the zinc atoms might participate in their catalytic function and in stabilizing the protein structure is not known (see Chapters 1 - 4). Moreover, there are still other properties of the enzymatic zinc ions which remain to be explained: (1) the failure of certain chelating agents, e.g. orthophenanthroline, to remove the zinc atoms from the enzyme molecule, (2) under certain conditions, the removal of zinc atoms from the enzyme molecule results in an inability for the enzyme to rebind the metal ions, (3) the asymmetric environment of chromophoric chelating agents binding to the zinc atoms, and (4) the poor correlation between the inhibition by many zinc-chelating agents on one hand, and the stability constant
of their ionic complexes on the other.

Further studies focused on: (a) the inter-relation between the four zinc atoms and the specific properties of both enzymes, (b) the attachment of chromophoric metal-chelating agents to zinc atoms, (c) the side-chain residues of the proteins being responsible for zinc binding and (d) the functional significance of exchanging zinc atoms with other metals, which may not only constitute a useful approach to this problem, but also provide a useful analogy for elucidating the diverse functions of metals in other metalloenzymes.

Sulphydryl residues are an essential constituent of the active sites of both LADH and YADH (see Chapter 1). Their identity has been established through chemical modification with site-specific reagents and the subsequent isolation and characterization of the peptides containing the modified amino acid residues. But the involvement of the active-site sulphydryl-groups in the binding of the coenzyme is a controversial subject. From the observation that the inactive carboxymethylated enzyme is still capable of binding two moles of NADH per mole of LADH, Li and Vallee (1965) suggested that the cysteinyl residues at the active sites are not indispensable to coenzyme binding. But the coenzyme protects the enzyme from carboxymethylation, indicating that the coenzyme binding may be localized and closely adjacent to the reactive sulphydryl residues. However, at this juncture, it is still not possible to distinguish whether the reactive sulphydryl residue is directly involved in catalytic function, or whether the carboxymethylation of the enzyme in altering the geometry of the active sites interferes with the catalytic activity.

Sulphydryl residues have also been suggested as the ligand sites for the catalytically essential zinc atoms of these enzymes (see Chapter 1). But,
by employing the carboxymethyl-cysteinyi residue as a label, the isolated
dipeptide does not bind the functional zinc atoms of the enzyme, and this
dipeptide is similar but not identical to the active site cysteinyi dipeptide
isolated from YADH (Harris, 1964). Thus, it is unlikely that the active
site of the carboxymethyl-cysteinyi residues is responsible for the en-
zymatic zinc binding. Recently, it has been shown that reduction and
alkylation of LADH in guanidine-hydrochloride solution resulted in almost
complete dissociation of the enzyme into subunits. It therefore, appears
that sulphhydril residue may have a role in subunit association (Green and
McKay, 1969). The catalytic activity of YADH has also been reported to be
strongly related to the number of sulphhydril groups in the enzyme molecule
(Wallenfels and Sund, 1957; Bühner and Sund, 1969).

The unique optical properties of the interaction between the enzyme
and coenzyme permit the direct and detailed examination of the mechanism
of the coenzyme binding by a variety of approaches (see Chapter 1 - 5). All
existing evidence shows that the adenine-, /pyrophosphate- and pyridine-
moieties of NAD$^+$ and NADH are essential for their interaction with LADH or
YADH. Detailed studies with coenzyme analogues, pioneered by Kaplan (1960)
show further the pitfalls inherent in the interpretation of experiments in-
volving coenzyme binding. This approach again suggests the overall configu-
ration of the coenzyme molecule is important for binding and its charge additionally influences its binding characteristics.

More recent data obtained from proton relaxation rates and electron
paramagnetic resonance studies, using spin-labeled analogue of coenzyme, add
an additional degree of confidence to deductions regarding possible sites for
the interaction between the coenzyme and the enzyme (Weiner, 1969); Mildvan
and Weiner, 1969). In addition to the previously-reported two binding sites of LADH for the coenzymes, there are, in fact, five or six weak sites involved in the binding of the coenzymes to LADH, which add significantly to the stability of the enzyme molecule, once the coenzyme is bound. Polarization of fluorescence studies further demonstrate that the two tight binding sites of the coenzyme may not be identical (Hoagstrom, et al., 1969), as previously suggested by Theorell and Bonnichsen (1951). One molecule of the coenzyme may bind in the active site and another in the additional site. After removal of zinc atoms from LADH, the enzyme is still capable of binding the coenzyme. Thus, the role of zinc atoms seems neither for coenzyme binding, nor for maintaining the gross conformation of the enzyme. However, since the loss of the enzymatic zinc atoms results in no catalytic activity, it is probable that zinc atoms must be an integral part of the catalytic portion of the active site, and be responsible for substrate binding or activation of the substrate. The actual manner by which the interaction between the individual components of the coenzyme molecule and the functional groups of the enzyme takes place remains conjectural.

In contrast, the interaction of substrates and their homologues which lack suitable chromophoric groups, has had to be studied indirectly by virtue of their effect on the kinetics of enzymatic reaction, and on the optical properties of the bound coenzyme at equilibrium (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961, b,c; Ulmer, et al., 1961; Sigman, 1967). Although the formation of a binary enzyme-substrate complex is in dispute, the existence of a ternary enzyme-coenzyme-substrate complex has been inferred from kinetic studies (see Chapter 1). Such a complex has been substantiated further by the effect of substrate homologues on the optical properties of the
enzyme-coenzyme complex. Thus, the fluorescence of the enzyme-coenzyme complex is enhanced in addition of substrate homologues (Winer and Theorell, 1960) and substrate competitive inhibitors (Theorell and McKinley-McKee, 1961b,c; Yonetani and Theorell, 1962). Likewise, the addition of acetamide increases the amplitude of the Cotton effect for the LADH-NADH complex (Ulmer, et al., 1961). It therefore, has been concluded that the interaction of the substrate and coenzyme is at a vicinal site of the enzyme, in close juxtaposition which permits molecular contact between the hydrogen-donating and hydrogen-accepting sites of the substrate and coenzyme.

However, in both instances the nature of the binding of the substrate to the active sites of the enzyme is from indirect evidence. Since the optical parameters under observation are generated by the coenzyme moiety of the complex and since the physico-chemical basis for the effects accompanying the addition of substrate homologues is not apparent, there is still ambiguity concerning the sites at which the substrate may bind. Since imidazole, and 2,2-bipyridine, zinc-chelating agents, compete for the binding to the enzyme with substrate (Plane and Theorell, 1961; Theorell and McKinley-McKee, 1961; Sigma, 1967) the substrate is therefore considered to be bound to the enzymatic zinc atoms. A number of other considerations, and additional data accumulated from inhibition studies using orthophenanthroline, have resulted in the conclusion that the substrate interacts at sites other than the zinc atoms (Vallee, et al., 1959; Mahler, et al., 1962).

Furthermore, the concept of direct hydrogen transfer between the substrate and the coenzyme has recently been challenged by Schellenberg (1965, 1966, 1969) and Schellenberg, et al. (1968). These authors have detected the transfer of hydrogen to tryptophan residues of various dehydrogenases from stereospecifically labeled substrates and coenzymes. It has, therefore, been
suggested that tryptophan serves as a hydrogen carrier between substrates and coenzymes.

The overall action of LADH and YADH may not proceed by the same mechanism. This is clear from evidence from kinetic data (see Chapter 1), and from the fact that β-mercaptoethanol is such a potent inhibitor of LADH, but has practically no effect at all on the activity of YADH (see Chapter 4) and that the two enzymes have different properties toward pyridoxal-5'-phosphate (see Chapter 3).

Taking into account all currently conceived mechanisms from every known enzyme, including orientation, proximity, acid-base, ion-pair, solvent, co-operative, charge, allosteric, and rack and strain effect, the nature of the extraordinary enzyme function has yet to be proved definitely for any one enzyme. An explanation for this apparent shortcoming cannot be given at present. There may be even more complicated ways in the enzyme catalytic process which have not yet been identified. Nevertheless, all the schemes proposed for the mechanism of action of enzymes are ingenious and have not only stimulated a search for the answers to such questions, but also have provided an essential base for further investigations.

Structure of Alcohol Dehydrogenases

However, there are still other obscurities in protein chemistry which act against obtaining the information necessary to correlate function and structure of proteins, e.g. there is no way to observe bond breaking in a transition state, or to measure the polarization of electrons in an orbital or to directive measure what are the forces which maintain the enzymatically active structure.

Although the nature of the forces responsible for the maintenance of the
conformation of protein molecule, and the chemical bonds which hold together the multi-chain protein have been the subject of current interest, to date, relatively little is known of how these forces operate in the native protein structure (Kauzmann, 1959; Linderström-Lang and Schellman, 1959; Reither, 1963; Brandts, 1964; Joly, 1965; Klotz, 1966; Sund and Weber, 1966; Tanford, 1968). At the outset, it is useful to list explicitly the known types of chemical bonds which might be important in maintaining the native protein structure in its unique folded state:

1. Covalent bonds - the cystine disulfide bonds are the first known type of bonds to be important in this connection, although side-chain imide or ester bonds may also play a role in stabilizing some protein conformation (Blumenfeld and Gallop, 1962).

2. Non-covalent bonds - these include (a) hydrogen bonds, (b) hydrophobic bonds, (c) ionic bonds, and (d) van der Waals forces.

In addition to the above two main categories, there is the finding that YADH dissociates into subunits when the zinc atoms are removed from the protein molecule by metal-chelating agents (Snodgrass, et al., 1960; Kägi and Vallee, 1960). Metal atoms in certain proteins are also thought to be important in stabilizing the protein conformation through the formation of bridges between the monomers, and the rupture of the metal bridges destroys a critical constellation required for the ensuing catalytic function.

However, direct evidence for showing which types of bond are more important in governing the stability of the protein conformation is not available. Definitive techniques commonly employed for these studies involve the use of heat, irradiation, freezing, detergents, guanidine-hydrochloride, urea, formamide, organic solvents, extremes of pH, metal-chelating agents and certain
neutral salts. The basic knowledge needed for interpreting the way by which these denaturating agents change the protein structure is still lacking. Usually, treatment of the protein with any of these denaturating agents causes: (a) loss of enzymatic activity, (b) alteration of the optical properties of the protein, (c) change of the conformation of the protein molecule, (d) dissociation of the multi-chain protein into subunits and (e) unfolding the protein structure. Under certain conditions, all these denaturating processes for several proteins have been shown to be reversed once the proteins are returned to their native environment in solution. But, a secondary reaction resulting in irreversible loss of structure may also occur during the denaturation (Reithel, 1963; Joly, 1965; Tanford, 1968).

The sensitivity of the structure of protein molecules to the action of various denaturating agents depends primarily on the nature of the denaturating agent and the types of force, or forces, which are involved in the stabilization of the protein structure. Thus, the rupture of the protein structure by these denaturating agents may occur in numerous ways: (a) if one portion of the protein structure is held in a folded conformation by one type of bond, e.g., interpeptide hydrogen bonds, whilst another portion of the native molecule is stabilized by another type of bond, e.g., hydrophobic bonds, then different types of denaturating agents act independently of each other in bringing about the denaturation; or (b) if the native protein structure is held together by all types of forces which work together, then different denaturating agents would help each other in destabilizing the protein structure.

The effectiveness of different denaturating agents on different proteins appears to be un-correlated; their mechanism of action is complicated (see
Chapter 5). Subsequent studies have been based on model and theoretical treatments (Kauzmann, 1959; Némethy and Scheraga, 1962; Scheraga, 1963; Brandts, 1964; Scheraga, et al., 1964). Urea is considered to weaken hydrophobic bonding by competitive clathrate formation around apolar side-chains. As in most proteins, 40% of the amino acid residues have non-polar side-chains. Thus, those proteins conformations which bring many of these non-polar groups from the aqueous phase are more stable than others, due to the formation of "hydrophobic micelles" in the protein molecules. Another viewpoint of the effect of urea on the protein structure is that it disrupts the native conformation of the protein by forming hydrogen bonds with the peptide groups (Gordon and Jencks, 1963). It has also been suggested that the mechanism of urea action on a protein may not be limited to disruption of \(-\text{NH} ... \text{O}=\text{C}-\) or breaking of hydrogen bonds (Klotz, 1966; Klotz and Shikama, 1968); the very fact that large concentrations of urea are necessary for a denaturating action, indicates that its influence may reside in its interference with ordered water structures surrounding apolar residues, i.e., that the effect is an alloplastic one.

The only information obtained for the effect of guanidine-hydrochloride on protein structure is that it abolishes non-covalent inter- and intramolecular interactions of polypeptide chains (Tanford, 1968). There is still no conclusive evidence available for the explanation of the effect of other denaturating agents on protein structure.

Indeed, the denaturation of LADH and YADH can be brought about by urea, guanidine-hydrochloride, sodium dodecyl sulfate, extremes of pH, neutral salts and metal-chelating agents (see Chapter 8). This denaturation involves both enzymes dissociating into inactive subunits. Since the active LADH and YADH
contain no cystine residues, it is therefore, obvious that the peptide chains of these two dehydrogenases are held together by forces other than covalent bonds. The marked negative temperature coefficients of the inactivation rates of both enzymes by urea and guanidine-hydrochloride seem to serve as a good indication that hydrophobic bonds are essential for maintenance of the active conformation of these enzymes (see Chapter 5).

Hydrophobic bonds are more stable at room temperature than they are at low temperatures because of the endothermicity of the transfer of non-polar group from water to a non-polar environment. Other evidence which supports this reasoning is the observation that high ethanol concentrations also accelerate the rates of inactivation of LADH by urea. However, it has also been found that LADH and YADH are inactivated by extremes of pH and high concentrations of neutral salts. Moreover, the urea inactivation is pH-dependence, and neutral salts have also a large effect. Certain neutral salts at low concentrations can either serve as a protector or accelerator of these enzymes against urea inactivation. These findings make the suggestion that only hydrophobic bonds are important in holding together the subunits of both enzymes unlikely. Salt linkages may also play a role in the subunit association, and in addition there is the involvement of the enzymatic zinc atoms in stabilizing the structure of these enzymes which has also been previously suggested by Snodgrass et al. (1960); Kagi and Vallee (1960); and Drum, et al. (1967, 1969).

In summarizing these observations, it cannot be stated with certainty to what extent these, or other chemical forces, may have contributed to stabilize the enzymatically active structure. It seems more likely, however, as postulated by Tanford (1968) that of all types of forces are important for holding together the protein conformation, for a protein structure is a co-
operative phenomenon in which all kinds of forces must be involved. It is by no means certain that an unequivocal identification of a particular force as most important in a given instance can always be made, and this cannot be established until more diagnostic direct techniques are developed.

Although the dissociation of LADH and YADH into subunits has been shown to be reversible, the species that can be reconstituted are not necessarily the completely dissociated enzymes (see Chapter 6). Prolonged exposure of both enzymes to denaturating agent results in no recovery of the enzymatic activity. It is likely, therefore, that the denaturing process by various agents may not proceed as a single stage. The loss of enzymatic activity can first be due to conformational change prior to the dissociation of the native protein into subunits (see Chapter 7 and 8). After the protein molecule has been dissociated into subunits, a further secondary, irreversible loss of the protein structure may also take place.

In some proteins, the number of polypeptide chains is equal to the number of active sites (see Chapter 8 and reviews by Reithel, 1963; and Sund and Weber, 1966). However, it seems that both LADH and YADH can be further degraded into smaller fragments, although kinetic evidence and data obtained by other approaches for the number of active sites argue against this (see Chapter 8). However, it is emphasised that this observation may be important as numerous proteins have been shown to contain a number of peptide chains which is greater than the number of active sites (see Chapter 8, and reviews by Reithel, 1963; and Sund and Weber, 1966). There are also proteins such as hemoglobin (Braunitzer, et al., 1964) or the isoenzymes of lactic dehydrogenase (Markert, 1963b) which - although they have equivalent numbers of peptide chains and active sites - contain non-identical peptide chains. Further
studies by techniques such as high resolution electron microscope may help to clear the matter.

Classical techniques of studying the properties of both LADH and YADH by methods such as the optical properties of coenzyme binding, or the use of inhibitors, have served as positive indicators of the affinity between the functional groups of these enzymes and their respective coenzymes and substrates. Since the direct observation of the mechanism of enzyme action is not possible, the identification of the chemical nature of active sites by other approaches must be simultaneously pursued. Introduction of a small amount of selected change in the local geometry with site specific reagents, and through a combination of studies with differential labeling, isotopic derivatizations, amino acid analysis and peptide isolation, are typical of methods which should prove to be very fruitful (Singer, 1967; Vallee and Riordan, 1969). Certain amino acid side-chains may play an insignificant role in the binding of substrates, coenzymes, or by functioning as a proton carrier or by maintaining the proper conformation suitable for catalysis. Thus, studies to centre on the functional role as well as the structural aspects of active sites are necessary and complementary.

Full identification of the absolute conformation of the coenzymes, their individual units, moieties and coenzyme analogues also seems important (Sarma, et al., 1968; Sarma and Kaplan, 1969; Patel, 1969). A significant difference in the conformation of ribose and 2-deoxyribose in nucleosides has been found by Jardetsky (1960) and Lemieux (1961). This may be enough to cause in-orientation which affects coenzyme binding or catalytic function and may also be responsible for the different results obtained by different approaches, using various coenzyme analogues and moieties.
Theoretically, at least, recent breakthroughs in X-ray crystallographical attack on the three-dimensional structure of the protein could generate an "entatic state" of protein side-chains, favourable to catalysis and the recognition of its existence in the protein. Current experimental developments are also being designed to evaluate the mechanism of action of some enzymes by intensive scrutiny of the geometry of the active sites. Some concrete ideas appear also to have taken shape. Because of the possibility of differences in structure between crystal and dissolved protein (Theorell, et al., 1966; Chance, et al., 1966; Maricic, et al., 1966), nuclear magnetic resonance studies should also provide an ever-widening base for extending knowledge of the chemical nature of mechanism of enzyme action. The promise that "a moment of truth" for the theory which attempts to explain the remarkable catalytic power of enzymes may be close at hand. Only when this ultimate goal has been achieved will it be brought to light how enzymes work in the body tissues and their important role in genetics.
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REVERSIBLE INACTIVATION OF LIVER AND YEAST ALCOHOL DEHYDROGENASE

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Many enzymes, including certain dehydrogenases, have been reversibly dissociated into inactive subunits (1-6). Dissociation has normally been affected with urea, guanidine, extremes of pH or salts. Reactivation generally required β-mercaptoethanol and neutral pH. In this connection liver and yeast alcohol dehydrogenase (LADH and YADH) are interesting because the native form of these enzymes contain two and four active centres respectively. Difficulties in dissociating or reversing inactivation of the LADH metallo-enzyme has been thought due to irreversible loss of zinc (6). However this does not appear to be the case as the reactivation after inactivation and dissociation of this enzyme shows.

EXPERIMENTAL AND RESULTS

Freshly dialysed solutions (60-100 μM) of the crystalline enzymes (Boehringer, Mannheim, Germany), in 0.1 μ phosphate buffer pH 7.3 were used. In assaying the enzymes (7) it was necessary to dialyse away mercaptoethanol from LADH (but not from YADH), as it is a potent inhibitor of this enzyme (8). Enzymatic activity was assayed in duplicate by withdrawing aliquots before and after inactivation and reactivation. Enzyme inactivation could be achieved by low or high pH, lithium chloride, or urea, and the effect reversed by back-titrating to neutral pH. Thus for acid
inactivation, 50 μl. of LADH (65 μM) were diluted to 3 ml. with 0.1 M phosphate buffer pH 7.3 and the activity determined. The solution was titrated with H₃PO₄ to pH 2.5 or 4.0. Assay showed that no activity remained. Immediately reactivation was carried out at 23°C by titrating with NaOH to pH 7.5, adding mercaptoethanol and allowing to stand for 1-4 hours. Then over a period of about 20 hr. the mercaptoethanol (0.1 M) was dialysed away using phosphate buffer at 4°C. 75% of the initial activity was recovered. Without mercaptoethanol 38% of the activity was recovered. Using the above conditions with mercaptoethanol, 29% of the activity was recovered if reactivation was carried out for the same time at 0°C instead of 23°C. These inactivations were done in a glass vessel at room temperature. If a cellulose nitrate vessel was used, 85% of the initially activity was recovered. The recovery of enzymatic activity also depended on the concentration of mercaptoethanol. It was only 58% with 0.05M mercaptoethanol. These results for LADH are listed in Table 1. As shown, more activity could be recovered after inactivation at pH 4.0 than at pH 2.5, where increased acidity produced further structural change. After inactivation of LADH by acid, the presence of NADH (71 μM) only increased the activity recovered a little (5-8%). However the addition of mercaptoethanol (0.15M in the presence of NADH, resulted in less activity being recovered than if mercaptoethanol was used alone. Under all conditions enzyme concentration was critical. If too high, then enzyme precipitation or aggregation was visible. This was particularly noticeable with high pH, where aggregation occurred readily, and reactivation had to be carried out quickly. Perhaps the increase reactivation given by a container of cellulose nitrate compared with glass, is due to the alkaline nature of the surface of glass.
### Table 1

Reactivation of LADH and YADH

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<th>Inactivation</th>
<th>Reactivation</th>
<th>Initial Activity</th>
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<td>pH</td>
<td>T°C</td>
<td>pH</td>
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<td>DH</td>
<td></td>
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</tr>
<tr>
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<tr>
<td>7.3</td>
<td>0, 6M LiCl</td>
<td>7.3</td>
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</table>

\[ \beta \text{-mercaptoethanol} \]

A cellulose nitrate instead of a glass container was used. Reactivation involved dilution of the inactivated enzyme solution, 6-fold for LiCl, 10-fold for urea.

Temperature was also important, and as listed in Table 1, it was found that inactivation by urea or lithium chloride at pH 7.3 was best carried out at 0°C, and reactivation at room temperature. Dilution was necessary for these reactivations. Although glycerol (10-25%o) protects LADH and YADH against urea inac-
For YADH, the experimental procedure was the same as for LADH, except that the activity recovered with YADH seemed independent of whether mercaptoethanol was dialysed away or not. In Table 1, reactivation of YADH after urea inactivation is not listed as this has been achieved previously (9). No doubt further experiments with YADH could give better conditions for inactivation and reactivation and result in the recovery of more activity.

**DISCUSSION**

Optimum reactivation depended on pH, temperature, concentration of enzyme, time course of reactivation, container and the presence of mercaptoethanol. More activity was recovered if inactivation was carried out at 0°C and reactivation with mercaptoethanol at 23°C and neutral pH. The period required to produce complete inactivation had to be restricted to a minimum, otherwise a secondary irreversible loss of structure frequently resulted. With high concentration of enzyme, precipitation occurred during reactivation, apparently because of intermolecular aggregation of dissociated protein. With very low enzyme concentration, low reactivation was also found. This seemed due to absorption onto the container surface. Reactivation of LADH after urea inactivation proved most difficult. Prolonged exposure of enzyme to high urea concentrations resulted in irreversible loss of structure. A suitable concentration of enzyme and urea, and a dilution such that the final concentration of urea was not too high to prevent reactivation was necessary.

The reversible inactivation at 0°C by 6M LiCl, parallels the hybridization under neutral conditions of lactic dehydrogenase subunits by saturated sodium chloride at 4°C, or by freezing and
It shows that salt (or urea) can be as important for these processes as high or low pH. The temperature-dependence of the urea experiments indicates that reversible and irreversible inactivation involve hydrophobic bonds with a negative temperature coefficient (11). This also suggests that dissociation into subunits, rather than conformation change alone occurs. The reactivation after inactivation of the alcohol dehydrogenases, suggests that the various methods of inactivation are not releasing the zinc moiety from the protein. In line with this zinc ions neither promoted reactivation nor protected against inactivation. The suggestion that activity cannot be regained after pH 5.0 inactivation, because of zinc removal (13), seems to confuse inactivation with zinc removal, occurring subsequently. Lack of success of previous attempts at LADH reactivation (6, 12) seems due to the conditions necessary being more critical than for many other dehydrogenases. Precipitation of enzyme on adjustment to neutral pH after acid inactivation and consequent lack of reversibility, result from the enzyme concentration being too high and bear out the critical nature of enzyme concentration in these processes.

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REFERENCES

II. THE DISSOCIATION OF THE ALCOHOL DEHYDROGENASES

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It has been established that liver and yeast alcohol
dehydrogenase (LADH and YADH) can be inactivated by low and high
pH, high salt concentrations, or urea, and then reactivated (1,2).
To ascertain the nature of the changes involved in the reversible
inactivation, molecular weight studies have been carried out on
the enzymes, inactivated under the conditions from which activity
can be recovered. While urea is known to reversibly dissociate
YADH into four subunits (2), the reversible dissociation of LADH
and YADH in the present experiments has been mainly established
by light scattering measurements.

EXPERIMENTAL AND RESULTS

The solutions of inactivated enzyme were prepared as
previously described (1). They were examined in the ultra-
centrifuge and by light scattering. The latter technique was
mainly used, as with care the molecular weight could be measured
immediately and subsequent changes with time followed.

The light scattering measurements were performed in a
Brice-Phoenix light scattering photometer, using a 50 ml.
cylindrical cell and the associated diaphragm system. Light of
5461A was employed. Solutions were clarified by spinning for 3 hr. in a preparative ultracentrifuge at 70,000 x g or by passing through a 100 μm millipore filter. The solutions were then introduced into the cell which had been cleaned by refluxing in acetone vapor. An enzyme concentration range of 0.5 - 2.0 mg/ml was used. The refractive index increment dn/dc was found at 23°C to be 0.16 for the protein in phosphate buffer, while in urea and guanidine-HCl it was 0.14 and 0.06 respectively. It was determined in a Brice-Phoenix differential refractometer using the mercury green line of 5461A. Table I lists the results obtained. The molecular weights were calculated using the dissymmetry method (3). The error in measurements was approximately 10%. 

LADH: The molecular weight of native enzyme is 84,000 (4). Whether the enzyme is free or mainly as binary complex with NADH, the light scattering molecular weight agrees with the accepted value, and shows that the presence of the coenzyme, neither aggregates nor dissociates LADH. At acid pH, or in the presence of urea or guanidine-HCl, dissociation into two, four or perhaps more subunits is indicated. At acid pH, reproducibility of the molecular weights was difficult to achieve due to dissociation being accompanied by aggregation. The situation was complicated as both these processes depended on time as well as pH, temperature, and enzyme concentration. Aggregation from the species measured occurred more readily at pH 4.0 than at pH 2.5, where the subunit appeared more stable. The fact that more activity can be recovered at pH 4 than at pH 2.5, also suggests enzyme-forms that cannot revert to active enzyme. The molecular weight of 320,000 at pH 4.0 results from aggregation caused by standing and ultracentrifugation used to remove turbidity. At alkali pH aggregation occurs rapidly (reactivation is difficult), and the
TABLE 1
Molecular Weight from Light Scattering

<table>
<thead>
<tr>
<th>pH</th>
<th>Additions</th>
<th>n₀</th>
<th>dn/dc</th>
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<td>&quot;</td>
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<td>-</td>
<td>&quot;</td>
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<td>21</td>
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<td>8M Urea + 0.1M</td>
<td>&quot;</td>
<td>0.11</td>
<td>11</td>
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<tr>
<td></td>
<td>4M Guanidine-HCl</td>
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<td>0.06</td>
<td>16</td>
</tr>
</tbody>
</table>

ADH

After spinning the solution for three hours in a preparative ultracentrifuge at 70,000 \(\times\) g.

**β-mercaptoethanol.**

High molecular weight at pH 12.0 demonstrates this. Table 1 also shows that in the presence of urea, the molecular weight measured is independent of whether mercaptoethanol is present or not.
YADH: As with LADH, aggregation is found at alkali pH. At acid pH or in the presence of guanidine-HCl dissociation into four subunits occurs. The latter result agrees with the reversible dissociation of YADH into four subunits by urea (2).

Using a Spinco Model E Ultracentrifuge, some experiments were also carried out at 20°C to determine sedimentation coefficients for the species present in solution. The ultracentrifuge was fitted with phase-plate Schlieren optics and a RTLC unit. However, the ultracentrifuge technique did not prove satisfactory due to aggregation often resulting from the centrifugal gradient, enzyme concentration and time used for experiments. Thus a comparison of native with inactive enzyme showed aggregation of LADH at pH 4.0 (6.1S; native 5.1S) and of YADH at pH 2.5. In agreement with the light scattering results, a smaller molecular size was indicated for LADH at pH 2.5 (3.2S).

DISCUSSION

For each of the conditions used for reversible inactivation the molecular weight measurements established dissociation of liver and yeast alcohol dehydrogenase. However the species involved in reactivation are not necessarily identified. Dissociation of both enzymes by detergents (5,6) and urea (2,7) has been reported. The number of subunits formed were the same as the number of active sites per molecule (four for YADH, and two for LADH) except for the urea inactivation of LADH. In this case, depending on whether mercaptoethanol was presented or not, two and four subunits were reported (7). The present results show dissociation of YADH into four subunits, and LADH into two, four or perhaps more subunits.

That the subunit molecular weights measured are not
necessarily those of the actual forms from which reactivation after inactivation occurs, is indicated by the reduced recovery of activity with standing. Reversible inactivation occurred best at neutral pH, while away from neutrality or if the enzyme concentration was too high, increasing irreversible aggregation resulted. Activity that could not be regained, thus seems due to irreversible structural change in the subunits. These may aggregate directly or may give further breakdown-products which may themselves aggregate. Because zinc did not promote reactivation, loss of zinc from the enzyme is considered part of the subsequent irreversible processes. The metal is regarded as essential for the structure of the subunits reactivating, with reversible inactivation occurring prior to the release of zinc from the subunits. Reversible inactivation appears characterised by dissociation into subunits without loss of zinc. For YADH, reactivation or reversible inactivation involves the 37,000 subunits (2). For LADH the experiments suggest reactivation occurs from the 42,000 or 21,000 species, but these can also break-down further or change irreversibly to forms which can irreversibly aggregate. At pH 4.0, four subunits of 21,000 molecular weight are obtained, while pH 2.5 is considered to stabilise a dimer of 42,000, and at higher pH, larger aggregates are formed. Urea and guanidine-HCl also dissociated LADH into four or more subunits, the molecular weights determined being in this case independent of whether mercaptoethanol is present or not.

The fact that inactivation with urea or lithium chloride occurs readily at 0°C, was already an indication of dissociation into subunits which the molecular weight measurements substantiate. The temperature dependence of reversible inactivation supported
this and indicated that hydrophobic bonds which are more stable at room temperature than lower temperatures, are important in the reversible and irreversible processes involved. Thus room temperature rather than 0°C promotes subunit association to act on the enzyme, and at 0°C dissociation is promoted and irreversible aggregation during inactivation minimized. That non-polar bonds predominate in holding the subunits in the native enzyme together was already suggested by the negative temperature coefficient for LADH and YADH inactivation by urea (8). Dissociation by detergents has also been considered as evidence for this (5,6).

While further experiments are necessary to definitely establish the reactivating species, the molecular weight measurements do show, that size as well as shape changes are involved during inactivation. The suggestion from the identity of sedimentation coefficients at pH 3.0 and 7.0, that acid inactivation only involves conformation change (6,9), underlines the limitation due to aggregation of the ultracentrifuge in this situation.

The dissociation of LADH into more than two subunits does raise several problems, as the enzyme contains two C-terminal and two acetylated N-terminal amino acids and is considered to contain two identical polypeptide chains (10-12).

REFERENCES


Eversible Inactivation and Dissociation of Liver and Yeast Alcohol Dehydrogenase

by Li-Yao Cheng and J. S. McKinley-McKee.

Biochemistry Department, University of Edinburgh, Edinburgh, 8)

Many enzymes including certain dehydrogenases have been reversibly dissociated into inactive sub-units. Dissociation has usually been effected with urea, guanidine, extremes of pH or salts. Reassociation generally required mercaptoethanol and neutral pH. Liver and yeast alcohol dehydrogenase (LADH and YADH) are interesting because the native form of these enzymes contains two and four active centres respectively. Also lack of success in dissociating or reversing inactivation of the LADH-metalloenzyme has been considered due to irreversible loss of zinc (Chilson, Kitto, Pudles & Kaplan, 1966).

The conditions for reversible inactivation of LADH and YADH by high and low pH, lithium chloride, and urea have been presented previously (Cheng & McKinley-McKee, 1967). Although preliminary experiments showed that dissociation, rather than conformation changes or proton equilibria, was involved, nevertheless detailed molecular weight measurements were required. Light-scattering has been the main method used, although some measurements have been made with the ultracentrifuge.

For YADH dissociation into four sub-units by urea (Ohta & Ogura, 1965) was confirmed. This was also found to be the case at pH 2-5, while aggregation occurred at pH 12. For LADH the molecular weight determined was 90000 at pH 7-0, whether coenzyme was added or not. At pH 2-5, values between 12000 and 50000 resulted, while at pH 4-0 or in 8M-urea or 4M-guanidine hydrochloride (with or without mercaptoethanol), molecular weights from 11000-21000 were obtained. These results indicate dissociation into two or four sub-units which may unfold, break down further or aggregate with time. At pH 12, the molecular weight was 400000, showing aggregation. Such a result shows a limitation in such determinations as, because of the time involved, dissociated enzyme can aggregate to inactive enzyme, particularly at extremes of pH. This can be much less in the shorter times over which reactivation is itself carried out. The molecular weights of course do show why under certain conditions recovery of activity is difficult.

The reactivation of the alcohol dehydrogenases suggests that the various methods of inactivation are not releasing the zinc moiety from the protein which is reactivated. Likewise, addition of zinc ions did not help reversal of inactivation, although Drum, Harrison, Li, Bethune & Vallee (1966), who recently reported the dissociation of LADH into four sub-units, have suggested that zinc promotes reactivation after urea and mercaptoethanol inactivation. However, mercaptoethanol is a potent inhibitor and the complex formed protects against inactivation. Thus it is not clear whether reactivation was measured, or residual activity detected after the removal of inhibitor by dialysis.


