PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Dynamic Aspects of Enzyme Specificity

K. Dalziel

Phil. Trans. R. Soc. Lond. B 1975 **272**, 109-122
doi: 10.1098/rstb.1975.0074

Phil. Trans. R. Soc. Lond. B. **272**, 109–122 (1975) [109]
Printed in Great Britain

Dynamic aspects of enzyme specificity

By K. Dalziel
Department of Biochemistry, University of Oxford

There are two aspects of enzyme specificity: recognition of the substrate by the formation of an enzyme–substrate compound and recognition of the transition state by catalysis of the reaction. Kinetic studies with inactive substrate analogues as potential competitive inhibitors, and structural studies of their compounds with enzymes, give information about the first of these specificity elements. Comparative kinetic studies with alternative substrates give information about both. There is a great deal of information from kinetic studies of dehydrogenases about the coenzyme specificities, substrate specificities and stereospecificities and mechanisms of these enzymes, particularly alcohol dehydrogenases. Recent X-ray diffraction studies of dehydrogenases have given insight into the molecular basis of some of their specificity elements. An attempt is made to correlate the available kinetic and structural data for alcohol and lactate dehydrogenases.

Introduction

The facts about enzyme specificity are obtained by detailed kinetic studies with highly purified enzyme and pure substrates and competitive inhibitors of systematically varied structure. A chemical explanation of the facts demands a knowledge of the three-dimensional structure at atomic resolution of the enzyme and, ideally, of the reactive enzyme–substrate complex.

A few months before his death in 1964, J. B. S. Haldane wrote the following sentences in the preface for a reprint of his book *Enzymes*, published in 1930. 'In that year, I confidently hoped that thirty years hence we should know the exact structures of a number of enzymes and be able to calculate their properties from the structures. This hope has not yet been fulfilled, partly because the active centres seem to include amino-acid residues in several adjacent and loosely-bonded peptide chains. It is one of my ambitions to live long enough to read a paper whose author explains, from the amino-acid sequences of two enzymes, why one must be an esterase and the other a peptidase.'

Had he lived another year, Haldane would have realized his ambition, in large measure, in the publication of the crystal structure of lysozyme (Blake *et al.* 1965). The evidence of substrate strain (Phillips 1967; Blake *et al.* 1967) might have given him particular satisfaction as confirmation of his hypothesis that the active centre of a hydrolase is designed to fit a configuration midway between substrate and products (Haldane 1930), that is, the transition state of the reaction (Pauling 1948; Wolfenden 1969). The complementary concept that the structure of the enzyme itself may also be altered in the enzyme–substrate complex, to create the active centre by orientation of the catalytic groups (Koshland 1963), has been substantiated in X-ray diffraction studies of carboxypeptidase (Lipscomb *et al.* 1970). The results of these and other structural studies of hydrolases underline the interdependence of the two most striking properties of enzymes, their specificity and their great catalytic powers.

X-ray diffraction studies of several dehydrogenases are now giving insight into the several specificity elements of these larger, oligomeric enzymes. Unlike the hydrolases, the

dehydrogenases form stable complexes with one of their two substrates, the coenzyme, that are amenable to structural study. The ternary complex of enzyme and both substrates, in which the catalytic step occurs, is too unstable for direct study by the methods presently available, however. As with the hydrolases, this problem can be circumvented to some extent by the use in inactive analogues of the substrate or its transition state which form stable complexes, but the degree to which the latter resemble reactive complexes is uncertain. Firm conclusions about the precise mode of binding of an active substrate and the identity of functional groups in the enzyme still rely on a detailed knowledge of dynamic specificity. Such knowledge is available for liver alcohol dehydrogenase, and to a lesser extent for some other dehydrogenases. My main purpose is to consider to what extent the facts of specificity, and kinetic evidence of mechanism, can be correlated with structural data for these enzymes.

The coenzyme specificities of dehydrogenases

A large group of dehydrogenases catalyses the oxidation of a variety of metabolites by transfer of a hydrogen atom and an electron to a nicotinamide–adenine dinucleotide coenzyme and, in most cases, release of a hydrogen ion. A typical overall reaction, such as that catalysed by an alcohol, lactate or malate dehydrogenase, is

$$NAD^+ + R_1R_2CHOH \rightleftharpoons NADH + H^+ + R_1R_2CO.$$

A second reaction is sometimes coupled to the dehydrogenation, for example phosphorylation in the case of glyceraldehyde 3-phosphate dehydrogenase and decarboxylation in the case of 6-phosphogluconate dehydrogenase. There is no net liberation of a hydrogen ion in such oxidative decarboxylation reactions, however, because molecular carbon dioxide is the product (Dalziel & Londesborough 1968).

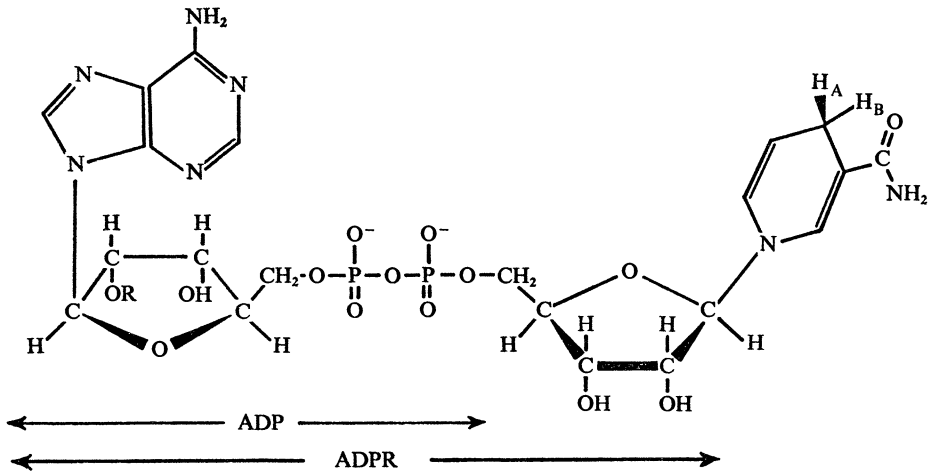


Figure 1. The structures of the natural coenzymes NADH, for which R = H, and NADPH, for which R = PO_3^{2-}. The structures of two hydrolysis products of NADH, adenosine diphosphate and adenosine diphosphoribose, are indicated.

The structures of the reduced forms of the two natural coenzymes are shown in figure 1. Most dehydrogenases are designed to use only one of these coenzymes, despite the similarity of their redox potentials. The biological significance of this specificity probably lies in the different metabolic functions of NADH and NADPH, and the consequent need for the cytoplasmic

NADP+-couple to be maintained in a more reduced state than the cytoplasmic NAD+-couple. This is achieved, in part, by the NADP+-specificity of the cytoplasmic dehydrogenases that catalyse the oxidative decarboxylation of 6-phosphogluconate, malate and isocitrate. The equilibrium constants for these reactions, at physiological hydrogen ion and carbon dioxide concentrations, are more favourable for coenzyme reduction than are those for the simple dehydrogenations catalysed by NAD+-specific lactate, malate and alcohol dehydrogenases, for example (Veech, Eggleston & Krebs 1969).

Westheimer & Vennesland and their colleagues showed that dehydrogenases catalyse direct transfer of the carbon-bound hydrogen of their substrates to NAD+ or NADP+ in a form that does not exchange with water, and that they can be divided into two classes according to their stereospecificity with respect to the prochiral C4 atom of the reduced nicotinamide ring of the coenzyme (Westheimer, Fisher, Conn & Vennesland 1951; Popják 1970). This is illustrated in figure 1. The hydrogen atom derived from the reduced substrate, and transferred to the oxidized substrate, is H_A in the case of alcohol, L-lactate and L-malate dehydrogenases, for example, and H_B for D-glyceraldehyde 3-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The absolute configurations corresponding to this A/B specificity as represented in figure 1 were established by Cornforth *et al.* (1966). For a particular reaction, this specificity appears to be independent of the source of the dehydrogenase or the structure of the substrate; for example, the alcohol dehydrogenases from yeast and horse liver, although quite different in molecular weight and other properties, are both A specific in the oxidation of primary and secondary alcohols (Levy & Vennesland 1957; Dickinson & Dalziel 1967*a*).

The molecular basis of coenzyme specificities

Information about the three-dimensional structures of four dehydrogenases and their complexes with NAD+ has now been obtained by X-ray diffraction studies. The enzymes are L-lactate dehydrogenase from dogfish muscle (Adams *et al.* 1973), alcohol dehydrogenase from horse liver (Brändén *et al.* 1973, 1975), cytoplasmic L-malate dehydrogenase from pig muscle (Hill, Tsernglou, Webb & Banaszak 1972) and D-glyceraldehyde 3-phosphate dehydrogenase from lobster muscle (Buehner *et al.* 1974).

Alcohol and malate dehydrogenase have two polypeptide chains in the molecule, while the other two enzymes have four. The subunits of the four enzymes are similar in size, but the amino acid sequence homologies are not extensive (Jörnvall 1973). Nevertheless, the crystal structure determinations show that the three-dimensional structures are strikingly similar. A considerable part of the polypeptide chain is folded in the same way in all these enzymes to form structurally homologous binding sites for the coenzyme, which is bound in an extended conformation with the adenine and nicotinamide ring structures well separated. Earlier suggestions that overlapping of these two moieties might be responsible for the A/B specificity are therefore not substantiated. It is not proved that the existence of A and B specific enzymes serves any metabolic function, and it is, perhaps, merely incidental to the positions, relative to the coenzyme binding site, in which different substrate binding sites were evolved from a precursor of the dehydrogenases.

Some of the probable atomic interactions between NAD+ and lactate and alcohol dehydrogenases are shown diagrammatically in figure 2. For lactate dehydrogenases they were derived mainly from X-ray diffraction studies of the binary complex with NAD+ and an abortive ternary complex with NAD+ and the oxidized substrate pyruvate (Adams *et al.* 1973). For

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

alcohol dehydrogenase, they were deduced by Brändén *et al.* (1973, 1975) from their studies of a complex of the enzyme and adenosine diphosphoribose (see figure 1). ADPR is a potent competitive inhibitor of alcohol dehydrogenase that binds to the enzyme, at neutral pH, much more firmly than ADP or NAD^+, but less firmly than NADH (Dalziel 1963*a*; Theorell & Yonetani 1964). Its conformation when bound to alcohol dehydrogenase is very similar to that of the ADPR moiety of NAD^+ bound to the other dehydrogenases (Ekland *et al.* 1974). The NAD^+ complex of alcohol dehydrogenase has not been studied because the crystals are not isomorphous with those of the free enzyme.

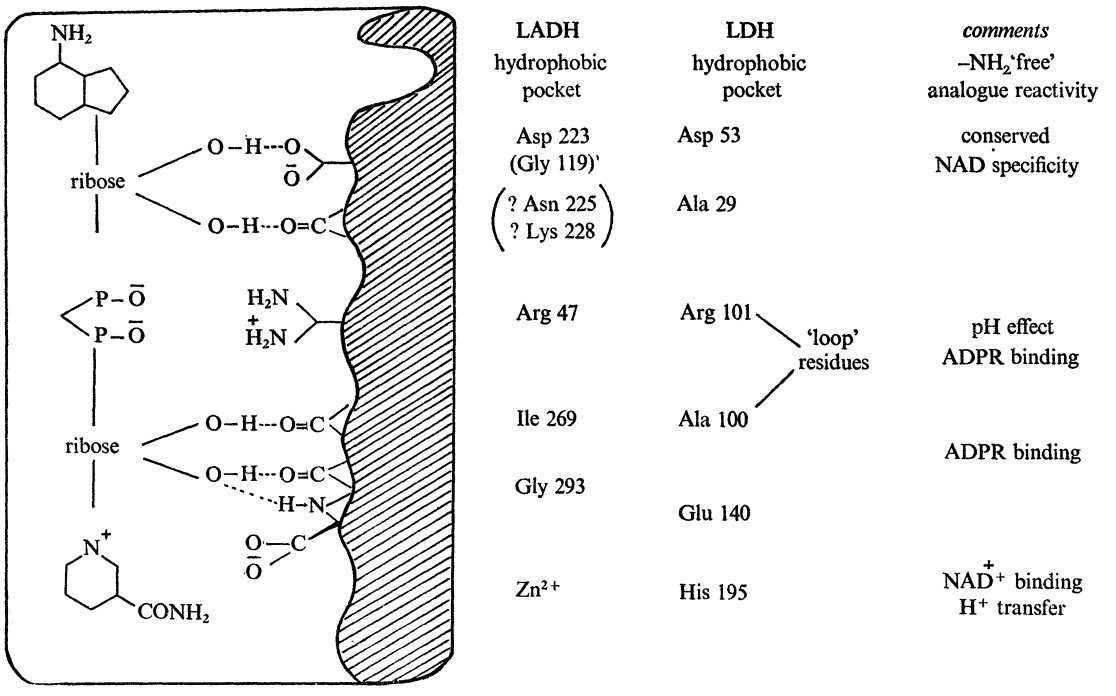


FIGURE 2. Some interactions between dogfish lactate dehydrogenase (LDH) and horse liver alcohol dehydrogenase (LADH) and their coenzyme NAD^+ indicated by X-ray diffraction studies (after Adams *et al.* 1973 and Brändén *et al.* 1974, 1975).

The diffraction data show that the adenine ring of the nucleotide is bound in a hydrophobic pocket in each enzyme, with the NH_2 group pointing outwards towards the solution. This explains the high activity of the enzymes with coenzyme analogues substituted at this group, and their low activity when substituents are introduced elsewhere in the adenine ring (Windmueller & Kaplan 1961).

The molecular basis of the NAD^+-specificity of these two enzymes can be discerned from the environment of the 2′-hydroxyl group of the adenosine-ribose of NAD^+ or ADPR. It is hydrogen-bonded to an aspartate residue in each enzyme. $NADP^+$ could not form this bond, and in addition its phosphate group in this position would suffer steric hindrance from the protein. In fact both these enzymes do exhibit activity with sufficiently large concentrations of $NADP^+$ (Fawcett & Kaplan 1962). It has been shown that alcohol dehydrogenase binds $NADP^+$ and NADPH very weakly, but with large enough concentrations of NADPH the rate of acetaldehyde reduction is as fast as with NADH (Dalziel & Dickinson 1965). An explanation of the inability of $NADP^+$-specific dehydrogenases to utilize the smaller coenzyme NAD^+ must await their

structural study; crystallographic studies of at least one, 6-phosphogluconate dehydrogenase, are in progress (Silverberg, Dalziel & Adams 1973).

In both enzymes, an arginine residue is suitably placed for ionic interaction with the pyrophosphate group of the nucleotide. With lactate dehydrogenase, however, this linkage and the hydrogen bond between the 3′-hydroxyl group of the nicotinamide-ribose and the main-chain carboxyl group of an alanine residue (see figure 2) are formed only in the ternary complex with NAD^+ and pyruvate, and not in the binary complex with NAD^+ (Adams *et al.* 1973). The arginine and alanine residues are part of a 'loop' of some twenty residues in the polypeptide chain of lactate dehydrogenase that moves towards the coenzyme on formation of the ternary complex, and for which there is no counterpart in alcohol dehydrogenase. This is consistent with the fact that ADPR binds much more weakly to lactate dehydrogenase (McPherson 1970) than it does to alcohol dehydrogenase.

Deductions regarding the probable mode of binding of the nicotinamide ring of NAD^+ to alcohol dehydrogenase are aided by the presence of two zinc atoms in the subunit, one of which is involved in the catalytic activity. It is known from inhibition and structural studies with zinc-chelating agents that the nicotinamide part of the coenzyme binds to the enzyme near this zinc atom (Yonetani 1963; Brändén *et al.* 1973). Assuming that the conformations of NAD^+ bound to alcohol and lactate dehydrogenases are similar, Brändén *et al.* (1975) deduced from the X-ray data for the ADPR complex that the nicotinamide ring of NAD^+ could be bound in a hydrophobic pocket near the essential zinc atom. Moreover, this can only occur without steric hindrance if the A side of the nicotinamide ring faces the zinc atom, in accordance with the A specificity of alcohol dehydrogenase and evidence that the zinc atom is a binding site for the substrate (Dunn & Hutchinson 1973). There is no enzyme residue in this pocket equivalent to histidine-195 in lactate dehydrogenase, which is essential for activity of the latter enzyme and suitably positioned in the ternary complex to accept the hydrogen ion from lactate. I shall discuss the probable significance of this difference between the two dehydrogenases in relation to the mechanisms of proton transfer later.

The substrate specificities of dehydrogenases

There is a great deal of evidence that catalysis of the oxidation of primary aliphatic alcohols by horse liver alcohol dehydrogenase proceeds essentially by the reversible ordered mechanism first suggested by Theorell & Chance (1951). The main steps are

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

$$E.NAD^+ + RCH_2OH \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E.NAD^+.RCH_2OH \underset{k'}{\overset{k}{\rightleftharpoons}} E.NADH.RCHO \underset{k'_2}{\overset{k'_{-2}}{\rightleftharpoons}} R.CHO + E.NADH\ (+H^+)$$

$$E.NADH \underset{k'_1}{\overset{k'_{-1}}{\rightleftharpoons}} NADH + E.$$

The proton is shown in parentheses because the stage at which it is liberated depends upon the pH of the medium. The maximum rate with saturating reactant concentrations is determined simply by the rate of dissociation of the coenzyme product (k'_{-1}), because the preceding unimolecular steps (k and k'_{-2}) are much faster.

The initial rate equation for alcohol oxidation in the steady state at any particular pH value has the form

$$\frac{[E]}{v_0} = \Phi_0 + \frac{\Phi_1}{[NAD^+]} + \frac{\Phi_2}{[RCH_2OH]} + \frac{\Phi_{12}}{[NAD^+][RCH_2OH]}.$$

The four parameters can be determined experimentally, and for the Theorell–Chance mechanism $\theta_0 = 1/k'_{-1}$ and $\theta_1 = 1/k_1$, while θ_2 is a function of the velocity constants k_2, k_{-2}, k, k' and k'_{-2}. Representation of the rate equation in terms of these parameters instead of Michaelis constants is to be preferred because the latter are more complex functions of the rate constants and are not dissociation constants. An exactly analogous rate equation applies to the reduction of acetaldehyde by this mechanism, of course.

Experimental values for these three initial rate parameters from studies of the oxidation of several primary alcohols and the reduction of the corresponding aldehydes are shown in table 1.

TABLE 1. KINETIC COEFFICIENTS FOR LIVER ALCOHOL DEHYDROGENASE AT pH 7 AND 25 °C

(After Dalziel 1962 and Dalziel & Dickinson 1966)

	S_2 = alcohol			S_2 = aldehyde		
alcohol substrate	θ_0 / s	θ_1 / μM s	θ_2 / μM s	θ_0 / s	θ_1 / μM s	θ_2 / μM s
$CH_3.CH_2OH$	0.37	1.1	66	0.0075	0.10	3.3
$CH_3.CH_2.CH_2OH$	0.31	1.2	19	0.0095	0.14	0.43
$CH_3.CH_2.CH_2.CH_2OH$	0.35	1.1	4	0.0075	0.10	0.17
$CH_3.CH(CH_3).CH_2OH$	0.34	1.7	40	0.0090	0.12	0.28

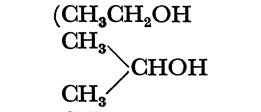
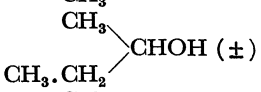
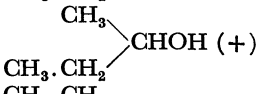
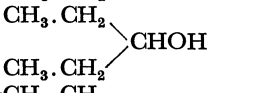
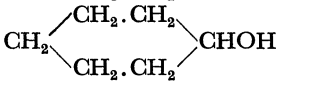
The constancy of θ_0 and θ_1 is obviously strong support for an ordered, Theorell–Chance mechanism with all these substrates, and the reciprocals of these constants agree very well with direct estimates of the velocity constants for the formation and dissociation of the enzyme–coenzyme complexes by rapid reaction techniques (Theorell & Chance 1951; Shore & Gutfreund 1970). The systematic decrease of θ_2 with increase of the *n*-alkyl chain length of the substrate means that the higher alcohols and aldehydes are the better substrates either because they bind to the enzyme–coenzyme complex more firmly than the lower homologues, or because the rate of hydride transfer in the ternary complex increases with the length of the alkyl chain. There is convincing evidence that both factors contribute. In particular, Brooks & Shore (1971) showed by measurements of the rate of formation of enzyme-bound NADH in the pre-steady-state phase of the reaction that the maximum specific rate of hydride tansfer with propan-1-ol, 650 s^{-1}, is five times faster than with ethanol, and is reached with a smaller concentration of the former alcohol. These results indicate that there is a hydrophobic binding site in the enzyme–coenzyme complex to which substrates with long alkyl chains bind most firmly, and form ternary complexes in which, through better orientation of the reacting groups, hydride transfer is faster than with ethanol.

Secondary alcohols, except for cyclohexanol, are much poorer substrates than ethanol (see table 2), and θ_0 and θ_1, as well as θ_2, vary from alcohol to alcohol. It can be concluded from these and other data (Dalziel & Dickinson 1966) that hydride transfer in the ternary complex is so much slower with secondary alcohols than with ethanol that it becomes the rate-limiting step with saturating coenzyme and substrate concentrations. This was confirmed by the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Table 2. Kinetic coefficients for liver alcohol dehydrogenase with secondary alcohols at pH 7 and 25 °C

(After Dalziel & Dickinson 1966, and Dickinson & Dalziel 1967*b*)

substrate	θ_0 / s	θ_1 / μM s	θ_2 / μM s
(CH_3CH_2OH	0.37	1.1	66)
	3.9	82	49×10^3
	1.7	41	9×10^3
	2.3	33	6×10^3
	1.05	23	2.5×10^3
	0.31	3.3	230

demonstration of a primary deuterium isotope effect on the maximum rate ($1/\theta_0$) of propan-2-ol oxidation, and the absence of a pre-steady-state burst of enzyme-bound NADH in stopped-flow experiments with this alcohol (Brooks & Shore 1971).

A great variety of other alcohols including aromatic alcohols, cyclohexanol derivatives, steroids and ω-hydroxy fatty acids are also substrates of liver alcohol dehydrogenase (Sund & Theorell 1963; Björkhem 1972) but have not been studied kinetically in such detail.

Information about the substrate binding site of liver alcohol dehydrogenase from X-ray diffraction studies is limited at present to inferences from the structure of the ADPR complex. No structural studies of complexes with substrate analogues have yet been made. The catalytic zinc atom is located at the bottom of a wide and deep hydrophobic pocket which has been tentatively identified as the substrate binding site, consistent with the very broad substrate specificity of the enzyme (Eklund *et al.* 1974; Brändén *et al.* 1975).

More is known about the substrate binding site of dogfish lactate dehydrogenase from X-ray studies of the abortive complex with NAD^+ and pyruvate and an inactive ternary complex with NAD^+ and oxalate (Adams *et al.* 1973). There is an ionic interaction between the carboxyl group of the substrate and an arginine residue in the enzyme, and this together with an interaction between the hydroxyl group of the substrate and histidine 195 (see figure 2) is sufficient to orientate L-lactate for A specific hydride transfer to the coenzyme. The methyl group of lactate is not involved in substrate binding; it points towards the solution, and this is consistent with the activity of lactate dehydrogenase from various species with phenyl pyruvate and several other 2-keto and 2-hydroxy acids. The kinetics of the lactate dehydrogenase reaction with substrates other than lactate and pyruvate have not yet been studied in sufficient detail to show how the rates of individual steps vary with substrate structure and indeed whether the mechanism is ordered; the evidence for an ordered mechanism with lactate and pyruvate is only substantial for the enzyme from bovine heart muscle (Schwert, Miller & Peanasky 1967).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Substrate stereospecificities

Most enzymes such as lactate dehydrogenase that catalyse a reaction at an assymmetric carbon atom are specific for one optical isomer. This is to be expected when the types of bond that the two non-reacting groups – methyl and carboxylate in lactate – are likely to form are quite different, since one of them at least must be involved in binding the substrate to the enzyme.

The reaction catalysed by alcohol dehydrogenases takes place at a prochiral carbon atom of primary alcohols, and Levy, Loewus & Vennesland (1957) showed that yeast alcohol dehydrogenase is absolutely stereospecific in the transfer of one of the two C1 hydrogen atoms of

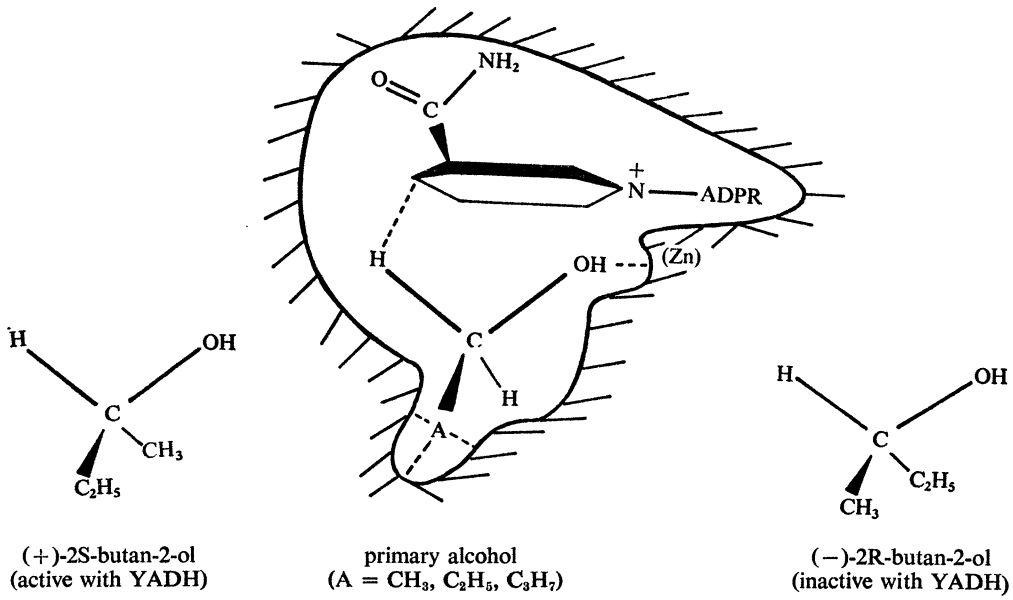


FIGURE 3. The stereochemistry of the active centres of yeast alcohol dehydrogenase (YADH) and liver alcohol dehydrogenase. Binding sites on the enzyme for the alkyl group, A, and the hydroxyl group – probably the catalytic zinc atom – of a primary alcohol are indicated. The configurations of the optical isomers of butan-2-ol are also shown. (After Dickinson & Dalziel 1967*a*, *b*.)

ethanol. A high degree of stereospecificity of this kind for horse liver alcohol dehydrogenase with geraniol as substrate has also been established (Donninger & Ryback 1964) and it has been inferred – but apparently not proved – that similar stereospecificity would be exhibited with ethanol.

The isomers of [1-^{2}H]ethanol (Lemieux & Howard 1963) and [1-^{3}H]geraniol (Donninger & Ryback 1964) from which isotope is specifically transferred to NAD^+ by the two enzymes have the R configuration in the nomenclature of Cahn, Ingold & Prelog (1956). The active centres of the two alcohol dehydrogenases can therefore be represented stereochemically as in figure 3; there are only two potential binding groups in a primary alcohol, the alkyl group A and the hydroxyl group. It is interesting to compare this stereochemistry with that of the active-centre of L-lactate dehydrogenase; the carboxylate group of L-lactate by which this substrate is bound to an arginine residue in the enzyme is stereochemically equivalent to the alkyl group of a primary alcohol, whilst the non-bonding methyl group of L-lactate is equivalent to the non-transferrable hydrogen atom of a primary alcohol in the active centre of the alcohol dehydrogenases (see figure 3).

Yeast alcohol dehydrogenase is also absolutely stereospecific for one enantiomorph of the optically active secondary alcohols butan-2-ol and octan-2-ol. Surprisingly, at first sight, we found that the liver enzyme is not, and indeed catalyses the oxidation of the two isomers of butan-2-ol at almost equal rates (Dickinson & Dalziel 1967*a*, *b*). This is to be expected, in fact, from the broad specificity of the enzyme and particularly its reactivity with a series of primary aliphatic alcohols, from which it is evident that the alkyl binding site on the enzyme can accommodate either alkyl group of a secondary alcohol. Each isomer of a secondary alcohol can therefore be bound in an active configuration. From the known stereospecificity of the enzyme with a primary alcohol, and the known configurations of the enantiomorphs of secondary alcohols (Levene, Walti & Haller 1926; Mills and Klyne 1954), it can be deduced that the (+)-2S-isomers of *n*-alkylmethyl carbinols, when bound to liver alcohol dehydrogenase, have the larger alkyl group attached to the alkyl binding site, while the (−)-2R-isomers will be bound by the methyl group (see figure 3). These conclusions are also in accordance with the fact that the (+)-2S-isomers are the better substrates, especially in the case of octan-2-ol, and that the reactivity of primary alcohols increases with the size of their *n*-alkyl groups (see table 1).

Since yeast alcohol dehydrogenase also catalyses the oxidation of propan-1-ol and butan-1-ol (although less effectively than that of ethanol), it is the absolute stereospecificity of this enzyme for the (+)-2S-isomers of butan-2-ol and octan-2-ol that requires explanation. The most obvious hypothesis is that, in contrast to the liver enzyme, it cannot accommodate an alkyl group larger than methyl in place of the non-transferrable hydrogen atom of ethanol (i.e. not bound to the alkyl binding site) because of steric hindrance (see figure 3). If this hypothesis is correct, secondary alcohols in which both alkyl groups are larger than methyl should not be acted upon by the yeast enzyme. We found this to be so: pentan-3-ol, (±) hexan-3-ol and heptan-4-ol are substrates for liver alcohol dehydrogenase, but not for the yeast enzyme (Dickinson & Dalziel 1967*b*), and this is true also of cyclohexanol (Merritt & Tomkins 1959).

These studies have given a great deal of information about the stereochemistry of the active centre of liver alcohol dehydrogenase, but it is premature to attempt to correlate the findings with the X-ray work in its present stage (C-I. Brändén, personal communication). It is clear that the substrate binding site of liver alcohol dehydrogenase is much less restrictive than that of the yeast enzyme. The latter is much the more active enzyme with ethanol and acetaldehyde, its best – and physiological – substrates, and it has been shown that the rate of hydride transfer in the ternary complex is faster than with the liver enzyme (Dickinson & Monger 1973). This may be attributed to the more restrictive substrate binding site of the yeast enzyme, which might permit more precise orientation of these small substrates for hydride transfer than is possible for the liver enzyme. The normal physiological role of liver alcohol dehydrogenase is not established; it may be involved in the oxidation of alcohols formed by bacterial action in the intestinal tract (Krebs & Perkins 1970), bile acid biosynthesis (Waller, Theorell & Sjövall 1965) and the ω-degradation of fatty acids (Björkhem 1972).

Mechanisms for proton release

In the overall reactions catalysed by simple dehydrogenases a proton is released from the substrate to the solution. If a basic group in the enzyme is involved in this process, proton liberation and hydride transfer to the coenzyme need not occur simultaneously, and it is now

clear that proton release occurs at different stages, and by different mechanisms, in the alcohol and lactate dehydrogenase reactions.

For liver alcohol dehydrogenase, a study of the variation with pH of the initial rate parameters for the oxidation of ethanol and the reduction of acetaldehyde (Dalziel 1963*b*) showed that at pH 8–10 the reaction must be formulated in the following way:

$$EH + NAD^+ \rightleftharpoons E^-.NAD^+ + H^+ \quad (1)$$

$$E^-.NAD^+ + C_2H_5OH \rightleftharpoons EH.NADH + CH_3CHO \quad (2)$$

$$EH.NADH \rightleftharpoons EH + NADH. \quad (3)$$

This conclusion was based on the variation with pH of the two velocity constants for step (1) and the equilibrium constant for step (2), which can all be calculated from initial rate measurements. The results also showed that at pH 7.0, only one half-equivalent of protons is released in

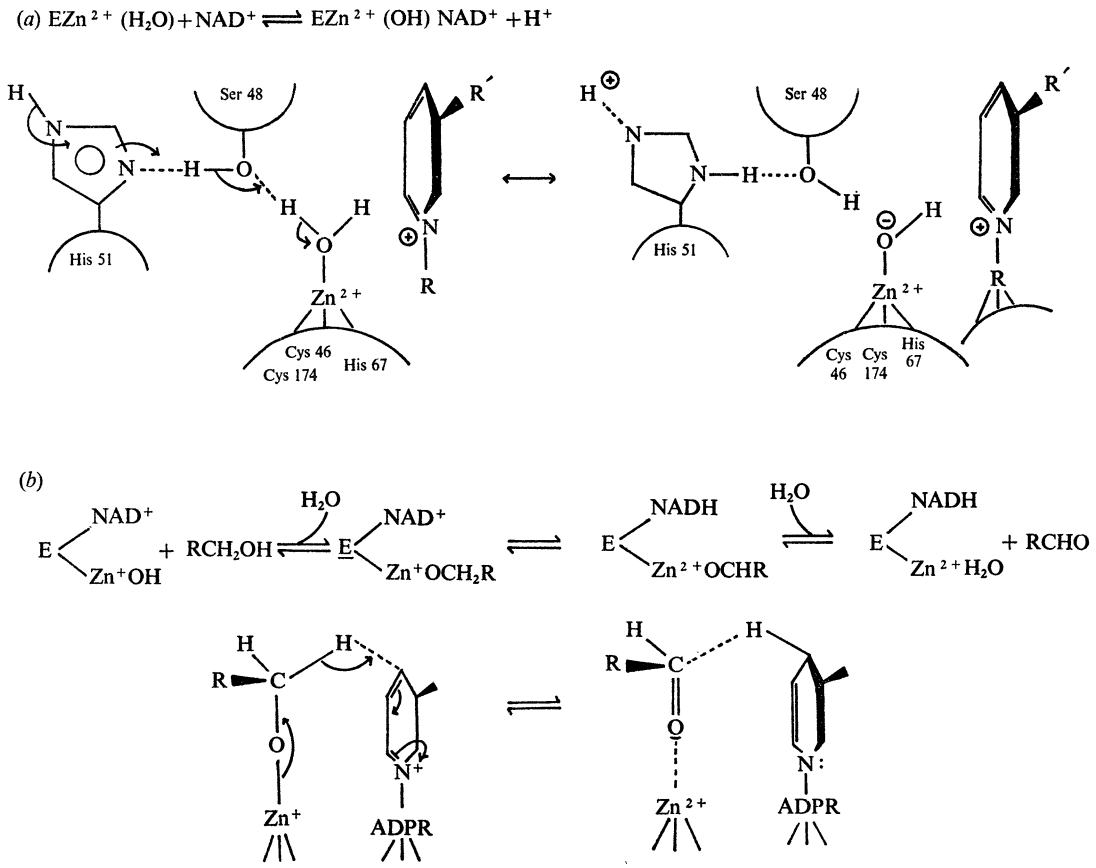


FIGURE 4. A tentative hypothesis for the mechanism of liver alcohol dehydrogenase (after Eklund *et al.* 1974 and Brändén *et al.* 1975).

step (1), and the other half-equivalent is released at some stage during step (2). Recent studies with trifluorethanol as an inactive substrate analogue indicate that this occurs on binding of the alcohol to form the ternary complex prior to hydride transfer (Shore, Gutfreund, Santiago & Santiago 1974). Both the kinetic studies and direct measurements of the equilibrium constant for step (1) by Theorell & McKinley-McKee (1961) and Taniguchi, Theorell & Åkeson (1967) indicate that proton release is caused by a shift of the pK_a value of a group in the enzyme

from about 9.0 in the free enzyme to about 7.0 in the complex with NAD^+. It was suggested by Theorell & McKinley-McKee (1961) that this group is a water molecule coordinated to the catalytic zinc atom. The kinetic studies also showed that protonation of a second group in the enzyme, with $pK_a \approx 6.7$, inhibits the combination of NAD^+ (Dalziel 1963*b*).

A tentative mechanism for proton release during the binding of NAD^+ and for the subsequent catalysis that accounts for these results, and involves both a histidine residue in the enzyme and the zinc-bound water molecule, has been proposed by Eklund *et al.* (1974) and Brändén *et al.* (1975) on the basis of their X-ray diffraction studies. The scheme, shown in figure 4, is an elaboration of an earlier proposal by Theorell (1967). The catalytic-zinc atom is liganded by three protein residues at the bottom of the deep hydrophobic pocket provisionally identified as the binding site for the substrate. A serine residue in this pocket can form a hydrogen bond with the water molecule that completes the tetrahedral coordination of the zinc, and also with a histidine residue, the second nitrogen atom of which points towards the solution. It is suggested that this system of hydrogen bonds facilitates the transfer of a proton from the zinc-bound water molecule in the hydrophobic active centre region to the solution, initiated by binding of the pyridinium ring of NAD^+ near the zinc atom. The alcohol then displaces the hydroxyl ion from the zinc, forming the alcoholate, and the oxidation occurs by electrophilic catalysis mediated by the pyridinium nitrogen atom of the coenzyme and the zinc acting as a Lewis acid.

In the lactate dehydrogenase reaction, proton release occurs at some stage during the overall reaction of the enzyme–coenzyme complex with the substrate. This can be deduced (Dalziel 1975) from the fact that the dissociation constants of the NAD^+ and NADH complexes of lactate dehydrogenase vary only slightly, and in the same way, with change of pH (Stinson & Holbrook 1973). There is evidence that proton release occurs after hydride transfer in the ternary complex, and accompanies pyruvate dissociation (Holbrook & Gutfreund 1973). As I have already described, the X-ray diffraction studies by Adams *et al.* (1973) indicate that the essential histidine residue of lactate dehydrogenase is suitably placed to accept the proton directly from lactate. The structural basis for the necessary change of the pK_a of this group remains to be discovered; it may be related to the large conformation change involving the 'loop' residues in lactate dehydrogenase that accompanies the combination of substrate analogues with the NAD^+ complex of the enzyme.

Conclusions

I have tried to indicate, all too briefly, some of the similarities and differences between the two dehydrogenases for which structural studies are most advanced, and their significance for our understanding of the several specificity elements and the overall mechanisms of these enzymes. We can expect a rapid increase in the extent and detail of our knowledge of structures and conformations of dehydrogenases and their stable complexes with coenzymes and substrate analogues from both crystallographic studies and spectroscopic studies in solution.

The striking success of structural studies increases, rather than diminishes, the need for more detailed and penetrating kinetic studies. Because of the optical properties of the reduced coenzymes, dehydrogenases are particularly convenient subjects for such studies. So far, attention has been directed mainly towards identifying and characterizing the molecular intermediates in the reaction pathway and determining the rates of the elementary reactions, by a

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

combination of kinetic studies of the overall reaction in the steady-state and the pre-steady-state and of elementary steps such as the reactions of enzyme with coenzyme.

Few studies of the thermodynamic properties of the transition states have been made. For liver alcohol dehydrogenase, the establishment of an ordered mechanism with primary aliphatic alcohols and aldehydes has allowed the construction of a partial energy diagram from measurements of initial rates at several temperatures (Dalziel 1963*c*). The results show that a

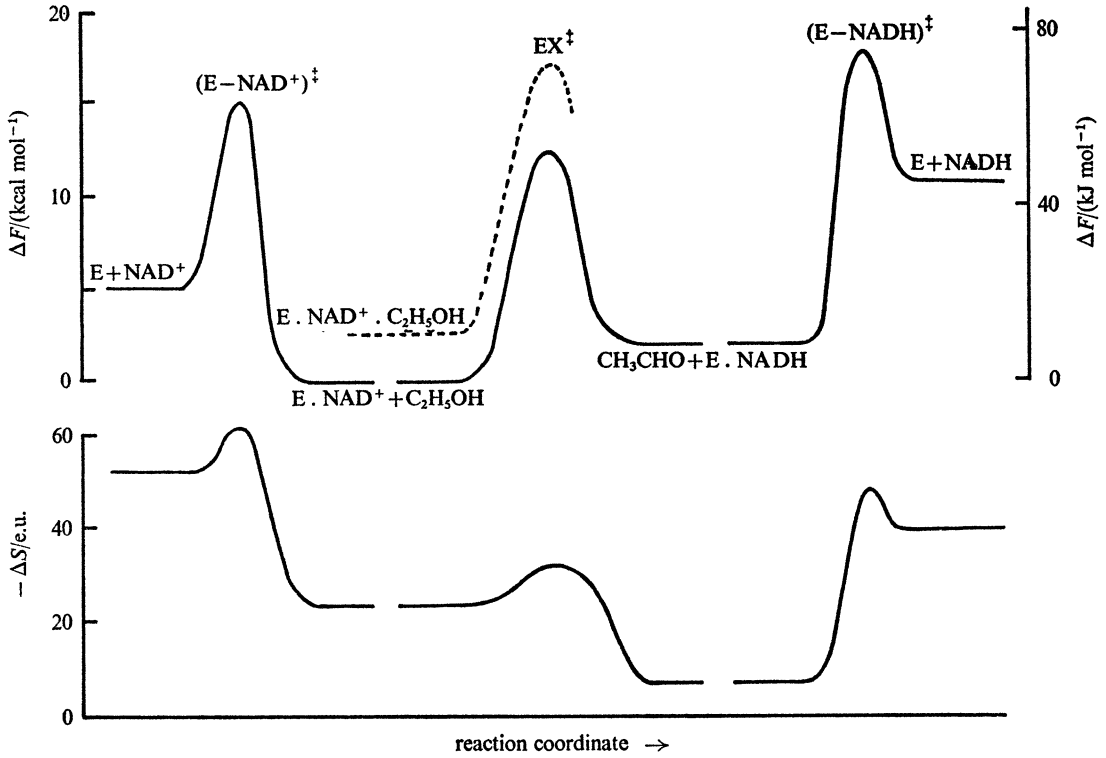


FIGURE 5. A partial energy diagram for the overall oxidation of ethanol catalysed by liver alcohol dehydrogenase (after Dalziel 1963*c* and Brooks *et al.* 1972).

large increase of entropy accompanies the formation of the enzyme–coenzyme complexes (see figure 5), and that while the activation entropy for the combination of the enzyme with oxidized or reduced coenzyme is the expected value for a bimolecular reaction, the reverse, dissociation reactions have large negative entropies of activation which are mainly responsible for the stabilities of the complexes. The activation energy for the reaction of bound coenzyme and substrate derived from initial rate measurements will correspond to the height of the highest energy barrier in the sequence of elementary steps preceding product–coenzyme dissociation. Direct studies of the temperature-dependence of hydride transfer in the ternary complex (Brooks, Shore & Gutfreund 1972) give a similar activation energy (see figure 3). Similar studies of other dehydrogenases would be of value.

I am grateful to Dr C-I. Brändén for allowing me to read the manuscript of his article for *The enzymes* (1975) before publication.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

References (Dalziel)

Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossmann, M. G., Smiley, I. E., Allison, W. S., Everse, J., Kaplan, N. O. & Taylor, S. S. 1973 *Proc. natn. Acad. Sci. U.S.A.* **70**, 1968–1972.

Björkhem, I. 1972 *Eur. J. Biochem.* **30**, 441–451.

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C. & Sarma, V. C. 1967 *Proc. R. Soc. Lond.* B **167**, 378–388.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Phillips, D. C. & Sarma, V. C. 1965 *Nature, Lond.* **206**, 757–761.

Brändén, C-I., Eklund, H., Nordström, B., Boiwe, T., Söderlund, G., Zeppezauer, E., Ohlsson, I. & Åkeson, Å. 1973 *Proc. natn. Acad. Sci. U.S.A.* **70**, 2439–2442.

Brändén, C-I., Jörnvall, H., Eklund, H. & Furugren, B. 1975 In *The enzymes* (ed. P. D. Boyer), vol. xi. (In the Press.)

Brooks, R. L. & Shore, J. D. 1971 *Biochemistry* **10**, 3855–3858.

Brooks, R. L., Shore, J. D. & Gutfreund, H. 1972 *J. biol. Chem.* **247**, 2382–2383.

Buehner, M., Ford, G. C., Moras, D., Olsen, K. W. & Rossmann, M. G. 1974 *J. molec. Biol.* **82**, 563–585.

Cahn, R. S., Ingold, C. K. & Prelog, V. 1956 *Experimentia* **12**, 81–124.

Cornforth, J. W., Cornforth, R. H., Donninger, C., Popják, G., Ryback, F. & Schroepfer, G. J. Jr 1966 *Proc. R. Soc. Lond.* B **163**, 436–464.

Dalziel, K. 1962 *Biochem. J.* **84**, 244–254.

Dalziel, K. 1963*a* *J. biol. Chem.* **238**, 1538–1543.

Dalziel, K. 1963*b* *J. biol. Chem.* **238**, 2850–2858.

Dalziel, K. 1963*c* *Acta chem. scand.* **17**, S27–33.

Dalziel, K. 1975 In *The enzymes* (ed. P. D. Boyer), vol. xi. (In the Press.)

Dalziel, K. & Dickinson, F. M. 1965 *Biochem. J.* **95**, 311–320.

Dalziel, K. & Dickinson, F. M. 1966 *Biochem. J.* **100**, 34–46.

Dalziel, K. & Londesborough, F. M. 1968 *Biochem. J.* **110**, 223–230.

Dickinson, F. M. & Dalziel, K. 1967*a* *Nature, Lond.* **214**, 31–33.

Dickinson, F. M. & Dalziel, K. 1967*b* *Biochem. J.* **104**, 165–172.

Dickinson, F. M. & Monger, G. P. 1973 *Biochem. J.* **131**, 261–270.

Donninger, C. & Ryback, G. 1964 *Biochem. J.* **91**, 11p.

Dunn, M. F. & Hutchinson, J. S. 1973 *Biochemistry* **12**, 4882–4892.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T. & Brändén, C-I. 1974 *F.E.B.S. Lett.* **44**, 200–204.

Fawcett, C. P. & Kaplan, N. O. 1962 *J. biol. Chem.* **237**, 1709–1715.

Haldane, J. B. S. 1930 *Enzymes*. London: Longmans Green. Reprinted 1965, p. 182. Cambridge, Ma: M.I.T. Press.

Hill, E., Tsernglou, D., Webb, L. & Banaszak, L. J. 1972 *J. molec. Biol.* **72**, 577–591.

Holbrook, J. H. & Gutfreund, H. 1973 *F.E.B.S. Lett.* **31**, 157–169.

Jörnvall, H. 1973 *Proc. natn. Acad. Sci. U.S.A.* **70**, 2295–2298.

Koshland, D. E. Jr 1963 *Cold Spring Harbor Symp. quant. Biol.* **28**, 473–482.

Krebs, H. A. & Perkins, J. R. 1970 *Biochem. J.* **118**, 635–644.

Lemieux, R. U. & Howard, J. 1963 *Can. J. Chem.* **41**, 308–316.

Levene, P. A., Walti, A. & Haller, H. L. 1926 *J. biol. Chem.* **71**, 465–469.

Levy, H. R., Loewus, F. A. & Vennesland, B. 1957 *J. biol. Chem.* **79**, 2949–2953.

Levy, H. R. & Vennesland, B. 1957 *J. biol. Chem.* **228**, 85–96.

Lipscomb, W. N., Reeke, G. N. Jr, Hartsuch, J. A., Quiocho, F. A. & Bethge, P. H. 1970 *Phil. Trans. R. Soc. Lond.* B **257**, 177–214.

McPherson, A. Jr 1970 *J. molec. Biol.* **51**, 39–46.

Merritt, A. D. & Tomkins, G. M. 1959 *J. biol. Chem.* **234**, 2778–2782.

Mills, J. A. & Klyne, W. 1954 In *Progress in stereochemistry* (ed. W. Klyne), vol. 1, p. 177. New York: Academic Press.

Pauling, L. 1948 *Nature, Lond.* **161**, 707–709.

Phillips, D. C. 1967 *Proc. natn. Acad. Sci. U.S.A.* **57**, 484–495.

Popják, G. 1970 In *The enzymes* (ed. P. D. Boyer), vol. 2, pp. 116–215. New York: Academic Press.

Schwert, G. W., Miller, B. R. & Peanasky, R. J. 1967 *J. Biochem.* **242**, 3245–3252.

Shore, J. D. & Gutfreund, H. 1970 *Biochemistry* **9**, 4655–4659.

Shore, J. D., Gutfreund, H., Santiago, D. & Santiago, P. 1974 In *Alcohol and aldehyde metabolising systems* (eds R. G. Thurman, T. Yonetani, J. R. Williamson & B. Chance), p. 45. New York: Academic Press.

Silverberg, M., Dalziel, K. & Adams, M. J. 1973 *Biochem. Soc. Trans.* **1**, 1132–1133.

Stinson, R. A. & Holbrook, J. H. 1973 *Biochem. J.* **131**, 719–728.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Taniguchi, S., Theorell, H. & Åkeson, Å. 1967 *Acta chem. scand.* **21**, 1903–1920.
Theorell, H. 1967 *Harvey Lectures*, series 61, 17–42.
Theorell, H. & Chance, B. 1951 *Acta chem. scand.* **5**, 1127–1144.
Theorell, H. & McKinley-McKee, J. S. 1961 *Acta chem. scand.* **15**, 1811–1833.
Theorell, H. & Yonetani, T. 1964 *Archs Biochem. Biophys.* **106**, 243–251.
Veech, R. L., Eggleston, L. V. & Krebs, H. A. 1969 *Biochem. J.* **115**, 609–619.
Waller, G., Theorell, H. & Sjövall, J. 1965 *Archs Biochem. Biophys.* **111**, 671–684.
Westheimer, F. H., Fisher, H. F., Conn, E. E. & Vennesland, B. 1951 *J. Am. chem. Soc.* **73**, 2403.
Windmueller, H. G. & Kaplan, N. O. 1961 *J. biol. Chem.* **236**, 2716–2726.
Wolfenden, R. 1969 *Nature, Lond.* **223**, 704–705.
Yonetani, T. 1963 *Acta chem. scand.* **17**, S96–S101.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES